

UNIVERSITÀ DEGLI STUDI DI MILANO-BICOCCA

Dipartimento di Biotecnologie e Bioscienze

Dottorato di ricerca in Biotecnologie Industriali-XXVIII Ciclo



**Development of a novel molecular assay based on  
Allele-Specific Loop mediated isothermal AMPLification  
for rapid detection of cKIT D816V point mutation  
in Core-Binding Factor Acute Myeloid Leukemia patients**

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Il lavoro presentato in questa tesi è stato realizzato presso i laboratori di Diagnostica Molecolare DiaSorin, sotto la supervisione della Dr.ssa Giulia Minnucci e del Dottor Francesco Colotta.

Il materiale contenuto nella seguente tesi è strettamente confidenziale. E' stata inoltre presentata richiesta di embargo tesi per un periodo di 3 anni dalla data di conseguimento del titolo

The logo for DiaSorin, featuring the company name in white serif font on a dark blue rectangular background.

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# ***1.RIASSUNTO***

## RIASSUNTO

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Le leucemie mieloidi acute Core-Binding Factor (CBF AML) rappresentano il 5-8% di tutte le AML. Le CBF AML sono causate dalla traslocazione t(8;21), dall'inversione pericentrica del cromosoma 16 e più raramente dalla traslocazione t(16;16). Tali alterazioni cromosomiche determinano rispettivamente la distruzione dei geni CBF $\alpha$  (21q22) e CBF $\beta$  (16q22) che codificano le due subunità proteiche del complesso Core-Binding Factor (CBF), un importante regolatore trascrizionale dell'ematopoiesi coinvolto nel differenziamento della linea cellulare mieloide.

Nel 2008 la World Health Organization (WHO) ha classificato le leucemie CBF AML nel gruppo di AML a prognosi favorevole. Tuttavia, nel 20-25% dei pazienti con t(8;21) e in circa il 30% dei pazienti con inv(16)/t(16;16) la prognosi peggiora a causa della presenza della mutazione D816V nel gene cKIT, infatti la leucemia tende a recidivare e diminuisce la probabilità di sopravvivenza del paziente. Pertanto le CBF AML associate alla mutazione cKIT D816V sono classificate nel gruppo di leucemie a rischio intermedio.

Il protooncogene cKIT codifica per il recettore dello Stem Cell Factor (SCF), una citochina che svolge un ruolo cruciale nella proliferazione e nel differenziamento delle cellule ematopoietiche. cKIT è una proteina transmembrana che appartiene alla famiglia dei recettori tirosin chinasi di tipo III. Essa è costituita da un dominio extracellulare con cinque motivi "immunoglobulin-like" che ospita il sito di legame dello SCF, un dominio transmembrana con funzioni di ancoraggio e un dominio juxtamembrana che svolge funzioni di regolazione inibendo l'attività tirosin chinasi in

assenza di SCF. La regione citoplasmatica contiene due domini separati da un inserto interchinasico: il dominio TK1 all'estremità N-terminale contenente il sito di legame dell'ATP e il dominio TK2 all'estremità C-terminale con funzione attivante fosfotransferasica.

cKIT D816V è una mutazione puntiforme in cui il codone GAC che codifica per l'acido aspartico in posizione 816, è sostituito dal codone GTC codificante per la valina. Tale mutazione causa l'attivazione del recettore cKIT indipendentemente dal legame dello SCF e la conseguente attivazione dei segnali di trasduzione che promuovono il differenziamento, la proliferazione e la sopravvivenza delle cellule. Inoltre, diversi studi hanno dimostrato che la mutazione D816V provoca resistenza all'Imatinib, un inibitore tirosin chinasi di prima generazione utilizzato nel trattamento dei pazienti CBF AML in cui il gene cKIT è overespresso. In tal caso i pazienti CBF AML sono trattati con inibitori di seconda generazione Dasatinib o Nilotinib che riescono a bloccare con una migliore efficienza l'attività del recettore.

La rilevazione della mutazione puntiforme cKIT D816V nei pazienti con CBF AML risulta dunque fondamentale per una corretta stratificazione prognostica e per definire un adeguato approccio terapeutico.

Ad oggi sono disponibili diverse tecniche di diagnosi molecolare basate su PCR per rilevare la mutazione D816V, come la PCR seguita dal sequenziamento diretto di Sanger, dalla digestione con l'enzima di restrizione HinfI (RFLP) o da dHPLC (Denaturing high performance liquid

chromatography). Tuttavia la maggior parte dei metodi basati su PCR richiede tempi di attesa molto lunghi, impiega strumentazioni costose e procedure laboriose. Al momento il sequenziamento Sanger rappresenta il metodo più utilizzato anche se raggiunge una scarsa sensibilità (10–15%). Negli ultimi anni sta emergendo una nuova tecnologia basata sul Next Generation Sequencing (NGS) che permette di rilevare le mutazioni puntiformi con una sensibilità maggiore, tuttavia essa risulta tecnicamente complessa e costosa per essere applicate nella diagnosi di routine.

Questo progetto di ricerca si propone di sviluppare un saggio molecolare per la rilevazione della mutazione cKIT D816V basato sulla tecnologia AS LAMP (Allele-specific Loop mediated isothermal AMPlification) che, rispetto ai metodi convenzionali, risulta più sensibile, rapida e meno costosa.

La reazione LAMP è stata descritta per la prima volta nel 2000 da Notomi, ed è basata sull'utilizzo di una DNA polimerasi dotata di attività di "strand displacement" in grado di amplificare il DNA con elevata efficienza (fino a  $10^{10}$  volte) a temperatura costante e in tempi estremamente rapidi. Inoltre LAMP utilizza quattro differenti primers, due "inner primers" (FIP e BIP) e due "outer primers" (F3 e B3) disegnati specificatamente per riconoscere sei distinte regioni sul DNA target. Il metodo AS LAMP rappresenta un'evoluzione della LAMP tradizionale che è stata brevettata da DiaSorin S.p.A. nel 2012. Questo metodo permette di rilevare mutazioni puntiformi con alta sensibilità e specificità grazie alla cooperazione di due reagenti: un

## **RIASSUNTO**

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backward loop primer che lega selettivamente la sequenza mutata complementare e catalizza la reazione di amplificazione, ed un blocker che ibridizza con alta affinità e specificità la sequenza wild-type e ne blocca l'amplificazione. Il saggio AS LAMP include l'amplificazione del gene housekeeping ABL utilizzato come controllo interno per escludere falsi negativi e per verificare le corrette condizioni di reazione (temperatura, pH, assenza di inibitori...). L'amplificazione del gene target e del controllo interno sono rilevati in tempo reale in due canali distinti, 500 e 530 nm rispettivamente, grazie all'impiego di due sonde opportunamente marcate. Il saggio AS LAMP è stato ottimizzato su plasmidi sintetici e successivamente validato su campioni clinici, confermando nel 100% dei casi i risultati ottenuti con i metodi basati su PCR.

In conclusione, il saggio AS-LAMP permette di rilevare la mutazione puntiforme D816V con alta sensibilità e specificità in soli 40 minuti, è semplice da utilizzare e non richiede strumentazioni particolarmente costose. Alla luce di queste considerazioni, AS LAMP è proposta come un valido e potente strumento nella pratica clinica per rilevare la mutazione cKIT D816V in pazienti affetti da CBF AML.



## ***2.ABSTRACT***

## **ABSTRACT**

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Acute myeloid leukemias with t(8;21) or inv(16)/t(16;16) are commonly referred to as Core-Binding Factor AML (CBF AML) and represent 5-8% of all AMLs . These genomic rearrangements are characterized by disruption of the CBF $\alpha$  gene at 21q22 and the CBF $\beta$  gene at 16q22, respectively. Both genes encode a subunit of Core-Binding factor (CBF) complex, an important transcriptional regulator of hematopoiesis involved in myeloid differentiation. In the current WHO classification, CBF AML are considered a favorable AML risk group based on high remission rate and survival probabilities. However, the presence of the cKIT D816V mutation has been reported to harbor an unfavorable prognostic implication in CBF AML.

The proto-oncogene cKIT encodes a receptor for Stem Cell Factor (SCF), a cytokine which plays an important role in survival, proliferation and differentiation of hematopoietic cells. cKIT belongs to the type-III receptor of the tyrosine kinase subfamily that is characterized by the presence of five extracellular immunoglobulin-like domains, a single transmembrane helix, a cytoplasmic juxtamembrane domain, and a kinase domain. The cKIT D816V mutation is found in 20–25% of patients with t(8;21) and in about 30% with inv(16)/t(16;16). cKIT D816V causes the independent-ligand activation of the tyrosine kinase receptor cKIT, and the activation of downstream pathways supporting cell proliferation and survival. According to the current National Comprehensive Cancer Network (NCCN) guidelines, CBF AML patients exhibiting D816V in cKIT gene, are classified in the intermediate risk category, since they present an high incidence of relapse and a low overall



## **ABSTRACT**

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survival. Moreover the replacement of the aspartic acid residue with valine at codon 816 causes resistance to Imatinib, the tyrosine kinase inhibitor (TKI) usually used in the treatment of CBF AML to suppress the overexpressed cKIT. In this case, patients are treated with TKIs of second generation such as Dasatinib or Nilotinib.

So, the single point mutation D816V in cKIT gene represents a critical prognostic factor for CBF AML patients and an important indicator to define the appropriate treatment.

Several PCR-based methods are developed for the detection of D816V mutation such as PCR followed by direct DNA sequencing, enzymatic digestion with HinfI enzyme (RFLP), or dHPLC (Denaturing high performance liquid chromatography). However, most of the PCR-based methods result expensive, time-consuming and labor-intensive. At the moment Sanger sequencing is still the gold-standard for D816V detection, even if it presents a low sensitivity (10–15%). In the last years is emerging the NGS technology that allows to detect single point mutation with higher sensitivity, however it results technically complex and expensive for being applied on a routine diagnosis in hospital laboratories.

The aim of this work is to improve the detection of cKIT D816V mutation in CBF AML patients by the development of rapid, user-friendly and cost-effective assay based on Allele-Specific Loop mediated isothermal AMPLification (AS LAMP) technology.

## **ABSTRACT**

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The AS LAMP assay is based on LAMP technology that amplifies target DNA at constant temperature thanks to the employment of the DNA polymerase with strand-displacement activity. AS LAMP assay is able to detect the single point mutation thanks to the cooperation of two elements: a backward loop primer (LB) specific for the mutated cKIT sequence and a blocker that binds only the wild type gene. While the blocker suppresses the aspecific amplification of wild type alleles, the LB primer efficiently recognizes the mutated codon and allows its amplification. The AS LAMP assay includes also the amplification of the housekeeping ABL gene as internal control that is informative to exclude false-negative results due to the failure of DNA extraction procedure. The target and the IC are detected in real-time in 500 nm and 530 nm channels respectively, thanks the use of two labeled probes specifically binding cKIT or ABL genes; so the amplification and detection are completed in a closed tube system that is crucial for preventing amplicon contaminations. The AS LAMP assay was optimized on synthetic plasmid containing the cKIT sequence and was successfully validated on negative and positive clinical samples, confirming in all cases the results obtained with PCR-based methods.

In conclusion the AS LAMP assay is able to detect the D816V mutation with high sensitivity and specificity in only 40 minutes and it is easy-to-use. For these reasons the AS LAMP assay represents a powerful tool for molecular diagnostics applications in clinical routine.

## ***3.INTRODUCTION***

### 3.1 HEMATOPOIESIS

Hematopoiesis is a complex orchestrated process that results in maturation of immature haematopoietic progenitor cells in the bone marrow, into differentiated cellular components of peripheral blood, including leukocytes, red blood cells and platelets (Speck et al., 2002). All of these cells derive from a single progenitor cell, termed the Hematopoietic Stem Cell (HSC), which undergoes a process of highly regulated division and differentiation (Reya T et al., 2001).

During embryonic development, hematopoiesis takes place in the blood islands of the yolk sac followed by a phase in the liver and spleen. This beginning stage of hematopoiesis is termed “primitive” and functions to produce red blood cells for oxygenation of the rapidly growing embryo. At the time of birth and throughout adulthood, the bone marrow is the primary site for hematopoiesis and is the location of most of the HSC population. A small percentage of HSCs are mobilized and found in circulating blood. HSCs differentiate into progenitor cells for both the lymphoid and myeloid lineages (Figure 1). Lymphoid progenitors develop lymphocytes, which are T cells, B cells, and natural killer (NK) cells. Myeloid progenitors produce dendritic cells, monocytes, macrophages, neutrophils, eosinophils, mast cells, basophils, platelets and erythrocytes.

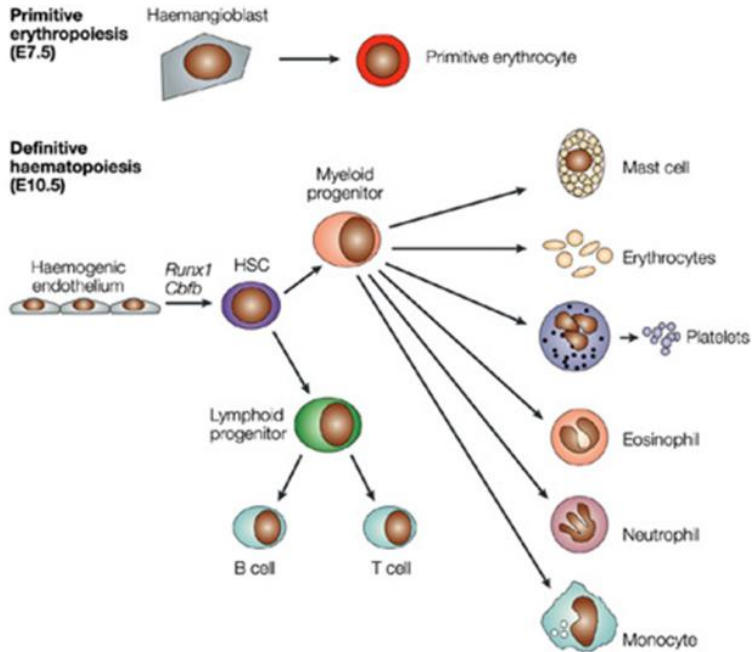


Figura 1: Schematic representation of the hematopoietic system

### 3.1.1 Role of core binding factor in hematopoiesis

The Core-Binding Factor (CBF) complex is a family of heterodimeric transcriptional regulator that contains one  $\beta$  subunit and one of three possible  $\alpha$  subunits (Solh et al., 2014). The  $\alpha$  subunit is the DNA binding element and is encoded by one of three mammalian genes: RUNX1 (also known as AML1 and PEBP2A2), RUNX2 (also referred to as AML3 and PEBP2A1) and RUNX3 (also referred to as AML2 and

## **INTRODUCTION**

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PEBP2A3). The CBF complex containing CBF $\beta$  and RUNX1 subunits is an important transcriptional regulator of hematopoiesis, involved in myeloid differentiation. RUNX1 recognizes the core DNA sequence TGT/cGGT which is present as a regulatory element in several hematopoietic cell-specific genes including those encoding interleukin-3 (IL-3), granulocyte-macrophage colony-stimulating factor (GM-CSF), colony-stimulating factor 1(CSF1/M-CSF) receptor, myeloperoxidase, neutrophil elastase, granzyme B, and T-cell antigen receptors (TCRs). The CBF $\beta$  subunit, stabilizes binding of the RUNX1 subunit to DNA, and also acts to protect RUNX1 from ubiquitin-proteasome-mediated degradation. The CBF complex can act as transcription activator or repressor, and this dual function is mediated through interaction with lineage specific transcription factor and with general coregulators (Ichikawa et al., 2013).

### 3.2 ACUTE MYELOID LEUKEMIA

Acute myeloid leukemia (AML) is an hematopoietic disorder resulting from genetic alterations in normal hematopoietic stem cells (Hartmut et al.2015). These alterations disrupt normal differentiation of myeloid precursor cells and cause excessive proliferation of abnormal immature leukemic cells, also known as blasts. As the disease progresses, blast cells accumulate in the bone marrow, blood, and organs and interfere with the production of normal blood cells. AML is a genetically heterogeneous disorder (Döhner et al., 2008). In about 55% of cases were detected cytogenetic abnormalities such as balanced translocations, inversions, deletions, monosomies and trisomies. The rest of the patients, that constitute approximately the 45% of the cases, do not appear to harbour any chromosomal abnormalities and therefore are classified as “normal karyotype” (NK AML). The most common mutated genes in NK AML are the nucleophosmin (NPM1) in 45% to 62% of the patients and the receptor kinase FLT3 in almost less than half of the patients. Based on cytogenetic analyses, AML can be divided into three prognostically relevant categories: favorable, intermediate or unfavorable, (Table 1). The first group includes the chromosomal translocations or inversions, which are associated with a favorable prognosis: t(15;17) PML-RAR $\alpha$ , t(8;21) AML1-ETO, and t(16;16)/inv(16) CBF $\beta$ -MYH11. The second one comprises complex aberrant karyotypes that confer a poor clinical outcome, such as deletion of chromosomes 5/5q, 7/7q, 17/17p, trisomy 8, 11, 13, 21, and also translocation t(6;9) DEX-NUP214 and inv(3) RPN1-EVI1.

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Finally, the intermediate prognosis group includes AML with karyotypic abnormalities such as t(9;11)(p22;q23), and others with normal karyotype.

**Table 1.** The prognostic classification of acute myeloid leukemia based on their cytogenetic and molecular abnormalities

Prognostic risks	Cytogenetic indicators	Molecular abnormalities
Favorable prognosis	inv(16) or t(16;16) t(8;21) t(15;17)	Normal cytogenetics: with isolated NPM1 or CEBPA mutation in the absence of FLT3-ITD mutation
Intermediate prognosis	Normal cytogenetic Exon 8 trisomy t(9;11) Others non identified	t(8;21), inv(16), t(16;16): with c-KIT mutation
Unfavorable prognosis	Complex karyotype (≥ 3 clonal chromosomal abnormalities) -5, 5q-, -7, 7q- 11q23 – without t(9;11) inv(3), t(3;3) t(6;9) t(9;22)	Normal cytogenetic: with FLT3-ITD mutation

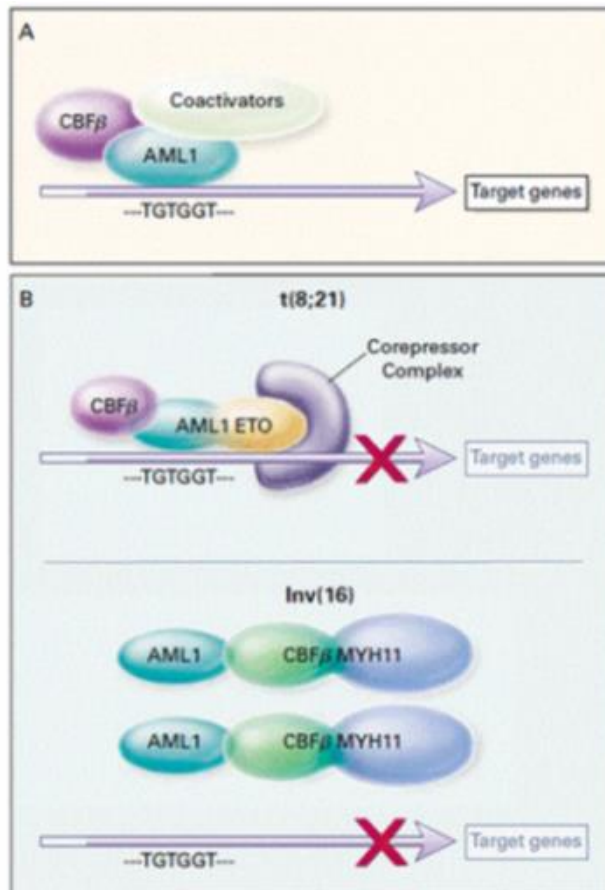


### 3.3 CORE-BINDING FACTOR ACUTE MYELOID LEUKEMIA

The Core-Binding Factor acute myeloid leukemias (CBF AML) are a subgroup of AML that includes patients with the t(8;21) and inv(16)/t(16;16) chromosomal rearrangements. These chromosomal rearrangements result in the formation of fusion proteins AML1-ETO and CBF $\beta$ -MYH11 respectively, that cause the CBF complex destruction (Figure 2) (Riera et al. 2013; Reilly et al. 2004).

Although these cytogenetic abnormalities belong to same AML subgroup, there are significant differences between patients with t(8;21) or inv(16)/t(16;16), regarding to demographic and clinical characteristics. Whereas t(8;21) occurs predominantly in younger patients and is found in up to 5% of AML cases, inv(16) or t(16;16) occurs in all age groups and accounts for 5% to 8% of all AML cases. The early symptoms of CBF AML are often non specific and may be similar to those of influenza or other common illnesses. Some generalized symptoms include fever, fatigue, weight loss or loss of appetite, shortness of breath, anemia, bruising or bleeding, petechiae, bone and joint pain, and persistent or frequent infections (Sangle et al. 2011).

## INTRODUCTION



**Figura 2: Panel A.** In normal cells, heterodimeric AML1-CBF $\beta$  transcription-factor complex binds to the DNA sequence TGTGGT in the transcriptional regulatory region of CBF $\alpha$  - regulated target genes and activates transcription through the recruitment of coactivators. **Panel B.** In AML cells with the t(8;21) translocation, the N-terminal part of AML1 fuses with the C-terminal portion of ETO. The resultant chimeric protein continues to interact with CBF $\beta$  and to bind to the core enhancer sequence; however, ETO recruits a nuclear corepressor complex and results in the dominant repression of AML1-regulated target genes. Similarly, the CBF $\beta$ -MYH11 chimeric protein encoded by the inv(16) mutation continues to interact with AML1; however, instead of allowing AML1 to interact with DNA, this chimeric protein recruits AML1 into functionally inactive complexes in the cytoplasm.

## INTRODUCTION

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CBF AML development is considered to be a multistep process that requires the collaboration of at least two classes of mutations. (Takahashi et al. 2011). Almost a decade ago, Gilliland and Griffin presented a paradigm model for this process, designated the “two-hit model”. This model comprises class I mutations that activate signal transduction pathways and confer a proliferation advantage on hematopoietic cells, and class II mutations that affect transcription factors and primarily serve to impair hematopoietic differentiation. Mutations leading to activation of the receptor tyrosine kinase (RTK) such as FLT3 and cKIT, or Ras signaling pathway, belong to class I mutations. Chromosomal aberrations such as t(8;21) and inv(16)/t(16;16) fall into class II mutations (D. Gary Gilliland and J. D. Griffin, 2002; Renneville et al., 2008).

### 3.3.1 Class II mutations

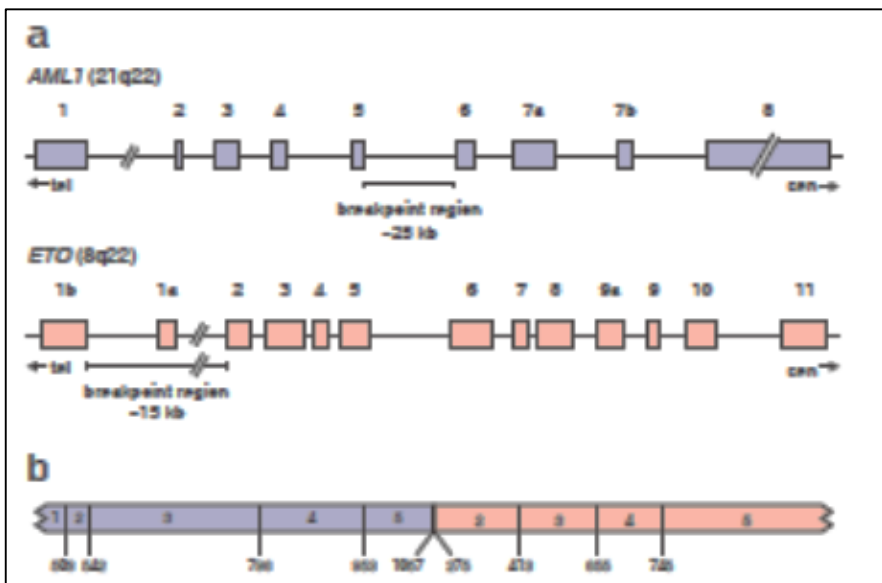
#### RUNX1-ETO t(8;21)

The chromosomal traslocation t(8;21)(q22;q22) is usually subclassified as French-American-British (FAB) M2, and was found in approximately 4–12% of AML. It fuses the N-terminal part of RUNX1 with the C-terminal portion of ETO generating the novel chimeric gene RUNX1-ETO, also know as AML1-ETO (Figure 3) (Kumar, 2011; Wang et al., 2011).

The final chimeric protein lacks the carboxyl terminal transactivation domain of RUNX1, that results in the dominant repression of RUNX1-regulated target genes. Moreover, ETO acts by associating with N-CoR

## INTRODUCTION

(nuclear receptor corepressor) through recruiting histone deacetylase (HDACs) and sin3A. The active ETO protein represses transcription by deacetylation of the AML1 target genes leading to disruption of normal hematopoiesis and inactivation of tumor suppressor genes needed for neoplastic transformation (Nick et al., 2012; Rulina et al.2010).



**Figure 3:** (a) Schematic diagram of the exon/intron structure of the AML1 and ETO genes, which are involved in t(8;21)(q22;q22). (b) Schematic diagram of the AML1-ETO transcript with AML1 exon 5 fused to ETO exon 2.

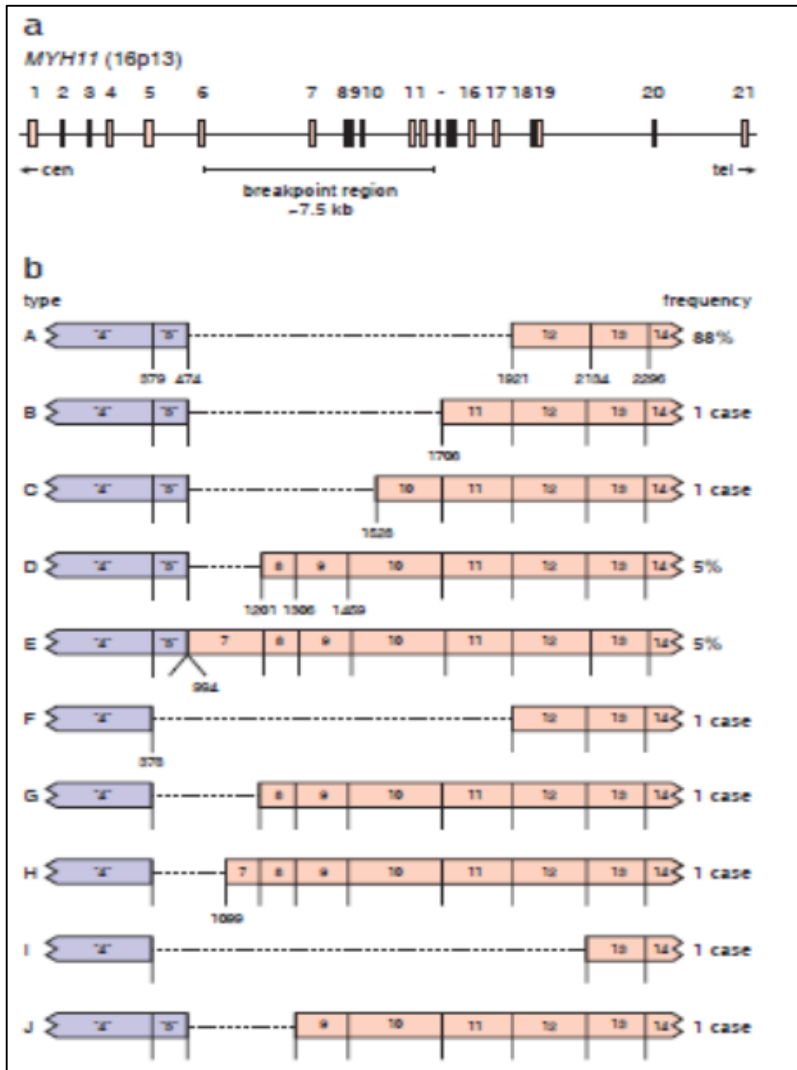
## **INTRODUCTION**

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### **CBFB MYH11 inv(16)/t(16;16)**

CBF leukemias with the inv(16) or t(16;16) are classified as FAB M4 and represent the 5% of AML. The aberrations inv(16) and t(16;16) lead the fusion of the CBFB gene on chromosome 16q22, with smooth muscle myosin heavy-chain gene (MYH11) on chromosome 16p13, leading to formation of two novel genes: CBFB-MYH11 and MYH11-CBFB. The breakpoint in the MYH11 gene is variable and gives origin to at least 10 different fusion variants (Figure 4). In contrast, the breakpoints in the CBFB gene are in intron 4 or 5 (Delabesse, 1999).

Only CBFB-MYH11 has leukemogenic potential resulting from the fusion of the majority of CBFB gene with the tail domain of MYH11. CBFB-MYH11 acts in a similar manner to RUNX1-ETO by associating with co-repressor complexes leading to recruitment of histone deacetylase and subsequent silencing of gene function. The breakpoints in MYH11 gene incorporate a myosin long tail functional domain that mediates homodimerization of the CBFB-MYH11 protein into high molecular weight structures. These filamentous structures also interacts with AML1, preventing RUNX1 localizing in the nucleus. These results suggest that CBFB-MYH11 disrupts hematopoiesis by sequestering the RUNX1 protein pool (Stephen M. et al. 2002).



**Figura 4:** (a) Schematic diagram of the exon/intron structure of the *MYH11* gene, which is involved in *inv(16)(p13;q22)*. (b) Schematic diagram of the 10 different types of *CBFB-MYH11* fusion transcripts. The different types of transcripts are mainly caused by breakpoints in different introns of the *MYH11* gene. Fusion transcripts type A, D and E together represent approximately 98% of all patients, while the other types concern single cases.

### 3.3.2 Class I mutations

Activating mutations in the hematopoietic tyrosine kinases cKIT and FLT3, and in K-RAS and N-RAS are found in more than 30% of CBF AML patients. These mutations, however, are not sufficient to cause AML, but confer proliferative advantage to hematopoietic progenitors and cooperate with mutated transcription factors CBF complex to cause an acute leukemia phenotype characterized by proliferation and impaired differentiation (Renneville et al., 2008). cKIT represents the most common mutated gene in CBF AML with an incidence that varies between 6.6% up to 46,1%. In CBF AML, cKIT mutations occur frequently within exon 17, which encodes the activation loop in the kinase domain, and in exon 8, encoding the extracellular portion of the cKIT receptor. The most frequent mutation in exon 17 is the point mutation A>T at codon 816 which results in the replacement of the aspartic acid by valine. cKIT D816V mutation is detected in 20–25% of patients with t(8;21) and in about 30% of patients with inv(16)/t(16;16). Moreover most studies have demonstrated that D816V mutation is associated with a poor prognosis of CBF AML patients resulting in higher incidence of relapse and a lower overall survival (Mu AM. et al.,2008). The most common FLT3 mutation is caused by the internal tandem duplication (ITD) within the juxtamembrane domain. It is infrequent in CBF leukemia, infact it has been detected in 2-9% of t(8;21) and 0 to 7% of inv(16)/t(16;16) patients. FLT3 tyrosine kinase domain (TKD) point mutations (most commonly D835) are more frequent with

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inv(16)/t(16;16) (6-24%) than t(8;21) (2–7%). Also FLT3 mutation have a negative impact among AML patients. Other common mutations in CBF leukemias, predominantly in those with inv(16), involve the oncogenes N-RAS (26–38%) and K-RAS (7–17%). However may not have an effect on prognosis. Finally a novel mutation V617F of the Janus kinase JAK2 has been found in a small percentage of CBF AML patients (3.6%). This incidence is significantly higher when compared to other AML patients (0.6%) (Paschka et al., 2013; Beghini et al., 2000).

### 3.3.3 Prognosis and treatment

CBF AML are classified by the World Health Organization (2008) in the AML group with a favorable prognosis. However CBF AML patients with the additional mutation D816V in cKIT gene exhibit an higher incidence of relapse and a lower overall survival. For this reason, according to the National Comprehensive Cancer Network (NCCN, 2014) guidelines, these patients are classified in the intermediate risk category. The mechanism underlying cKIT gene mutations adversely affects the prognosis, involves the independent-ligand activation of cKIT receptor which activates downstream pathways, supporting cell proliferation and survival. Currently, AML therapies are based solely on the patient risk of relapse. Thus, patients with an intermediate risk of relapse receive hematopoietic stem cell transplantation therapy, while low-risk patients only receive chemotherapy treatment (Goyama S. et al., 2011). In this case, with a cytarabine and



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anthracyclines based therapy, complete remission is achieved in approximately 90% of the CBF AML patients (Michael et al.,2015, Bhatt et al.,2013).

Moreover, several studies have proved that CBF AML patients with the additional mutation cKIT D816V, express high level of cKIT gene; in order to suppress the cKIT activity it is recommended the treatment with tyrosine kinase inhibitors (TKIs). Imatinib, also known as STI-571 or Gleevec, is a potent inhibitor of wild type cKIT that binds to the ATP-binding pocket of tyrosine kinases and inhibits the inactive form of cKIT. In wild type protein, the aspartic acid at position 816, within the activation loop, plays a crucial role for maintaining the inactive conformation of cKIT, by forming a hydrogen bond with N819. The replacement of the aspartic acid residue with valine, breaks this bond and thereby destabilizes the inactive conformation, thus preventing the imatinib binding. As result, patients with D816V mutation are resistant to imatinib, and they must be treated with second generation tyrosine kynase inhibitors, Dasatinib or Nilotinib, that are able to bind the active conformation of cKIT (Mol et al.2004).

### **3.4 STEM CELL FACTOR RECEPTOR cKIT**

#### **3.4.1 Gene structure and regulation of cKIT mRNA expression**

The viral oncogene *v-Kit* was identified in 1986 as the transforming gene of Hardy-Zuckerman 4 feline sarcoma (Besmer et. al., 1986). Shortly thereafter, the human homolog cKIT, was cloned and sequenced (Yarden et al, 1987). The gene for cKIT was found to be located on chromosome segment 4q11 in humans, and is comprised of 21 exons, spanning more than 34 kb of DNA. The first exon encodes the translational initiation codon and the signal peptide. The remainder of the extracellular part of cKIT is encoded by exons 2–9. The transmembrane region is encoded by exon 10, while the remaining exons encode the intracellular part of the receptor. The promoter region of the c-KIT gene includes no typical TATA-boxes specific for many eukaryotic promoters, but consensus binding sites for AP-2, basic helix-loop-helix proteins, Sp1, Ets, and Myb. This sequence was found to contain functional binding sites for the transcription factors Myb and Ets-2, which serve as regulators of cKIT expression in hematopoietic cells. Apart from regulation of expression levels by transcription factors, cKIT has also been reported to be regulated by miRNA. In particular miR-221 and miR-222 have been described as regulators of cKIT expression in hematopoietic cells, and have also been reported to be potential regulators of cKIT

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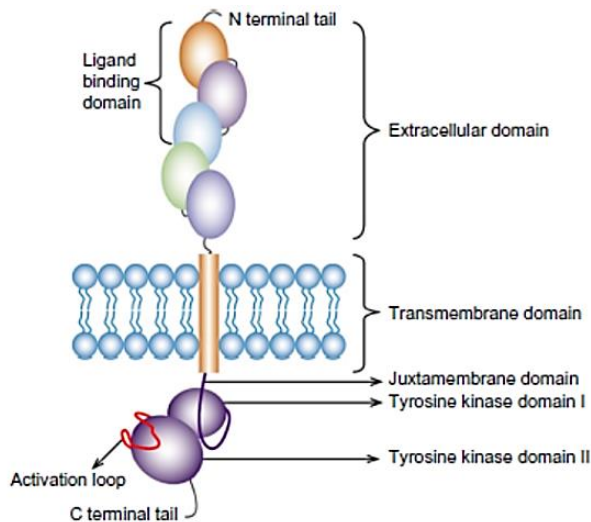
expression in gastrointestinal stromal tumors. In humans there are four protein isoforms that are produced due to alternative splicing. Two of them are discriminated by the presence or absence, of 12 base pair insertion encoding 4 amino acids between nucleotides 512 and 513 of the cDNA sequence. The four amino acids (GNNK) are added to the extracellular domain, relatively close to the transmembrane domain. The GNNK<sup>+</sup> and GNNK<sup>-</sup> isoforms are coexpressed in some cell types, but the GNNK<sup>-</sup> sequence is typically more abundant. Although the mechanism is unknown, the presence of the GNNK insert abolishes the low level of constitutive cKIT signal transduction that normally occurs in the absence of SCF. Two other forms are characterized by the presence or absence of a single serine residue at position 715, within the inserted sequence bisecting the kinase domain; both the Ser<sup>+</sup> and Ser<sup>-</sup> isoforms are also coexpressed (Lennartsson et al.,2012).

### 3.4.2 Protein structure

The cKIT protein is a member of type III receptor tyrosine kinase (RTK) family. This group also includes colony stimulating factor 1 receptor (CSF1R, also called FMS), FMS-like tyrosine kinase 3 (FLT3), and platelet-derived growth factor receptor  $\alpha$  (PDGFR $\alpha$ ) and  $\beta$ . This class of kinases (also called the PDGF-R family) plays a key role in the regulation of cell proliferation and differentiation (Verstraete et al., 2012). The receptor cKIT is a transmembrane protein with an extracellular domain (ED) comprised of five

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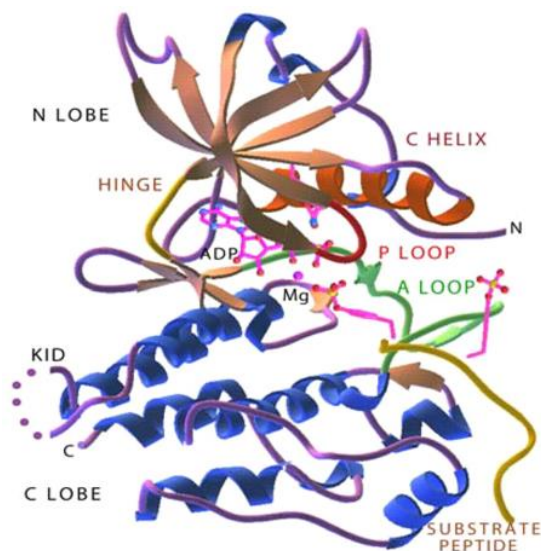
immunoglobuline-like (Ig-like) domains, followed by a single spanning transmembrane region (TMD). The intracellular part of cKIT starts with the juxtamembrane domain (JMD), a region of great importance for regulation of cKIT kinase activity. The kinase domain is comprised of two subdomains, tyrosine kinase domain 1 (TKD1) and 2 (TKD2), which is interrupted by a kinase insert of 77 hydrophilic amino acids. Finally, the COOH-terminal tail ends the protein. Most of the phosphorylation sites that occur upon ligand stimulated activation of c-Kit reside either in the juxtamembrane region, the kinase insert, or in the COOH-terminal tail (Figure 5).



**Figura 5:** cKIT belongs to the class III receptor tyrosine kinases (RTK) family. It is characterized by an extracellular region that is divided into five immunoglobulin-like modules, a transmembrane domain (TMD), a regulatory juxtamembrane (JM) region, a conserved tyrosine kinase domain (TKD) containing a kinase-insert region between the N- and C-kinase lobes, and a carboxy-terminal tail.

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cKIT consists of 976 amino acids with the characteristic bi-lobed architecture of all protein kinases (Figure 7). Residues 582-671 comprise the small N-terminal lobe (N-lobe), and residues 678-953 include the large C-terminal lobe (C-lobe). The cleft created between the two lobes contains the catalytic site. The core protein size is of 110 kDa (Mol et al., 2003).



**Figure 7:** Structure of active cKIT protein. The C $\alpha$  ribbon illustrates the two-domain kinase fold and key structural elements, including the C-helix, phosphate-binding P-loop, adenine-recognition hinge loop, and kinase activation A-loop. The positions of the ADP, metal ion, and substrate peptide are also shown.

Heterogeneous N-linked glycosylation results in a mature protein of between 145 and 160 kDa. There are up to nine N-glycosylation sites, most of which are concentrated in extracellular domain, closest to the plasma membrane. Additional known post-translational modifications of cKIT

include phosphorylation on both tyrosine and serine residues, that in some cases have been shown to function in fine tuning of receptor responses. Finally, ligand-induced ubiquitination of cKIT is known to regulate both internalization and degradation of cKIT.

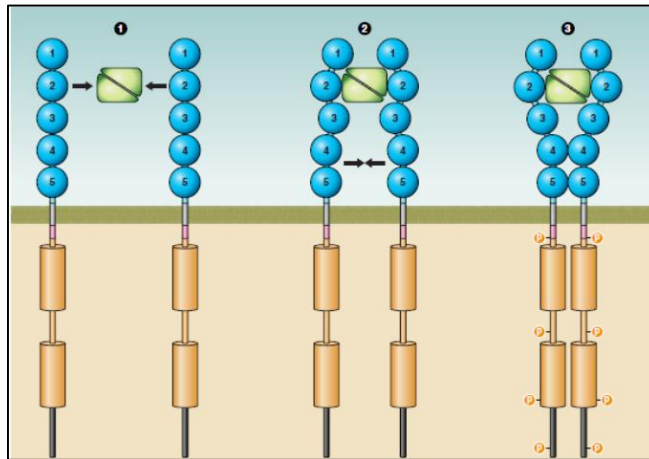
### 3.4.3 SCF-induced cKIT activation

Stem cell factor (SCF) is the specific ligand for cKIT receptor. It is a growth factor expressed by fibroblasts and endothelial cells throughout the body, promoting proliferation, migration, survival, and differentiation of hematopoietic progenitors, melanocytes, and germ cells (Virginia et al., 1997). It maps to chromosome 12 in humans and is encoded by nine exons. SCF exists as two alternative splice forms, one soluble isoform and one membrane-bound isoform that differ only in exon 6. Both isoforms bind cKIT and activate its downstream signal pathways (Ashman et al., 1999).

cKIT activation process begins with the binding of the SCF homodimer, at two cKIT monomers in close proximity of each other. Only the first three Ig-like domains are required for SCF binding. Further, SCF binding induces a conformational change that enables homotypic interactions between Ig-like domains 4 and 5 in two adjacent cKIT molecules. As result the transmembrane regions of cKIT moves into close proximity of each other (15 Å). The proximity of two kinase domains within the dimer enables their activation and subsequent transphosphorylation. Initially, this occurs in the juxtamembrane region (Tyr568 and 570). Next, autophosphorylation

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proceeds in an orderly manner, on other tyrosine residue in the kinase insert region (Tyr703,721,730 and 747), kinase domain (Tyr823 and 900) and COOH-terminal tail (Tyr936) (Figure 8).



**Figura 8. Activation of cKIT receptor induced by SCF.** (1) The SCF binding induces the oligomerization of the receptor. (2) The Ig-like domains of the ED rotate to form an efficient receptor-ligand complex. (3) This causes trans-phosphorylation of tyrosine residues in the TKD and JMD.

There are several levels of cKIT downregulation that function in concert: removal from the cell surface and intracellular degradation, inactivation of the kinase domain by serine phosphorylation, and tyrosine dephosphorylation. Upon phosphorylation the receptor can be ubiquitinated by ubiquitin E3 ligases, among which protein Cbl is the most important. This protein binds with activated cKIT using adaptor proteins and is phosphorylated by SFK (Src family kinase). It seems that just the

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SFK-dependent phosphorylation of Cbl and the subsequent ubiquitination of cKIT lead to internalization of the receptor and its degradation in lysosomes. The tyrosine dephosphorylation is regulated mainly by the protein tyrosine phosphatases SHP-1; other phosphatases are also involved but their mechanism is still unclear.

### 3.4.4 Signal transduction downstream of cKIT

SCF binding to cKIT receptor activates downstream signal transduction pathways supporting cell proliferation and survival (Lennartsson et al., 2005). These pathways do not operate in isolation but they are integrated into a very complex signaling circuit. Below are described the currently known signal transduction pathways (Liang et al., 2013).

- ✚ Ras/Erk signal transduction pathway. Ras/Erk pathway plays a very important role in cell differentiation and survival. Sos and adapter protein Grb2 form a complex through SH2, and then raise to membrane to act on Ras. Activated Ras can activate the Ser/Thr kinase Raf-1 which can then activate the dual specificity kinases Mek1 and Mek2. Phosphorylation of Mek1 and Mek2 can re-activate Erk1 and Erk2. Subsequently, dimeric Erks moves to the nucleus and regulate gene activities.
- ✚ PI3K signal transduction pathway. After cKIT receptor activation, PI3K dimerizes through SH2 and then recruits to membrane. SH2 which contains an adapter protein of 85 kDa connects 110 kDa esterase



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subunits to cKIT receptor. As PI3K produces a series of biological signals on the membrane, signal transduction molecules downstream of PI3K are activated and cell survival and angiogenesis are regulated.

- ✚ PLC- $\gamma$  signaling transduction pathway. Phospholipase (PLC) PLC- $\gamma$  contains two SH2 domains, a SH3 domain, a PH domain and a catalytic domain. PLC- $\gamma$  can catalyze phosphoinositide PIP2 to generate second messenger diacylglycerol (DAG) and soluble inositol 1,4,5-trisphosphate (IP3). DAG can activate PKC; IP3 then binds to the endoplasmic reticulum to stimulate the release of  $\text{Ca}^+$ .
- ✚ Src kinase signal transduction pathway. Src kinase is related to a series of cell functions including cell survival, angiogenesis, proliferation, motility, migration and invasion. Src kinase which contains SH2, SH3, the N-terminal kinase domain and a fourteen alkylation site plays the role of anchoring to membrane. And Src kinase activation has to do with GNNK sequence. More importantly, GNNK- form can be faster and more intense to activate this kinase.
- ✚ JAK/STAT signaling pathway. JAKs are cytoplasmic tyrosine kinases. The cKIT receptor can be fast and transient to activate JAK2. Then, activated JAKs can make the transcription factor STATs phosphorylation and dimerization. Finally, dimerized STATs transfer to the nucleus and regulate cell proliferation, differentiation and apoptosis.

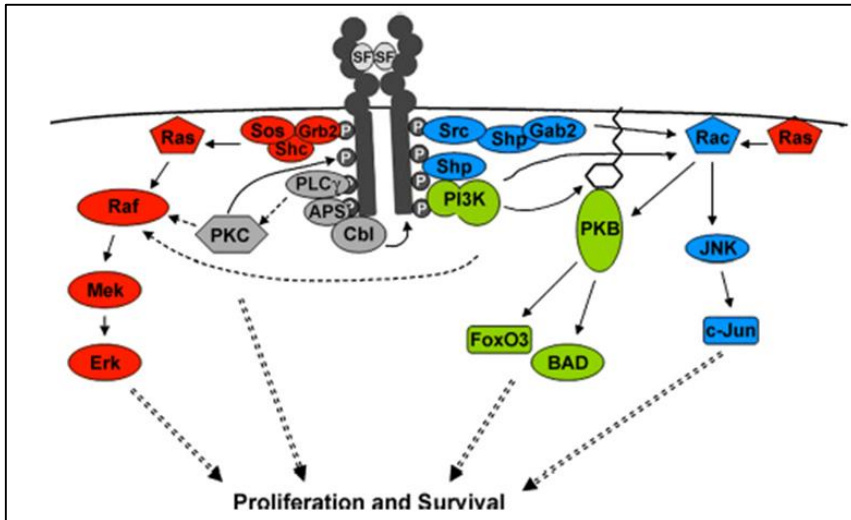


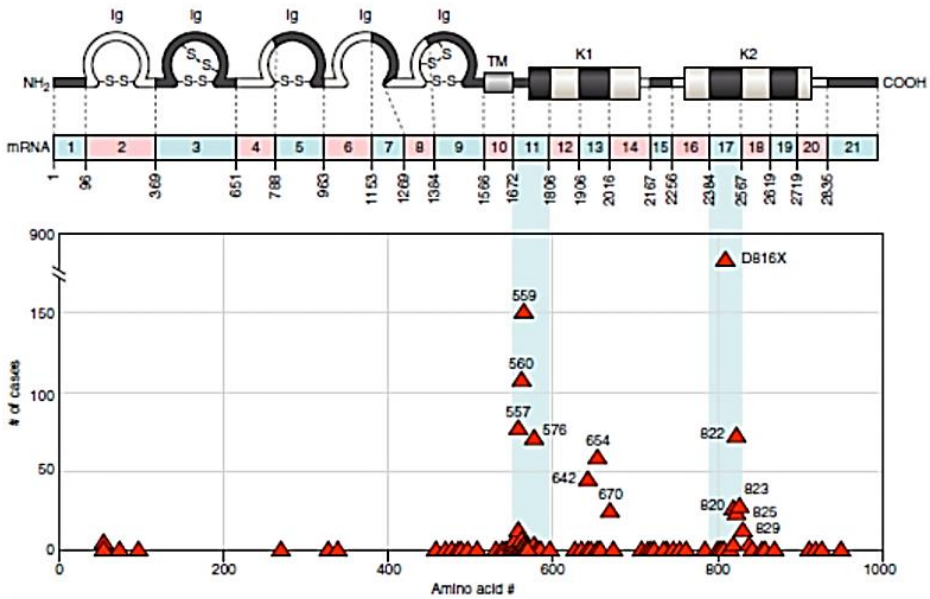
Figura 9: Activation and downstream signaling of cKIT.

### 3.4.5 Role of cKIT in hematopoiesis

cKIT is expressed by early hematopoietic cells (including stem cells and progenitor cells), and it is lost during cells differentiation. Early hematopoietic cells are dependent on cKIT mediated signals for their proliferation and survival. This occurs in synergy with other cytokines and factors. In two cases, cKIT expression is not lost during maturation, namely, the mast cell and the dendritic cell. They express high levels of cKIT even as fully differentiated cells, and they are dependent on cKIT for their proliferation, survival, and function (Oliveira et al.,2003).

### 3.4.6 cKIT gene mutations

Gain-of-function mutations in cKIT causing constitutive, ligand-independent activation of the receptor, have been associated with mast cell tumor, mastocytosis, gastrointestinal stromal tumors (GIST), germ cell tumors, and acute myeloid leukemia (AML). Moreover, in rare cases cKIT mutations are found involved in small cell lung carcinoma, malignant melanomas and colorectal cancer (Pittoni et al.,2011). More than 500 different mutations of cKIT have been described in human tumors (Figure 10).



**Figure 10:** Schematic representation of the cKIT tyrosine kinase receptor and mutation frequency

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Mutations often occur in membrane proximal immunoglobulin-like domain (exon 8 and exon 9), near membrane domain (exon 11) and the tyrosine kinase domain (exon 17) and include missing, point mutation, duplication and insertion.

cKIT mutations occur at high frequency in CBF AML subtypes. The most common mutations in CBF AML are single point substitutions D816V and N822K in exon 17, encoding the activation loop of the kinase domain, and insertion or deletion within exon 8 which encodes the extracellular portion of the receptor. The mutation of D816 causes a structural change in the activation loop, but also weakened the binding of the juxtamembrane region to the kinase domain. Consequently, the juxtamembrane region, which has an inhibitory effect on the kinase activity, is no longer able to efficiently suppress the enzymatic activity of cKIT. In addition, the extended juxtamembrane region potentially could make contact with another cKIT receptor, thereby promoting dimerization in the absence of ligand. Exon 8 mutations involve small deletions or insertions affecting amino acids 417 to 419. One well-characterized exon 8 mutant (T417I) replaces a threonine residue at amino acid 417 with isoleucine and includes a small deletion of amino acids 418 to 419.

The majority of GIST mutations have been found in the juxtamembrane region (exon 11), between the amino acids 550–560. In wild type cKIT the juxtamembrane region is associated with the kinase domain and suppresses its kinase activity. Mutations in this region lead a release of this suppression

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and activation of the tyrosine kinase. Less common mutations also occur in exon 9, encoding the extracellular region, and exon 17 that encodes for the regions around the A loop of the kinase domain (Longley et al., 2001).

### **3.5 MOLECULAR TECHNIQUES FOR DETECTION OF cKIT D816V**

cKIT D816V mutation represents a critical prognostic indicator for CBF AML patients as well as important indicator to define the appropriate treatment of patients (Margaret et al., 2010). Several molecular methods have been developed for the detection of point mutation D816V in cKIT gene (De Matteis et al., 2015; Kähler et al., 2007; Tan et al., 2006). The main techniques used are based on Polymerase Chain Reaction (PCR), that utilizes the Taq polymerase enzyme, and synthetic oligonucleotides to amplify DNA fragments through cycles of denaturation, annealing and elongation, performed in a thermocycler (Akin et al., 2006). Most of the assay described in literature uses genomic DNA, isolated from bone marrow (BM) mononuclear cells or peripheral blood (PB), as target. However, few studies reported in the literature use RNA; in this case a previous step of retrotranscription is requested to synthesize cDNA from RNA. Below, few variants of PCR-based methods, commonly used to detect the point substitution D816V in cKIT gene, have been described.

#### **3.5.1 Amplification Refractory Mutation System**

Amplification Refractory Mutation System (ARMS) PCR is based on the principle that a 3' mismatch between a PCR primer and target DNA prevents PCR amplification (Matteis et al. 2015). For ARMS, target DNA is

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amplified into separate and simultaneous reactions; each reaction contains an allele-specific primer, either wild type or mutant, and a second primer common to both reactions. PCR is performed under stringent conditions, to prevent PCR amplification if a mismatch is present. Genotype is based on amplification in either one of the reactions alone (homozygous normal or mutant) or both reactions (heterozygous).

### **3.5.2 Nested PCR**

In nested PCR, two pairs of PCR primers with one set internal to the other (nested) are used to sequentially amplify a single locus. The first pair is used to amplify the locus as in any PCR assay; a dilution of the first PCR reaction then is amplified with the nested primers. Thus, nested PCR enhances specificity while also increasing sensitivity. The problem with nested PCR is the high risk of amplicon contamination when the first-round PCR products are used to set up the second round of PCR with the nested primers. For this reason, many clinical laboratories do not use nested PCR procedures (Arock et al., 2015).

### **3.5.3 Real-Time PCR**

Real-time PCR is based on the generation of a fluorescent signal by the PCR process, which is detected during PCR cycling. Different real-time PCR methods use alternative ways to generate fluorescent signal during PCR. These include an intercalating dye such as SYBER Green that binds DNA or

an oligonucleotide used as a primer or probe and labeled with a fluorescent dye (Lawley et al., 2005).

### **3.5.4 Restriction Fragment Length Polymorphism**

Hinfi RFLP assay, commonly reported in the literature, is based on detection of the creation of a new restriction site by A>T change at cDNA nucleotide position 2468. The presence of the Asp816Val mutation, which creates an additional Hinfi site, leads to a new DNA fragment that is detected by Hinfi digestion of cKIT exon 17 PCR products. As a result of Hinfi digestion of the mutated sequence, the 147 bp DNA fragment is detected. Although this assay is reasonably sensitive, it is important to realize that it will miss rare mutations caused by other nucleotide changes not affecting the restriction enzyme recognition pattern of the amplified PCR fragment (Nagata et al., 1995).

### **3.5.5 Denaturing-High Performance Liquid Chromatography**

The PCR is followed by denaturation and rehybridization. In the presence of the mutation, two types of complexes can be formed. The first one is the homoduplexes, where the wild type sense strand is hybridized with its wild type antisense strand, and the same occurs with PCR products harboring the point mutation D816V; the second one is the heteroduplexes, where the wild type sense strand is hybridized with the mutant antisense strand,



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and the mutant sense strand is hybridized with the wild type antisense strand. After the formation of these complexes, the next step is running the samples on an HPLC machine under partially denaturing conditions. The mobility of the heteroduplexes and homoduplexes are different, therefore the number of peaks indicate the presence of mutation. This method does not provide information about the nature and location of the mutation, but only shows its presence within the PCR product. Therefore, the samples showing aberrant mobility need to be analyzed further using other methods, like DNA sequencing in order to determine the type of mutation (Battochio et al.,2010)

### **3.5.6 High Resolution Melting (HRM)**

A standard PCR is performed in the presence of a specialized double-stranded DNA (dsDNA) binding dye. This specialized dye is highly fluorescent when bound to dsDNA and poorly fluorescent in the unbound state. This change allows the user to monitor the DNA amplification during PCR. After completion of the PCR step, the amplified target is gradually denatured by increasing the temperature in small increments, in order to produce a characteristic melting profile. The amplified target denatures gradually, releasing the dye, which results in a drop in fluorescence. When set up correctly, HRM is sensitive enough to allow the detection of a single base change between otherwise identical nucleotide sequences (Lu et al. 2014)

### 3.5.7 Direct Sanger sequencing

Traditional DNA sequencing relies on the Sanger sequencing method, which was developed by Sanger et al in 1977. The targeted DNA fragment is amplified by standard PCR. Then, a sequencing reaction is added using only one primer (forward or reverse). After denaturation of the DNA fragment, the primer anneals at a lower temperature, leading to primer extension and fluorescence labeling of the template. In each round of primer extension, the reaction is terminated by incorporation of fluorescently labeled dideoxynucleotide triphosphates (ddNTPs). Thereby the label on the terminating ddNTP corresponds to the nucleotide of the terminal position. Subsequent to the sequencing reaction, redundant ddNTPs are removed by a cleaning step of the DNA template. The sequence is determined by high-resolution electrophoretic separation. Therefore, products are again denatured and then applied to a capillary-based polymer gel. The sequencing read-out is provided by a four-color detection of the emission spectra, which is generated by laser excitation of fluorescent-labeled fragments. When fragments of distinct length exit the capillary, the sequencing analysis software transforms the position of the respective fluorescence signal into a DNA sequence and also generates error probabilities for each base call. Sanger sequencing is still seen as the gold-standard for D816V detection. However, it exhibits disadvantages. First of all, time and labor for this method are very extensive. In addition, Sanger sequencing shows

limitations in detection sensitivity (only 10%–15%), therefore low amounts of mutated alleles could be overlooked generating false-negative results (Akin et al., 2004).

### **3.5.8 Next Generation Sequencing (NGS)**

Over the past few years, there have been significant advances in DNA sequencing technologies with the emergence and rapid evolution of next-generation sequencing (NGS), also known as massively parallel sequencing (Su et al., 2015). NGS method is a high-throughput technology able to sequence large numbers of different DNA at once. Massive parallel sequencing produces millions of short DNA fragment reads at the same time. The reads are assembled to larger sequence fragments (contigs) and mapped back to the reference genome, allowing discrimination of mutated and wild type DNA. Currently NGS remains expensive; moreover the required bioinformatics know-how and time to perform data analysis, hinder rapid routine diagnostic applications. Methodological differences in the commercially available NGS platforms as template preparation, sequencing, imaging, and data analysis additionally complicate the introduction of generalized diagnostic procedures. Until the accuracy of NGS has improved, Sanger sequencing will remain the method of choice for sequencing diagnostic approaches (Kristensen et al., 2015).

Although PCR-based methods are widely used as a routine procedure for the detection of D816V mutation, they present few limitations. The main

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limitation remains the high cost of reagents and instrument, as well as the time-consuming and the labor-intensive. In addition the most of PCR-based methods need to open the tube after DNA amplification, take out part of amplification product and carry it on the agarose gel electrophoresis, however this open-system can easily contaminate the laboratory environment. Other methods, including NGS, are relatively technically complex and expensive for being applied on a routine diagnosis in hospital laboratories (Ilyas et al., 2015).

## ***4.AIM OF THE THESIS***

## **AIM OF THE THESIS**

CBF AML are haematologic disorders characterized by the chromosomal aberrations AML1-ETO t(8;21) and CBFβ-MYH11 inv(16)/t(16;16). These two chromosomal alterations destroy the Core-Binding Factor (CBF) protein, that is an important transcriptional regulator of hematopoiesis involved in myeloid differentiation. Patients with CBF AML disease achieve the complete remission if treated with chemotherapy, and therefore are stratified into the AML group with a favorable prognosis. However, approximately 25% of these patients exhibits a poorer prognosis with an higher incidence of relapse and an higher mortality rate, due to the presence of the point mutation D816V in the proto-oncogene cKIT (Döhner et al., 2010).

cKIT D816V is a gain-of-function mutation that causes the independent-ligand activation of the tyrosine kinase receptor cKIT and the activation of downstream pathways supporting cell proliferation and survival. According to the National Comprehensive Cancer Network (NCCN) guidelines, CBF AML patients exhibiting also the D816V mutation, are classified in the intermediate risk category and they need hematopoietic stem cell transplantation (Cairolì et al., 2006). In addition, in these patients the use of tyrosine kinase inhibitors (TKIs) is recommended to suppress the constitutive activation of cKIT receptor; several studies have revealed that D816V mutation destabilizes the inactive conformation of cKIT and prevents the bind of the tyrosine kinase inhibitor Imatinib, therefore the patients

## **AIM OF THE THESIS**

must be treated with TKIs of second generation, Dasatinib or Nilotinib, able to bind the active conformation of cKIT (Park et al- 2011; Shah et al. 2006).

The detection of D816V mutation in CBF AML patients is crucial to provide a better prognostic stratification and to identify the most appropriate treatment. Several PCR-based methods are currently employed for routine detection of D816V mutation, such as PCR followed by direct DNA sequencing, enzymatic digestion with HinfI enzyme (RFLP), or dHPLC (Denaturing high performance liquid chromatography). Sanger sequencing is still the gold-standard for D816V detection, even if time-consuming and with low sensitivity (10%–15%). In the last years the NGS technology allows to detect single point mutation with higher sensitivity, however it results technically complex and expensive for being applied on a routine diagnosis in hospital laboratories. This work is aimed to develop a rapid, user-friendly and cost-effective molecular assay for the detection of D816V mutation, and suitable for the routine testing in hospital laboratories. The assay is based on AS LAMP technology, that amplifies target DNA at constant temperature thanks to the employment of DNA polymerase with strand displacement activity





# ***5.MATERIALS AND METHODS***

### 5.1 ALLELE-SPECIFIC LAMP

#### 5.1.1 Principle of AS LAMP

Loop mediated isothermal AMPlification (LAMP) is an innovative technology, developed by Notomi in 2000, that allows to amplify DNA with high specificity, efficiency and rapidity under isothermal conditions (Notomi T et al., 2000; Parida M et al., 2008, Shijun F et al., 2011).

LAMP reaction is based on the use of 4 primers specifically designed to recognize 6 distinct regions on the target gene: a pair of outer primers (F3 and B3) and a pair of inner primers (FIP and BIP).

- ✚ FIP: Forward Inner Primer (FIP) consists of the F2 region (at the 3' end) that is complementary to the F2c region, and the same sequence as the F1c region at the 5' end.
- ✚ F3 Primer: Forward Outer Primer consists of the F3 region that is complementary to the F3c region.
- ✚ BIP: Backward Inner Primer (BIP) consists of the B2 region (at the 3' end) that is complementary to the B2c region, and the same sequence as the B1c region at the 5' end.
- ✚ B3 Primer: Backward Outer Primer consists of the B3 region that is complementary to the B3c region.

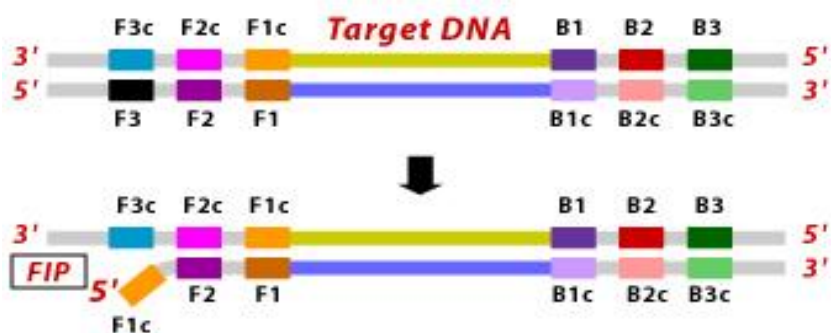
The reaction proceeds at a constant temperature thanks the use of a DNA polymerase with strand displacement activity. Amplification of genes can be

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achieved in a single step, by incubating the mixture of samples, primers, DNA polymerase and target DNA at constant temperature. It provides high amplification efficiency, with DNA being amplified  $10^9$ - $10^{10}$  times in 15-60 minutes.

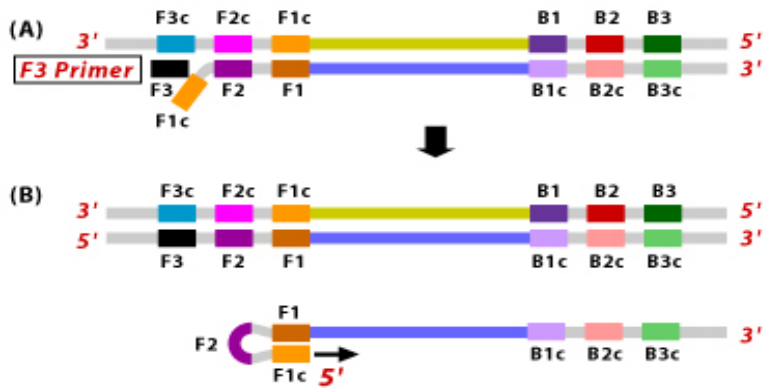
The reaction steps are described below:

1. F2 region of FIP hybridizes to F2c region of the target DNA and initiates complementary strand synthesis.

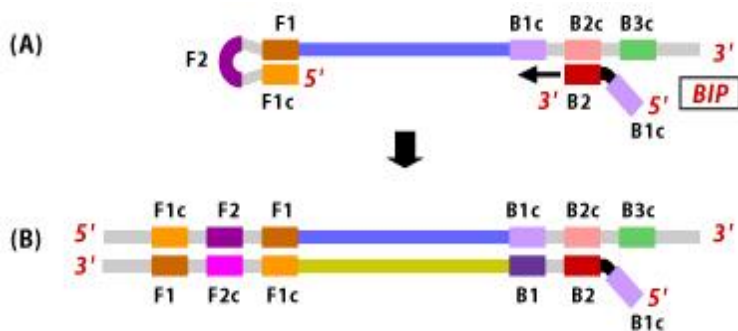


2. Outer primer F3 hybridizes to the F3c region of the target DNA and extends, displacing the FIP linked complementary strand. This displaced strand forms a loop at the 5' end.

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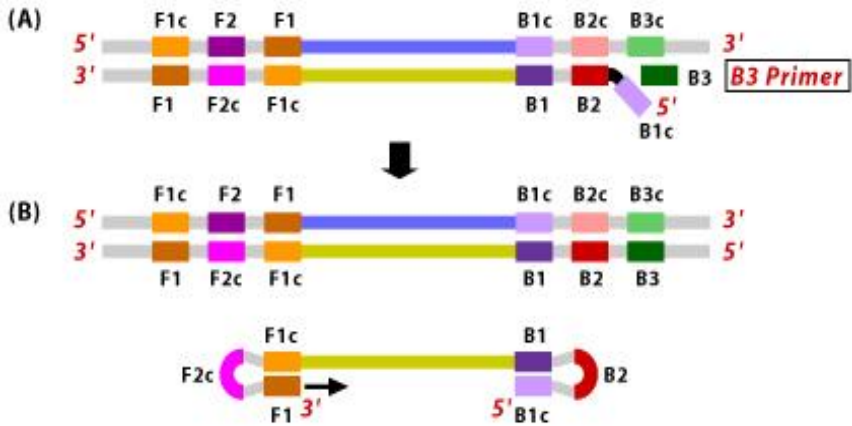


- This single stranded DNA with a loop at the 5' end serves as a template for BIP. B2 hybridizes to B2c region of the template DNA. DNA synthesis is initiated leading to the formation of a complementary strand and opening of the 5' end loop.



- The outer primer B3 hybridizes to B3c region of the target DNA and extends, displacing the BIP linked complementary strand. This results in the formation of a dumbbell shaped DNA.

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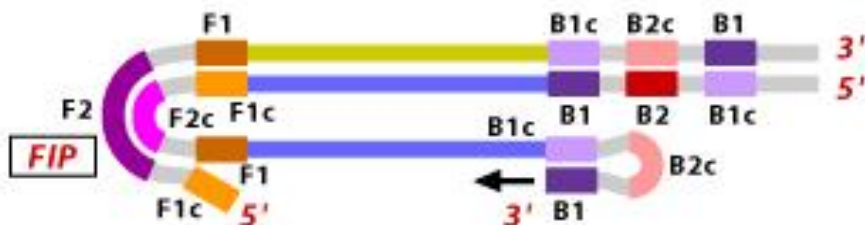


- The nucleotides are added to the 3' end of F1 by DNA polymerase, which extends and opens up the loop at the 5' end. The dumbbell shaped DNA now gets converted to a stem loop structure. This structure serves as an initiator for LAMP cycling, which is the second stage of the LAMP reaction.

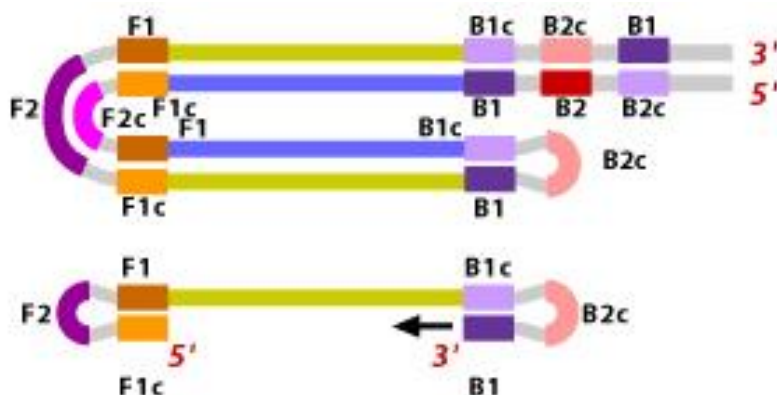


- To initiate LAMP cycling, the FIP hybridizes to the loop of the stem-loop DNA structure. Strand synthesis is initiated here. As the FIP hybridizes to the loop, the F1 strand is displaced and forms a new loop at the 3' end.

## MATERIALS AND METHODS

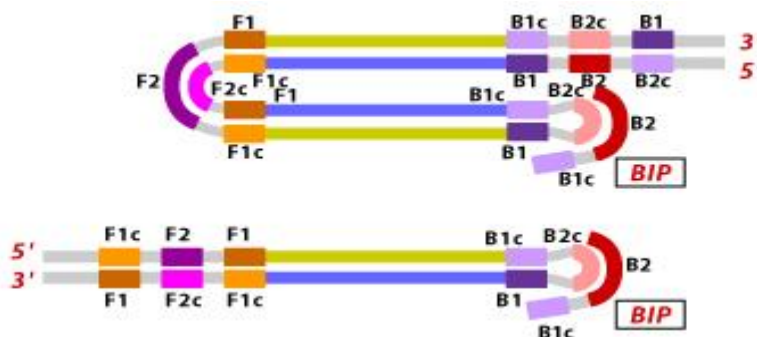


7. The nucleotides are added to the 3' end of B1. The extension takes place displacing the FIP strand. This displaced strand again forms a dumbbell shaped DNA. Subsequent self-primed strand displacement DNA synthesis yields one complementary structure of the original stem loop DNA and one gap repaired stem loop DNA.



8. Both these products then serve as template for a BIP primed strand displacement reaction in the subsequent cycles. Thus, a LAMP target sequence is amplified 13 fold every half cycle.

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The final products obtained are a mixture of stem-loop DNA with various stem lengths and various cauliflower like structures with multiple loops. The structures are formed by annealing between alternatively inverted repeats of the target sequence in the same strand.

Allele-Specific LAMP is a modified version of LAMP technology patented by DiaSorin in 2010. It was initially described as simple, robust and easy method for the molecular diagnosis of JAK2 V617F mutation, in patients with chronic myeloproliferative neoplasms (Minnucci G et al., 2012).

In AS LAMP assay two specific reagents cooperate to identify a single nucleotide changes in DNA sequence: a Backward Loop primer (LB) and a blocker. They specifically hybridize with mutated and wild type sequence respectively. If the target in the reaction is wild type, the blocker forms a stable duplex with the stem-loop structure, preventing the annealing and extension of the LB primer and, therefore, suppressing the amplification. If the target in solution is mutated, the blocker does not anneal, while the LB primer binds its target and finally extended.

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### 5.1.2 AS LAMP reaction

The LAMP reaction was carried out with a total of 25  $\mu$ L reaction mixture containing 1.6  $\mu$ M of each FIP and BIP primers, 0.2  $\mu$ M of F3 and B3 primers and 0.8  $\mu$ M of LB primer and blocker . Then were added 1.4 mM of each dNTPs, the 1X Reaction Buffer (pH 8.8 at 25°C) containing 20 mM Tris-HCl, 10 mM KCl, 2 mM MgSO<sub>4</sub>, 10 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.1% Triton® X-100, 8U of *Bst* DNA polymerase large fragment (New England Biolabs), water (made up to the final volume of 20  $\mu$ L/tube) and finally 5  $\mu$ l of samples. The reaction mixture was incubated at 65°C for 40 minutes.

### 5.1.3 Detection of LAMP products

Several methods have been reported in literature to monitor the isothermally amplified DNA (Nyan et al., 2015; Zhang et al., 2014). The most common methods used are the turbidimetry and agarose gel electrophoresis. Turbidimetry detection method consists in the measurement of turbidity of the reaction mix, due to the precipitation of magnesium pyrophosphate. This insoluble salt is formed by the interaction between Mg<sup>2+</sup> present in solution and the inorganic pyrophosphate produced by the incorporation of dNTPs in the DNA growing strands. Thus, the amount of magnesium pyrophosphate is proportional to the amount of amplified DNA. The turbidity is visible by the naked eye and is measurable on a turbidimeter as a transmittance signal either at end-point for a



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qualitative analysis or in real-time, allowing quantitative applications. Turbidimetry is the ideal detection method for LAMP technology, at least for fast clinical applications and point-of-care approaches since it does not require expensive fluorescent reagents and complex detectors (Mori Y et al.2010).

One more possible detection approach is the addition to the reaction tube of Yo-Pro or others fluorescent dyes, that produce a green fluorescent signal when they were intercalated into the dsDNA. Additionally, the LAMP products were also monitored using 2% agarose gel electrophoresis stained with ethidium bromide and visualized under UV light. Gel electrophoresis requires opening of the reaction tubes and therefore significantly increases the risk of carry-over contamination.

In this work detection of AS LAMP products was performed by annealing analysis and fluorescent probes-based method. The annealing analysis provide information about the annealing temperature of the amplified products. AS LAMP reaction was carried out in the presence of the intercalating dye Yo-Pro. During the amplification the instrument LIAISON<sup>®</sup> lam records the total fluorescence generated by Yo-Pro binding to double-stranded DNA as temperature changes, and plots the fluorescence in real time as a function of temperature. The first derivative of this plot,  $dF/dT$ , is the rate of change of fluorescence in the reaction, and a significant change in fluorescence accompanies the annealing of the double-stranded LAMP products. A plot of  $-dF/dT$  vs. temperature display these changes in

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fluorescence as distinct peaks. The annealing temperature of each product is defined as the temperature at which the corresponding peak occurs.

In order to make the LAMP detection easier and improve the applicability of the LAMP assay in routine molecular diagnosis, AS LAMP assay cKIT D816V was developed using specific probes labelled with different fluorophores and detected with LIAISON® lam instrument (DiaSorin) (Figure 11). LIAISON® lam instrument monitors in real time changes in fluorescent signal, in up to three channels (500 nm, 530 nm and 570 nm), allowing the detection of different targets simultaneously.



**Figura 11.** LIAISON® lam instrument

### 5.2 SAMPLES COLLECTION AND PREPARATION

#### 5.2.1 Plasmids

In order to perform preliminary test on AS LAMP assay, plasmids harboring the wild type or mutated cKIT sequence were synthesized. Both types of plasmids were manufactured by GeneArt. They contain a fragment of 420 bp of cKIT cDNA including the codon 816 (NCBI Reference Sequence NG\_007456.1). One of these plasmids expresses the GAC wild type codon that encodes for aspartic acid at position 816, while the second one, contains the GTC mutated codon, encoding for valine. The fragments were cloned into pMA-T vectors using SfiI/SfiI cloning sites; then plasmids were purified from transformed E.coli K12 cells and the final constructs were verified by sequencing.

#### 5.2.2 Cell lines

Several negative cell lines were used to test the AS LAMP assay specificity. Cell lines were ordered from DSMZ (Braunschweig, Germany) and cultured according the manufacturer's instructions. The following cell lines, all carrying only wild type cKIT allele, were used: HL60 (acute myeloid leukemia), KASUMI-1 (acute myeloid leukemia), K562 (chronic myeloid leukemia in erythroid blast crisis), TOM1 (B cell precursor leukemia), 697 (B cell precursor leukemia), ME-1 (acute myeloid leukemia), MV4 (acute

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monocytic leukemia), REH (B cell precursor leukemia), RS4-11 (B cell precursor leukemia), KCL22 (chronic myeloid leukemia in blast crisis).

### **5.2.3 Positive and negative clinical samples**

To validate the AS-LAMP assay, patients with CBF AML disease, harboring the mutation cKIT D816V were tested. Despite the rarity of the point mutation D816V, a total of 30 positive clinical samples were collected from several hospitals. In particular 12 samples were selected with t(8;21) and 18 with inv(16), in which the presence of the additional mutation D816V was confirmed by Direct Sanger Sequencing. In order to evaluate the specificity of the AS LAMP assay, also 30 negative clinical samples from healthy donors, were tested. In both positive and negative clinical samples genomic DNA was extracted from bone marrow or, less frequently, from peripheral blood, using the QIAmp DNA Mini Kit (Qiagen). Then the concentration and quality of extracted DNA were evaluated using the Nanodrop spectrophotometer (ThermoScientific).

### **5.2.4 DNA Extraction**

DNA was extracted from cell pellets using QIAmp DNA Mini Kit (Qiagen). Pellets containing  $5 \times 10^6$  of cells were resuspended in 200  $\mu$ l of PBS in a 1.5 ml microcentrifuge tube, adding 20  $\mu$ l of proteinase K and 4  $\mu$ l of RNase A (100mg/ml). 200  $\mu$ l of Buffer AL was added to the sample in order to lyse

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cells during 10 minutes of incubation at 56° C. 200 µl of ethanol (100%) were added to each sample. The mixture was transferred to QIAamp Mini spin column and centrifuged at 8000 rpm for 1 minute to eliminate the cell lysate, whereas genomic DNA remained attached to the column. The column was subsequently washed with buffers AW1 and AW2 (500 µl each) in order to remove denatured proteins, RNA and salts. After 1 minute of incubation with water, the genomic DNA was eluted from QIAamp Mini spin column by centrifugation and stored at -20°C until used.

### **5.2.5 Determination of samples concentration and purity**

DNA concentration and purity were checked using the Nanodrop spectrophotometer (ThermoScientific). DNA concentration is estimated by measuring the absorbance at 260nm, multiplying by the dilution factor, and using the relationship that an  $A_{260}$  of 1.0 = 50µg/ml pure dsDNA. Direct measurement of nucleic acid samples at  $OD_{260}$  can be converted using the Beer-Lambert equation which relates absorbance to concentration:

$$A = E * b * c$$

where A is the absorbance represented in absorbance units (A), E is the wavelength-dependent molar absorptivity coefficient (or molar extinction coefficient) with units of  $M^{-1} \cdot cm^{-1}$ , b is the path length in cm, and c is the analyte concentration in mol/L or molarity (M).

To evaluate DNA quality, measure of ratio of the absorbance 260/280nm and 260/230nm were achieved. Good-quality DNA will have an  $A_{260}/A_{280}$

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ratio of about 1.8 and  $A_{260}/A_{230}$  ratio ranging between 1.8 and 2.2. A reading of lower ratio  $A_{260}/A_{280}$  indicate the presence of protein or other contaminants that absorb strongly at or near 280 nm, while low  $A_{260}/A_{230}$  values may indicate a problem with the extraction procedure, due to presence of residual phenol.

## ***6.RESULTS***

## RESULTS

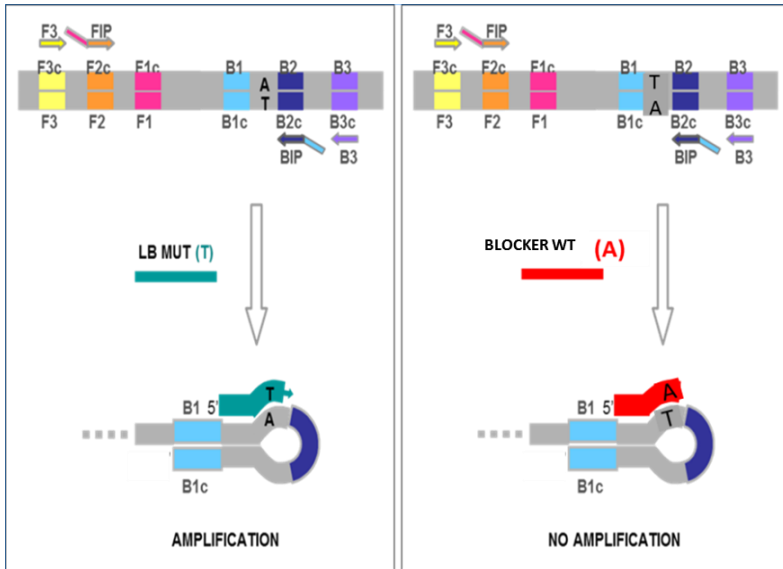
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According to the “two-hit model”, proposed by Gilliland and Griffin, CBF AML disease is due to two different classes of mutations. The class II mutation is a chromosomal aberrations, such as t(8;21) or inv(16)/t(16;16), that affects the CBF transcription factors and initially impairs the hematopoietic differentiation. The class I mutation is the constitutive activating mutation D816V of the receptor tyrosine kinase cKIT that confers a proliferation advantage to hematopoietic cells.

The mutation cKIT D816V is caused by just one oligonucleotide exchange, from GAC to GTC, leading the transition from the aspartic acid to valine at the codon 816 (D816V).

In this work the AS LAMP is proposed as rapid, simple and easy to use method to improve the detection of point mutation cKIT D816V, in routine laboratory diagnosis of CBF AML disease. As reported in the literature (Minnucci et al., 2012; Aonuma et al., 2013) in the AS LAMP technology, two specific reagents cooperate to identify the single nucleotide changes: the backward loop primer (LB) and a blocker. The LB primer specifically hybridizes with the mutated GTC codon; it increases number of starting points for DNA synthesis, allowing a more specific amplification of mutated DNA and an higher amplification efficiency (Nagamine et al., 2002). The blocker is partially overlapped to LB primer, and specifically binds the wild type codon (GAC); this blocker cannot function as primers for DNA polymerase, therefore prevents amplification of wild type DNA and competitively excludes the LB primer (Figure 12).





**Figure 12.** The principle of AS-LAMP cKIT D816V assay

The AS LAMP cKIT D816V assay development can be divided in three different phases.

- 1) The first one was involved in the design of two LAMP primers sets, each one specific for the target cKIT D816V and the internal control, and a blocker able to prevent the wild type cKIT gene amplification.
- 2) The second phase was aimed to optimize the duplex AS LAMP assay by identification of optimal reaction conditions (pH, temperature and time of reaction), and reagent concentrations for dNTPs, betaine, DNA polymerase and MgSO<sub>4</sub>.
- 3) In the last phase the performances of AS LAMP assay were evaluated.

## **6.1 AS LAMP ASSAY DESIGN**

### **6.1.1 cKIT primers design**

The first step was focused on LAMP primers design specific for DNA sequence of cKIT gene. For this purpose was used the LampZero software developed by DiaSorin; we manually designed the oligonucleotides, carefully selecting the LAMP primers sets that don't form secondary structure folding, and potentially unable to produce non-specific hybridization events. The presence of intramolecular and intermolecular secondary structures, such as hairpin, self dimer and cross dimer, can determine a decrease in the yield of amplification or even an absence of amplification. Secondary structures in fact compete for the annealing of the primer with the target DNA sequence, drastically decreasing the effective concentration of primers available for the amplification reaction. We have been collected more than thirty sets, each one of 4 primers: a pair of outer primers (F3 and B3) and a pair of inner primers (FIP and BIP), specifically designed to recognize 6 distinct regions on cKIT gene around the D816 codon (Figure 13).

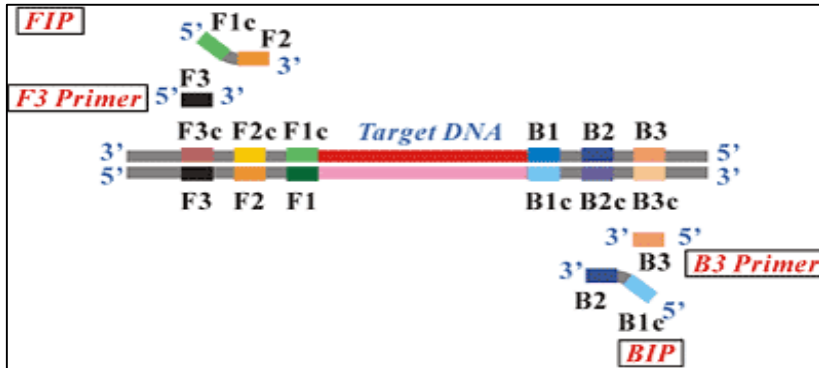


Figure 13. Outer and inner primers for LAMP reaction

To initially evaluate their specificity for cKIT gene, each primer set was tested in a LAMP reaction using synthetic plasmid harboring the cKIT sequence as target. LAMP reactions were carried out in a total of 25  $\mu$ L reaction mixture containing standard primers concentrations: 1.6  $\mu$ M of FIP and BIP, 0.2  $\mu$ M F3 and B3. The target amplification was detected in real time using the intercalating dye Yo-Pro; Yo-Pro was added in the same reaction solution and was incorporated in the DNA during LAMP reaction, producing a green fluorescent signal. As result all primers sets had amplified the cKIT gene after 37 minutes; in some cases aspecific amplifications were observed into No Template Controls (NTCs) due to primers dimers production, although they were amplified very late. For the AS LAMP assay development, the interest was focused on primer set which allows a more rapid cKIT gene amplification and prevents aspecific amplification (Figure 14A).

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Once the LAMP primer set (including F3, FIP, B3 and BIP) has been defined, the primer designing proceeded with the backward loop primer (LB) selection. LB primer contains sequence complementary to the single stranded loop region, between the B1 and B2 regions, on the 5' end of the dumbbell-like structure. It was carefully designed to hybridize with D816V codon (GTC) to facilitate mutated cKIT sequence amplification. In order to test the LB specificity, several LAMP reactions have been performed using the previously selected primers set and one of the new LB primers designed; all results were compared to the LAMP reaction without LB primer. Two synthetic plasmids containing the wild type or mutated cKIT sequences, have been used as targets. As expected all LB primers have accelerated the reaction, reducing the amplification time of mutated sequence from about 37 to 22 minutes. Despite the wild type cKIT amplification was disadvantaged, due to the mismatch between LB and codon GAC, aspecific amplifications of wild type sequence are observed after 30 minutes. The LB primer that produces the widest time gap (about 8 minutes) between mutated and wild type sequence amplification has been selected for the final AS LAMP assay (Figure 14B).

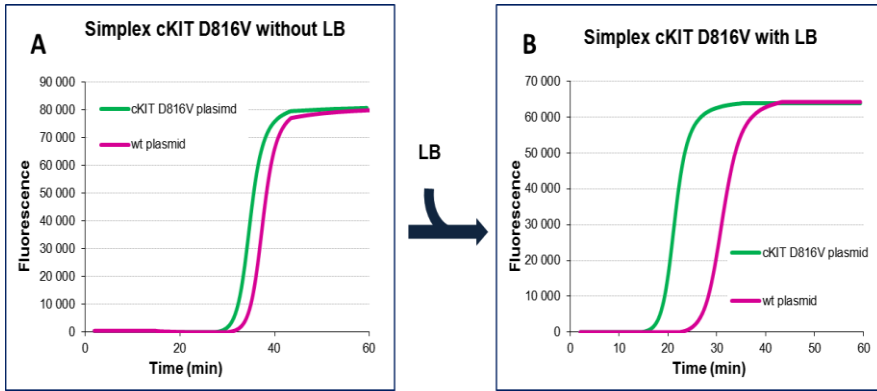


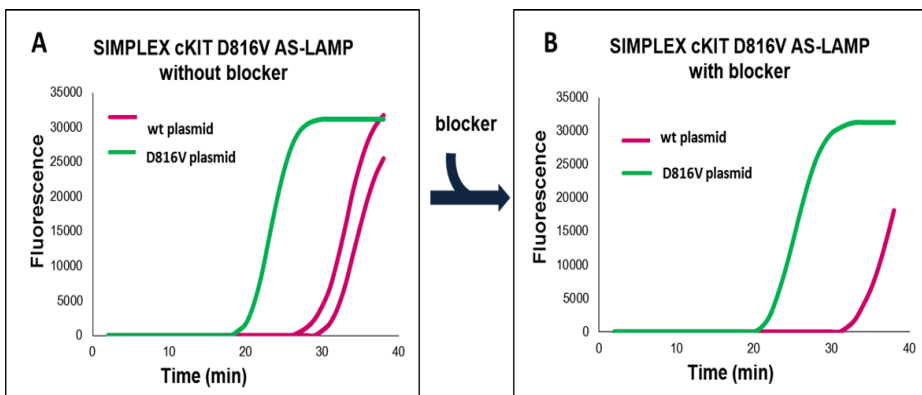
Figure 14. Simplex cKIT D816V with (B) and without LB (A)

### 6.1.2 Blocker design

In order to suppress the aspecific amplification of wild type cKIT sequence, the LAMP assay has been implemented with the use of a blocker. This blocker has been carefully designed to bind the wild type cKIT gene, surrounding the D816 codon (GAC), and partially overlapped to LB primer. As result the blocker competitively excludes the LB primer from the wild type sequence, and suppresses its amplification. At the same time, due to the presence of single-base mismatch, the blocker does not anneal with mutated sequence that can be amplified by the LB primer. For an efficient clamping reaction, the blocker must bind the wild type sequence ahead of binding of the mismatched LB primer. For this purpose the  $T_m$  of blocker is higher than  $T_m$  of LB (Ørum, 2000). A panel of blockers in the size range from 13 to 18 nucleotides have been designed. Next, several AS LAMP

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reactions have been performed, each one with a different blocker at the same LB primer concentration. As result all blockers have caused a considerable delay in the wild type cKIT amplification; in particular the selected blocker allows the wild type cKIT amplification only after 30 minutes, i.e. 8 minutes later than the mutated sequences (Figure 15).



**Figure 15.** Simplex cKIT D816V with (B) and without blocker (A)

Despite the presence of the blocker, few non-specific amplifications have been observed. In order to improve the selective amplification of mutated cKIT sequence, a really challenging work to find the precise proportions of LB and blocker concentrations, has been performed. In more details, it has been observed that using high blocker concentration, both wild type and mutated DNA were clamped; on the contrary with low blocker and high LB primer concentrations no blocking has been produced. As result of the optimization, also the aspecific amplifications, after the 30 minutes of reaction, have been eliminated.

### **6.1.3 ABL primers design**

As second step, the simplex AS LAMP assay has been implemented by the amplification of the endogenous ABL gene as internal control. The internal control (IC) is a nontarget DNA sequence which is coamplified simultaneously with the target. In the mixture without the IC, a negative result could mean that there is no target sequence present in the reaction tube. But, it could also mean that the reaction has been inhibited, due to malfunction of instrument, incorrect LAMP mixture, poor DNA polymerase activity, or not least the presence of inhibitory substances in the reaction tube. Conversely, in a reaction with an IC, a control signal should always be produced even though there is no target sequence present.

The AS LAMP duplex assay has been developed in order to have the IC which is coamplified with the target sequence in the same reaction tube. The primers set specific for the IC targets the housekeeping ABL gene, which is always present in human cells. The DiaSorin LampZero software has been used to manually design several LAMP primers sets, including the forward and backward loop primers. Between these, some primers sets are synthesized and tested at standard LAMP concentrations. Once primers sets performances were evaluated, we have focused on the most weary IC; this is aimed to limit the production of IC amplicon in the duplex reaction and to reduce the competition of the IC and the target for dNTPs and DNA polymerase.

## **6.2 DETECTION SYSTEMS**

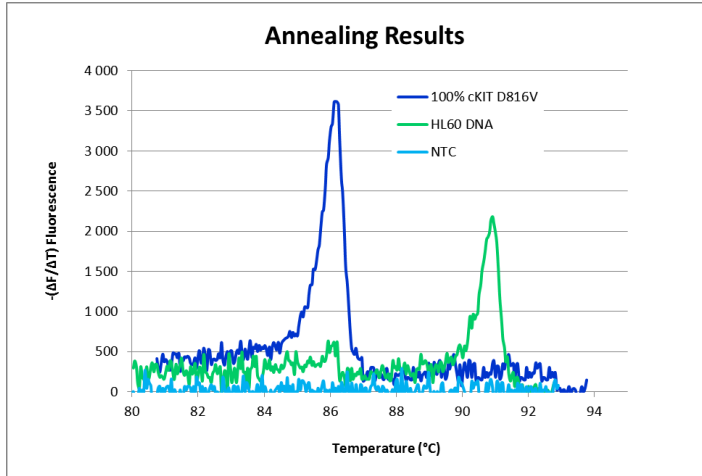
Several methods to detect the LAMP products have been reported in the literature; the most common methods used are the turbidimetry and the agarose gel electrophoresis. Turbidimetry consists in the measurement of turbidity of the reaction mix due to the precipitation of magnesium pyrophosphate. It is the ideal detection method for LAMP technology, at least for fast clinical applications and point-of-care approaches since it does not require expensive fluorescent reagents and complex detectors. The agarose gel electrophoresis identifies the LAMP products by size. Though gel electrophoresis is relatively inexpensive, it is time-consuming and non-automated. It is also low in specificity, since molecules of the same or similar weights cannot be easily differentiated. For these reasons it is not suitable for endpoint analysis for most laboratory purposes.

For the duplex AS LAMP assay two alternative detection methods were used: the annealing analysis and the more innovative fluorescent probes-based method that allow to detect and differentiate simultaneously both target and IC. Moreover for these methods there is no need to open the tube after the LAMP reaction was completed; the closed tube systems prevent amplicon carryover and so result more suitable for the routine laboratory diagnosis. The fluorescent probes-based method has the additional advantage to monitor the LAMP products in real time in contrast to other methods that require analysis at the end of the LAMP reaction.



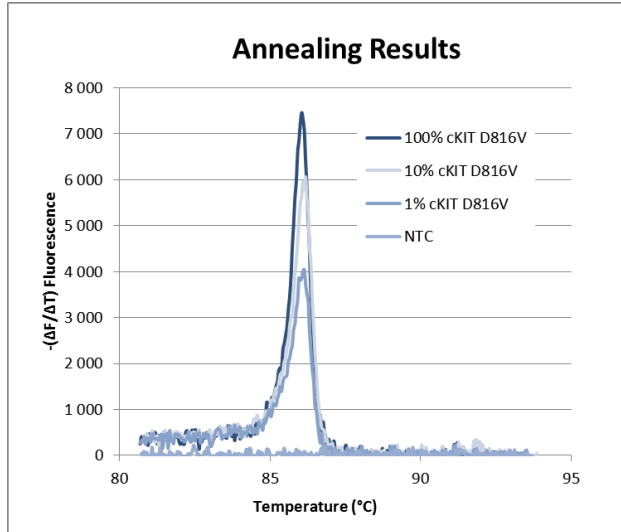
### 6.2.1 Annealing analysis

The annealing analysis has been performed by LIAISON<sup>®</sup> lam instrument (DiaSorin) to identify and differentiate the DNA products of AS LAMP amplification characterised by different annealing temperatures. The annealing temperature of DNA is the temperature at which 50% of its structure is in double strand form. It depends on length and base composition of the sequence, so each DNA products will have a characteristic annealing temperature. The AS LAMP assay was performed in the presence of the intercalating dye Yo-Pro. Annealing curves are generated after the denaturation at 100°C of the LAMP product followed by slowly cooling the amplified DNA from 95°C to 80°C. As the temperature decreases, amplified complementary DNA strands anneal and the intercalating dye is incorporated, increasing the fluorescence signal. Annealing curve raw data is generally represented by plotting fluorescence over temperature, but to make analysis more convenient, the negative first derivatives ( $-dF/dT$ ) are used, revealing annealing temperatures at peaks. The negative first derivatives of the annealing curves in the duplex AS LAMP assay showed two annealing peak referring to two simultaneous amplifications. The amplification of cKIT D816V mutation exhibits an annealing curve with a peak at about 86°C ( $\pm 0.25^\circ\text{C}$ ) while the amplification of the ABL internal control shows a peak at the higher temperature of about 91°C ( $\pm 0.37^\circ\text{C}$ ) (Figure 16).



**Figure 16: Annealing analysis.** Duplex AS LAMP assay generates an annealing peak for 100% mutated plasmid cKIT D816V at 86°C and a different peak at 91°C for ABL internal control.

This analysis can reveal the presence of primer dimers or non-specific amplification. Due to their small size, primer dimers usually anneal at lower temperatures than the desired product, while non-specific amplification may result in LAMP products that melt at temperatures above or below of the desired product. Moreover a direct relationship between the amount of mutated target tested and the height of peak obtained after annealing analysis has been consistently observed. Mutated plasmid cKIT D816V was serially diluted in negative DNA extracted from HL60 cell line and amplified in the duplex AS LAMP assay. The results are shown in the figure 17.



**Figure 17.** Annealing results of serially dilution of mutated plasmid cKIT D816V on negative DNA. It generates an annealing peak at 86°C and height of peak decreases proportionally to the amount of mutated plasmid.

### 6.2.2 Fluorescent probes

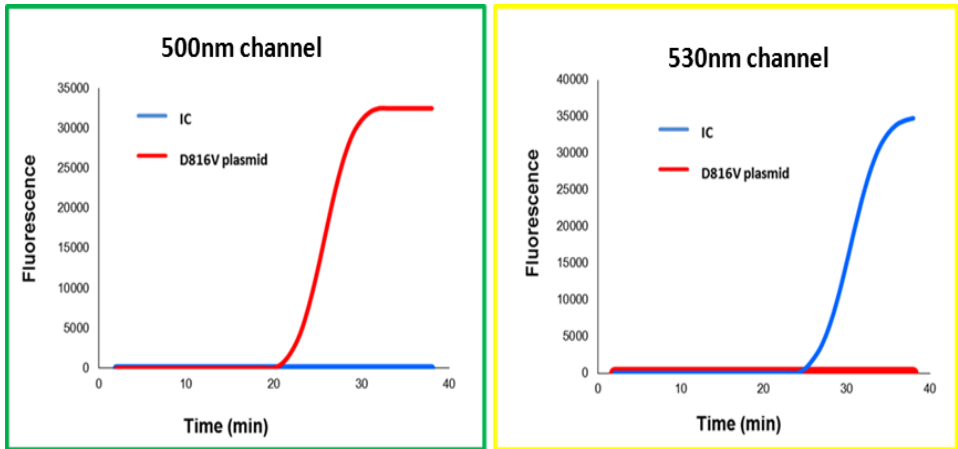
Detection method based on the use of sequence-specific fluorescent probes allows to discriminate between cKIT D816V and IC, monitoring in real time their amplifications. Two different fluorescent probes, specific each one for cKIT or ABL sequence have been designed and then synthesized by Eurofins Genomics. These probes were engineered with a blocking group at 3' end to prevent the extension and a fluorophore at 5' end; in particular the green fluorophore has been used to label probe specific for the cKIT sequence and yellow fluorophore for probe binding the

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IC. The probes anneal their specific target sequences without competing with other primers, and monitor the DNA amplification in real time. The isothermal AS LAMP reaction was performed on LIAISON® lam instrument (DiaSorin). The reaction was carried out in 25 µl total reaction mixture volume containing 1.6 µM of each FIP and BIP primers, 0.2 µM of F3 and B3 primers, 0.8 µM of LB primer and blocker and 0.8 µM of each fluorescent probes. Then are added 1.4 mM of each dNTPs, the 1X Reaction Buffer 20 mM Tris-HCl, 10 mM KCl, 2 mM MgSO<sub>4</sub>, 10 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.1% Triton® X-100, 8U of *Bst* DNA polymerase large fragment (New England Biolabs), water (made up to the final volume of 20 µL/tube) and finally 5 µl of samples. The reaction mixture was incubated at 65°C for 40 minutes. As target were used 100% of mutated plasmid cKIT D816V and 100 ng/rx of negative DNA extracted from HL60 cell line. During incubation on LIAISON® lam instrument at constant temperature, the real time monitoring of fluorescence in two separate channels was achieved. As result the target cKIT D816V and the IC were simultaneously detected in 500 nm and 530 nm channels respectively (Figure 18).

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**Figura 18. Fluorescent probes-based method.** Mutated plasmid cKIT D816V was amplified in 500nm channel and IC in 530nm channel.

The use of sequence-specific fluorescent probes offers the benefits of combined amplification and detection in a single reaction tube. This system is user-friendly and hastens the time-to-result, characteristics that make it suitable for the routine detection of D816V mutation in hospital laboratories. Moreover it is a closed-tube system that prevents re-opening of the tube post amplification, thus limiting the possibility of contamination by amplicon carryover.

### **6.3 AS LAMP ASSAY OPTIMIZATION**

Duplex AS-LAMP cKIT D816V assay optimization was performed in order to reduce primer dimers formation and to increase the efficiency and specificity of the amplification process.

An initial optimization has been involved in the definition of the fine balance between concentrations of both cKIT D816V and IC primers sets to ensure the most sensitive and most efficient assay. Indeed an high concentration of primer set for IC can abolish the cKIT D816V signal, due to competition for reagents, and cause false negative results, especially if the target is present in extremely low concentration. At the same time excessive concentration of cKIT D816V primers can allow aspecific amplification.

The second step has been involved in the fine-tuning of the reaction conditions, including temperature, pH and reagents concentrations (enzyme, MgSO<sub>4</sub>, dNTPs, betaine) to determine the most efficient amplifying conditions of the AS LAMP assay. The AS LAMP cKIT D816V assay optimization was carried out on LIAISON® lam instrument and the DNA amplification was detected in real time using fluorescent probes.

### **6.3.1 Temperature**

Polymerase Chain Reaction (PCR) is the most common DNA amplification method employed for molecular diagnosis and biomedical research. It uses thermostable polymerase enzymes and a cyclic heating and cooling to obtain strand separation and annealing, respectively. The advent of LAMP technology has revolutionized the acid nucleic amplification because it is performed under isothermal condition. LAMP technology makes use of enzymes able to perform strands separation without the need for the additional heating steps, making the process simpler and rapid. The enzyme selected for the AS LAMP cKIT D816V assay is the *Bst* polymerase (New England Biolabs) that is characterized by a strong strand displacement activity and an optimum reaction temperature between 60°C and 65°C. In order to determine the optimal temperature and time of reaction, the duplex AS LAMP cKIT D816V reaction was performed at different temperatures ranging from 55°C to 70°C. Triplicates of 100% mutated plasmid cKIT D816V and negative DNA extracted from HL60 cell line were incubated at each temperatures for 40 minutes. As shown in the table 2 significant differences have been highlighted in terms of threshold time when the temperature was modified.

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ASSAY TEMPERATURE	REPS DETECTED	AVG	SD
55°C	0/3	none	None
56°C	0/3	none	None
57°C	2/3	32.10	3.15
58°C	3/3	30.65	2.27
59°C	3/3	31.67	1.85
60°C	3/3	29.54	2.34
61°C	3/3	28.37	1.97
62°C	3/3	28.59	1.04
63°C	3/3	27.82	1.43
64°C	3/3	23.61	0.24
<b>65°C</b>	<b>3/3</b>	<b>19.65</b>	<b>0.37</b>
66°C	3/3	22.29	0.78
67°C	3/3	25.27	0.53
68°C	3/3	24.45	1.49
69°C	2/3	33.50	1.76
70°C	0/3	none	None

**Table 2. Optimization of AS LAMP assay at different temperatures ranging from 55°C to 70°C.** Three replicates of plasmid cKIT D816V were tested at each temperature; here, the average (Avg) and standard deviation (SD) of the threshold times recorded in 500nm channel were reported.

The target was amplified before 25 minutes at temperature ranging between 64°C and 68°C. At temperatures below 56°C and above 70°C, the AS LAMP assay gave no product. A reaction temperature of 65°C was selected as the final temperature for the AS LAMP cKIT D816V assays



because it allows a more rapid amplification of the target and a lower standard deviation.

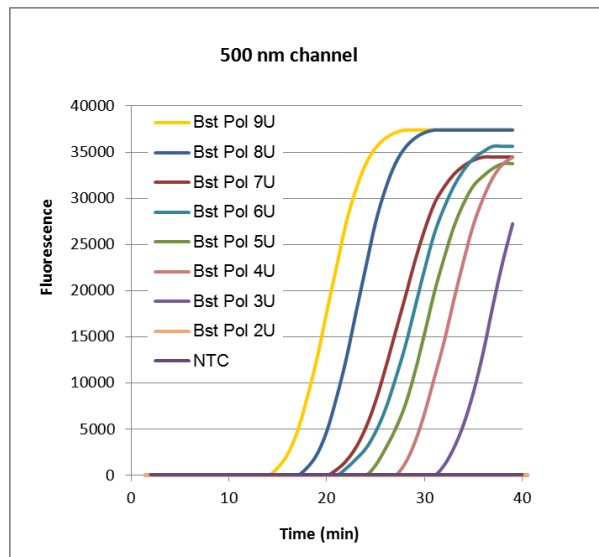
Regarding the IC, no amplifications were obtained above 58°C and roughly equivalent values of threshold times were observed at temperatures from 63 to 65°C (data not shown). Although LAMP products were detected as early as 20 min at 65°C, reaction time was optimized and set at 40 min to ensure positive detection of templates with lower concentration.

### 6.3.2 Enzyme

*Bst* polymerase is a DNA polymerase that was isolated in 1968 (Stenesh et al. 1968) from the thermophilic bacterium *Bacillus stearothermophilus* (*Bst*). *Bst* enzyme (New England Biolabs) catalyzes the polymerization of nucleotides into duplex DNA in the 5'→3' direction in the presence of magnesium ions; it has heat-resistant property and a strand-displacement type DNA polymerase activity, which synthesizes a new DNA strand while dissociating the hydrogen bond of the double stranded template DNA by itself. Moreover it lacks the 5'→3' exonuclease activity (Nagamine et al., 2001; Wozniakowski et al. 2012) and can be heat inactivated at temperatures above 80°C. Finally it is suitable for amplification of difficult DNA templates, including repetitive sequences, GC-rich regions and problematic secondary structures. The effect of *Bst* DNA polymerase concentration on duplex AS LAMP assay was tested. In particular were tested *Bst* concentrations ranging from a minimum of 2U and a maximum

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of 9U. One unit is defined as the amount of enzyme that will incorporate 10 nmol of dNTP into acid insoluble material in 30 minutes at 65°C. As expected in both 500 and 530 nm channels, the reaction slows down when the enzyme concentration decreases (Figure 19).

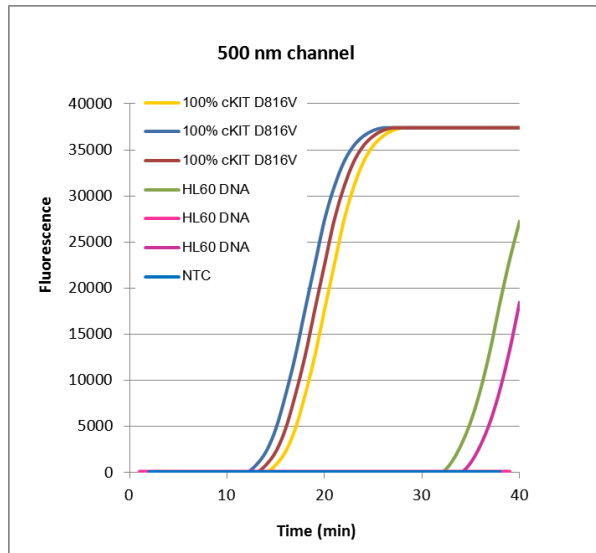


**Figure 19. Optimization of AS LAMP assay at different Bst DNA polymerase concentrations ranging from 9U to 2U.** Plasmid cKIT D816V was amplified in 500 nm channel with different threshold times: 20.17 min (9U); 21.16 min (8U); 27.51 min (7U); 28.13 min (6U); 30.15 min (5U); 32.14 min (4U); 37.18 min (3U); none (2U).

Moreover these results showed that the AS LAMP assay could work with the presence of a minimum of 3U of Bst DNA polymerase and that 8U of enzyme gave the optimal amplification of plasmid cKIT D816V with a threshold time of about 20 minutes. Higher Bst polymerase concentration

## RESULTS

was tested and, as expected, gave the faster plasmid amplification cKIT D816V; however it wasn't selected as final concentration for duplex AS LAMP assay because produce aspecific amplification (Figure 20).



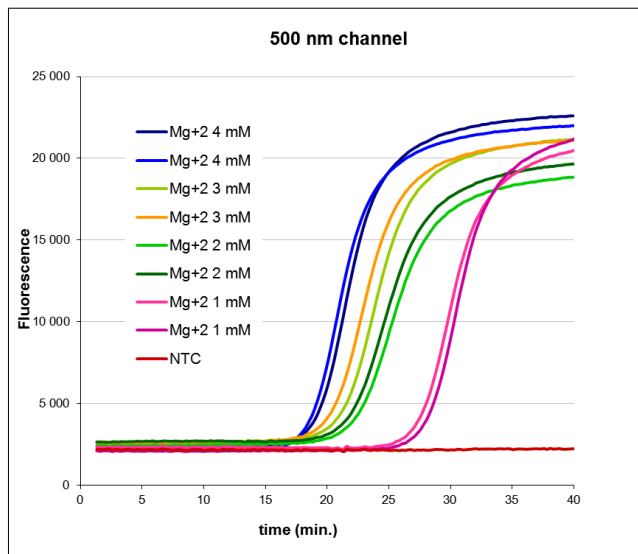
**Figure 20. AS LAMP assay with 9U of Bst DNA polymerase.** Three replicates of plasmid cKIT D816V was amplified in 500 nm channel with an AVG of 18.13 and a SD of 0.75. Two non-specific amplification of negative HL60 DNA were amplified after 37 minutes.

### 6.3.3 Magnesium

Since free  $Mg^{+2}$  availability affects primer annealing and Bst DNA polymerase activity, the effect of  $MgSO_4$  concentrations on the duplex AS LAMP reaction was determined. Duplicates of mutated plasmid cKIT D816V were amplified in the presence of 1 mM, 2 mM, 3 mM, or 4 mM of  $MgSO_4$ .

## RESULTS

In the presence of higher  $Mg^{+2}$  concentrations was observed a faster amplification of both target (Figure 21) and IC.

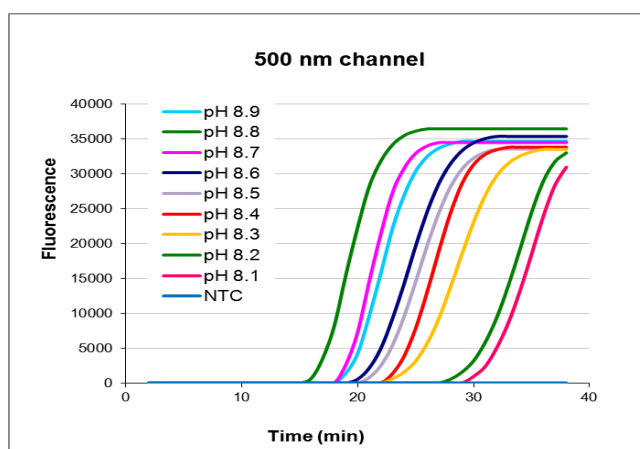


**Figure 21. Optimization of AS LAMP assay at different  $MgSO_4$  concentrations ranging from 4 mM to 1 mM.** Plasmid cKIT D816V was amplified in 500 nm channel with different threshold times. Below has been reported the AVG value: 20.82 min (4 mM); 22.87 min (3 mM); 24.63 min (2 mM); 30.32 min (1 mM).

Even though  $Mg^{+2}$  at 4 mM gives the faster target amplification, it wasn't selected as the final concentration for the AS LAMP assay, because it caused aspecific primer binding and aspecific amplifications. The  $Mg^{+2}$  concentration selected is 3 mM.

### 6.3.4 Buffer

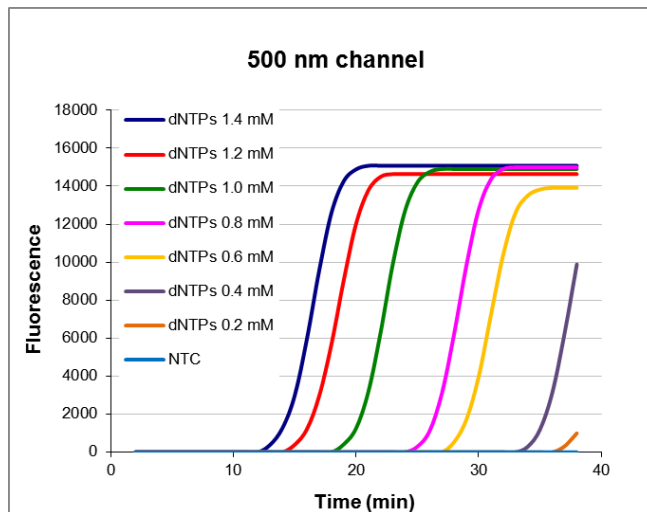
The buffer regulates the pH of the reaction, which affects the *Bst* DNA polymerase activity. The reaction buffer 1X (New England Biolabs) containing 20 mM Tris-HCl, 10 mM  $(\text{NH}_4)_2\text{SO}_4$  10 mM  $\text{KCl}_2$  mM  $\text{MgSO}_4$  0.1% Triton® X-100 (pH 8.8 at 25°C) is the recommended buffer for *Bst* polymerase. The effect of pH variations on duplex AS LAMP cKIT D816V was investigated in a range from 8.9 to 8.1 in order to enhance DNA polymerase activity. As shown in the figure 22, the buffer at pH 8.8 gave the faster amplification of plasmid cKIT D816V and the DNA polymerase activity decreases at lower pH, as well as at higher pH than 8.8.



**Figure 22. Optimization of AS LAMP assay with buffers at different pH ranging from 8.9 to 8.1.** Plasmid cKIT D816V was amplified in 500 nm channel with different threshold times: 22.15 min (pH 8.9); 19.15 min (pH 8.8); 21.14 min (pH 8.7); 25.14 min (pH 8.6); 25.12 min (pH 8.5); 27.18 min (pH 8.4); 29.18 min (pH 8.3); 33.13 min (pH 8.2); 35.18 min (pH 8.1).

### 6.3.5 Deoxynucleotide triphosphate

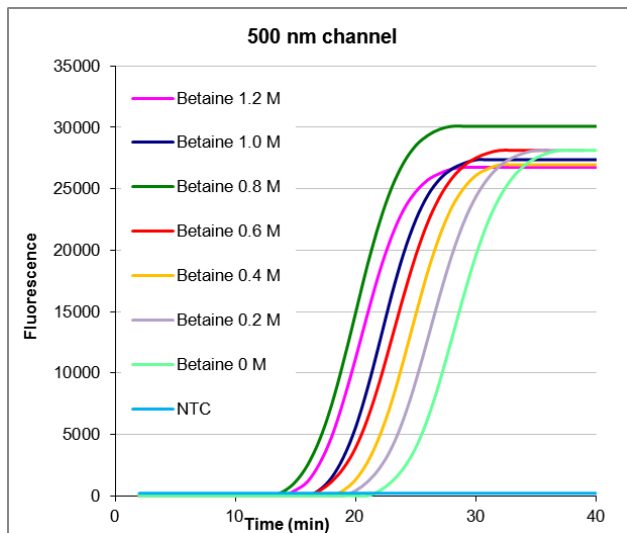
A premixed, balanced mixture of dATP, dGTP, dCTP and dTTP (New England Biolabs) was used in the duplex AS LAMP development. The AS LAMP assay was tested in the presence of several concentrations of dNTPs ranging from 0.2 to 1.4. As result the dNTPs concentrations between 0.6 and 1.4 mM have amplified the target DNA (Figure 23). Using more than 1.0 mM of dNTPs, aspecific amplifications were observed in 500 nm channel, so 1 mM was selected as final concentration.



**Figure 23. Optimization of AS LAMP assay with different dNTPs concentrations ranging from 1.4 mM to 0.2 mM:** Plasmid cKIT D816V was amplified in 500 nm channel with different threshold times: 16.59 min (1.4 mM); 18.57 min (1.2 mM); 22.63 min (1.0 mM); 28.62 min (0.8 mM); 31.72 min (0.6 mM); start amplification after 37 min (0.4 mM); none (0.2 mM).

### 6.3.6 Betaine

The addition of betaine has been reported to enhance the specificity of DNA amplification by reducing the formation of secondary structure in GC-rich regions and making DNA templates accessible for DNA polymerase. The betaine (Sigma-Aldrich) was tested in AS LAMP assay at a final concentration ranging from 1.2 to 0.2 M. As shown in the figure 24 the LAMP reaction speeds up when betaine concentrations was increased. Betaine at 0.8 M gave the faster amplification and so it was selected as final concentration.



**Figure 24. Optimization of AS LAMP assay with different betaine concentrations ranging from 1.2 M to 0 M:** Plasmid cKIT D816V was amplified in 500 nm channel with different threshold times: 21.15 min (1.2 M); 23.16 min (1.0 M); 20.14 min (0.8 M); 24.15 min (0.6 M); 25.14 min (0.4 M); 27.16 min (0.2 M); 29.16 min (0 M);

### 6.3.7 Positive and negative controls

The AS LAMP assay must be validated by a positive and negative controls. The inclusion of positive and negative controls helps to identify the presence or absence of false positive and false negative results respectively.

**POSITIVE CONTROL:** the use of positive control helps to exclude the possibility of false negative (when no amplification signal is achieved in the positive control reaction), which may be brought about either the poor quality of one or more of the reagents, or by erroneous omission of one of the reagents. Positive control was prepared with a template that is certain to amplify if the reaction goes as planned; it contains a high copy number of plasmid cKIT D816V that was amplified at about 10 minute in 500 nm channel.

**NEGATIVE CONTROL:** the negative control helps to exclude the possibility of false positive (when amplification signal is achieved in the negative control reaction), which may be due to amplicon contamination. Negative control was prepared with an high copy number of plasmid containing the specific sequence of ABL gene. The negative control was amplified at about 12 minutes in 530 nm channel.



## **RESULTS**

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To summarize the optimized duplex AS LAMP cKIT D816V assay was prepared with primers specific for target and IC and the blocker annealing the wild type cKIT sequence. The optimal reaction mixture contains 8U of *Bst* DNA polymerase, 3.0 mM MgSO<sub>4</sub>, buffer at pH 8.8, 1.0 mM of each dNTPs and 0.8 M of betaine. Finally were added 5 µL of DNA sample (100 ng/rx). Known positive and negative controls were included in each run. The reaction was performed at 65 °C for 40 min in LIAISON® lam instrument and the products were monitored in real time in 500 nm and 530 nm channels thanks the addition of fluorescent probes specific for target and IC.

## **6.4 ANALYTICAL PERFORMANCES**

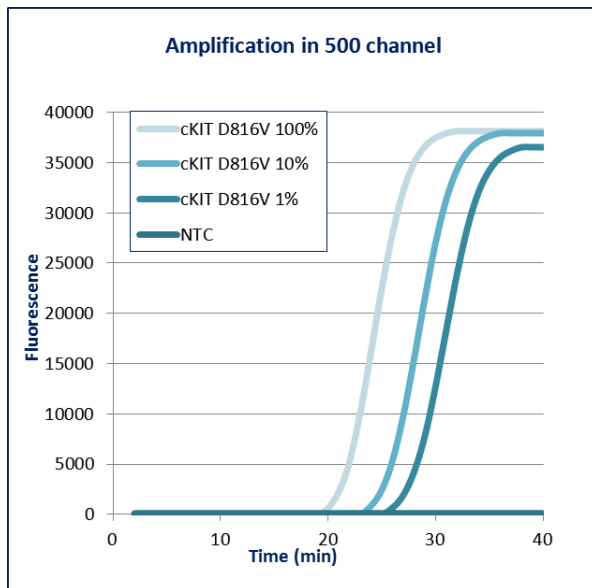
Since no cell line with the point mutation cKIT D816V was available, two recombinant and synthetic plasmids, harboring mutated or wild type cKIT sequences, were used to evaluate the analytical sensitivity and specificity. Before carrying out the AS LAMP reaction, plasmid aliquots were heated at 100°C for 10 minutes to allow DNA denaturation, rapidly cooled in ice for 10 minutes and finally added to reaction mix.

### **6.4.1 Analytical sensitivity**

To evaluate analytical sensitivity of duplex AS LAMP assay, tenfold serial dilutions of mutated plasmids were prepared in wild type plasmid from 100% to 1%, and tested. This sensitivity level was achieved using 30,000 copies of plasmid for reaction, approximately corresponding to 100 ng of genomic DNA (based on 3.7 pg of DNA content per human haploid cell). A total of 50 replicates for each type of dilution was tested and LAMP products were detected by fluorescent probes. The AS LAMP duplex assay proved to be reproducible on different plasmids dilutions and to be sensitive up to 300 copies for reaction in 100% of cases. All data were validated by the correct amplification of both positive and negative controls. In order to mimic the positive cell line, the analytical sensitivity was also evaluated on progressive dilutions of recombinant plasmid cKIT D816V into genomic DNA, from 100% to 1%. The genomic DNA was

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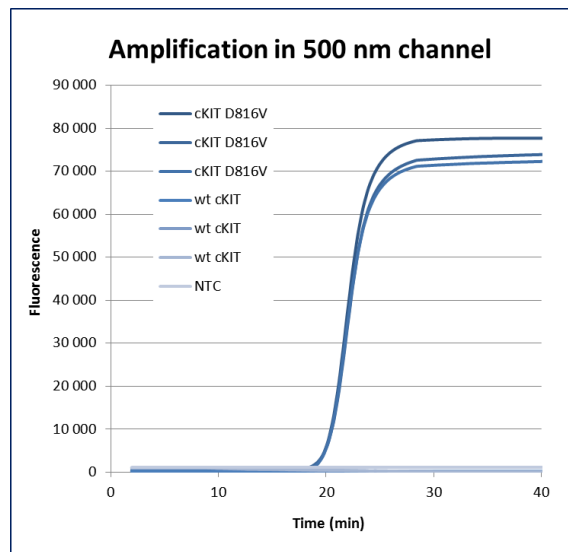
extracted from HL60 cell line, negative for cKIT D816V point mutation, and diluted up to 20 ng/ $\mu$ L. Also in this case the 100% of sensitivity was obtained on 50 replicates of each type of dilution. Moreover comparable value of threshold time has been obtained using dilution of synthetic plasmid cKIT D816V into wild type plasmid or genomic DNA. (Figure 25).



**Figure 25. Analytical sensitivity of AS LAMP assay on recombinant plasmid cKIT D816V diluted into genomic DNA.** 100% plasmid cKIT D816V (30.000 cps/rx) was amplified at 23 minutes; 10% plasmid cKIT D816V (3.000 cps/rx) was amplified at 26 minutes; 1% plasmid cKIT D816V (300 cps/rx) was amplified at 29 minutes; NTC (No Target Control)

### 6.4.2 Analytical specificity

Analytical specificity of AS LAMP assay was tested on recombinant plasmid containing wild type cKIT sequence. A total of 50 replicates of 100% wild type plasmid (30.000 copies per reaction) were tested and no amplification within 40 minutes of reaction were observed in 500 nm channel (Figure 26).

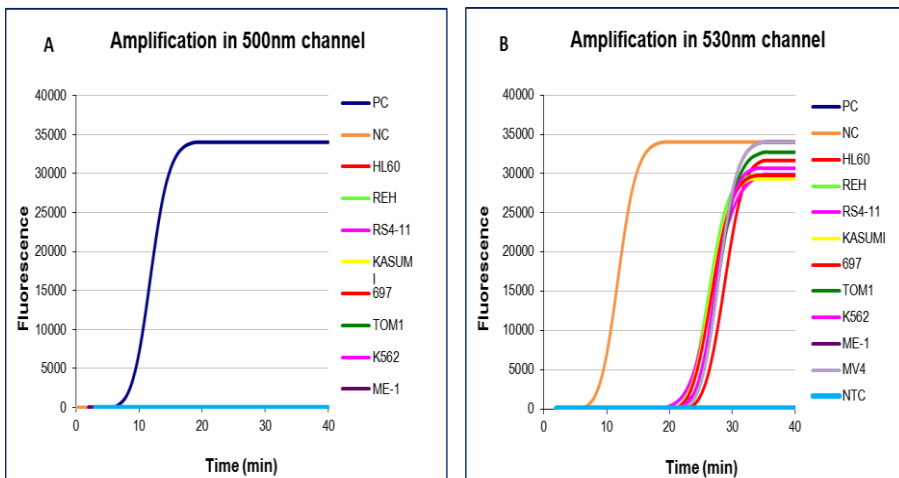


**Figure 26. Analytical specificity of AS LAMP assay on wild type plasmid cKIT.** Triplicates of 100% plasmid cKIT (30.000 cps/rx) was tested and no amplification had produced. 100% mutated plasmid cKIT D816V (30.000 cps/rx) was correctly amplified at 23 minutes.

In order to evaluate the analytical specificity, several cell lines negative for D816V point mutation were tested on AS LAMP assay. The AS LAMP was performed using 100 ng per reaction of genomic DNA extracted from nine different cell lines (REH, RS411, MV4, 697, K562, TOM1, KASUMI, ME-1 and

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HL60). After 40 minutes of reaction, no cell line was amplified in 500 nm channel (Figure 27A). All results have been validated through the correct amplification of the internal control in 530 nm channel, that excludes the presence of inhibitors and ensures the use of correct reaction conditions (Figure 27B).



**Figure 27. Analytical specificity of AS LAMP assay on negative cell lines.** Nine negative cell lines REH, RS411, MV4, 697, K562, TOM1, KASUMI, ME-1 and HL60 were tested. No cell line was amplified in 500 nm channel (A), while IC was correctly amplified in 530nm channel (B). Positive control (PC) was amplified in 500 nm channel at about 10 min and negative control (NC) in 530 channel at 12 minutes.

To assess the specificity of the AS LAMP assay, was further tested 200 replicates of No Template Control (NTC). NTC includes reaction mix to which water has been added instead of extracted nucleic acid. It helps to check primer dimer formation and to verify that no contaminating nucleic acid has been introduced into the master mix during sample processing. No

## **RESULTS**

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amplification was observed in both 500 nm and 530 nm channels. All results were validated by the correct amplification of both positive and negative controls.

These data proved that the AS LAMP is able to discriminate sequences which differ only for a single base nucleotide with a high level of reliability. Moreover, despite AS LAMP is a duplex reaction that uses a total of eleven primers, of which five specific for cKIT D816V and six for ABL sequences, no primer dimer formation and non specific primers annealing are obtained within 40 minutes of reaction.

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**6.5 VALIDATION ON CLINICAL SAMPLES**

AS LAMP assay was validated on positive and negative clinical samples. A total of 30 positive clinical samples of CBF AML patients harboring the additional mutation D816V, were collected from several hospitals. In particular 12 samples with AML1-ETO t(8;21) traslocation and 18 with CBF-MYH11 inv(16) were obtained. Between these, 13 patients express the CBF-MYH11 A isoform, 2 patients CBF-MYH11 D isoform and 3 patients the CBF-MYH11 E isoform. In each of these samples, the presence of the additional mutation D816V was previously confirmed by PCR and Direct Sanger Sequencing.

The genomic DNA was extracted from bone marrow or, less frequently, from peripheral blood, using the QIAmp DNA Mini Kit (Qiagen). Then, the concentration and quality of extracted DNA were evaluated using the Nanodrop spectrophotometer (ThermoScientific). All clinical samples were diluted up to 20 ng/ $\mu$ L and tested on AS LAMP assay. The results were validated by the correct amplification of the IC control. In the following table were listed all samples and their  $A_{260}/A_{280}$  and  $A_{260}/A_{230}$  ratios, achieved by analysis at Nanodrop; in addition has been indicated the threshold time in which each sample was amplified in 500 nm channel with AS LAMP reaction.

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SAMPLE	TYPE OF SAMPLE	CLASS II MUTATION	CLASS I MUTATION	A <sub>260</sub> /A <sub>280</sub>	A <sub>260</sub> /A <sub>230</sub>	Tht (min)
1	BM	AML1-ETO t(8;21)	cKIT <sup>D816V</sup>	1.90	1.93	19.85
2	PB	AML1-ETO t(8;21)	cKIT <sup>D816V</sup>	1.88	2.01	20.77
3	BM	AML1-ETO t(8;21)	cKIT <sup>D816V</sup>	1.88	0.47	21.47
4	BM	AML1-ETO t(8;21)	cKIT <sup>D816V</sup>	2.12	1.22	27.70
5	BM	AML1-ETO t(8;21)	cKIT <sup>D816V</sup>	1.97	1.80	23.97
6	PB	AML1-ETO t(8;21)	cKIT <sup>D816V</sup>	1.92	1.90	28.25
7	BM	AML1-ETO t(8;21)	cKIT <sup>D816V</sup>	1.94	2.00	31.22
8	BM	AML1-ETO t(8;21)	cKIT <sup>D816V</sup>	1.91	1.91	30.68
9	BM	AML1-ETO t(8;21)	cKIT <sup>D816V</sup>	1.89	1.51	27.52
10	BM	AML1-ETO t(8;21)	cKIT <sup>D816V</sup>	1.89	0.75	19.32
11	BM	AML1-ETO t(8;21)	cKIT <sup>D816V</sup>	2.07	1.87	20.45
12	PB	AML1-ETO t(8;21)	cKIT <sup>D816V</sup>	1.88	1.82	25.72
13	BM	CBFB-MYH11 A inv(16)	cKIT <sup>D816V</sup>	1.91	1.86	25.92
14	PB	CBFB-MYH11 A inv(16)	cKIT <sup>D816V</sup>	1.92	2.12	30.28
15	BM	CBFB-MYH11 A inv(16)	cKIT <sup>D816V</sup>	1.93	1.85	30.67
16	BM	CBFB-MYH11 A inv(16)	cKIT <sup>D816V</sup>	2.04	0.20	25.18
17	PB	CBFB-MYH11 A inv(16)	cKIT <sup>D816V</sup>	2.08	1.55	28.85
18	BM	CBFB-MYH11 A inv(16)	cKIT <sup>D816V</sup>	2.01	1.32	19.60
19	BM	CBFB-MYH11 A inv(16)	cKIT <sup>D816V</sup>	1.71	1.50	24.35
20	PB	CBFB-MYH11 A inv(16)	cKIT <sup>D816V</sup>	1.92	2.07	30.32
21	BM	CBFB-MYH11 A inv(16)	cKIT <sup>D816V</sup>	1.92	2.11	24.25
22	BM	CBFB-MYH11 A inv(16)	cKIT <sup>D816V</sup>	1.96	2.05	28.90
23	PB	CBFB-MYH11 A inv(16)	cKIT <sup>D816V</sup>	1.99	1.03	21.45
24	PB	CBFB-MYH11 A inv(16)	cKIT <sup>D816V</sup>	1.94	1.70	22.93
25	PB	CBFB-MYH11 A inv(16)	cKIT <sup>D816V</sup>	1.94	0.86	29.52
26	BM	CBFB-MYH11 D inv(16)	cKIT <sup>D816V</sup>	2.04	0.79	27.95
27	BM	CBFB-MYH11 D inv(16)	cKIT <sup>D816V</sup>	1.92	1.87	20.08
28	PB	CBFB-MYH11 E inv(16)	cKIT <sup>D816V</sup>	1.96	0.42	19.42
29	BM	CBFB-MYH11 E inv(16)	cKIT <sup>D816V</sup>	1.86	1.92	29.82
30	BM	CBFB-MYH11 E inv(16)	cKIT <sup>D816V</sup>	1.93	1.88	28.28

**Table 3. List of clinical samples positive for cKIT D816V mutation.** 12 samples have AML1-ETO t(8;21) traslocation and 18 CBFB-MYH11 inv(16). Between these, 13 patients express the CBFB-MYH11 A isoform, 2 patients CBFB-MYH11 D isoform and 3 patients the CBFB-MYH11 E isoform.



## **RESULTS**

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In all CBF AML samples, the duplex AS LAMP assay confirmed the presence of single point mutation D816V. The mutation has been detected within 40 minutes of reaction, with a threshold time ranging from a minimum of 19.32 and a maximum of 31.22 minutes. Moreover results proved that the use of impure DNA with  $A_{260}/A_{230}$  ratio lower than 1.8 does not affect the LAMP amplification, since they are correctly amplified.

To assess the relative specificity a total of 30 negative clinical samples were tested. The genomic DNA (100 ng/rx) was extracted from healthy donors and tested on AS LAMP assay. The 100% of concordance with home brew methods was found and all results have been validated through the correct amplification of the internal control in 530 nm channel with an average of 27 ( $\pm 2.35$ ) minutes.

The AS LAMP proved to be sensitive and specific always confirming the results obtained with PCR and Direct Sanger Sequences on positive and negative clinical samples. The AS LAMP assay takes approximately 40 minutes to process extracted DNA samples in comparison with over three and half hours requested by PCR-based method.

## **6.6 FREEZE-DRYING**

A freeze-dried formulation for the AS LAMP assay was optimized in order to make the product user-friendly and handier in the routine diagnosis in hospital laboratories. The lyophilisation of AS LAMP assays has been developed to produce one cake that contains primers, enzyme, dNTPs and dextran. The reconstitution of the product is easily obtained by using a specific resuspension buffer. Freeze-drying is a process in which a product is frozen and then dried. With this method the product is preserved by the removal of water and it maintains the original structure and activity. The freeze-drying process consists of three stages: freezing, primary drying and secondary drying. Freezing is done by cooling the lyophilizer shelves, which are in contact with the vials containing the reaction mix. This freezing process separates the water from the product, and also blocks activity of the enzyme. The results is an amorphous solid product and water crystals. The shelf temperature used for lyophilization of reaction mixture is  $-40^{\circ}\text{C}$ . Primary drying, also named sublimation, is the phase in which the pressure is lowered and heat is added in order to allow the water sublimation. The vacuum speeds sublimation. The cold condenser provides a surface for the water vapor to adhere and solidify. About 95% of the water in the material is removed in this phase. During Secondary drying, also named adsorption, the ionically-bound water molecules are removed. By raising the temperature higher than in the primary drying phase, the bonds are broken

## RESULTS

between the material and the water molecules. Freeze dried materials retain a porous structure. After the freeze-drying process was completed, the vacuum was broke with an inert gas before the material was sealed. The lyophilized formulation of AS LAMP assay was tested and compared with liquid format (Figure 28).

AS LAMP ASSAY: FREEZE-DRIED FORMAT			
SAMPLE	REPS	AVG	SD
100% cKIT D816V	50/50	24.19	0.75
10% cKIT D816V	50/50	26.82	1.56
1% cKIT D816V	150/150	30.15	2.27
NEG DNA	0/200		
NTCs	0/200		

AS LAMP ASSAY: LIQUID FORMAT			
SAMPLE	REPS	AVG	SD
100% cKIT D816V	30/30	23.67	1.17
10% cKIT D816V	30/30	26.37	1.43
1% cKIT D816V	100/100	29.65	3.14
NEG DNA	0/100		
NTCs	0/100		

**Figure 28.** Comparison between freeze-dried and liquid format of AS LAMP assay.

No significant delay or decrease in sensitivity were seen in freeze-dried formulation, nor aspecific amplification was achieved in more than 200

## RESULTS

negative DNA and 200 NTCs. All results were validated through the correct amplification of the internal control in 530 nm channel.

To evaluate stability, the lyophilis were stored at +4°C for six months (Thekiso, O. et al., 2009). Each month were tested triplicates of 100%, 10%, 1% mutated plasmid cKIT D816V and HL60 DNA, then the averages of thresholds time were represented in the following graphs (Figure 29). These results proved that the lyophilized product was robust for 6 months since no significant variation of threshold time after 6 months were obtained.

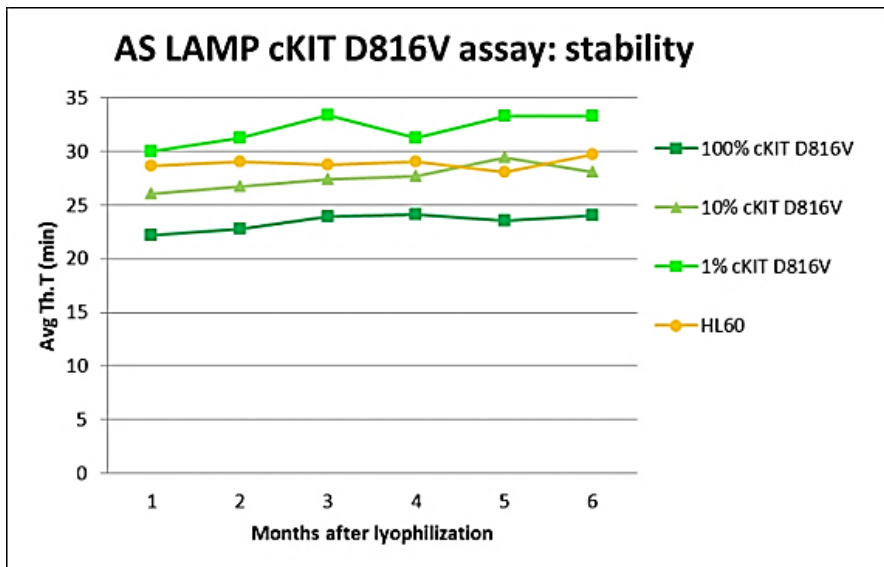


Figure 29. Stability of AS LAMP assay for six months.

## ***7.DISCUSSION***

## DISCUSSION

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Acute myeloid leukemias (AML) harboring t(8;21) translocation, pericentric inversion of chromosome 16 inv(16), or the less frequent balanced translocation t(16;16), are commonly referred to as Core-Binding Factor (CBF) AML. Compared with other AML groups, patients with CBF AML are considered a favorable risk group based on their high remission rate and survival probabilities. However, about 20–25% of CBF AML patients with t(8;21), and more than 30% of CBF AML with inv(16)/t(16;16), harbor the additional mutation D816V in the cKIT gene, that is a negative prognostic factor (Zhao et al., 2012). According to the current National Comprehensive Cancer Network (NCCN) guidelines, CBF AML patients exhibiting D816V in the cKIT gene, are classified in the intermediate risk category, with a higher incidence of relapse and a lower overall survival (Cairoli R et al., 2013; Paschka et al., 2006; Beghini et al., 2004).

The cKIT receptor is a type III receptor tyrosine kinase expressed on hematopoietic stem cells. The ligand for cKIT is the stem cell factor (SCF), a hematopoietic cytokine, which plays an important role in maintaining survival of hematopoietic cells, promoting hematopoietic cell proliferation and differentiation, and regulating growth and development of hematopoietic cells. Gain-of-function mutation D816V in exon 17 of cKIT gene is found to be associated with CBF AML, which leads to uncontrolled cell proliferation and apoptosis resistance (Lennartsson et al., 2005). Moreover, cKIT represents an important therapeutic target for tyrosine kinase inhibitors, such as Imatinib. Imatinib is a small molecule that binds to

## **DISCUSSION**

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the ATP-binding pocket of different tyrosine kinases and stabilizes the inactive conformation of the cKIT kinase domain, thus preventing the ligand-independent activation of cKIT. Unfortunately the point mutation D816V confers resistance to Imatinib. Aspartic acid at position 816 is a critical residue contributing to the structure of the activation loop (A-loop) of the tyrosine kinase enzymatic domain. Disruption of this bond by replacement of aspartic acid by valine destabilizes the inactive conformation of the kinase domain and causes the ligand-independent constitutive activation and autophosphorylation of cKIT (Cairolì et al., 2005).

National Comprehensive Cancer Network (NCCN) guidelines introduced in 2011 the cKIT D816V mutational analysis to provide a correct risk stratification of CBF AML patients and to give information for tyrosine kinase inhibitor (TKI) therapy planning. A great gap still seems to remain between the demand of the cKIT D816V mutational analysis and the availability of commercial kits. The major problem is the absence of a standardized molecular assay able to identify a single point mutation with the sensitivity required by clinical needs.

In order to perform the diagnosis, many hospital laboratories have developed home-brew assays based on PCR followed by Sanger sequencing (Kristensen et al., 2014). Currently, this is the gold-standard method for D816V detection, despite its numerous limitations. The limitations are mainly related to the low sensitivity (10-15%) and the execution of the test,

## DISCUSSION

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which requires a long reaction time, along complex and multistep procedures that must be performed by specialized personnel with costly equipment.

In this study an alternative method for the analysis of the point mutation cKIT D816V, based on AS LAMP technology, has been developed. The AS LAMP assay is an improvement of LAMP technology, developed by DiaSorin in 2012. LAMP method amplifies DNA under isothermal conditions, employing the *Bst* DNA polymerase and a set of four primers that recognize six distinct regions on the target DNA. A pair of inner primers (FIP and BIP), constituted by two portions (F1c and F2; B1c and B2), respectively complementary to sense and antisense target sequence, initiates LAMP. A pair of outer primers (F3 and B3) then displaces the amplified strand with the help of *Bst* DNA polymerase which has a high displacement activity, to release a single-stranded DNA, which then form a stem-loop structure that initiates the amplification process.

In this study, LAMP technology has been implemented with two elements that play a key role in the detection of point mutation D816V in cKIT gene: a backward loop primer (LB) specific for the mutated cKIT sequence and a blocker that binds exclusively to the wild type gene. While the blocker suppresses the non-specific amplification of wild type alleles, the LB primer efficiently recognizes the mutated codon and allows its selective amplification. Thanks to the cooperation between LB and blocker, the AS LAMP assay is able to discriminate the mutated cKIT sequence with



## **DISCUSSION**

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high sensitivity and specificity, even though it differs from wild type by just one oligonucleotide.

AS LAMP assay has several advantages if compared to the conventional PCR-based methods.

AS LAMP reaction is performed under isothermal conditions employing a DNA polymerase with strand displacement activity; therefore there is no requirement for cycling, that in conventional PCR is necessary for DNA denaturation, primer annealing and elongation. This characteristic allows AS LAMP to reach the final result in a very short time and to use simple and cost-effective equipment.

AS LAMP is a highly specific reaction as the use of four different primers, recognizing six distinct regions on the target sequence, confers AS LAMP a higher specificity than PCR, which conventionally uses only two primers.

Another great advantage of AS LAMP is the ability to detect more than one target simultaneously. This has allowed the development of a duplex reaction that includes the amplification of the housekeeping ABL gene as internal control. The internal control has a crucial role for the exclusion of false-negative results due to the failure of the DNA extraction procedure. Therefore, it controls that the reaction conditions (temperature, pH) are correct and verifies the absence of inhibitors which might interfere with the DNA amplification. In the duplex AS LAMP assay the target and internal control amplification occurs simultaneously in a single tube. This represents a novel improvement if compared to PCR-based methods, which amplifies

## **DISCUSSION**

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target and control in parallel in two separate tubes, making the procedure more laborious and expensive.

In AS LAMP assays the target and IC are detected in real time in 500 nm and 530 nm channels respectively using the Liaison lam instrument (DiaSorin), due to the presence of two labeled probes specifically binding cKIT or ABL genes. This detection system eliminates the need for gel electrophoresis or other post-amplification detection steps that require opening of the reaction tubes. This closed tube system is advantageous as it prevents laboratories contamination due to amplicon carryover.

Because of the high amplification efficiency, the AS LAMP reaction is completed in only 40 minutes as opposed to 6-7 hours required for the home-brew method. Moreover, as previously described, it allows to complete amplification and detection in a single step further reducing time to result.

In conclusion, the AS LAMP assay greatly improves the cKIT D816V mutational analysis that, as recently highlighted by NCCN, is essential to allow the correct risk stratification of CBF AML patients and to provide important therapeutic information. AS LAMP cKIT D816V assay has demonstrated to be a reliable diagnostic tool thanks to its rapidity, easy set-up and lower cost compared to current traditional methods. The absence of commercial assay in the market further highlights the potential of this new product that could successfully substitute the current methods, which are inadequate to clinical needs in different aspects previously described

## **DISCUSSION**

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(Notomi et al.; 2015; Spinelli et al., 2014; Guan L et al. 2014). Future plans include the employment of AS LAMP technology for the detection of different point mutations involved in the risk stratification of leukemias. This is an evolving field in diagnostics and AS LAMP represents an innovative and reliable solution for future clinical needs.



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