Università degli Studi di Milano-Bicocca
Dipartimento di Biotecnologie e Bioscienze
Dottorato di ricerca in Biotecnologie Industriali
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Development of an innovative molecular assay for the simultaneous detection of the BCR-ABL Major and Minor fusion transcripts by the use of Loop Mediated Isothermal Amplification reaction (Q-LAMP)

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Ringraziamenti

"Quanto più già si sa, tanto più bisogna ancora imparare. Con il sapere cresce nello stesso grado il non sapere, o meglio il sapere del non sapere"

Nietzsche

La via del sapere è infinita , il viaggio è lungo e non esiste meta che possa soddisfare la nostra mente...ringrazio ***Giulia*** per avermi dato questa consapevolezza e per avermi spinto a ricominciare il viaggio.
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Il lavoro presentato in questa tesi è stato realizzato presso i laboratori di Diagnostica Molecolare DiaSorin, sotto la supervisione della Dott.ssa Giulia Minnucci e del Dott. 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 (vedi pag. 143).

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RIASSUNTO

Il progetto di dottorato illustrato nella presente tesi è volto allo sviluppo di un test diagnostico molecolare innovativo, basato sulla tecnologia RT-QLAMP® (Retro Transcription-Loop Mediated Isothermal Amplification), che permette la simultanea rilevazione dei due principali trascritti di fusione di BCR-ABL, p210 e p190, associati rispettivamente alla leucemia mieloide cronica e alla leucemia linfoblastica acuta.

Le leucemie sono patologie caratterizzate da una proliferazione neoplastica incontrollata di cellule staminali ematopoietiche. Il gene di fusione BCR-ABL codifica per una proteina tirosin-chinasica oncogenica responsabile della trasformazione neoplastica osservata nella leucemia mieloide cronica (LMC) e nella leucemia acuta linfoblastica (LAL). L'isoforma BCR-ABL p210 è il segno distintivo della LMC, rilevabile in più del 95% dei casi, mentre nelle LAL BCR-ABL positive, il gene di fusione può presentare entrambe le isoforme p190 (60% dei casi) e p210 (40% dei casi) (Cimino et al., 2006).
La proteina di fusione BCR-ABL possiede una attività tirosin chinasica costitutivamente attivata e delocalizzata rispetto alla controparte normale. La deregolazione e la delocalizzazione dell'attività chinasica portano all'attivazione inappropriata di vie di trasduzione del segnale che si traducono in forti stimoli proliferativi e antiapoptotici responsabili della trasformazione cellulare.
Il gene di fusione BCR-ABL è il risultato della traslocazione reciproca tra il cromosoma 9 e il cromosoma 22 t(9;22), responsabile della formazione del cromosoma aberrante Philadelphia. Il punto di rottura sul cromosoma 9

avviene in corrispondenza dell'esone 2 del gene ABL, mentre i punti di rottura sul cromosoma 22 possono avvenire in diverse posizioni sul gene BCR. I diversi possibili punti di rottura sul cromosoma 22 determinano la creazione di isoforme BCR-ABL differenti. Le isoforme più frequenti sono le cosiddette p210 Mbcr (Major breakpoint cluster region) e p190 mbcr (minor breakpoint cluster region) cui fanno seguito altre forme rare come la p230 µbcr (micro breakpoint cluster region) (Pane F et al., 2002).
Grazie alla disponibilità di farmaci molecolari (inibitori della tirosin-chinasi), che sono in grado di inattivare selettivamente la proteina chimerica BCR-ABL, la gestione delle leucemie Philadelphia positive è drasticamente migliorata nel tempo (Mediterr J et al., 2014).

Il rilevamento molecolare dei trascritti BCR-ABL è essenziale per diagnosticare la LMC e la LAL Philadelphia positiva, consentendo così di somministrare la terapia corretta (Jabbour et al., 2014; Baccarani et al., 2013). Infine, la discriminazione dell'isoforma, possibile esclusivamente con metodi molecolari, agevola la scelta del test quantitativo specifico per il monitoraggio molecolare durante il trattamento.
Ad oggi le tecniche molecolari più diffuse per la rilevazione dei trascritti sono basate sulla metodica di RT-PCR (Reverse Transcription-Polymerase Chain Reaction). Si tratta di un metodo con un tempo di esecuzione elevato e costituito da numerosi passaggi , retro trascrizione - amplificazione - rilevazione su gel, che devono essere eseguiti da personale specializzato e in laboratori attrezzati. Il rischio di cross-contaminazione dovuto al carattere multistep della tecnica e l'assenza di un controllo interno di

reazione possono inoltre portare alla generazione di risultati falsi positivi o falsi negativi (Dongen et al., 1999).

Lo scopo di questa tesi è stato quello di sviluppare un nuovo saggio diagnostico basato su una metodica non PCR in grado di rilevare in un unico passaggio, partendo direttamente dall' RNA, i principali trascritti di fusione BCR-ABL in modo rapido, specifico e molto sensibile. Questo metodo rappresenta una valida alternativa alle metodiche convenzionali, in quanto ne supera le principali limitazioni e viene incontro ai bisogni espressi dai clinici.

La tecnologia LAMP, scelta per lo sviluppo del nuovo saggio, è caratterizzata dalla capacità di amplificare le sequenze di DNA/RNA target in condizioni isoterme grazie all'utilizzo in reazione di una DNA polimerasi termostabile dotata di attività strand-displacement. L'assenza di cicli termici permette alla reazione di amplificazione di avvenire in tempi molto più brevi rispetto alla PCR conferendo alla metodica la caratteristica velocità.

Per la rilevazione di ogni target è necessario il disegno di 6 diversi primers che riconoscono in maniera specifica 8 regioni distinte del trascritto di interesse , inoltre l'utilizzo di sonde marcate con fluorocromi permette l'amplificazione e la rilevazione in real-time del segnale in un'unica provetta, evitando passaggi di separazione su gel e diminuendo in questo modo il rischio di cross-contaminazione del campione. La tecnologia LAMP rappresenta quindi un semplice, rapido e specifico metodo per lo sviluppo di nuovi saggi diagnostici, grazie anche alla facilità di esecuzione e al basso costo della strumentazione richiesta.

RIASSUNTO

La fase iniziale dello sviluppo è stata incentrata sul disegno di numerosi set di primers necessari per l'amplificazione specifica dei trascritti p210 e p190. Inizialmente la selezione dei primers e la loro ottimizzazione è stata effettuata con l'utilizzo di plasmidi contenenti la regione di trascritto di interesse.

Le condizioni di reazione LAMP sono state ottimizzate in modo da ottenere due saggi in simplex specifici e molto sensibili capaci di rilevare anche basse dosi di trascritto.

Per superare una delle principali limitazione della tecnica standard, si è resa necessaria l'introduzione in reazione di un controllo interno.

Il gene di controllo, rappresentato da un gene housekeeping, permette la valutazione della qualità del campione di RNA testato e la sua amplificazione permette di validare il risultato dei campioni negativi.

Numerosi geni sono stati valutati in letteratura come possibili candidati. Le caratteristiche prese in considerazione durante la selezione sono state l'assenza di pseudogeni, la localizzazione cromosomica e il livello di espressione genica.

Tre geni sono stati alla fine selezionati (RARα, ABL e GUSβ) e per ognuno di questi sono stati disegnati e selezionati, con l'utilizzo di un prototipo di software per il disegno LAMP creato da Diasorin, vari set di primers. Il disegno è stato incentrato su diverse regioni di ogni gene e i differenti set generati sono stati selezionati e ottimizzati in modo da garantire un'amplificazione specifica ma tardiva del controllo interno rispetto all'amplificazione del target.

L'amplificazione del controllo interno successiva a quella dei campioni positivi, permette di evitare una competizione durante la reazione favorendo la rilevazione anche di basse dosi di trascritto.

Solo i set specifici per GUSβ si sono rivelati ideali per essere utilizzati alle condizioni di reazione precedentemente ottimizzate per l'amplificazione dei target p210 e p190, ed è stato quindi scelto questo gene come controllo interno della reazione LAMP.

Un altro importante obbiettivo di sviluppo è stato quello di ottimizzare il saggio in modo che le reazioni di retro trascrizione e amplificazione avvenissero nella stessa provetta, permettendo al saggio di amplificare i trascritti di fusione direttamente a partire da RNA e garantendo maggiore rapidità di esecuzione rispetto alla PCR. Inoltre l'assenza di più passaggi di reazione diminuisce notevolmente i rischi di cross-contaminazione del campione.

A questo scopo sono stati presi in considerazione numerosi enzimi: tre specifici per il passaggio di retro trascrizione, due dotati di attività strand-displacement per lo step di amplificazione e un enzima ingenierizzato internamente da Diasorin in grado di svolgere entrambe le funzioni.

Le prove sono state condotte a partire da RNA estratto da linee cellulari positive e negative per i due principali trascritti di fusione utilizzando i set di primers e le condizioni di reazione ottimizzate precedentemente su plasmide. L'approccio a un enzima si è rivelato molto efficace ed è stato quindi selezionato per lo sviluppo del saggio LAMP.

Avendo identificato il set di primers specifico per l'amplificazione del controllo interno e l'enzima candidato per ottenere la simultanea retro

trascrizione e amplificazione del campione a partire da RNA, l'obbiettivo successivo è stato quello di ottimizzare le reazioni LAMP dapprima in duplex, in modo da permettere la co-amplificazione all'interno della stessa reazione sia del trascritto p210 o p190 che del controllo interno, e successivamente in triplex, in modo da permettere la co-amplificazione all'interno della stessa reazione di entrambi i trascritti di fusione e del controllo interno.

La sensibilità analitica del saggio è stata valutata su diluizioni seriali di RNA positivo per p210 o p190 in RNA negativo per i due trascritti, mentre la sua specificità è stata testata sia su campioni di H_2O che su campioni di RNA negativo per i due trascritti.

Il saggio RT-QLAMP si è dimostrato essere altamente sensibile e specifico, in grado di rilevare la presenza del trascritto di fusione fino a livelli pari a 0.001% per p210 e 0.01% per p190 utilizzando la metà della quantità di RNA richiesta dalla PCR. Non è stata inoltre rilevata nessuna amplificazione aspecifica entro 60 minuti di reazione su un alto numero di replicati.

Queste performance sono state inoltre confermate utilizzando due diversi metodi di estrazione dell'RNA comunemente utilizzati nei principali laboratori oncoematologici; estrazione con l'utilizzo del kit commerciale Qiagen RNeasy Mini kit (Qiagen) ed estrazione manuale con l'utilizzo di fenolo e cloroformio (TRIzol®).

Per assicurare una maggiore stabilità del prodotto e permetterne la conservazione e il trasporto in modo pratico ed economico, il formato ideale del saggio è di tipo liofilo. Per garantire le performance analitiche del

saggio anche nel diverso formato si è resa necessaria una ri-ottimizzazione delle condizioni di reazione.

E' stato inoltre condotto uno studio sui potenziali inibitori della LAMP derivanti dal processo di estrazione del campione. I test sono stati condotti aggiungendo il potenziale inibitore, a concentrazioni note per essere inibenti per PCR, all'interno del lisato cellulare o dell'RNA già estratto. Tra le sostanze testate vi sono l'eparina, l'emoglobina, le IgG, lattoferrina, Ficoll e fenolo. I risultati di questo studio mostrano come la LAMP non sia significativamente inficiata dai principali inibitori di PCR rendendo questa tecnologia molto più robusta e affidabile.

Per testarne il valore diagnostico e l'applicabilità nella pratica clinica il nuovo saggio è stato validato su campioni clinici positivi e negativi per i due trascritti analizzati con il metodo standard RT-PCR (forniteci dall'Ospedale Papa Giovanni XXIII di Bergamo) e su campioni di soggetti sani provenienti da banche sangue (forniteci dall'ospedale Humanitas di Milano).

I test sono stati effettuati utilizzando RNA estratto dai leucociti isolati dal sangue intero di 30 pazienti positivi per p210, 30 positivi per p190 e 30 donatori sani.

Tutti i campioni positivi sono stati correttamente identificati dal saggio LAMP e nessun falso positivo è stato riscontrato dall'analisi sui donatori sani. Inoltre durante la validazione clinica è stata confermata anche la robustezza del saggio in quanto sono stati amplificati correttamente anche campioni parzialmente degradati, presentanti concentrazioni inferiori a quella ottimale (<100 ng/μL) e caratterizzati da rapporti non ottimali 260/230 nanometri (<1.6). Il saggio mostra quindi una alta specificità,

robustezza e una concordanza del 100% con il metodo di riferimento (RT-PCR).

Per dimostrare la superiorità delle performance del nuovo saggio nella routine dei laboratori di diagnostica molecolare, il prototipo del saggio è stato testato in collaborazione con i tecnici di laboratorio degli ospedali Papa Giovanni XXIII di Bergamo, Sant'Orsola di Bologna e Policlinico Tor Vergata di Roma.

I test sono stati effettuati in parallelo con il metodo home-brew per la diagnosi di campioni all'esordio. I risultati ottenuti sono 100% concordanti con il metodo di riferimento. È stato rilevato un solo caso di discordanza (campione positivo per LAMP e negativo per PCR), in cui l'analisi è stata effettuata con l'uso di un terzo metodo diagnostico, la PCR quantitativa, dimostrando una bassa positività del campione.

Inoltre sono stati testati e rilevati correttamente tre campioni di follow up caratterizzati da livelli molto bassi di trascritto, rilevabili solo con PCR quantitativa. Questi casi dimostrano la maggior sensibilità del nuovo metodo rispetto alla tecnica di PCR comunemente utilizzata.

Concludendo, questa tesi illustra lo sviluppo e l'ottimizzazione di un innovativo test diagnostico molecolare basato sulla tecnologia RT-QLAMP® (Retro Transcription-Loop Mediated Isothermal Amplification) e la sua applicazione in campo diagnostico per la rilevazione simultanea dei trascritti p210 e p190 associati alla fusione genica BCR-ABL .

L'ottimizzazione è stata condotta sia su controlli plasmidici che su RNA estratto da linee cellulari ed il saggio finale è stato validato su campioni

clinici. La tecnologia LAMP si è dimostrata molto sensibile e specifica, semplice e rapida.

L'amplificazione e la rilevazione dei trascritti avvengono in real-time, in un'unica provetta e a partire direttamente da RNA.

I campioni clinici negativi sono validati grazie all'amplificazione del controllo interno di reazione e le possibilità di cross-contaminazione del campione sono drasticamente diminuite.

Inoltre il metodo si è dimostrato molto robusto essendo insensibile ai principali inibitori della PCR.

Il nuovo saggio diagnostico supera tutte le limitazioni della metodica diagnostica attualmente in uso e rappresenta un valido metodo alternativo per la diagnosi molecolare delle leucemie mieloidi croniche e linfoblastiche acute.

ABSTRACT

The PhD project presented in this thesis is focused on the development of an innovative molecular diagnostic test, based on RT-QLAMP® technology (Reverse Transcription Loop-Mediated Isothermal Amplification), which allows the simultaneous detection of the two main fusion transcripts of BCR ABL, p210 and p190, associated with the chronic myeloid leukemia and acute lymphoblastic leukemia.

Leukemias are diseases characterized by an uncontrolled proliferation of malignant hematopoietic stem cells. The BCR-ABL fusion gene encodes an oncogenic tyrosine kinase protein responsible for the neoplastic transformation observed in chronic myeloid leukemia (CML) and acute lymphoblastic leukemia (ALL). The isoform p210 is the hallmark of CML, detectable in more than 95% of cases, while in ALL BCR-ABL positive, the fusion gene may be present in both isoforms p190 (60% of cases) and p210 (40 % of cases) (Cimino et al., 2006). The fusion protein BCR-ABL has a constitutively activated tyrosine kinase activity, delocalized than the normal counterpart. Deregulation and delocalization of the kinase lead to an inappropriate activation of signal transduction pathways that results in strong proliferative and anti-apoptotic stimuli, responsible for the cell transformation.

The fusion gene BCR-ABL is the result of a reciprocal translocation between chromosome 9 and chromosome 22 t (9;22), responsible for the formation of an aberrant Philadelphia chromosome. The breakpoint on chromosome 9 takes place in correspondence of exon 2 of ABL gene, while the break points

on chromosome 22 can take place in different positions on the BCR gene. The different possible breakpoints on chromosome 22 determine the creation of BCR-ABL different isoforms. The most frequent isoforms are p210 MBCR (Major breakpoint cluster region) and p190 mBCR (minor breakpoint cluster region), followed by other rare isoforms such as the p230 µbcr (micro breakpoint cluster region) (Pane F et al., 2002). Thanks to the availability of molecular drugs (tyrosine kinase inhibitors), which are able to selectively inactivate the chimeric protein BCR-ABL, the management of Philadelphia positive leukemias is drastically improved over time (Mediterr J et al., 2014).

The molecular detection of BCR-ABL is essential to diagnose CML and Philadelphia positive ALL, making possible the administration of proper treatment (Jabbour et al., 2014; Baccarani et al., 2013). Finally, the discrimination of the isoform, only possible using molecular methods, facilitates the choice of the specific quantitative test for the molecular monitoring during the treatment.

To date, the most widely used molecular techniques for the detection of the transcripts are based on the RT-PCR method (Reverse Transcription-Polymerase Chain Reaction). It is a time-consuming procedure consisting of several steps, retro transcription - amplification - gel detection, which must be performed by skilled personnel and in equipped laboratories. The risk of cross-contamination, due to the multistep feature of the technique, and the absence of an internal reaction control, may also lead to false positive or false negative signals generation (Dongen et al., 1999).

ABSTRACT

The aim of this thesis was to develop a new molecular diagnostic method, based on a non-PCR technique, able to detect in a high sensitive, specific and rapid single step, starting directly from RNA, the two main BCR-ABL fusion transcripts. This method represent a valid alternative to the conventional methods, overcoming their main limitations and meeting clinical needs.

The LAMP technology, selected for the new assay development, is characterized by the ability to amplify sequences of target DNA / RNA under isothermal conditions thanks to the use of a thermostable DNA polymerase with strand-displacement activity. The absence of thermal cycles allows to have an amplification in a very short time compared to the PCR.

6 different primers, that specifically recognize 8 distinct regions of the transcript of interest, are necessary for the detection of each target in LAMP tests. Moreover the use of fluorescent labeled probes allows the amplification and the real-time signal detection in a single tube, avoiding gel separation steps and decreasing the risk of cross-contamination of the sample. The LAMP technology thus represents a simple, rapid and specific method for the development of new diagnostic assays, considering also its simplicity of execution and the low cost of the required instrumentation.

The initial phase of the assay development has been focused on the design of several primer sets required for the specific amplification of p210 and p190 transcripts. Initially the selection and optimization of the primers has been carried out with the use of plasmids containing the region of interest of the specific transcript. The LAMP reaction conditions were optimized in

order to obtain two specific and very sensitive simplex assays able to detect even very low doses of transcript.

To overcome one of the major limitation of the standard technique, it was necessary the introduction of an internal reaction control.

The control gene is represented by an housekeeping gene and allows the evaluation of the tested RNA sample quality. The control gene amplification in negative samples is very important to validate the result.

Numerous genes have been evaluated in the literature as possible candidates. The main features taken into consideration during the selection were the absence of pseudogenes, the chromosomal location and the level of gene expression. Three genes have been finally selected (RARα, Able GUSβ) and for each gene were designed and selected various primer sets thanks to the use of a software prototype for LAMP primer design created by DiaSorin. The design was performed on different regions of each gene and all the generated primer sets were selected and optimized in order to ensure a specific amplification of the internal control. The internal control amplification is delayed respect to the target one in order to avoid competition during the reaction, promoting in this way the detection of even small doses of transcript.

Only GUSβ primer sets have proved to be optimal for the use under the reaction conditions previously optimized for the target p210 and p190 amplification. GUSβ has been therefore selected as internal control of the LAMP reaction.

Another important development objective was to optimize reverse transcription and amplification in the same tube, allowing the fusion

transcripts amplification directly from RNA and ensuring faster execution than the PCR. Moreover, the absence of multiple reaction steps significantly decreases the risks of cross-contamination of the sample. For this purpose numerous enzymes have been evaluated: three enzymes specific for the reverse transcription step, two enzymes presenting strand-displacement activity for the amplification step and an enzyme engineered by DiaSorin presenting both functions.

The tests were performed starting from RNA extracted from BCR-ABL positive and negative cell lines, using primer sets and reaction conditions previously optimized on plasmids. The one enzyme approach has proven to be very effective and it was selected for the LAMP assay development.

After the internal control implementation and the selection of the candidate enzyme, the next objective was to optimize the LAMP reaction first in a duplex format, to allow the co-amplification within the same reaction of both p210 or p190 transcripts and the internal control, and finally in a triplex format, to allow the co-amplification within the same reaction of both fusion transcripts and the internal control.

The analytical sensitivity of the assay was evaluated on serial dilutions of positive p210 or p190 RNA into negative RNA for the two transcripts, whereas the specificity was tested on both water samples and on negative RNA samples.

The assay RT-QLAMP has proved to be highly sensitive and specific, able to detect the presence of the fusion transcript down to 10^{-4} dilution level for p210 and p190, using half of the RNA amount required by PCR. Moreover

no unspecific amplification was detected within 60 minutes on a high number of replicates.

These performances have been also confirmed by using two different RNA extraction methods commonly used in the major onco-hematology laboratories; the commercial Qiagen RNeasy Mini Kit (Qiagen) and the manual phenol-chloroform extraction method (TRIzol).

To ensure greater product stability and to allow the storage and transport in a practical and affordable way, we decided to move from the liquid format to the lyophilized format. In order to maintain the same analytical performances of the assay, in freeze-dried format , it was necessary an additional work, optimizing again the reaction conditions.

To evaluate the robustness of the technology, BCR-ABL Q-LAMP assay was tested in the presence of the most common PCR inhibitors found in literature. The tests were conducted by adding the potential inhibitor to cell lysate or extracted RNA using concentrations known to be inhibitory for the PCR. The tested inhibitors were heparin, hemoglobin, IgG, lactoferrin, ficoll and phenol, carried over from cell isolation, and genomic DNA, isopropanol, ethanol and guanidine isothiocyanate, carried over from RNA extraction process. The results of this study showed that LAMP is not significantly affected by the main inhibitors of PCR rending this technology much more robust and reliable. Moreover the assay was tested in the presence of non optimal RNA samples concentration, 10 fold lower than the optimal one, showing the ability to detect correctly and at very similar threshold time values all the target transcripts (GUSβ, p190 and p210).

These data together proved the very high robustness of the LAMP amplification method.

To test the diagnostic value and the applicability in clinical practice, the new assay was validated on BCR-ABL positive clinical samples (provided from Papa Giovanni XXIII Hospital of Bergamo) and negative samples of healthy blood donors (provided from the Humanitas hospital of Milan). All the clinical samples were previously analyzed by the standard RT-PCR method.

The tests were performed using RNA extracted from leukocytes isolated from whole blood of 30 patients positive for p210, 30 patients positive for p190 and 30 healthy donors.

All positive samples were correctly identified by LAMP assay and no false positive results were detected on healthy samples. During the clinical validation the very high robustness of the assay was confirmed due to the ability to amplify correctly even partially degraded samples, samples having concentrations less than optimal (<100 ng/uL), and samples presenting not optimal spectrophotometric absorbance ratios (260/230 nanometers <1.6). The assay showed a high specificity, robustness and a 100% agreement with the reference method (RT-PCR).

To demonstrate the superiority of the performance of the new assay in the routine molecular diagnostic, the prototype of the assay was tested directly by the laboratory technicians of the most important onco-hematology centers in Italy : hospital Papa Giovanni XXIII of Bergamo, hospital Sant'Orsola of Bologna and hospital Policlinico Tor Vergata of Rome . The tests were carried out in parallel with the home-brew method for the diagnosis of samples at the disease onset. The results obtained were 100%

concordant with the reference method. It was found only one discordant sample (the sample was detected positive by LAMP and negative by PCR), in this case the analysis was carried out with the use of a third diagnostic method, the quantitative PCR, demonstrating a low positivity of the sample. They have also tested and successfully detected three follow-up samples characterized by very low levels of transcript, detectable only by PCR. These cases demonstrate the greater sensitivity of the new method respect to the commonly used PCR technique.

In conclusion, this thesis describes the development and optimization of an innovative molecular diagnostic test based on RT-QLAMP® technology (Retro Transcription Loop Mediated Isothermal Amplification) and its application in the diagnostic field for the simultaneous detection of BCR-ABL p210 and p190 transcripts. The optimization was performed on both plasmid controls and RNA extracted from cell lines. Moreover the final assay was validated on clinical samples. The LAMP technology has proven to be very sensitive and specific, simple and rapid. The amplification and detection of the transcripts occur in real-time, inside a single tube and starting directly from RNA. The negative clinical samples were validated by the amplification of the internal control and the possibility of cross-contamination of the sample was dramatically decreased. In addition, the method has proved to be very robust due to the insensitivity to the major PCR inhibitors.

The new diagnostic assay overcomes all the limitations of the currently used diagnostic methods and represents a valid alternative for the

molecular diagnosis of chronic myeloid leukemia and acute lymphoblastic leukemia.

INTRODUCTION

1. The BCR-ABL translocation

The history of the hybrid BCR-ABL gene started in 1960, when an abnormal, shortened chromosome 22, termed the Philadelphia chromosome, was described in the leukemic cells of a patient affected by chronic myeloid leukemia (Nowell PC and Hungerford DA, 1960).

At molecular level, the Philadelphia chromosome comes from a balanced gene translocation resulting in the juxtaposing of the 5' part of the BCR gene to the 3' part of the ABL gene. It is one of the most frequent chromosomal abnormalities found in chronic myeloid leukemia (CML) and in acute lymphoid leukemia (ALL) (Groffen J et al., 1984).

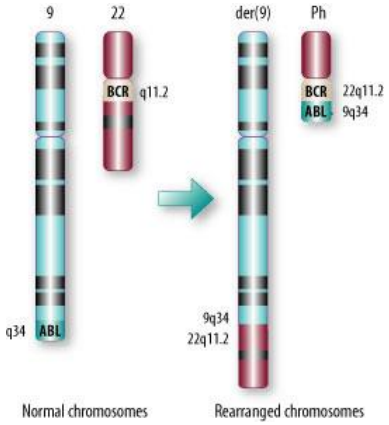


Figure 1: Generation of the Philadelphia (Ph) chromosome via breakage and reunion of chromosomes 9 and 22 at bands 9q34 and 22q11.2, respectively

The juxtaposition of the ABL oncogene from 9q34 and the BCR locus from 22q11.2 results in a chimeric gene that expresses a chimeric BCR-ABL messenger RNA and fusion protein.

The c-ABL proto-oncogene is located on chromosome 9 band q34 (Heisterkamp N et al., 1983). It is highly conserved during phylogenesis, and encodes for a protein ubiquitously expressed in all mammalian tissues and cell types examined (Rosenberg N et al., 1988). c-ABL is the normal cellular homolog of V-ABL, the transforming oncogene of Abelson Murine Leukemia virus, in which part of c-ABL is fused with murine retroviral gag sequences (Goff SP et al., 1980; Abelson HT et al., 1970).

Cloning of the human c-ABL has shown that it spans approximately 230 kb, contains 11 exons and it is oriented with the 5' end toward the centromere (Shtivelman ET et al., 1985). The gene has two alternative first exons, exon la and exon lb, that are separated by the first intron, a very long region of more than 200 kb (Bernards A et al., 1987). The alternative splicing of these two 5' exons generates two different c-ABL mRNAs: a 6 kb transcript, termed la, including exon la through 11, and a 7 kb transcript, termed lb, that begins with exon lb, skips exon la and the first intron and joins to exon 2 through 11 (Ben-Neriah Y et al., 1986).

The two ABL mRNA transcripts produce two distinct ABL proteins with the same molecular mass of 145 kd (P145), but distinct N-terminal depending on whether the la or lb first exon is included (Shtivelman E et al., 1986; Franz WM et al., 1989).

c-ABL is a member of the family of non receptor protein-tyrosine kinases of which src is the prototype. In addition to the kinase domain, this gene

family shares other domains called SH2 and SH3 (src homology 2 and 3) that are important in the interaction and regulation of proteins involved in signal transduction (Ben-Neriah Y et al., 1986).

A unique feature of c-ABL is the presence of a large carboxi-terminal non catalytic domain that contains sequences important for DNA and cytoskeleton binding and a region involved in nuclear translocation signaling (Daley GQ and Ben Neriah Y, 1991). P145, like others tyrosine kinases, may be involved in control of cell growth and differentiation (Konopka JB and Witte ON, 1985).

The BCR gene is located on chromosome 22q11.2, spans 130 kb, contains 21 exons and is oriented with the 5' toward the centromere (Heisterkamp N et al., 1985).

Two different BCR mRNAs are transcribed: a 4.5 kb transcript and a 6.7 kb transcript [38]. They are translated into a ubiquitously expressed protein with a molecular mass of 160 kd (P160 BCR) and kinase activity (Stam K et al., 1987).

The carboxi-terminal portion of P160 BCR has been shown to be associated with GAP activity for the RAS related GTP binding protein P21 (Diekmann D et al., 1991). This finding could have new important implications for the role of BCR in leukemogenesis.

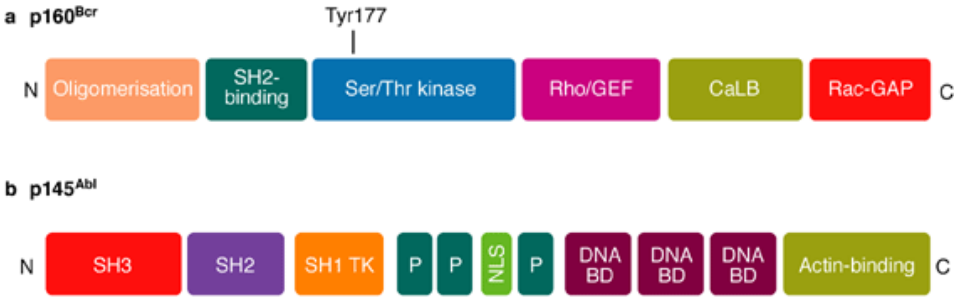


Figure 2: The c-Bcr and c-Abl proteins

Structural motifs have been highlighted in p160Bcr and p145Abl proteins. (a) c-Bcr comprises an oligomerisation domain, a domain thought to mediate binding to Src-homology 2 (SH2)-domain-containing proteins, a serine/threonine kinase domain, a region with homology to Rho guanine-nucleotide-exchange factor (Rho-GEF), a region thought to facilitate calcium-dependent lipid binding (CaLB) and a region showing homology to Rac GTPase activating protein (Rac-GAP). Bcr is phosphorylated on many tyrosine residues, notably Tyr177, which when phosphorylated mediates the binding of the adaptor molecule Grb2. (b) c-Abl comprises an SH3 and SH2 domain, an SH1 tyrosine kinase (TK) domain, several proline-rich domains (P), a nuclear localization signal (NLS), several DNA-binding domains (DNA BD) and an actin-binding domain.

2. The BCR-ABL fusion gene

The BCR-ABL gene is a hybrid gene formed on chromosome 22 by the juxtaposition of BCR and c-ABL sequences. Depending on chromosomal breakpoint locations, different parts of these two genes may be included in the oncogenic fusion gene (Pane F et al., 2002).

Breakpoints on the BCR gene are usually clustered in three well defined regions:

- ***The major breakpoint cluster region (M-BCR)*** results in two different kind of junctions: e13a2 (formerly b2a2) and e14a2 (formerly b3a2) giving rise to two BCR-ABL hybrid genes encoding two chimeric 210 kDa proteins (P210) differing for the 25 aminoacids codified by exon 14.

This type of transcript is detectable in:

- more than 95% of Cronic Myeloid Leukemia (CML) cases (Faderl S et al., 1999)
- about 30-40% Ph positive adult acute lymphoid leukemias (ALLs) (Gleissner B et al., 2002)
- 15% Ph positive ALL childhood cases (National Cancer Institute, 2010)
- very rare (<2%) in Acute Myeloid Leukemias (AMLs)

- ***The minor breakpoint cluster region (m-BCR)*** results in an e1a2 junction, which is translated into a smaller 190 kDa protein (P190).

The transcript is detectable in:

- 50-70% Ph positive adult acute lymphoid leukemias (Gleissner B et al., 2002)
- 85% Ph positive ALL childhood cases (National Cancer Institute, 2010)
- rarely detected in CML patients: it appears during the blastic phase of the disease in association with p210

- ***The micro breakpoint cluster region (μ-BCR)*** correspond to an e19e2 junction which is translated into a 230 kDa protein (P230) and it is associated with neutrophilic-CML. Progenitor cells expressing the p230 oncoprotein retain the capacity to progress through the normal stages of granulocytic differentiation and show neutrophilia. (Nowell PC and Hungerford DA, 1960; Uppal G and Gong J, 2015). Neutrophilic-chronic myeloid leukemia (CML-N) is a rare myeloproliferative disorder in which the large majority of circulating myeloid cells consist of mature granulocyte. Clinically, the disease is much more benign than 'classical' CML.

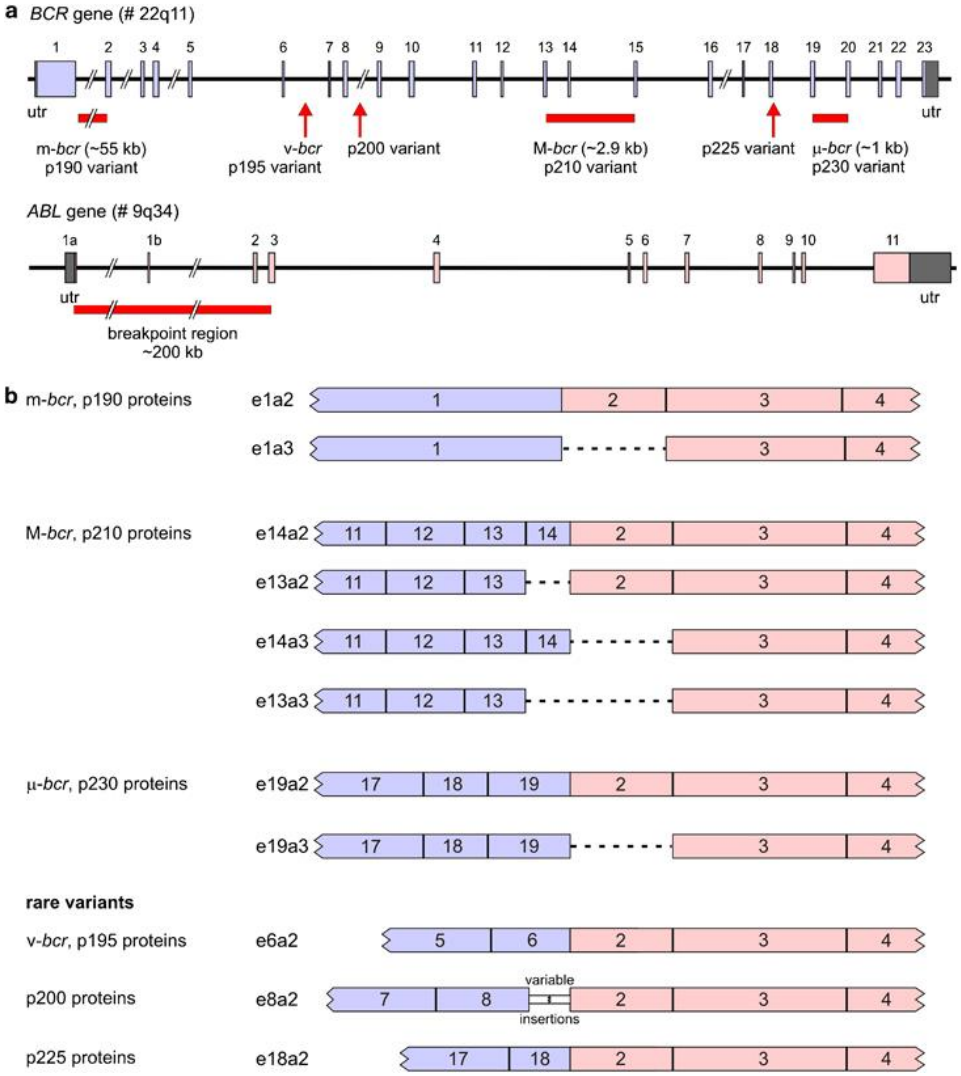


Figure 3: Translocation t(9;22)(q34;q11) and fusion products

(a) The ABL gene contains one large breakpoint region (~ 200 kb), whereas three breakpoint regions have been found in the BCR gene: m-bcr, M-bcr and -μbcr, which are associated with the p190, p210 and p230 BCR–ABL fusion proteins. In addition

several rare breakpoints have been reported (see arrows), including the v-bcr breakpoint. (b) The three well-defined breakpoint regions in the BCR gene can produce at least eight different fusion transcripts, because of alternative splicing in the ABL gene (splicing to exon 2 or exon 3) and because the M-bcr consists of two intronic regions (intron 13 and intron 14). Additional BCR–ABL fusion transcripts have been described, but they are rare or only reported once.

3. The BCR-ABL fusion protein

All forms of BCR-ABL fusion proteins display an increased and deregulated tyrosine kinase activity and the p190 form has been shown to have more transforming potential than p210.

Different functional domains of BCR-ABL seems to be involved in the transformation process. Activation of the tyrosine-kinase activity of c-ABL has been shown to be due by interaction of the coiled-coil motif encoded by the first BCR exon with the SH2 domain of ABL (Muller AJ et al., 1991; Pendergast AM et al., 1991).

The two adjacent BCR-ABL molecules phosphorylate their respective partners on tyrosine residues. The uncontrolled kinase activity of BCR-ABL then usurps the physiological functions of the normal ABL enzyme by interacting with a variety of effector proteins leading to a deregulated cellular proliferation, decreased adherence of leukemia cells to the bone marrow stroma and reduced apoptotic response to mutagenic stimuli.

The tyrosine kinase encoded by the Src-homology 1 (SH1) domain of the ABL component of BCR-ABL is the most crucial for oncogenic transformation.

Fusion of BCR sequences to ABL may also be important in changing the intracellular distribution of BCR-ABL. Normal c-ABL binds DNA and actin thanks to its C-terminal nuclear localization signal (NLS) and it is distributed in both the nucleus and cytoplasm. BCR is distributed cytoplasmically and is involved in the maintenance of the cytoskeleton.

BCR-ABL protein has higher actin binding activity than normal c-ABL and accumulates in the cytoplasm. Disregulation of actin and subcellular localization of BCR-ABL protein with cytoplasmic accumulation may be critical in altering normal cell growth and differentiation (McWhirter JR and Wang JYJ, 1991; McWhirter JR and Wang JYJ, 1993).

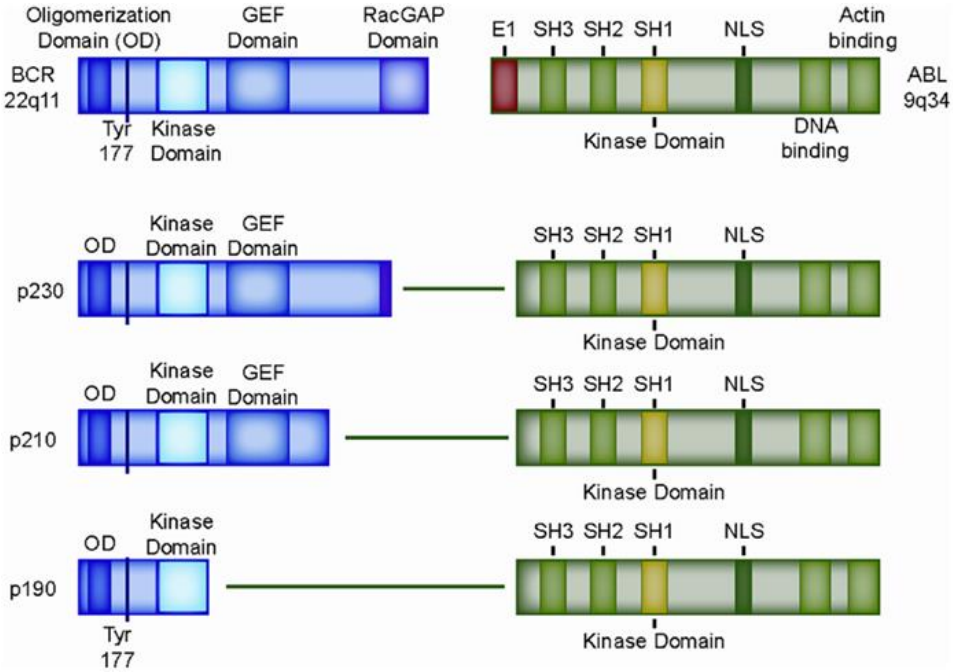


Figure 4: Structure of the most common BCR–ABL1 fusion genes *Domain structure of wild type BCR and wild type ABL1 protein, as well as retained domains in the three most common BCR–ABL1 variants, p230, p210, and p190. OD: oligomerization domain (coiled-coil domain) mediating oligomerization, Tyr177: tyrosine 177, which, when phosphorylated, serves as a docking site for the adaptor protein GRB-2; SH2-domain: SRC homology 2 (binding to phosphorylated tyrosine residues, including BCR exon 1), SH3-domain: SRC homology 3 (binding to proline rich peptides). SH1-domain: SRC homology 1 (ABL1 catalytic domain); GEF-domain: guanine nucleotide exchange factors (G-protein signaling); E1: exon 1 of ABL1, contains the inhibitory N-terminal "cap" that binds the catalytic domain (SH1) of ABL1 and prevents autophosphorylation; NLS: nuclear localization signal.*

4. Philadelphia positive leukemias

BCR–ABL is an active constitutive tyrosine kinase that activates multiple downstream signalling pathways resulting in the survival and proliferation of leukemic cells. The presence of this genetic alteration is considered a trigger element for Chronic Myeloid Leukaemia (CML) and a negative prognostic factor for Acute Lymphoblastic Leukaemia (ALL) (Pane F et al., 2002; Faderl S et al., 1999).

The leukemias are a group of malignant disorders of haematopoietic stem cells characteristically associated with increased number of primitive white cells (called blasts) in the bone marrow. Uncontrolled growth of immature haematopoietic cells at the expense of normal marrow function results in bone marrow failure.

Clinically and pathologically, leukemias are subdivided into a variety of large groups. The first division is between acute and chronic forms:

- ***Acute leukemia*** is characterized by a rapid increase in the number of immature blood cells. Crowding due to such cells makes the bone marrow unable to produce healthy blood cells. Immediate treatment is required in acute leukemia due to the rapid progression and accumulation of the malignant cells, which then spill over into the bloodstream and spread to other organs of the body. Acute forms of leukemia are the most common forms of leukemia in children.
- ***Chronic leukemia*** is characterized by the excessive production of relatively mature, but still abnormal, white blood cells. Typically taking

months or years to progress, the cells are produced at a much higher rate than normal, resulting in many abnormal white blood cells. Whereas acute leukemia must be treated immediately, chronic forms are sometimes monitored before treatment to ensure maximum effectiveness of therapy. Chronic leukemia mostly occurs in older people, but can theoretically occur in any age group.

Additionally, the diseases are subdivided according to which kind of blood cell is affected. Leukemias can be divided into lymphoblastic or lymphocytic leukemias and myeloid or myelogenous leukemias:

- In ***lymphoblastic or lymphocytic leukemias***, the cancerous mutation takes place in hematopoietic precursors of lymphocyties. Most lymphocytic leukemias involve a specific subtype of lymphocyte, the B cell.
- In ***myeloid or myelogenous leukemias***, the cancerous mutation takes place in hematopoietic precursors of red blood cells, granulocyties, monocyties and platelets.

4.1. Chronic myeloid leukaemia (CML)

4.1.1. Epidemiology

Chronic myeloid leukaemia is a clonal haematopoietic stem cell disorder affecting about one individual per 100000 population per year with a slight male preponderance (1.7:1) , and accounts for 15% of all new cases of leukaemia in the Western hemisphere.

Before the development of targeted therapy with tyrosine kinase inhibitors (TKIs), the median survival was 5–7 years. More than 90% of the cases are diagnosed in chronic phase and the 50% of the diagnosis is occasional (Jabbour et al., 2014).

4.1.2. Clinical features

Chronic myeloid leukaemia is a triphasic disease: most patients present the chronic phase in which symptoms can be easily controlled, but without effective medical intervention it will progress through a period of increasing instability known as acceleration, to terminal transformation to an acute leukemic-like illness or so-called blast crisis.

One of the strongest pieces of evidence of the stem cell origin of chronic myeloid leukaemia is that the final transformation phase can result in both lymphoblastic (25%) and myeloblastic (50%) subtypes with a further 25% manifesting bi-phenotypic or undifferentiated phenotypes.

Damage to the bone marrow, by way of displacing the normal bone marrow cells with higher numbers of immature white blood cells, results in a lack of blood platelets, which are important in the blood clotting process. This means people with leukemia may easily become bruised, bleed excessively, or develop pinprick bleeds (petechiae).

White blood cells may be suppressed or dysfunctional. This could cause the patient's immune system to be unable to fight off a simple infection or to start attacking other body cells. Because leukemia prevents the immune system from working normally, some patients experience frequent infections, ranging from infected tonsils, sores in the mouth,

or diarrhea to life-threatening pneumonia or opportunistic infections. Finally, the red blood cell deficiency leads to anemia, which may cause dyspnea and pallor.

Some patients experience other symptoms, such as feeling sick, having fevers, chills, night sweats, feeling fatigued and other flu-like symptoms. Some patients experience nausea or a feeling of fullness due to an enlarged liver and spleen; this can result in unintentional weight loss.

If the leukemic cells invade the central nervous system, then neurological symptoms (notably headaches) can occur.

All symptoms associated with leukemia can be attributed to other diseases. Consequently, leukemia is always diagnosed through medical tests (Melo JV et al., 2003).

4.1.3. Diagnosis

At the onset more than a half of the patients doesn't present any symptoms and the disease is diagnosed only via a cell blood count analysis.

The CML diagnosis has to be confirmed by the detection of BCR-ABL translocation. Conventional cytogenetic occasionally fails, for technical reasons, in detecting BCR-ABL fusion gene. In this case it can be identified by fluorescent - in situ - hybridisation (FISH) using specific chromosome markers and by reverse transcription PCR (RT-PCR).

This extensive investigation not only confirms the diagnosis, but also allows disease staging and prognostic scoring. The definitions of acceleration and blast crisis are largely dependent on the proportion of blasts in the blood

and bone marrow, but vary in the two commonly used systems (WHO and European Leukaemia Net) (Jabbour E and Kantarjian H, 2012).

	WHO criteria[5]	European Leukaemia Net criteria[6]
Accelerated phase		
Blasts in peripheral blood or bone marrow	10–19%	15–29% or blasts plus promyelocytes in peripheral blood or bone marrow >30% with blasts <30%
Basophils in peripheral blood	≥20%	≥20%
Platelets	<100 x 10^9/L not attributable to treatment, or platelets >1000 x 10^9/L uncontrolled on treatment	<100 x 10^9/L not attributable to treatment
Additional chromosomal abnormalities	Occurring on treatment	Occurring on treatment
White cell count and spleen size	Increasing and uncontrolled on treatment	..
Blast crisis		
Blasts in peripheral blood or bone marrow	≥20%	≥30%
Blast proliferation	Extramedullary, except spleen	Extramedullary, except spleen
Large foci of blasts	Bone marrow or spleen	..

Table 1: Definitions of accelerated phase and blast crisis according to present classification systems

4.1.4. Treatment options

4.1.4.1. Historical

The first treatment of chronic myeloid leukaemia occurred in the 19th century with use of arsenic containing compounds (Conan-Doyle A, 1882). In the early 20th century, splenic irradiation reduced the degree of splenomegaly but was replaced in the 1960s by the alkylating agents, after

the first ever randomised study of chronic myeloid leukaemia, which showed improved survival in patients given busulfan (Witts LJ, 1968).

Later, there was recognition that busulfan did not always normalise the blood count, and, more importantly, that it might be mutagenic and induce blast crisis, so it was replaced by the ribonucleotide reductase inhibitor, hydroxycarbamide.

Busulfan and hydroxycarbamide improved the blood count and gave symptomatic relief, but they did not delay the onset of disease progression, which occurred at a median of 4–5 years after diagnosis. Cytogenetics showed that patients remained 100% Ph positive.

In the 1970s, two entirely different treatment strategies namely, interferon α and allogeneic stem cell transplantation, showed not only the achievement of Ph negativity, but also, and most importantly, that this was associated with prolonged survival (Bonifazi F et al., 2001).

Interferon α induced some level of Ph negativity in a significant proportion and complete cytogenetic remission in about 10–15% of patients (Talpaz M et al., 1986). Subsequently, several randomized clinical trials compared interferon α with busulfan, hydroxycarbamide, or both, and showed that interferon α improved median life expectancy to 6–7 years (Hehlmann R et al., 1994; Allan NC et al., 1995).

Interferon α is given subcutaneously and is accompanied by a range of side-effects that interfere with quality of life, and for many individuals long-term use was impossible. The addition of subcutaneous cytarabine to interferon α increased the proportion of patients achieving complete cytogenetic remission, and one study showed that this addition had a survival

advantage compared with interferon α alone, but was associated with increased toxicity. The mechanism of action of interferon α is poorly understood but the drug at least in part reflects a degree of immune modulation.

4.1.4.2. Allogeneic stem cell transplantation

Allogeneic stem cell transplantation for chronic myeloid leukaemia was first described in the context of syngeneic donors and later in sibling and volunteer unrelated donors (Hansen JA et al., 1998). Preparative myeloablation with total body irradiation or busulphan, followed by infusion of normal donor stem cells induce durable complete cytogenetic remission in most patients. Allogeneic stem cell transplantation resulted in long term survival and probable cure, particularly if the transplant was performed in chronic phase (Pavlu J et al., 2011).

Unfortunately, the associated transplant-related mortality restricted transplant to younger patients and those with fully HLA-matched donors. In the 1980s, removal of donor T lymphocytes from the transplant product before infusion reduced the incidence and severity of the alloimmune-mediated graft versus host disease. Although highly successful in the short term, this approach increased the risk of relapse, which is direct evidence of the long-suspected graft versus leukaemia effect. The cure induced by transplant was not only due to the use of high dose chemo-radiotherapy, but also to the ongoing surveillance and destruction of residual leukemic cells by the donor derived immune system. The risk of further graft versus

host disease was largely abrogated by the sequential use of graduated doses of donor lymphocyte infusions.

By the 1990s, allogeneic stem cell transplantation was first line treatment for all eligible patients in first chronic phase and early acceleration phase. Intravenous combination chemotherapy, normally reserved for the treatment of acute leukaemia, was used to induce a second chronic phase before transplant in patients with blast crisis.

Since the advent of TKIs, the numbers of transplants done for chronic myeloid leukaemia have greatly declined: one of the major barriers to early transplant had been the fear of transplant related mortality and a trial of an oral targeted drug seemed much preferable to allo-grafting. For most patients, this trust in TKIs was justified and allogeneic stem cell transplantation became second line, then subsequently third or fourth line therapy, restricted to patients who had failed multiple TKIs, or whose disease had progressed. Recent data of regular molecular monitoring suggest that patients destined to fail TKIs can be recognised early, and this information might return allogeneic stem cell transplantation to an earlier time-point in the disease course (Apperley JF et al., 1986).

4.1.4.3. Tyrosine kinase inhibitors (TKI)

CML has a well defined molecular target and relatively selective therapies aimed at that target, which is not the case for the majority of cancers and chemotherapies today (Pane F et al., 2002). Bcr-Abl was regarded as highly attractive target for drug intervention since the Bcr-Abl fusion gene encodes a constitutively activated kinase.

Drug discovery that specifically targeted the ATP binding site of a single kinase was regarded as quite a challenging task since hundreds of protein kinases were known in the human genome (Faderl S et al., 1999). In the presence of TKI the binding of ATP is blocked, phosphorylation is prevented and Bcr-Abl expressing cells either have a selective growth disadvantage or undergo apoptotic cell death (Gleissner B et al., 2002; National Cancer Institute, 2010).

In 1996 was reported the first in-vitro data for the effect of the highly selective 2 phenylaminopyrimidine ABL1 TKI, then known as signal transduction inhibitor 571 (STI571), on chronic myeloid leukaemia cell lines. In a phase 1 study in advanced phase disease, STI571, now known as Imatinib, not only controlled blood counts and restored chronic phase, but also induced cytogenetic responses in a substantial proportion of patients (Druker BJ et al., 2001). A phase 2 study showed a high rate of complete cytogenetic remission in patients in chronic phase who had previously failed (absence of efficacy, intolerance, or both) interferon α (Kantarjian H et al., 2002).

Within 2 years of the phase 1 study, the IRIS study (international randomised study of interferon and cytarabine versus STI571) was completed in 1100 newly diagnosed patients, the results of which revolutionized the management of chronic myeloid leukaemia (O'Brien SG et al., 2003).

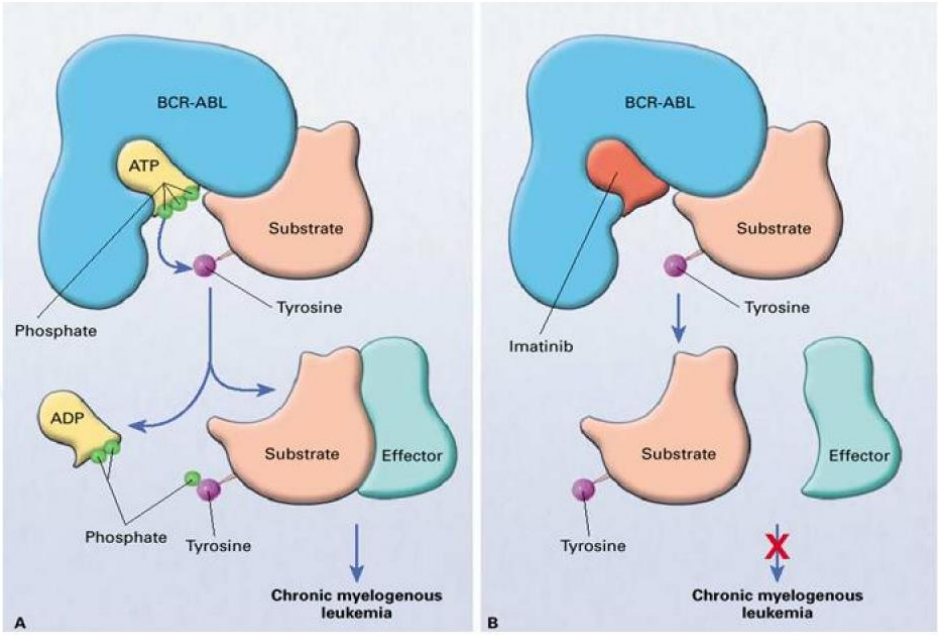


Figure 5: Imatinib mechanism of action

In the presence of TKI the binding of ATP is blocked, phosphorylation is prevented and Bcr-Abl expressing cells either have a selective growth disadvantage or undergo apoptotic cell death.

Shortly after the introduction of imatinib, investigators began to describe a number of in vitro derived cell lines with resistance to the drug. This was rapidly followed by the clinical description of Imatinib resistant cells in patients, which has resulted in efforts to better understand the biology behind these observations.

In general, Imatinib resistance can be subdivided into Bcr-Abl dependent and independent mechanisms. Bcr-Abl dependent mechanisms include over expression or amplification of the Bcr-Abl gene and point mutations within

the Bcr-Abl kinase domain. Bcr-Abl independent mechanisms include factors influencing the concentration of imatinib within the cell, for example by alterations in drug influx and efflux and activation of Bcr-Abl independent pathways, such as members of the Src kinase family. However, the more frequent mechanism seems to be the development of point mutations in the ABL kinase domain, which results in decreased binding of TKI.

Kinase domain mutations were first described in 11 patients in advanced phase who relapsed on imatinib; six of whom had an identical cytosine to thymidine mutation at ABL nucleotide 944 resulting in a single aminoacid change at position 315, later designated T315I (Gorre ME et al., 2001). So far more than 90 different nucleotide substitutions have been described, although 15 aminoacid substitutions account for more than 85% of the mutations observed in clinical practice (Apperley JF, 2007).

The functional relevance of these mutations is as yet unclear, in particular as to whether they contribute to disease progression (perhaps by conferring a growth advantage to affected cells) or are simply a surrogate marker of increased genomic instability associated with advanced disease. The mutations are not induced by TKI exposure but are selected through treatment to form an increasing proportion of the leukemic cells, finally manifesting as drug resistance and being relatively easily detected by direct sequencing. Attempts to detect mutations at diagnosis are of little value in chronic phase, of similarly little value is routine screening for mutations in patients who are responding well to treatment (MR^3, or complete cytogenetic remission). By contrast, mutation analysis is mandatory in any

patient with acquired resistance or who progressed to advanced phase disease, and can direct subsequent treatment.

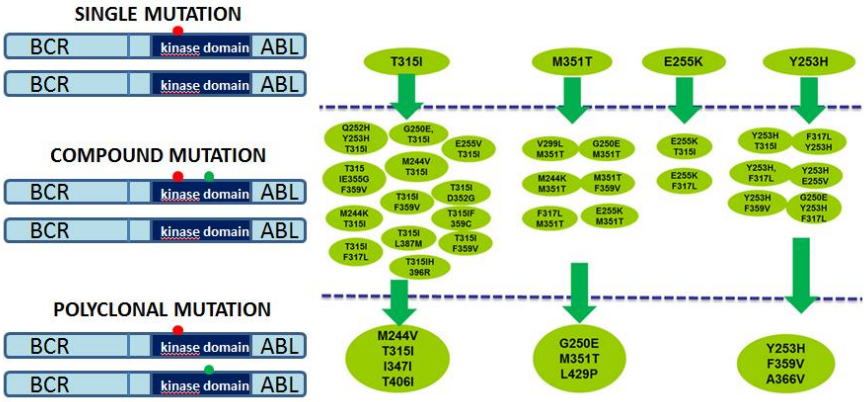


Figure 6: Frequent mutations of BCR–ABL gene

Legend: three common mutations were listed above including single mutation, compound mutation and polyclonal mutation. Single mutation means single missense mutation detectable in BCR–ABL molecule. Compound mutation refers to two or more missense mutations in the same BCR–ABL molecule. Polyclonal mutation is defined as two or more missense mutations in different BCR–ABL molecules. The arrow shows successive acquisition of mutations

4.1.4.4. Second generation TKIs

The identification of imatinib resistance led to a focused effort to develop additional TKIs with efficacy against kinase specific mutations. Dasatinib and nilotinib entered clinical trials within 12 months of each other and bosutinib 2 years later.

All the three drugs seem equally efficacious in the second line setting and only two criteria help to guide choice. First, a small number of kinase domain mutations are sensitive to one or other of the second generation

TKIs. These mutations emerged in the phase 2 studies in imatinib failure, either through absence of efficacy in patients with certain pre-existing mutations or by identification of new mutations at the time of resistance to second generation TKI (Müller MC et al., 2009; Hughes T et al., 2009).

For instance, patients with the imatinib-resistant F317L/V/I/C and G252H mutations are relatively insensitive to dasatinib and those with imatinib-resistant T253H, E255K/V, and F317L/V/I/C mutations are poorly sensitive to nilotinib. The V299L mutation, sensitive to nilotinib, was identified in patients resistant to, or relapsing on, dasatinib.

The second criterion is the toxicity profile of each of the second generation TKI. Pre-existing co-morbidities are clearly important in the selection of second and subsequent line drugs, but as always these decisions must be reached after balancing the risk of drug side effects against disease progression.

4.1.4.5. Third generation TKIs

Ponatinib is described as third generation because it is the only family member with activity against T315I. The phase 1 study showed impressive activity in a group of heavily pre-treated patients with 63% achieving complete cytogenetic remission (Cortes JE et al., 2012). The phase 2 study enrolled patients in all stages of disease and subdivided these according to the presence or absence of the T315I mutation. Although most patients had received two or more previous TKIs, the results were encouraging.

A phase 3 randomised study of ponatinib and imatinib in newly diagnosed patients began in 2013, but was closed a few months later because maturing data from the phase 2 study suggested an increased incidence of

arterial thrombotic events in patients given ponatinib (Cortes JE et al., 2013). For this reason further development of ponatinib as front-line treatment for chronic phase disease is now on-hold
pending further information about toxicity.

Name	Alternative name	Target	Year approved	Clinical complication	Side effect
Imatinib	Gleeve STI571 PDGFR	BCR–ABL c-KIT	2001	CML in CP, AP or BC after failure of interferon therapy	Nausea, skin rashes, vomiting, hepatotoxity, diarrhea, myalgia muslecramps,
			2003	Newly diagnosed CP	
Dasatinib	BMS-354825	BCR–ABL Src family	2006	CML in CP, AP, BC after resistance to or intolerance of imatinib	Myelosuppression, thrombocytopenia, constitutional complaints, electrolyte abnormalities, effusions, leukotrichia, reversible pulmonary hypertension
			2010	Newly diagnosed CP	
Nilotinib	AMN107 Tasigna	PDGFR BCR–ABL	2007	CML in CP or AP after resistance to or intolerance of imatinib	Nasopharyngitis, edema, nausea, pleural effusion, fatigue, rash, diarrhea, vomiting, abdominal pain and anorexia, asthenia, vascular and cardiac disease, acute pancreatitis
			2010	Newly diagnosed CP	
Bosutinib	SKI-606	BCR–ABL Src family	2012	CML in CP, AP or CP after resistance to or intolerance of prior CP	Self-limited diarrhea, rash, vomiting, anemia, thrombozytopenia, neutropenia,
Potinib	AP24534	BCR–ABL FTL3 Src family RET	2012	CML in CP, AP or CP after resistance to or intolerance of prior TKI CP	Myelosuppression, rash, fatigue, dose-related pancreatitis

Table 2: BCR–ABL inhibitors approved by FDA until 2012

4.1.5. Management of newly diagnosed patients

The choice of drug for newly diagnosed patients is one of the major dilemmas in chronic myeloid leukaemia, because imatinib, dasatinib, and nilotinib are all licensed for this indication. The outcome for patients treated with imatinib are well established through regular updates from the IRIS study (Druker BJ et al., 2006) together with the use of imatinib 400 mg daily as the standard arm of clinical trials
investigating increased imatinib doses, combinations of imatinib with interferon or cytarabine, or second generation TKIs . About 70–80% of patients will achieve durable complete cytogenetic remission (Cortes JE et

al., 2010; Hehlmann R et al., 2011; Preudhomme C et al., 2010; Larson RA et al., 2012; Steegmann JL et al., 2012).

However, long-term follow-up within IRIS reported imatinib discontinuation in 37% of patients at 5 years and 45% of patients at 8 years (Deininger M et al., 2009). Some patients discontinued because of commercial availability, but others because of primary or acquired resistance and adverse events.

Chronic low-grade debilitating side-effects lead to imatinib discontinuation because second generation TKIs can offer equivalent efficacy with reduced toxicity (Hochhaus A et al., 2012).

All three second generation TKIs have completed phase 3 randomised studies against imatinib. Their major benefit compared with imatinib is the speed of achievement of deep responses—ie, MR^3, MR^4, and $MR^{4.5}$—probably in a higher proportion of patients.

However, imatinib remains the most popular first line therapy, predominantly because of efficacy in the majority, but also because after more than 14 years experience, severe or late unexpected toxic effects have not occurred. By contrast, nilotinib and ponatinib are associated with an increased risk of vascular obstruction, and dasatinib might induce, albeit infrequently, pulmonary hypertension (Aichberger KJ et al., 2011; Kim TD et al., 2013; Montani D et al., 2012). Until these risks have been substantiated and quantified, restricting the

use of second generation TKIs for patients who are not responding optimally to imatinib or with high risk prognostic scores at diagnosis seems reasonable. A further contributing factor is cost; generic imatinib should be widely available within 2–3 years and this together with awareness that

patients not responding to imatinib can be recognised by RQ-PCR assays within 3–6 months of treatment, suggest first-line imatinib as the most cost-effective strategy.

4.1.6. Defining and monitoring responses to treatment

The first goal of treatment is normalisation of the blood count, ie. complete haematological remission. If, however, treatment is stopped at this point the blood count rapidly becomes abnormal so it is clear that the total tumour load has not been greatly affected. The second goal is therefore to deepen the response by achieving Ph negativity or complete cytogenetic remission. Conventional cytogenetic examination requires cells to be in mitosis and is technically challenging and laborious. At the time of complete haematological remission, this is not easily achieved with blood and requires a sample derived from an invasive bone marrow aspirate. Even then it is difficult to visualise more than 20 metaphases. If none contain the Ph chromosome, the disease is said to be absent to a level of one in 20 (sensitivity 5%). The sensitivity of chromosome analyses can be enhanced to one per 1000 normal cells (sensitivity 0.1%) by FISH, which can be used in both interphase and metaphase nuclei.

RT-PCR for BCR-ABL transcripts was initially developed to detect early relapse after allogeneic stem cell transplantation. Early assays were qualitative but further developments permitted quantification of residual disease.

Modern quantitative RT-PCR (RQ-PCR) can reliably detect residual disease to a sensitivity of 0.01% and often to 0.001% BCR-ABL/ABL. The present

method uses specific primers to detect transcripts arising from BCR-ABL and compares these with the numbers of transcripts from a control gene (typically BCR, ABL, and GUSβ). The results are expressed as a ratio of BCR-ABL transcripts to the control transcripts, multiplied by 100 to give the result as a percentage, where 10%, 1%, 0.1%, 0.01%, and 0.001% correspond to a reduction in tumour load of 1, 2, 3, 4, and 5 logs (Cross NCP et al., 2012).

RQ-PCR is now the accepted method for monitoring response to TKIs. Unfortunately, inter-laboratory results cannot be compared easily as the equipment, reagents, control genes, and techniques differ between laboratories.

The international RQ-PCR standardisation project began in 2006 with the aim of using a common

reference baseline to develop a standardised scale (Hughes T et al., 2006). The original baseline material was derived from a pool of 30 newly diagnosed patients, tested in three laboratories with a median result identified for each. This median was then used to derive a laboratory specific conversion factor to standardise the results (Hughes TP et al., 2003).

Other laboratories were invited to participate but subsequently the baseline material was exhausted and now conversion factors are created by a comparison of local results with those of the reference laboratory in Adelaide, Australia (Branford S et al., 2008).

At present, about 200 laboratories worldwide report their results on the international scale, but many more do not. Efforts continue to create

internationally accepted reference material to allow more general uptake of the scheme (White HE et al., 2010).

In 2006, a group of chronic myeloid leukaemia experts came together through the European Leukemia Net, a European Union funded Network of Excellence, to develop recommendations for disease management (Baccarani M et al., 2006).

They advised both cytogenetics and RQ-PCR for monitoring and determined the goals of treatment as complete haematological remission by 3 months and complete cytogenetic remission by 18 months. Updated recommendations in 2013 formally defined a optimal responder as a patient who achieved complete haematological remission, partial cytogenetic remission (Ph+≤35%) and a RQ-PCR ≤10% by 3 months, complete cytogenetic remission (equivalent to a 2 log reduction in tumour load) by 6 months, and a 3 log reduction (RQ-PCR <0.1% known as major molecular remission or MR^3), by 12 months (Baccarani M et al., 2009).

4.2. Acute lymphoblastic leukemia (ALL)

4.2.1. Epidemiology

Acute lymphoblastic leukemia (ALL) is the most common malignancy diagnosed in children, representing nearly one third of all pediatric cancers. The annual incidence of acute lymphoblastic leukemia is approximately 6000 new cases in the United States (3400 male and 2600 female). The peak incidence occurs in children aged 2-5 years; approximately 60% of cases occur at age <20 years. The survival rate of childhood ALL is

approaching 90% (appendix, figure 1), although the treatment of infants and adults needs improvement. In adults the risk of developing the disease begins around age 50 and increases thereafter (Pui CH et al., 2008; Hunger SP et al., 2012; Bassan R and Hoelzer D, 2011; Pui CH et al., 2009; Pieters R et al., 2007).

The disease consists in clonal proliferation of lymphoid precursor with arrested maturation. It can originate in lymphoid cells of different lineages, thus giving rise to B-cell (88% incidence) or T-cell leukemias (12% incidence). The ALL subtypes are (Hoelzer et al., 2002):

- Pro-B ALL, 60% of all ALL
- Pre-B ALL, about 20 % of all ALL
- Mature-B ALL, about 5% of all ALL
- Early-T ALL
- Thymic-T ALL — about 10% of all ALL
- Mature-T ALL

4.2.2. Clinical features

Patients with acute lymphoblastic leukemia (ALL) present with either symptoms relating to direct infiltration of the marrow or other organs by leukemic cells, or symptoms relating to the decreased production of normal marrow elements.

Fever is one of the most common symptoms of ALL, and patients with ALL often have fever without any other evidence of infection. Patients with ALL often have decreased neutrophil counts, regardless of whether their total white blood cell (WBC) count is low, normal, or elevated. As a result, these

individuals are at an increased risk of infection. The prevalence and severity of infections are inversely correlated with the absolute neutrophil count, which is defined as the number of mature neutrophils per unit of volume. Infections are common when the absolute neutrophil count is less than 500/µL, and they are especially severe when it is less than 100/µL (Dieter Hoelzer et al., 2002).

Symptoms of anemia are common and include fatigue, dizziness, palpitations, and dyspnea upon even mild exertion. Other patients present with signs of bleeding. Bleeding can be the result of thrombocytopenia due to marrow replacement. Additionally, approximately 10% of patients with ALL have disseminated intravascular coagulation (DIC) at the time of diagnosis. These patients may present with hemorrhagic or thrombotic complications.

Some patients present with palpable lymphadenopathy and splenomegaly. Others, particularly those with T-cell ALL, present with symptoms related to a large mediastinal mass, such as shortness of breath. Infiltration of the marrow by massive numbers of leukemic cells frequently manifests as bone pain. This pain can be severe and is often atypical in distribution (Hiroto Inaba et al., 2013).

4.2.3. Genetic basis of ALL

High-resolution profiling of genetic alterations has transformed our understanding of the genetic basis of ALL. It has been known for several decades that the majority of childhood ALL cases harbour gross chromosomal alterations (figure 7) (Harrison CJ, 2009).

In B-ALL, these include high hyperdiploidy with non-random gain of at least five chromosomes (including X, 4, 6, 10, 14, 17, 18, and 21), hypodiploidy with fewer than 44 chromosomes, and recurring translocations, including t(12;21)(p13;q22), encoding ETV6-RUNX1 (TEL-AML1); t(1;19) (q23;p13), encoding TCF3-PBX1 (E2A-PBX1); t(9;22)(q34;q11), encoding BCR-ABL; MLL rearrangement involving 11q23 with a wide range of partner genes; and rearrangement of MYC into antigen receptor gene loci. Dysregulation of TAL1, TLX1, TLX3, and LYL1, particularly by rearrangement into T cell antigen receptor loci, is common in T-ALL. These alterations are of key importance in both the pathogenesis and clinical management of ALL (figure 3).

Many chromosomal rearrangements disrupt genes that regulate normal hematopoiesis and lymphoid development (eg, RUNX1 and ETV6), activate oncogenes (eg, MYC), or constitutively activate tyrosine kinases (eg, ABL1). Several of these alterations are significantly associated with outcome, particularly in B-ALL, and are used in risk stratification.

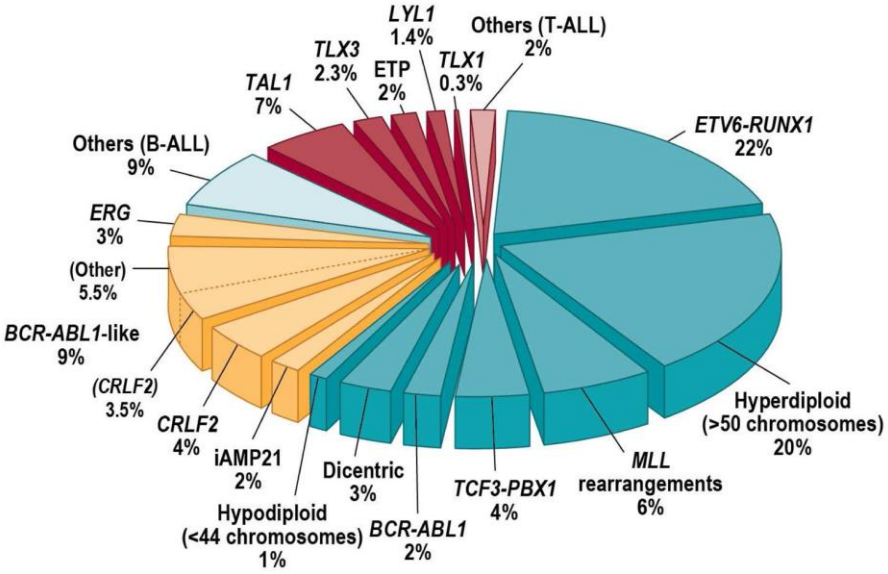


Figure 7: Cytogenetic and molecular genetic abnormalities in childhood ALL

t(9;22) BCR ABL is present in 35-30% cases of ALL in adults, p190 (60% of cases) or p210 (40% of cases), and in 5-8% cases of ALL in children , p190 (85% of cases) or p210 (15% of cases). This translocation has the worst prognosis. In particular p190 is associated with a probability of relapse of 88% versus 12% of p210 (Hoelzer et al., 2002).

Aberration	Target (mRNA or DNA)	Frequency of Applicability (%)[b] Children	Adults
Precursor-B-ALL			
t(9;22)(q34;q11)	*BCR-ABL* (mRNA)	5-8	30-35
t(1;19)(q23;p13)	*E2A-PBX1* (mRNA)	5-8	3-4
t(4;11)(q21;q23)	*MLL-AF4* (mRNA)	3-5[c]	3-4
11q23 aberrations	aberrant *MLL* (mRNA)	5-6[c]	< 5
t(12;21)(p13;q22)	*TEL-AML1* (mRNA)	~30	1-3
TOTAL		**40-45**	**40-45**
T-ALL			
TAL1 deletion	*SIL-TAL1* (DNA/mRNA)	10-25	5-10
t(8;14)(q24;q11)	*c-MYC-TCRA/D* (DNA)	5-10	5-10
t(11;14)(p15;q11)	*LMO1-TCRD* (DNA)		
t(11;14)(p13;q11)	*LMO2-TCRD* (DNA)		
t(1;14)(p34;q11)	*TAL1-TCRD* (DNA)		
t(10;14)(q24;q11)	*HOX11-TCRD* (DNA)		
TOTAL		**25-30**	**10-15**

Table 3: Frequent chromosomal aberrations in B-ALL and T-ALL

4.2.4. Diagnosis

Morphological identification of lymphoblasts by microscopy and immunophenotypic determination of lineage commitment and developmental stage by flow cytometry are essential for correct diagnosis of ALL (Pui CH et al., 2008). Chromosomal analysis still plays an important role in the initial cytogenetic work-up. RT-PCR, FISH, and flow cytometry are used to identify leukaemia-specific translocations, sub-microscopic chromosomal abnormalities, and cellular DNA content, respectively.

4.2.5. Risk assignment

The most important prognostic factors (Hoelzer et al., 2002) in ALL are:

- clinical characteristic as age, whole blood count
- immunophenotype
- cytogenetic/molecular abnormalities
- response to treatment, including time to achieve a Complete Remission (CR) (after 2-4 weeks) and level and course of MRD during induction and consolidation

4.2.5.1. Clinical and biological factors

Age (infant or ≥10 years old), presenting leukocyte count (≥50×109/L), race (Hispanic or black), male sex, and T-cell immunophenotype have been considered adverse clinical prognostic factors in children, although their effect is diminished by contemporary risk adapted therapy and improved supportive care (Pui CH et al., 2009). Infants with MLL rearrangement, especially those < 6 months old with a leukocyte count >300×109/L at diagnosis, still have a dismal prognosis. Cytogenetic and molecular risk factors have been discussed above.

Racial/ethnic differences in prognosis have been linked not only to socioeconomic factors but also to differences in genomic alterations (Harvey RC et al., 2010; Xu H et al., 2012). For example, germline single nucleotide polymorphisms of PDE4B70 and ARID5B71 were shown to be associated with Native American genetic ancestry, and somatic CRLF2 rearrangements in ALL blasts were overrepresented in children from a Hispanic ethnic background; these alterations were found to contribute to inferior outcomes in Hispanics. Adverse prognosis conferred by genetic

ancestry was mitigated by adding a course of delayed intensification therapy (Yang JJ et al., 2011).

Adolescents and adults have a greater prevalence of biologically high-risk leukaemia (eg, BCR-ABL1 and MLL rearrangement), a low incidence of favorable subtypes (eg, ETV6- RUNX1 and hyperdiploidy), and poorer adherence and tolerance to therapy. Older age (especially ≥60 years) and high presenting leukocyte count are also poor prognostic factors in this population. Recent studies showed that they had better outcomes when treated on paediatric rather than adult regimens (Schafer ES and Hunger SP, 2011; Pui CH et al., 2011).

4.2.5.2. Response to therapy

Early treatment response is predictive of the risk of relapse and is used to assign patients to subsequent risk-adapted therapy (Campana D, 2012). Methods that track residual leukaemic cells by flow cytometry (detecting aberrant immunophenotypes) and by PCR amplification (detecting leukaemia-specific immunoglobulin and T-cell receptor genes or fusion transcripts) allow the recognition of ALL cells present at levels well below those detectable by microscopic morphologic assessment, ie, minimal residual disease (MRD). MRD is currently the most powerful prognostic indicator in childhood and adult ALL, even in patients with low-risk features at presentation (Borowitz MJ et al., 2008; Gokbuget N et al., 2012).

The kinetics of MRD clearance in response to identical remission-induction chemotherapy differed between B- and T-ALL; negative MRD on day 33 was the strongest prognostic factor in B-ALL, while negative MRD on day 78 was

also predictive in T-ALL, regardless of positive MRD on day 33 (Conter V et al., 2010; Schrappe M et al., 2011).

The detection of the MRD (Szczepanski et al., 2002; Van Dongen JJM et al., 1999) is performed by:

1. flow cytometric immunophenotyping using aberrant or "leukemia-associated" phenotypes
2. PCR techniques using chromosome aberrations that result in fusion gene transcripts or aberrant expression of transcripts
3. PCR techniques using patient-specific junctional regions of rearranged immunoglobulin (Ig) and T-cell receptor (TCR) genes

4.2.6. Treatment

Treatment of ALL typically spans 2–2.5 years, comprising 3 phases: remission-induction, intensification (or consolidation), and continuation (or maintenance) (Pui CH et al., 2008). Most of the drugs used were developed before 1970. However, their dosage and schedule of administration in combination chemotherapy have been optimized on the basis of leukaemic-cell biological features, response to therapy (MRD), and patient pharmacodynamic and pharmacogenomic findings, resulting in the current high survival rate. Central nervous system (CNS)-directed therapy is administered to prevent relapse caused by leukaemia cells sequestered in this sanctuary site. Allogeneic haematopoietic stem-cell transplantation is considered for patients at very high risk.

The treatment of the ALL disease consist in:

- chemotherapy, divided in three phases:

1) induction

2) consolidation

3) maintenance

- molecular drugs in presence of targeted markers (i.e. Imatinib for Ph positive patients) (Ching-Hon Pui, 2006), usually as front-line treatment or in association with chemotherapy or in case of relapse after Stem Cell Transplantation
- stem cell transplantation (STC), usually after chemotherapy, in case of high risk (Ph positive) patients or in case of relapse.
- antibody treatment consisting in monoclonal antibodies specific for antigens expressed by the ALL blast cells

Antigen	Expression on >20% [a]	Clinical Application Antibody	Stage	*n*	Tx	Results	Author
CD19	95% B-precursor 94% Mature B-ALL	AntiCD19+PAP	de novo child	14 14	CH AB+CH	43% CR d14 93% CR d14	Seibel et al [41]
		AntiCD19+Ricin	de novo adult	46	AB+CH	No effect on MRD	Szatrowski et al [42]
		AntiCD19+Genistein	ref/rel child	15	AB	2 CR, 2 PR	Messinger et al [43]
CD20	41% B-precursor 86% Mature B-ALL	Rituximab	de novo adult	19	AB+CH	93% CR 86% OS 1y	Thomas et al [29]
CD52	66% B-/T-lineage	Campath	ref/rel. adult	5	AB	0 CR, 0 PR	Faderl et al [44]

[a] Data from the GMALL (German Multicenter Studies for Adult ALL) central immunophenotyping, E.Thiel, S.Schwartz et al, Berlin, Germany.

Table 4: Antigen expression and treatment results with monoclonal antibodies in acute lymphocytic leukemia (ALL)

4.2.6.1. Remission-induction therapy

Four to 6 weeks of remission-induction treatment eradicates the initial leukaemic cell burden and restores normal haematopoiesis in 96–99% of children and 78–92% of adults. The chemotherapy agents typically include a glucocorticoid (prednisone or dexamethasone), vincristine, and asparaginase, with or without anthracycline. This regimen appears to be sufficient for standard-risk ALL if intensified post-remission treatment is given. Patients at high or very high risk receive four or more drugs.

Patients with BCR-ABL-positive ALL have been considered to have a poor prognosis but benefit from early administration of a tyrosine kinase inhibitor (eg, imatinib, dasatinib). When this agent is added to multiagent chemotherapy, complete remission rates are >90% and event-free survival is superior to that of historical controls (Schultz KR et al., 2009; Ravandi F et al., 2010). Unlike imatinib, dasatinib targets both ABL1 and Src kinases; it also has more potent activity against BCRABL1, is active against imatinib-resistant BCR-ABL1 (except for T315I mutation), and has better CNS penetration (Porkka K et al., 2008).

4.2.6.2. Intensification (consolidation) therapy

Intensification (consolidation) therapy is administered after remission-induction to eradicate

residual leukaemic cells. This phase commonly uses high-dose (ie, 1–8g/m2) methotrexate (MTX) with mercaptopurine, frequent pulses of vincristine and glucocorticoid, uninterrupted asparaginase for 20–30 weeks, and

reinduction therapy with agents similar to those used during remission-induction.

4.2.6.3. Hematopoietic stem cell transplantation and cellular therapy

Allogeneic haematopoietic stem cell transplantation (HSCT) is considered for children with very high-risk ALL and/or persistent disease. Contemporary HSCT protocols with high resolution HLA typing, case-based conditioning, and improved supportive care have reduced relapse-related mortality, regimen-related toxicity, and infection (Balduzzi A et al., 2005; Marks DI et al., 2010; Leung W et al., 2011).

A level of MRD ≥10−4 before HSCT is strongly associated with relapse, and new strategies are needed to reduce the disease burden before and/or after HSCT (Bader P et al., 2009). Patients with BCR-ABL1-positive ALL who obtain remission after multi-agent chemotherapy with ABL1 kinase inhibitors and young children (age <6 years) with B-ALL in delayed remission after induction failure can be treated without HSCT(Schultz KR et al., 2009; Ravandi F et al., 2010; Schrappe M et al., 2012). The benefit of HSCT for infants with ALL is controversial; the role of HSCT, if any, is limited to a small high-risk group (Dreyer ZE et al., 2011; Mann G et al., 2010). Although many adult centers have considered HSCT during first complete remission a key element of therapy, treatment with paediatric-based regimens will decrease its use (Schafer ES and Hunger SP, 2011).

4.2.6.4. Continuation therapy

Continuation therapy typically lasts 2 years or longer and comprises mainly daily mercaptopurine and weekly methotrexate with or without pulses of vincristine and dexamethasone. Mercaptopurine and thioguanine are structural analogs of hypoxanthine and guanine, respectively, and inhibit de novo purine synthesis. Although thioguanine requires fewer steps to form the active metabolite thioguanine nucleotides and has greater in vitro cytotoxicity to lymphoblasts, randomized studies have not consistently shown a benefit of thioguanine in event-free survival or overall survival, and protracted doses were associated with death during remission, veno-occlusive disease, portal hypertension, and thrombocytopenia (Harms DO et al., 2003; Pui CH and Howard SC, 2008).

Thus, mercaptopurine is preferred for continuation therapy. Thiopurine methyltransferase (TPMT) catalyzes S-methylation of thiopurines to inactive methylated metabolites. Patients with homozygous or heterozygous TPMT deficiency experience moderate to profound myelosuppression when treated with thiopurines (Relling MV et al., 2011). Further, an adherence rate <95% to planned mercaptopurine doses is associated with relapse.129 Therefore, uninterrupted, pharmacogenetics-based mercaptopurine dosing is important.2 After thioguanine nucleotides are incorporated into DNA, DNA mismatch repair enzymes exert cytotoxicity. Deficiency of such enzymes (eg, MSH2) renders leukaemic cells thiopurine-resistant (Diouf B et al., 2011).

Clinical characteristics	Higher age: > 50 yrs, > 60 yrs High WBC: > 300,00/µl in B-lineage
Immunophenotype	Pro B (B-lin., $CD10^{-}$) Early T (T-lin., $CD1a^{-}$, $sCD3^{-}$) Mature T (T-lin., $CD1a^{-}$, $sCD3^{+}$)
Cytogenetics/molecular genetics	t(9;22)/BCR-ABL or t(4;11)/ALL1-AF4
Treatment response	Late achievement of CR: > 3, 4 weeks MRD positivity

Table 5: Adverse prognostic factors in adult acute lymphocytic leukemia (ALL

MATERIAL AND METHODS

1. Loop Mediated Isothermal Amplification (LAMP)

LAMP is an isothermal DNA amplification method which relies on the use of a thermo-stable DNA polymerase with strand displacement activity and at least 4 primers (F3, B3, FIP, BIP) specifically designed to recognize 6 different regions on the target gene.

F3 and B3 are the most external primers, which allow the strand-displacement activity of the enzyme at the beginning of the reaction. The inner primers are FIP (Forward Inner Primer) and BIP (Backward Inner Primer), made by 2 parts (F1 and F2; B1 and B2), respectively complementary to the sense and antisense sequence. These characteristics allow the self-annealing of the single strand product and the formation of the stem-loop structure, which represents the starting point for the LAMP reaction (Notomi et al., 2000; Fu et al., 2011).

The mechanism of LAMP amplification reaction includes three steps: production of the starting structure, cycling amplification and elongation, and recycling.

All four primers are operative in the initial steps of the reaction, but in the later cycling steps only the inner primers are required for strand displacement synthesis.

At a constant reaction temperature, inner primer FIP hybridizes to F2C in the target DNA and initiates complementary strand synthesis (Structure 1).

Outer primer F3 hybridize to F3C in the target and initiates strand displacement of the newly DNA chain (Structure 2), releasing a FIP-linked

complementary strand, which forms a looped-out structure at one end (Structure 4).

This single stranded DNA serves as template for BIP initiated DNA synthesis and subsequent B3-primed strand displacement DNA synthesis leading to the production of a dumbbell-form DNA which is quickly converted to a stem-loop DNA (Structure 6).

The dumbbell serves as the starting structure for LAMP exponential amplification, the second step of LAMP reaction.

The stem-loop DNA structure contains a double stem-loop which is the starting point for the whole LAMP amplification process as it promotes an amplification from its self-annealed 3' terminus and from a newly annealed internal primer (FIP or BIP) (Structure 7).

During amplification, FIP hybridize to the loop in the stem-loop DNA and primes strand displacement DNA synthesis, generating as an intermediate one gapped stem-loop DNA with an additional inverted copy of the target sequence in the stem, and a loop formed at the opposite end via the BIP sequence (Structure 8).

Subsequent self-primed strand displacement DNA synthesis yields one complementary structure on the original stem loop DNA and one gap repaired stem loop DNA (structure 10) with a stem elongated to twice as long and a loop at the opposite end (Structure 9).

Both of these products than serve as templates for BIP-primed strand displacement in the subsequent cycles, the elongation and recycling step. Thus, in LAMP the target sequence is amplified 3-fold every half cycle. Amplification proceeds promoting itself, each strand being displaced by

elongation of the new loops formed. The final product is a mixture of stem-loop DNA with various stem length and cauliflower-like structures with multiple loops formed by annealing between alternately inverted repeats of the target sequence in the same strand. In order to boost the reaction and to increase the sensitivity of the method, it is possible to add 2 more primers, LF and LB (Loop Forward and Loop Backward), that anneal to the single strand sequence of the loops. Loop primers hybridize to the stem loop, except for the loops that are hybridized by the inner primer, and prime strand displacement DNA synthesis. Therefore the loop primers act as reaction boosters annealing and extending on single strand regions on the loop region of the dumbbell structure.

By these peculiar amplification dynamics and exploiting the continuous displacement by the enzyme of the already produced DNA strands with no need for denaturation steps, it is possible to amplify the initial DNA amount up to 10^{10} times in less than one hour and with very high specificity, due to the many independent target recognition events.

Several methods can be used to detect positive LAMP reactions. Reaction results are detectable either by fluorescence or turbidimetry. Many fluorescence approaches are employable, like intercalation dyes or fluorescent probes, in order to visualise directly in real-time the products of LAMP reaction .

LAMP constitutes an attractive alternative to PCR for sequence detection, with higher sensitivity and specificity performances and allowing simplex and multiplex applications in a fast, easy and cheap molecular diagnostic platform.

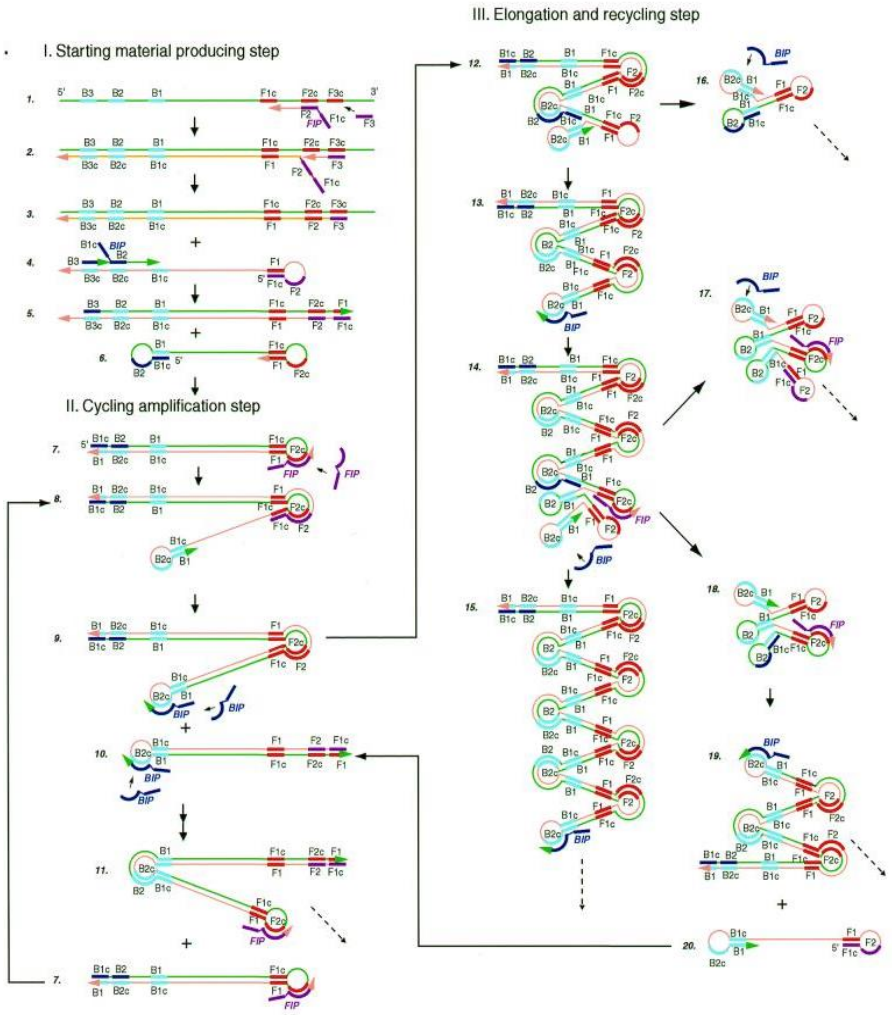


Figure 8: schematic representation of the mechanism of the LAMP

Steps in the LAMP reaction. This figure shows the process that starts from primer FIP. However, DNA synthesis can also begin from primer BIP.

2. BCR-ABL Q-LAMP Assay

2.1. Primer design

A LAMP reaction employs at least 4 primers, that recognize 6 different regions on the target gene. F3 and B3 are the most external primers, which allow the strand-displacement activity of the enzyme at the beginning of the reaction. The inner primers are FIP (Forward Inner Primer) and BIP (Backward Inner Primer), made by 2 parts (F1 and F2; B1 and B2), respectively complementary to the sense and antisense sequence. This characteristic allows the self-annealing of the single strand product and the formation of the stem-loop structure, which represents the starting point for the LAMP reaction.

In addition, 2 more primers, LF and LB (Loop Forward and Loop Backward), that anneal to the single strand sequence of the loops, can be employed in order to increase the speed of amplification.

In this work we have introduced a fluorescent probe, substituting one of the loop primers, which allows the real time detection of the amplification.

The primer sets were initially designed with the software *Primer Explorer*. This free-access software is specifically created to design primers for a LAMP reaction.

It is able to design the necessary 4 primers and also the loop primers, using the “nearest neighbour” method for Tm calculation.

Among the great number of sets produced in the output we selected the ones in which the forward and backward portions were designed in-between the translocation.

We further analyzed those sets with the software *Visual OMP* (DNASoftware, Ann Arbor, USA). The characteristics of this software is to provide also predictions of secondary structures, that can help in excluding primer sets that can give primer dimers amplification.

It performs the calculation of Tm by the "nearest neighbour" method, but considering also many factors present in the reaction, such as temperature, primers, salts and glycerol concentration.

2.2. Primer sets selection

In silico selection was based on the absence of primer dimers and non-specific binding to other portions of the target sequence.

Experimental selection was performed testing each primer set at standard conditions on 100 copies/reaction of the specific plasmid to test the speed of amplification and on water samples to test the specificity. The primer sets that passed the selection had 100% of specificity and the amplification of the plasmid should be completed within 25 minutes.

2.3. Labelled probes

In this work we have introduced a fluorescent probe, substituting one of the loop primers, which allows the real time detection of the amplification and the transcripts discrimination.

For the triplex assay were used three different customized fluorophores that can be specifically read by the channels of the Liason IAM instrument.

Customized fluorophores were produced by Cyanagen (Bologna, Italy), with different emission wavelength so that they could be read in three different channels of Liason IAM instrument without cross-talk effect.

For the triplex reaction p210 was labelled with CHROMIS 570, p190 with CHROMIS 500 and GUSβ with CHROMIS 530. CHROMIS 500 (C500) has an absorption wavelength of 496 nm and emits at 506 nm, CHROMIS 530 (C530) has an absorption wavelength of 529 nm and emits at 561 nm, CHROMIS 570 (C570) has an absorption wavelength of 573 nm and emits at 612 nm.

The primers and probes were synthesized by SGS DNA.

2.4. LAMP reaction

p190 and p210 simplex LAMP reactions were performed on Liason IAM instument (DiaSorin) at 65°C for 50 minutes using standard LAMP reaction conditions. Each reaction mixture contained the primer set specific for the translocation (F3/B3 0.2 µM, FIP/BIP 1.6 µM, LF or LB 0.8 µM) and the specific probes 0.8 µM, dNTPs 1.4 mM each, 8 mM MgSO4, Tris-HCl 20 mM,

KCl 10 mM, (NH4)2SO4 10 mM, tween 20 0.1%, enzyme 0.32 U/µL, 5 µL of plasmid and distilled water (up to the final volume of 25 µL).

BCR-ABL duplex and triplex assays with labelled probes were performed on Liason IAM instument (DiaSorin) at 66°C for 60 minutes using optimized LAMP reaction conditions. Each reaction mixture contained LAMP reagents, 5 µL of RNA 100 ng/uL and distilled water (up to the final volume of 25 µL).

2.5. Liason IAM instrument

The Liason IAM instrument was specifically developed by DiaSorin for the execution of the Q-LAMP assays. It performs isothermal amplification and detection of nucleic acids, by real-time measuring of changes in fluorescence in up to three channels.

Liason IAM detection system consists of three LED for excitation and three photodiodes for detection of three fluorescence channels. 8 optical fibers (one for each well) transmit the light for excitation of the fluorophores to the wells and consequently conduct the light emitted by the reaction for the elaboration.

The instrument is integrated with a *Liason IAM Software* that recognises in which channel occurs the amplification and, according to the specific assay performed, is able to return a clear and objective result about the state of the patient (positive or negative) and, if positive, which specific transcript has been identified.

2.6. Freeze-drying

The freeze drying process was performed on VirTis AdVantage Plus (SP Scientific) freeze dryer.

The protocol consists of different phases that were optimized during the development of the final products:

1. freezing, in which the reaction mix is taken at low temperatures (< -35°C) in order to immobilize the material and define the structure that will be dried;
2. primary drying through sublimation. In this phase vacuum is needed to reach the triple point of water, when the sublimation can occur;
3. secondary drying, in which the temperature increases to 20°C and a further drying of the product is obtained by desorption.

In order to create the solid organized structure that will remain at the end of the process Dextran70 is added to the reagents mix.

The BCR-ABL triplex assay reaction mix is lyophilized into two cakes, one cake containing dextran70 plus dNTPs, enzyme and primers, and the other cake containing dextran70 and labelled probes.

Once freeze dried, the cakes can be easily reconstituted adding the two remaining elements of the liquid reaction mix: LAMP buffer and manganese.

3. Reverse Transcription Polymerase Chain Reaction (RT-PCR)

The European BIOMED Concerted Action promoted the standardization of RT-PCR protocol and primer sets that can be used for molecular classification of acute leukemia at diagnosis and for MRD (Minimal Residual Disease) detection during follow-up to evaluate treatment effectiveness (Dongen et al., 1999).

The RT-PCR protocol consists in three main steps: reverse transcription of target RNA into cDNA, amplification of the target cDNA and finally separation and identification of the PCR products using gel-electrophoresis techniques.

The target cDNA amplification consists in:

1) PCR for molecular genetic classification of acute leukemia at diagnosis: This is generally performed by single PCR (primers A -B) followed by agarose gel electrophoresis
2) Sensitive PCR for Minimal Residual Disease detection: This can be achieved by nested PCR with a set of external primers (A -B) and internal primers (C -D), followed by evaluation of the size of the obtained PCR products in agarose gel electrophoreses

The standardized RT-PCR protocol, used in the all the most important onco-haematology centres, is reported below.

1. *RT-reaction with random hexamers*
   - 1 μg of RNA (or 0.1 μg of mRNA) in 9.5 μl of H_2O
   - incubation at 70°C for 10 min
   - cool on ice and add other reagents to final volume of 20 μl:
     RT buffer: 20 mM Tris HCl, 50 mM KCl, pH 8.3
     $MgCl_2$: 5 mM
     DTT: 10 mM
     random hexamers: 5 μM
     RNAasin: 20 units
     RT enzyme: 200 units Superscript (200 units per μl)
     dNTP: 1 mM
   - temperatures and incubation times:
     room temperature for 10 min
     42°C for 45 min
     99°C for 3 min
     4°C at end of RT step

2. *Single PCR or first round of nested PCR*
   - final volume of 50 μl
   - 2–3 μl of cDNA (ie 10–15% of RT mixture)
   - primers: 400 nM final concentration
   - dNTP: 200 μM final concentration
   - PCR buffer: 20 mM Tris HCl, 50 mM KCl, pH 8.3
   - $MgCl_2$: 2.5 mM (to be optimized in each laboratory)
   - *Taq* enzyme[a]: 1 unit per 50 μl volume[b]

3. *PCR temperatures and cycle times*
   - initial melting: 95°C for 30 s
   - PCR cycles[c]:
     94°C for 30 s (melting)[b]
     65°C for 60 s (annealing)
     72°C for 60 s (extension)
   - number of cycles: 35
   - no final extension needed
   - stop of PCR: 16°C (or room temperature)[d]

4. *Second round PCR for nested PCR*
   - 1 μl of first round PCR
   - same volume, reagents and cycle conditions as for first round PCR, using the internal (nested) C ↔ D primers

[a]During the final phase of the study it appeared that usage of *AmpliTaq* Gold (PE Biosystems, Foster City, CA, USA) further improves the sensitivity.
[b]If the GC content of the PCR product is high (>70%), it is advised to use 2 units of *Taq* enzyme per 50 μl and to increase the melting temperature to 95°C.
[c]The melting, annealing and extension times might be shortened, if a new generation of rapid PCR machine is used.
[d]For evaluation of the PCR products, 15 μl of the PCR mixture is used for agarose gel electrophoresis.

Table 6 : Standardized RT-PCR protocol

4. Detection of BCR-ABL t(9;22) by RT-PCR

The retro-transcription step and the single PCR were performed as described by the BioMed standardized RT-PCR protocol (Dongen et al., 1999). 2 different reactions are needed, one for the detection of p210 and one for the detection of p190.

A third reaction is separately performed for the amplification of the negative control.

Primer code	5' Position (size)	Sequence (5'-3')
BCR-e1-A	1479 (21)	GACTGCAGCTCCAATGAGAAC
ABL-a3-B	458 (21)	GTTTGGGCTTCACACCATTCC

Table 7: Primers for RT-PCR analysis of t(9;22)(q34;q11) with the BCR-ABL p190 fusion gene

Primer code	5' Position (size)	Sequence (5'-3')
BCR-b1-A	3086 (22)	GAAGTGTTTCAGAAGCTTCTCC
ABL-a3-B	458 (21)	GTTTGGGCTTCACACCATTCC

Table 8: Primers for RT-PCR analysis of t(9;22)(q34;q11) with the BCR-ABL p210 fusion gene

PCR products are run in 1,5% agarose gel and visualized with ethidium-bromide staining (Van Dongen). The discrimination of the transcript is based on the length of the PCR product.

	A + B
P190 e1a2	521
P210 b2a2	342
P210 b3a2	417

Table 9: Sizes of PCR products of BCR-ABL p190 and p210 primer sets in RT-PCR testing

When a single PCR was used the sensitivity reaches 10^{-3} dilution of p210 patient RNA in HL60 RNA.

When a second round of amplification (nested PCR) was performed using the internal primers a sensitivity level of 10^{-4} was obtained.

When a single PCR was used the sensitivity reaches 10^{-3} dilution of p190 patient RNA in HL60 RNA

When a second round of amplification (nested PCR) was performed using the internal primers a sensitivity level of 10^{-4} was obtained.

5. cDNA Plasmid

For the evaluation of preliminary sensitivity of the BCR-ABL Q-LAMP assay we used plasmids containing the cDNA sequence of the p210 and p190 transcripts and of the internal control.

The size of the insertions for p210 and p190 were respectively 331 bp and 269 bp and 355 bp for GUSβ.

The plasmids were produced by Life Technologies (GeneArt® Gene Synthesis). The cDNA sequence is synthetized, cloned into pMA-T vector and transformed into E. Coli. The construct is then purified and sequence-verified.

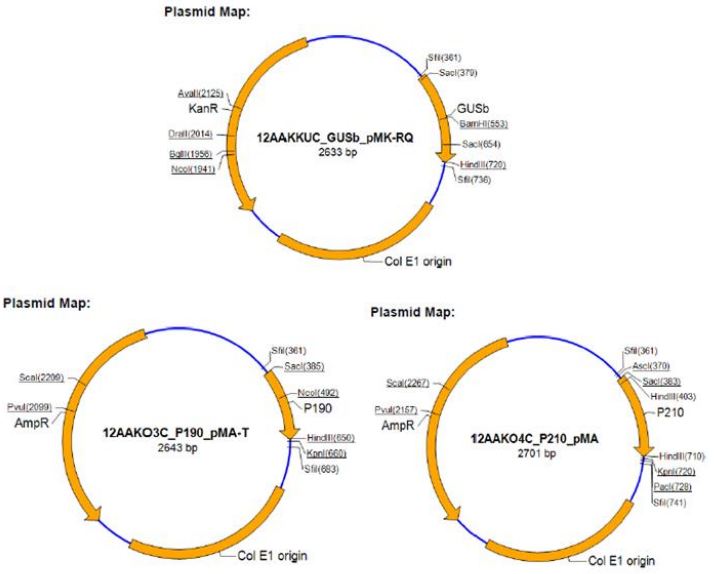


Figure 9: **Plasmid's map provided by Life Technologies for p210 (A), p190 (B) and GUSβ (C)**

The copy number per µL of each plasmid was calculated based on the length and on the concentration. Plasmids were diluted into a buffer containing Tris HCl and Yeast RNA.

In order to assess the sensitivity, plasmids were denatured by heating them at 100°C for 10 minutes and immediately chilled on ice to prevent re-naturation.
In the final format of the assay the not-denatured plasmids have also been used as positive and negative controls.

6. Cell culture

A stable culture was established for positive cell lines TOM-1 (t(9;22) (q34;q11) p190-DMSZ no. ACC 578) and K-562 (t(9;22) (q34;q11) p210-DMSZ no. ACC 10), carrying the p190 and p210 translocations, and for negative cell lines such as HL-60 (AML, M2-DMSZ no. ACC 3), NB-4 cell line (t(15;17) (q22;q11-12.1)-DMSZ no. ACC 207) , KASUMI-1 (t(8;21) (q22;q22)-DMSZ no. ACC 220), MV4-11 (t(4;11) (q21;q23)-DMSZ no. ACC 102), RS4;11 (t(4;11) (q21;q23)-DMSZ no. ACC 508), REH (t(12;21) (p13;q22)-DMSZ no. ACC 22) and 697 (t(1;19)(q23;p13)-DMSZ no. ACC 42).

Cell lines NB-4, HL-60, K562 e KCL-22 were plated in medium RPMI 1640 (Gibco Life Tecnologies, Gaithersurg, MD), supplemented with 10% FBS (v/v) (Fetal Bovine Serum) (Lonza, Gaithersurg, MD) and Penicillin-Streptomycin-Amphotericin B Mixture (Lonza). Cell lines 697, Kasumi-1, MV4-11, REH e TOM-1 plated in medium RPMI 1640 (Gibco Life Tecnologies, Gaithersurg, MD), supplemented with 20% FBS (v/v) (Fetal Bovine Serum) (Lonza, Gaithersurg, MD) and Penicillin-Streptomycin-Amphotericin B Mixture (Lonza). Cell line RS4;11 was plated in medium

MEMα (Gibco Life Tecnologies, Gaithersurg, MD), supplemented with 10% FBS (v/v) (Fetal Bovine Serum) (Lonza, Gaithersurg, MD) and Penicillin-Streptomycin-Amphotericin B Mixture (Lonza). All the cell lines were stored at 37 °C in a humidified atmosphere containing 5% CO2.

7. Clinical samples

The final evaluation of relative sensitivity was performed on 80 positive clinical samples, previously tested with PCR. Relative specificity was tested on 60 healthy donors. These clinical samples were collected at two different clinical sites (Bergamo and Rome) from subjects who gave their informed consent.

For the real-life study the BCR-ABL final assay was tested on 100 p190, 100 p210 positive clinical samples at the disease onset and 100 BCR-ABL negative clinical samples. These clinical samples were tested in parallel with the reference method at three different clinical sites (Bergamo, Rome and Bologna).

8. RNA extraction

Two different RNA extraction methods were used, in order to evaluate their impact on the assay performances.

8.1. Qiagen RNeasy Mini Kit

Total RNA from cultured cell lines and healthy donors was extracted byRNeasy Mini Kit (Qiagen, Hilden, Germany) starting from 10 x 10^6 cells lysed in 600 µL of RLT Buffer.

Total RNA from clinical samples was extracted from mononuclear cells isolated by Ficoll-Hypaque gradient centrifugation and lysed in guanidinium iso-thiocyanate (GITC).

For the extraction we used the standard protocol described in the RNeasy Mini Handbook (Fourth Edition, June 2012), adding a centrifugation step of 4 minutes at 4000 rpm before the elution step, for a better removal of ethanol.

8.2. Phenol-Chloroform

10 x 10^6 cells were resuspended in 1 mL of TRIzol® for the RNA extraction from cultured cells and healthy donors, while the cells isolated from the clinical samples were resuspended in guanidinium iso-thiocyanate (GITC).

The extraction was performed as described by the manufacturer's protocol, with the following modifications:

1. All incubation steps were performed on ice, instead of room temperature
2. RNA precipitation was performed in isopropanol overnight at -20˚C, instead of RT for 10 minutes
3. Residual ethanol was removed by a second centrifugation step of 1 minute prior to air drying the pellet on ice.

9. Extracted RNA quality evaluation

After each extraction we evaluated the quality of RNA by *Nanodrop 2000* (Thermo Scientific) reading.

The two important parameters which allow the determination of the quality of RNA are A260/A280 and A260/A230 ratio.

The 260/280 nm ratio represents the ratio of the readings at 260 nm and 280 nm and provides an estimate of the purity of RNA with respect to contaminants that absorb in the UV spectrum, such as proteins.

Values between 1.8 and 2.1 indicate a highly pure RNA.

The 260/230 nm ratio gives an indication of contamination by some reagent used during the extraction, such as TRIzol and guanidinium iso-thiocyanate. Expected values are commonly in the range of 2.0-2.2.

In order to evaluate the integrity of the extracted RNA we used the *Bioanalyzer 2100 Instrument* (Agilent Technologies) with the Agilent RNA 6000 Nano Kit.

Samples were analysed as per manufacturer's instructions for use. 1 µL of sample (usually 100 ng) was loaded on the chip for the electrophoretic analysis, using the Eukaryote Total RNA Nano II Series assay protocol. The integrity of RNA is determined by calculating the ratio between the peaks of 28s and 18s rRNAs, that indicates good RNA quality if around 2. But the main instrument to evaluate RNA integrity is the RIN (RNA Integrity Number) software algorithm which allows the classification of total RNA, based on a numbering system from 1 to 10, with 1 being the most degraded and 10 being the most intact.

RESULTS

1. p210 and p190 LAMP primer set design and screening

To allow the amplification of p210 and p190 transcripts using the LAMP technology I have manually designed, with the support of Visual-Omp (VOMP) software, 108 different primers that can be combined together to obtain 30 sets specific for p210 and 35 sets specific for p190. Each primer set is constituted by a number of 6 primers.

The strategy of the design was focused to create two primer sets with a common backward part, localized on exon 2 of ABL, and two different forward parts specific for the two different transcripts. This strategy minimize the number of primers used in the reaction. Each primer set has also a specific probe labeled with different fluorochromes to allow the transcripts discrimination.

The p190 forward primers are designed on exon 1 of BCR gene, whereas the p210 forward primers are localized on exon 13 of BCR gene in order to amplify both p210 forms (b2a2,b3a2).

Plasmids containing the cDNA corresponding to the p190 and p210 fusion region were generated to screen all the designed primer sets.

Primer sets able to amplify 100 copies/reaction of the specific plasmid in a very specific way were selected as possible optimal candidates. At the end of the process the two primer sets were identified, one specific for p190 and the other for p210, that were able to amplify the plasmid in the shortest time, about 20 minutes, using standard LAMP reaction conditions.

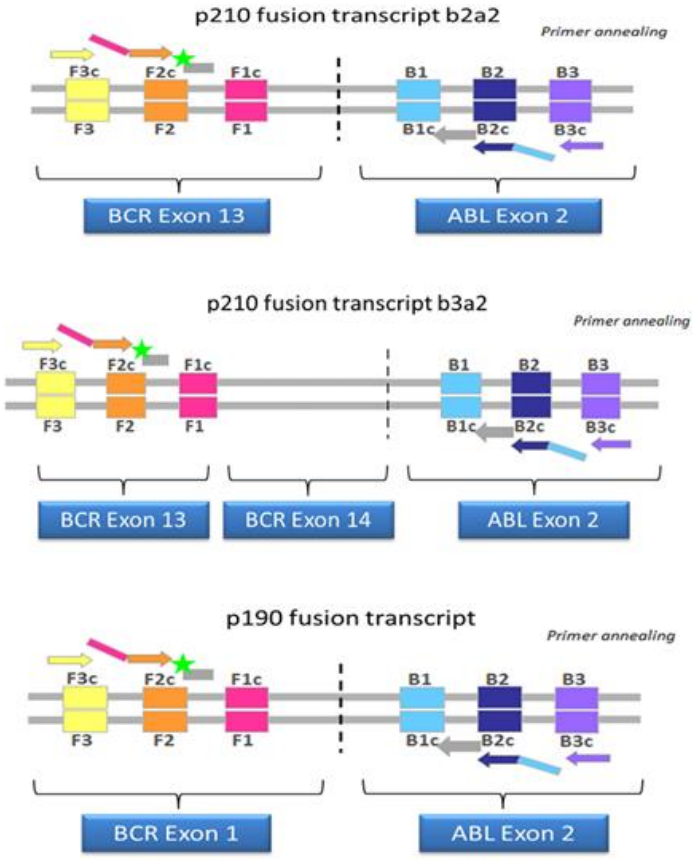


Figure 10: p210 and p190 primer design strategy

The backward primer sets (B3-BIP and LB) are designed on ABL exon 2 in order to be used for both p190 and p210 amplification. The forward primer sets are specific for p190, designed on BCR exon 1, and p210, designed on exon 13, in order to detect both p210 forms with only one forward set. Target specific probes labeled with different fluorochromes are designed in order to discriminate p190 and p210 amplification.

			Primer combinations	
Target region	No. primer sets	Set type	p210 detection	p190 detection
Exon 13 BCR	6	Forward p210	30	35
Exon 1 BCR	7	Forward p190		
Exon 2 ABL	5	Backward both p190 and p210		

Table 10 : p210 and p190 primer sets combinations

Forward and backward sets were designed using both VOMP software and manual design . 5 different primer sets were generated for ABL , 7 for exon 1 BCR and 6 sets for exon 13 BCR. 30 different combinations were found for p210 detection and 35 for p190 detection.

Target	Plasmid amplification:NO (No.sets)	Plasmid amplification :YES (No. sets)
p210	5	25
p190	7	28

Target	Amplification before 25 min (No.sets)
p210	10
p190	13

Target	Specific amplification (No.sets)	Non specific amplification (No.sets)
p210	4	6
p190	8	5

Table 11 : p210 and p190 primer sets screening results

In order to screen different primer combinations, we performed a Q-LAMP assay at 65°C for 1 hour using standard LAMP conditions on 100 copies/reaction of target plasmid (previously denatured at 95°C for 10 minutes and then chilled on ice). We selected the set of primers able to amplify the target sequence rapidly and specifically. Screening criteria were: the ability to amplify the target within 25 minutes and the absence of primer dimers using water as non template control (NTC).

2. p210 and p190 primer set optimization

In order to obtain a very sensitive amplification of the transcripts of interest , primer sets concentrations and LAMP reaction conditions were optimized.
The optimization was performed on plasmids dilutions, ranging from 1000 to 10 copies/reaction, to evaluate assay sensitivity, and on water samples (NTC) to evaluate assay specificity. The modified parameters were: the reaction temperature, the reaction buffer pH value, the final concentration of primers, dNTPs and enzyme.

The first part of the optimization was focused on the selection of the best LAMP conditions to perform our simplex assays.
We tried a high number of variables in order to find the most sensitive and specific condition that permits the 10 plasmid copies/reaction amplification.
Changing the reaction temperature (Fig. 10) and the buffer pH (Fig.11) we observed a different threshold time of the 10 copies/reaction amplification and an effect on the assay specificity. We chose a temperature of 66°C for 50 minutes and a buffer pH of 7.9 that give the faster amplification time.

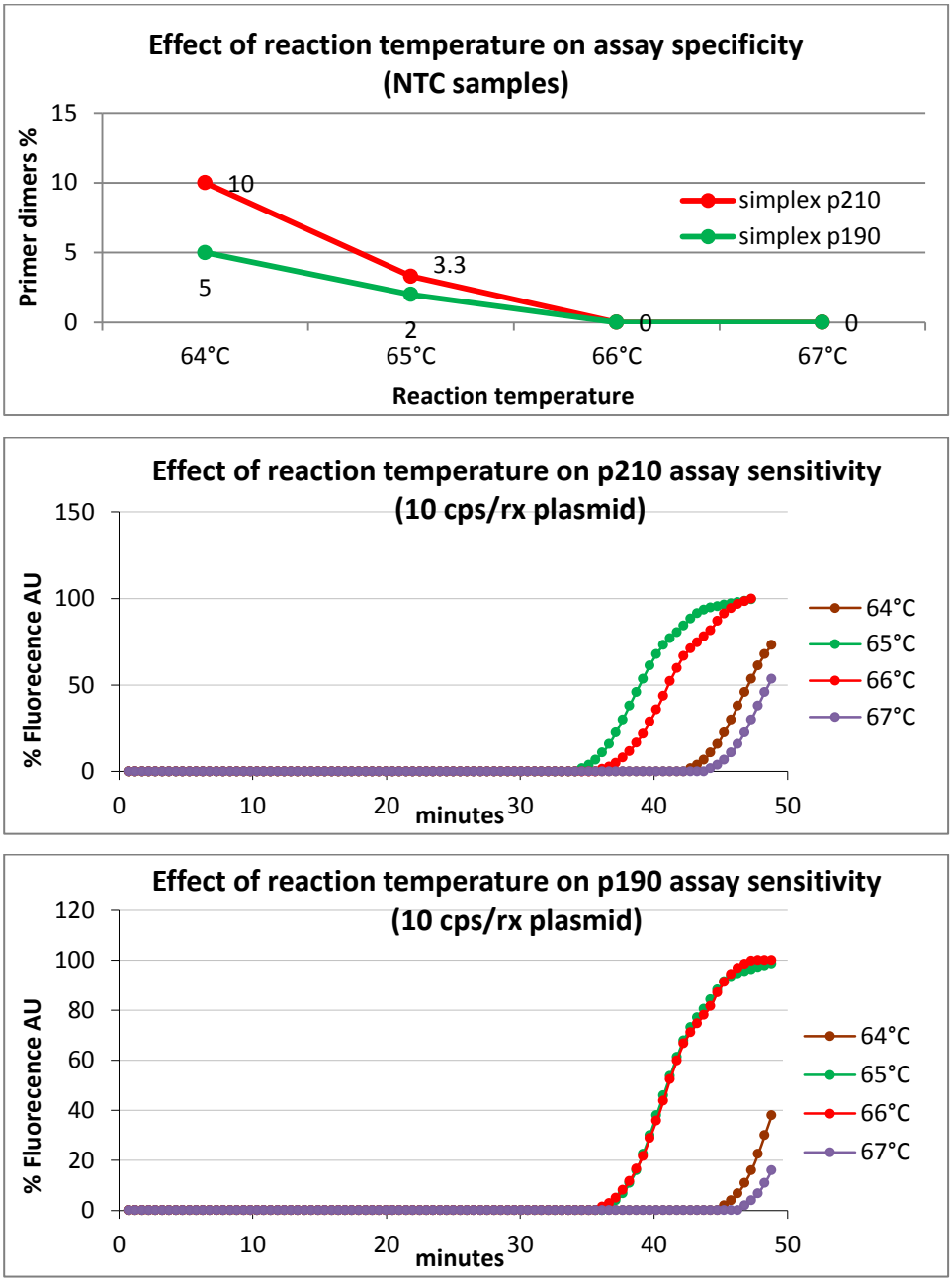
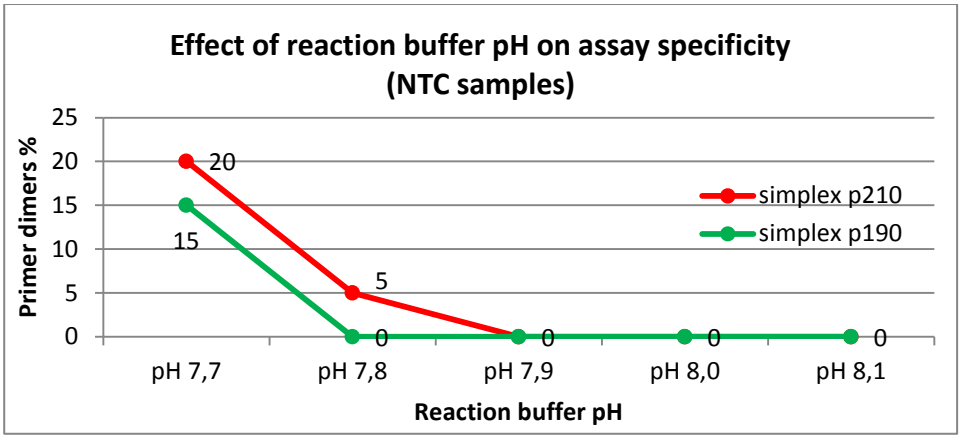
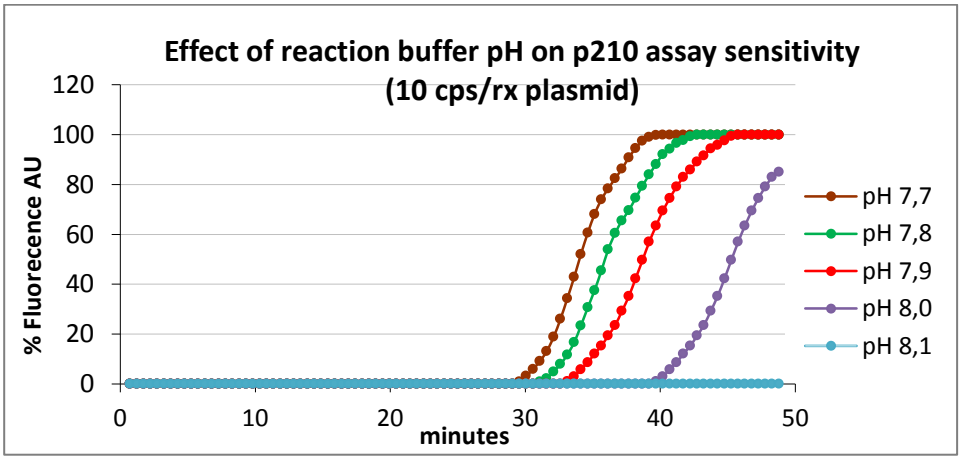


Figure 11: Effect of reaction temperature on the assay performance

p210 and p190 simplex assays were run on plasmids (10 cps/rx) and NTC (water samples) using different reaction temperatures. Increasing the reaction temperature we can observe a higher assay specificity, consisting in the absence of primer dimer amplification, and a variation in the assay sensitivity level, consisting in different Tt values of the 10 cps/rx plasmid. Reaction temperature 66°C was selected as optimal LAMP condition to allow a rapid and specific target amplification.





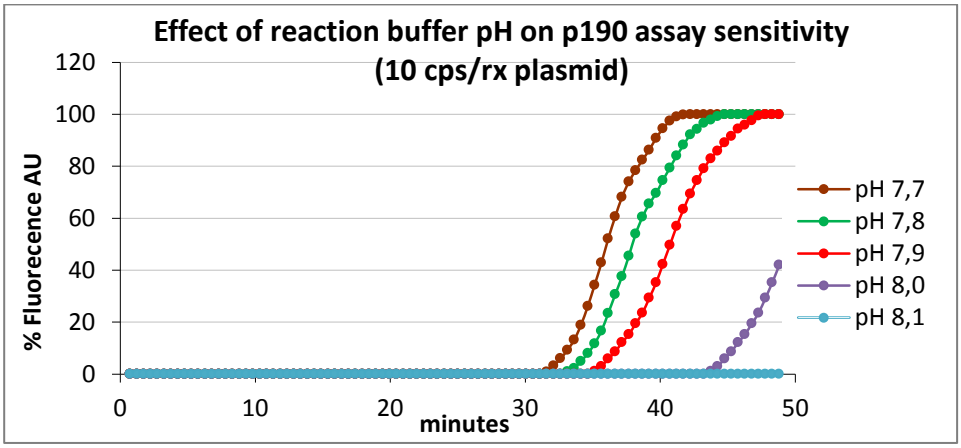


Figure 12: Effect of reaction buffer pH on the assay performance

p210 and p190 simplex assays were run on plasmids (10 cps/rx) and NTC (water samples) using different reaction buffer pH. Increasing the buffer pH we can observe a delay of the reaction and an increase of the assay specificity, whereas decreasing the buffer pH we obtain a reaction speed up and a lower assay specificity. Reaction buffer pH 7.9 was selected as optimal LAMP condition to allow a rapid and specific target amplification.

Once established the optimal reaction temperature and the buffer pH we continued the optimization acting on the other parameters.

An increase or a decrease of primers, enzyme and dNTPs concentrations can lead to a different sensitivity and specificity level of our simplex assay.

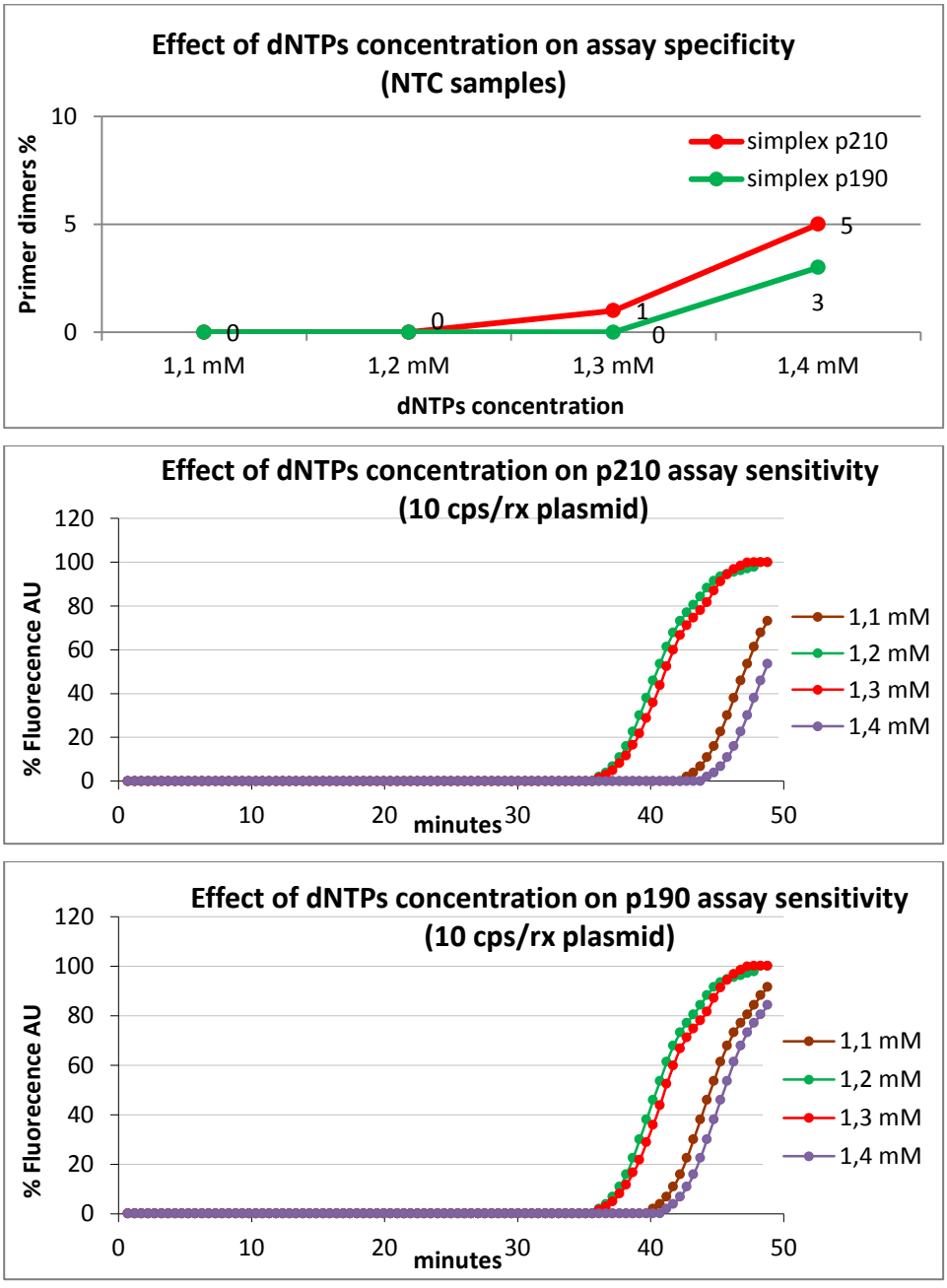
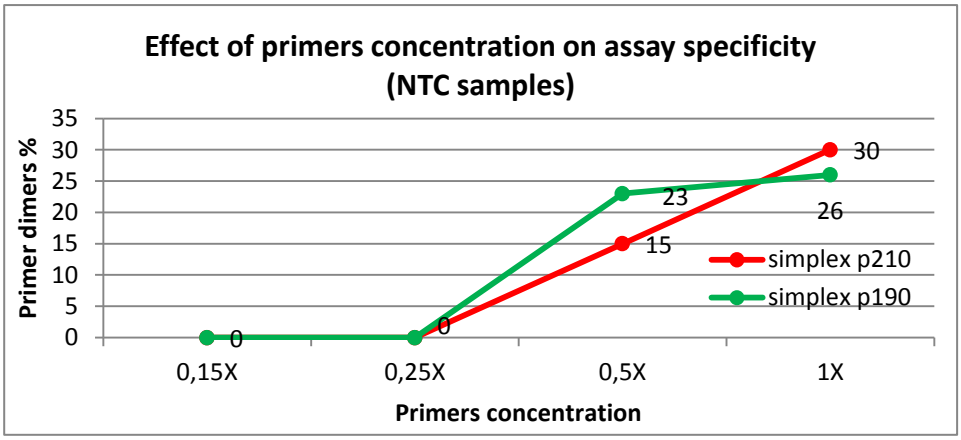
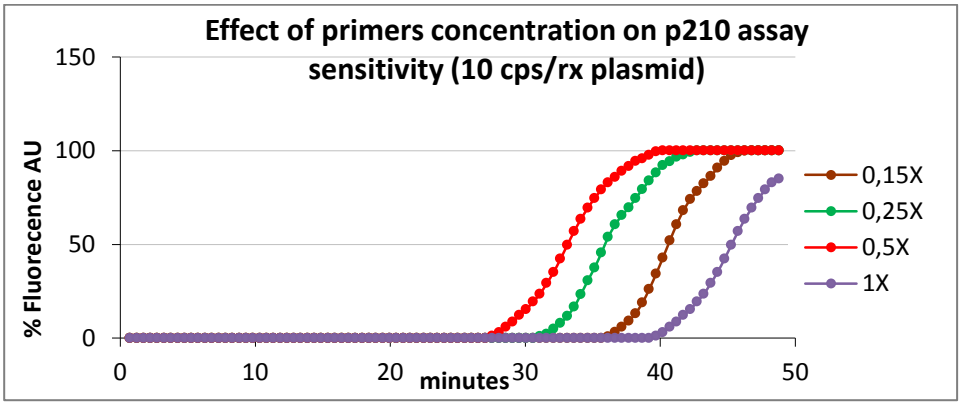


Figure 13: Effect of dNTPs concentration on the assay performance

p210 and p190 simplex assays were run on plasmids (10 cps/rx) and NTC (water samples) using different dNTPs concentrations. Too low or too high dNTPs concentration cause a delay in the reaction whereas increasing dNTPs concentration a decreasing of the specificity was observed. dNTPs concentration 1.2 mM was selected as optimal LAMP condition to allow a rapid and specific target amplification.





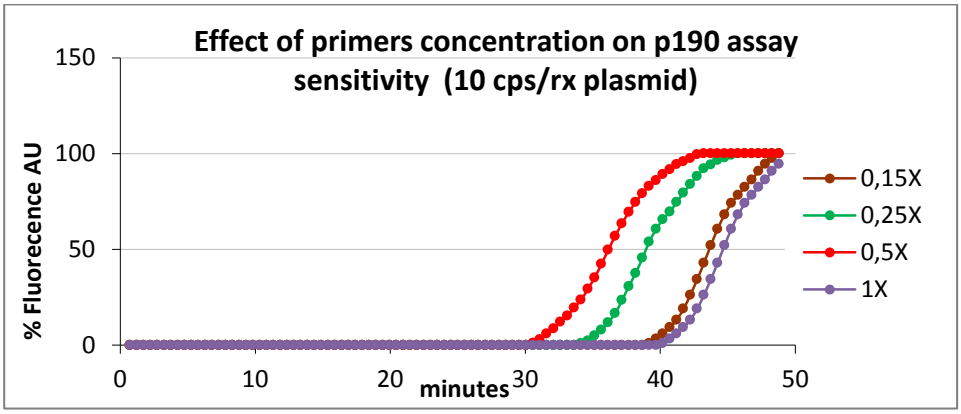
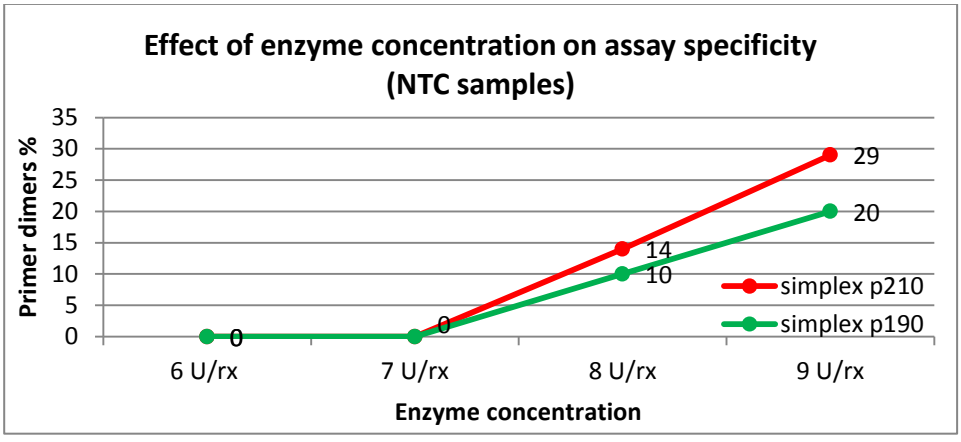


Figure 14: Effect of primers concentration on the assay performance

p210 and p190 simplex assays were run on plasmids (10 cps/rx) and NTC (water samples) using different primers concentration. 1X concentration refers to the standard LAMP primers concentrations. Increasing the primer concentration we can earlier detect the 10 cps/rx plasmid, but too high concentrations can inhibit the reaction. Regarding the specificity, the higher the primer concentration, the lower the specificity. Primer concentration 0.25 X was selected as optimal LAMP condition to allow a rapid and specific target amplification.



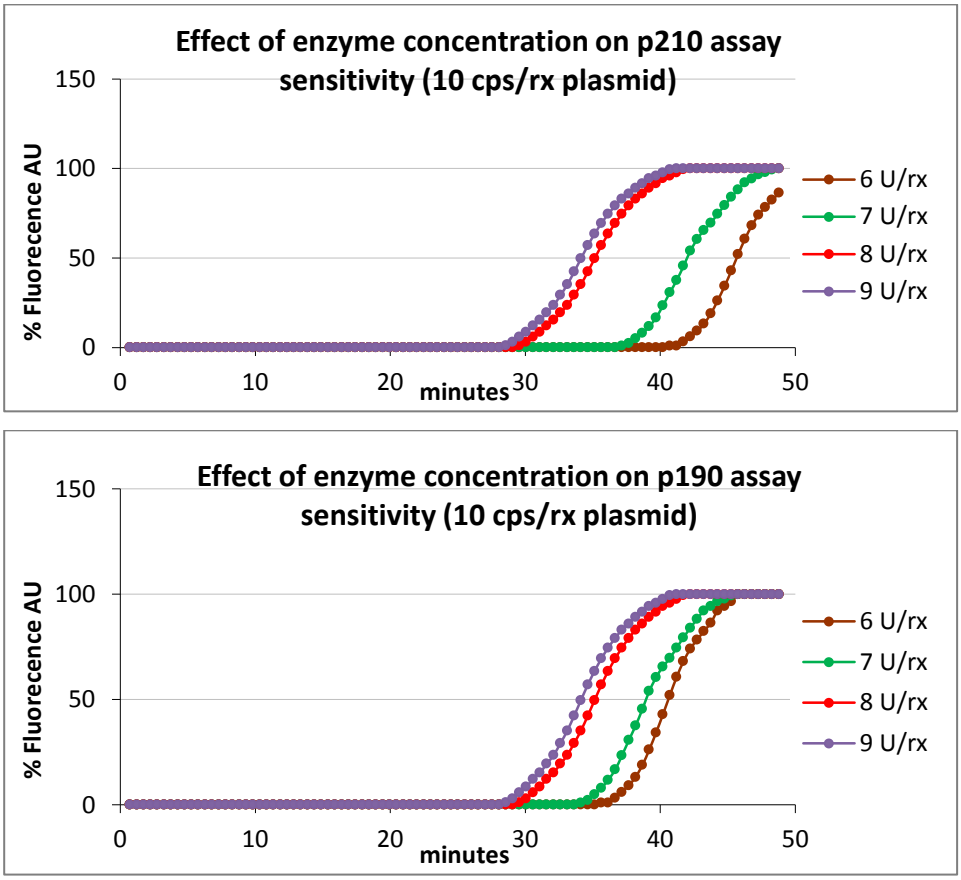


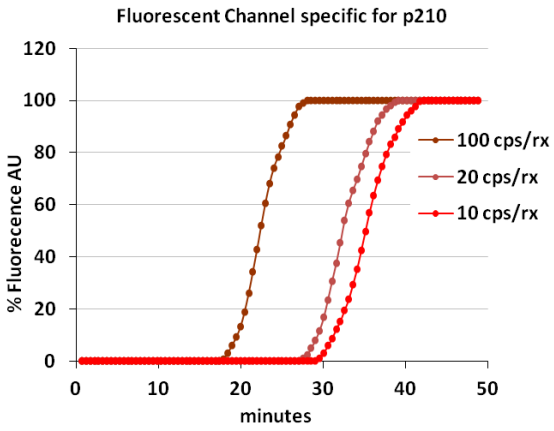
Figure 15: Effect of Bst polymerase enzyme concentration on the assay performance

p210 and p190 simplex assays were run on plasmids (10 cps/rx) and NTC (water samples) using different enzyme *concentrations. Increasing the* enzyme *concentration we obtain a speed up of the reaction and a decrease in assay specificity.* Enzyme *concentration 7 U/rx was selected as optimal LAMP condition to allow a rapid and specific target amplification.*

At the end of the optimization we obtained two very sensitive and specific p190 and p210 simplex assays using all the LAMP conditions previously selected.

Parameter	Optimized condition
Reaction temperature	66 °C
Buffer pH	7.9 pH
dNTPs concentration	1.2 mM
Enzyme concentration	7 U/rx
Primers concentration	0.25 X

Table 12: p190 and p210 simplex assays optimized LAMP reaction conditions



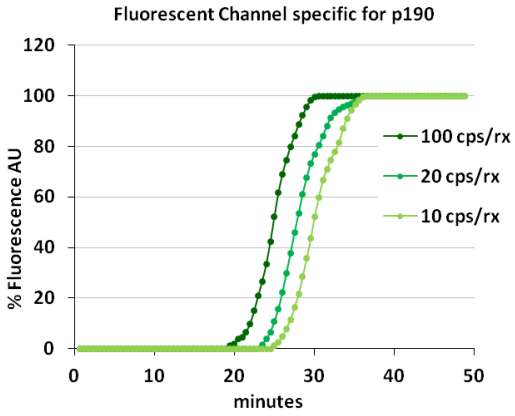


Figure 16: Optimized p210 and p190 simplex assays

The optimization was performed on serial plasmids dilutions (100-20-10 cps/rx) specific for the different transcripts and on NTC samples (H_2O). LAMP conditions were changed in order to allow a specific 10 cps/rx amplification before 50 minutes for both the target transcripts.

3. Internal control primer set design and screening

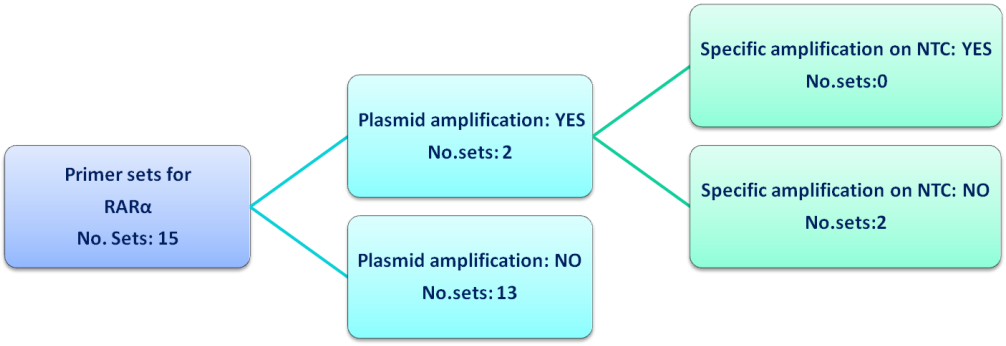
The use of an internal control in a LAMP reaction is very important to assess the quality and the integrity of the RNA sample tested, to control the extraction process and finally to evaluate the presence of reaction inhibitors. For this purpose we have designed primer sets specific for the amplification of a housekeeping gene. Different genes were evaluated in literature as possible optimal candidates (Beillard E et al., 2003); the selection was performed on the basis of the chromosomal location, the presence of eventual pseudogenes and the gene expression levels.

Only three genes were selected as possible internal control of LAMP reaction:

- RARα, encoding a nuclear receptor called Retinoic acid receptor alpha (RAR-α)
- ABL, Abelson murine leukemia viral oncogene homolog 1, encoding a cytoplasmic and nuclear protein tyrosine kinase
- GUSβ, encoding the Beta-glucuronidase enzyme

We designed, with the use of a prototype of LAMP primer design software, different primer sets for the specific amplifications of the three genes. Forward and backward primers were localized on different exons of the transcript in order to avoid genomic amplification.

Only GUSβ primer sets were able to amplify in an efficient and specific way the plasmid at the previously established reaction conditions. For this reason GUSβ was selected as internal control gene.



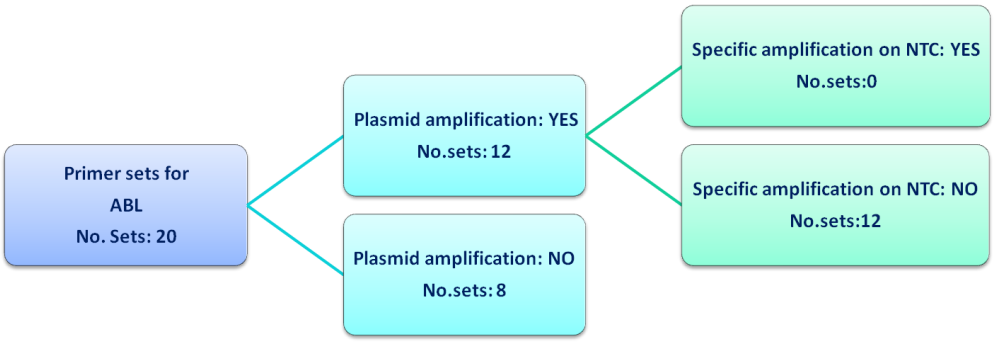


Table 13: ABL and RARα primer sets design and screening results

Forward and backward sets were designed using the DiaSorin LAMP design software. 20 different primer sets were generated for ABL on different exons. 12 sets for exon 3-4, 5 sets for exon 4-5 and 3 sets for exon 6-7. 15 different primer sets were generated for RARα on different exons. 10 sets for exon 5-6 and 5 sets for exon 6-7.

The screening of the best primer set was performed on 100 copies/reaction of plasmid and water samples using LAMP conditions optimized for the sensitive amplification of p210 and p190. Only 12 sets for ABL and 2 sets for RARα were able to amplify the plasmid but the amplification was not specific.

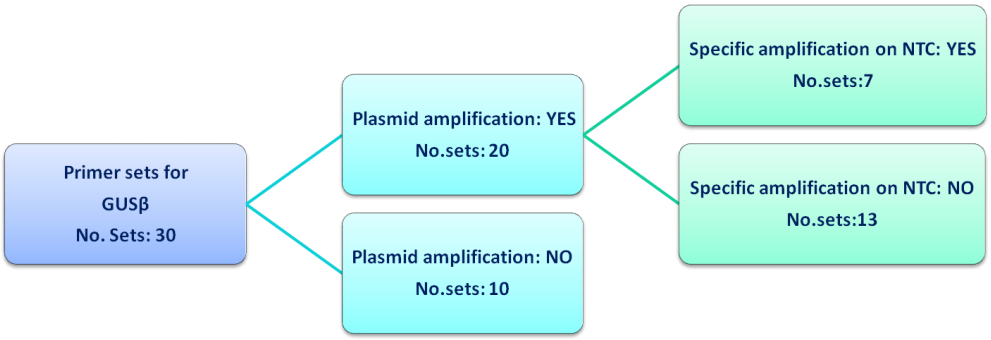


Table 14: GUSβ primer sets design and screening results

Forward and backward sets were designed using the DiaSorin LAMP design software.

30 different primer sets were generated for GUSβ on different exons. 5 sets for exon 1-2, 10 sets for exon 9-10 and 10 sets for exon 11-12. The screening of the best primer set was performed on 100 copies/reaction of plasmid and water samples using LAMP conditions optimized for the sensitive amplification of p210 and p190. The criteria for the primer sets screening were the ability to amplify the target plasmid and the absence of primer dimers using water nuclease-free as non template control (NTC). 7 sets were able to amplify the target in a specific manner. The selected set is designed on exon 9 and exon 10.

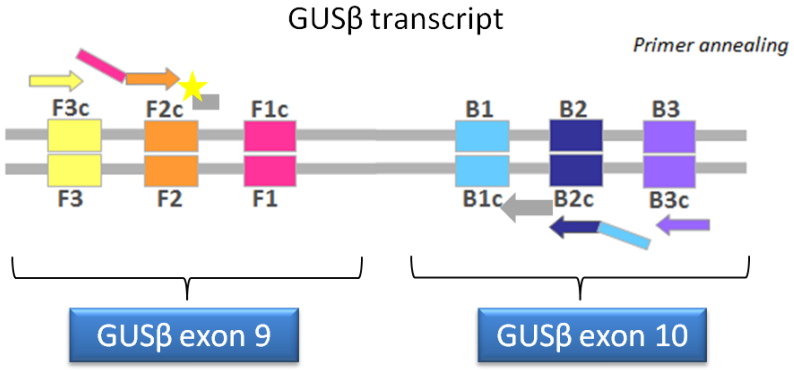


Figure 17 : Internal control primer design strategy

The backward primer set (B3-BIP and LB) is designed on GUSβ exon 9 while the forward primer set (F3-FIP and LF) is designed on exon 10. Specific probe labeled with fluorophore is designed in order to discriminate GUSβ amplification from p190 and p210 amplification.

4. LAMP enzyme evaluation

One of the major limitation of the standard diagnostic method currently used in all the Hospitals, is the necessity to perform a separated reverse-transcription step in order to produce cDNA used for the amplification reaction. To avoid this step our assay is able to amplify the target transcripts directly from RNA.

Two main different strategies were evaluated: the use of two enzyme in a single reaction, one specific for the RT step and the other for the amplification, and the use of one unique enzyme presenting both the activities. This last enzyme was engineered by the DiaSorin group.

The enzymes specific for the RT step (AMV by Promega, SuperScript III by Life Technologies and MMLV by Promega) were selected on the basis of the most used commercial RT protocols whereas the enzymes used for the amplification step (BST by NEB, SD by Bioron) are polymerases presenting strand-displacement activity.

The two different approaches were evaluated. We used primer sets and LAMP conditions previously optimized on plasmids. For the two enzyme approach we added an incubation time of 15 minutes, at the optimal temperature of the selected RT enzyme, before the amplification phase.

The tests were performed on water samples and RNA samples extracted from cell lines positive for p210 (K562) or p190 (TOM1) and negative for both the transcripts (HL60).

The one enzyme allowed the most efficient and specific amplification of the target transcripts and the internal control and it was selected as the optimal RT-LAMP enzyme.

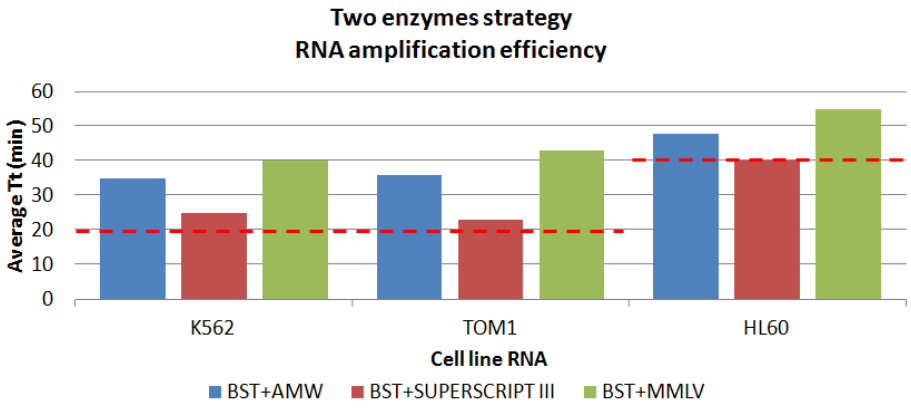


Figure 18: p210, p190 and GUSβ amplification directly from RNA using the two enzymes approach (Bst DNA polymerase enzyme)

The average Tt values for the RNA extracted from different cell lines are shown in the graph. Red lines indicated Tt values comparable to those obtained using plasmids. The reaction was performed using Bst, as amplification enzyme, in combination with 3 different RT enzymes (AMV, Superscript III and MMLV). The reaction conditions used were the previously optimized on plasmids. The reaction time was 15 min (to allow reverse transcription step) + 60 min of LAMP reaction.

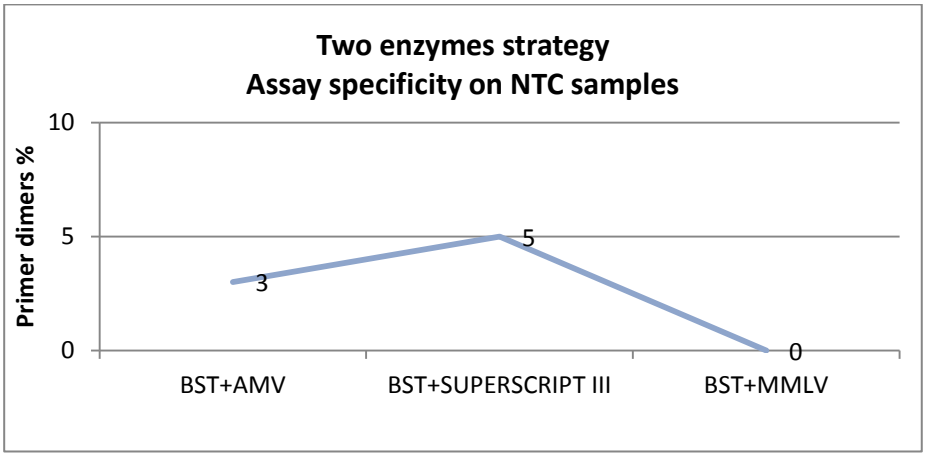


Figure 19: p210, p190 and GUSβ reaction specificity using the two enzymes approach (Bst DNA polymerase enzyme)

In the graph are shown the primer dimers percentages obtained performing p210, p190 and GUSβ reaction on NTC samples using the two enzyme approach. The combination of Bst with AMW and Superscript III produced not specific amplification, only the combination with MMLV seems to be specific.

The two enzyme approach, using Bst as amplification enzyme, permits the amplification directly from RNA in all the three combination tested.

However only the combination with Superscript III enzyme showed an efficient amplification, with Tt comparable to those obtained using plasmids. This combination is associated with the higher primer dimers percentage on NTC samples. For this reason this approach was discarded.

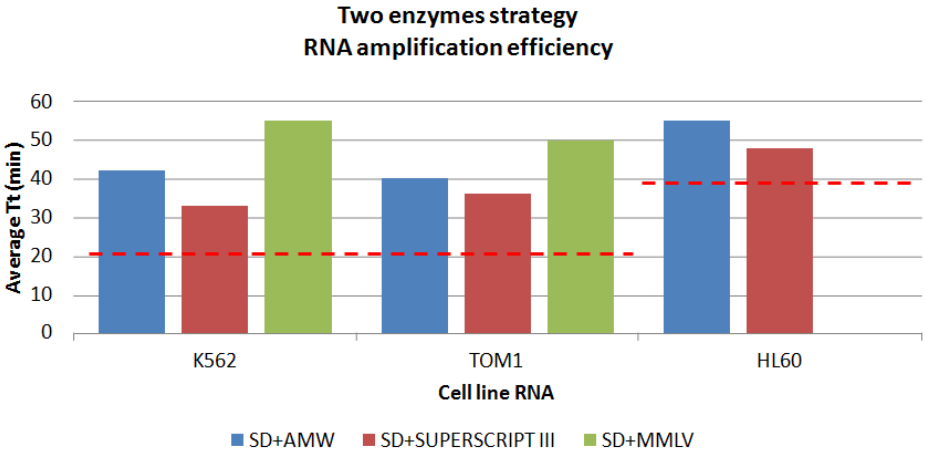


Figure 20: p210, p190 and GUSβ amplification directly from RNA using the two enzymes approach (SD DNA polymerase enzyme)

In the graph are shown the average Tt values for the RNA extracted from different cell lines. Red lines indicated Tt values comparable to those obtained using plasmids. The reaction was performed using SD, as amplification enzyme, in combination with 3 different RT enzymes (AMV, Superscript III and MMLV). The reaction conditions used were the previously optimized on plasmids. The reaction time was 15 min (to allow reverse transcription step) + 60 min of LAMP reaction.

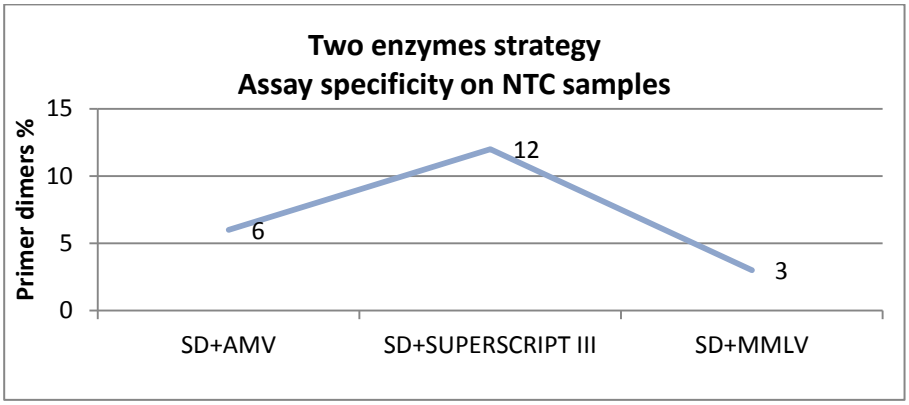


Figure 21: p210, p190 and GUSβ reaction specificity using the two enzymes approach (SD DNA polymerase enzyme)

In the graph are shown the primer dimers percentages obtained performing p210, p190 and GUSβ reaction on NTC samples using the two enzyme approach. All the tested combination of SD with RT-enzymes produced no specific amplification on NTC samples. No specific combinations were found.

The two enzyme approach, using SD as amplification enzyme, permitted the amplification directly from RNA only in 2 out of 3 tested combinations. SD+MMLV didn't amplify HL60 RNA and showed a very delayed amplification on the K562 and TOM1 RNA. The other two combinations amplified the tested RNA with delayed Tt respect to the Bst approach. Moreover we detected primer dimers formation using all three combinations.

However only the combination with Superscript III enzyme showed an efficient amplification, with Tt comparable to those obtained using plasmids, but it is the combination associated with the higher primer

dimers percentage on NTC samples. For this reason this approach was discarded.

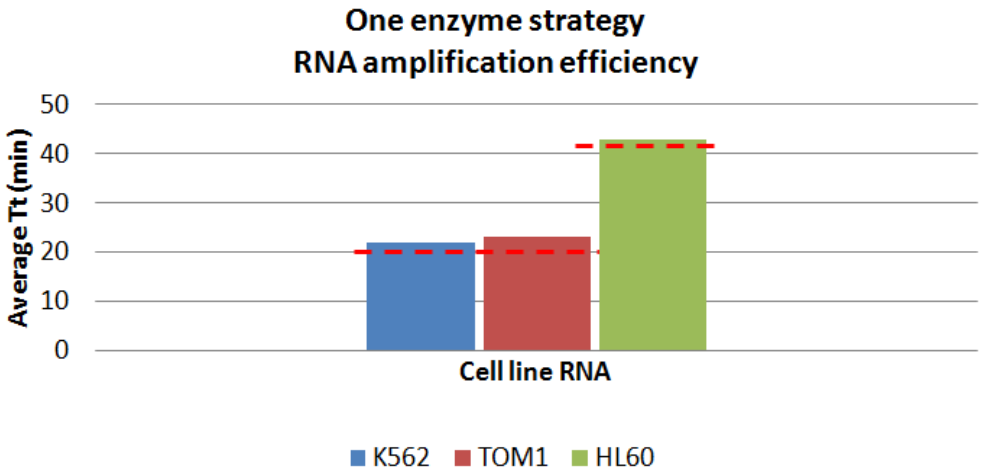


Figure 22: p210, p190 and GUSβ amplification directly from RNA using the one enzyme approach (Diasorin enzyme)

In the graph are shown the average Tt values for the RNA extracted from different cell lines. Red lines indicated Tt values comparable to those obtained using plasmids. The reaction was performed using only Diasorin enzyme to perform both reverse-transcription and amplification steps. The reaction conditions used were the previously optimized on plasmids.

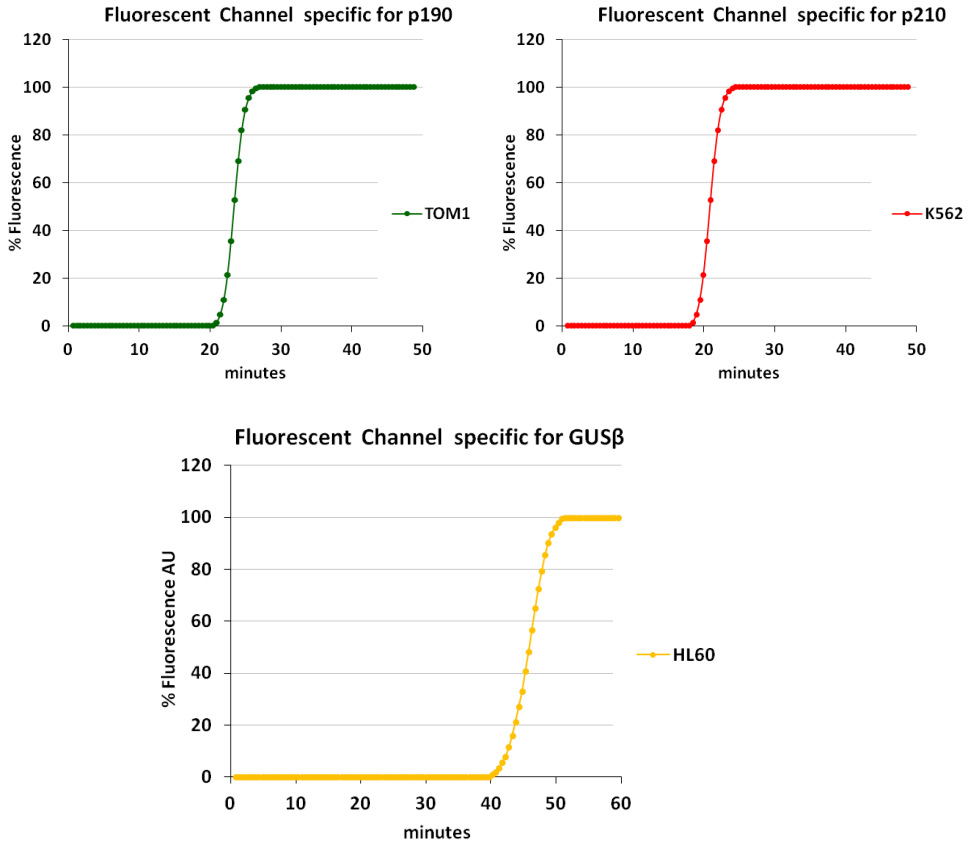


Figure 23: p210, p190 and GUSβ amplification directly from RNA using the single enzyme approach.

Amplification plots of p190 (green), p210 (red), GUSβ (yellow) are shown. The amplifications were obtained directly on RNA extracted from cell lines positive and negative for the specific translocation. We used 500 ng/rx of RNA. TOM1 cell line is positive for p190 , K562 cell line is positive for p210, HL60 is negative for both the transcripts.

The one enzyme approach, using Diasorin enzyme, permitted the amplification directly from RNA in all the cases with Tt values very similar to those obtained using plasmids. The reaction time is reduced respect to the two enzyme approach due to the lack of the 15 min for RT-phase and we didn't observe any primer dimer on NTC samples.

This approach was the best candidate to develop our final assay in order of simplicity of execution, efficiency of amplification and specificity of the reaction.

5. Multiplex assay format set-up

The ability of the final assay to amplify both p190 and p210 transcripts and the internal control in one unique reaction is another important feature that renders our assay competitive compared to the currently used protocol. As first step we tried to optimized two different duplex assays, specific for the simultaneous amplification of the p210 or p190 transcripts and the internal control.

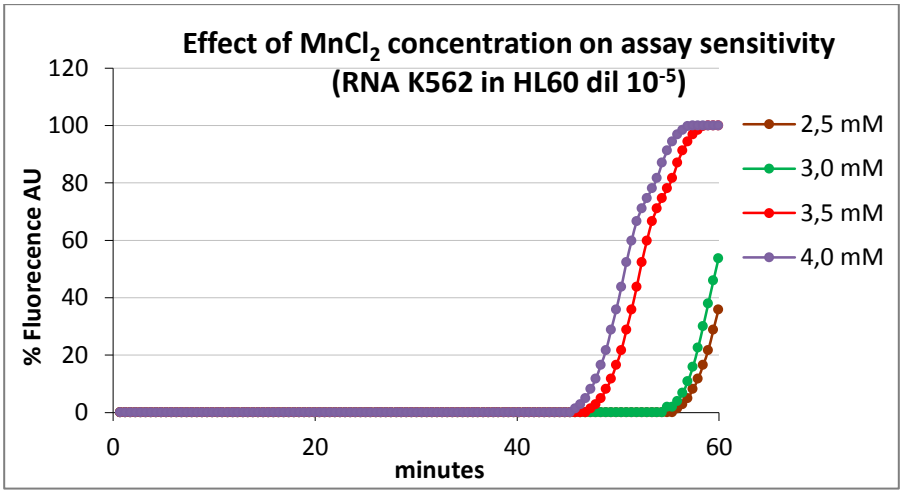
The optimization was performed on serial dilutions of positive RNA into negative RNA, in order to obtain very sensitive assays able to detect the target transcripts down to a dilution of 10^{-4} or 10^{-5}. The dilutions have been made in negative RNA to mimic clinical samples.

To reach this sensitivity level, we needed to modify LAMP reaction conditions. In particular we focused on two aspects: the manganese chloride concentration and primers concentration.

The optimization of the enzyme co-factor concentration ($MnCl_2$) was necessary in order to improve the RT step efficiency and therefore improve the sensitivity of the assay.

Moreover the final concentration of each single primer of the duplex assay was optimized to obtain an amplification of the GUSβ transcript delayed respect to the target one. In this way we can avoid any competition during the amplification in order to detect also very low quantity of the target transcripts. The amplification of the internal control has to occur only in negative samples in order to validate the results.

We also monitored the specificity of the assay testing water samples, for primer dimers detection, and negative RNA, for non specific amplification detection.



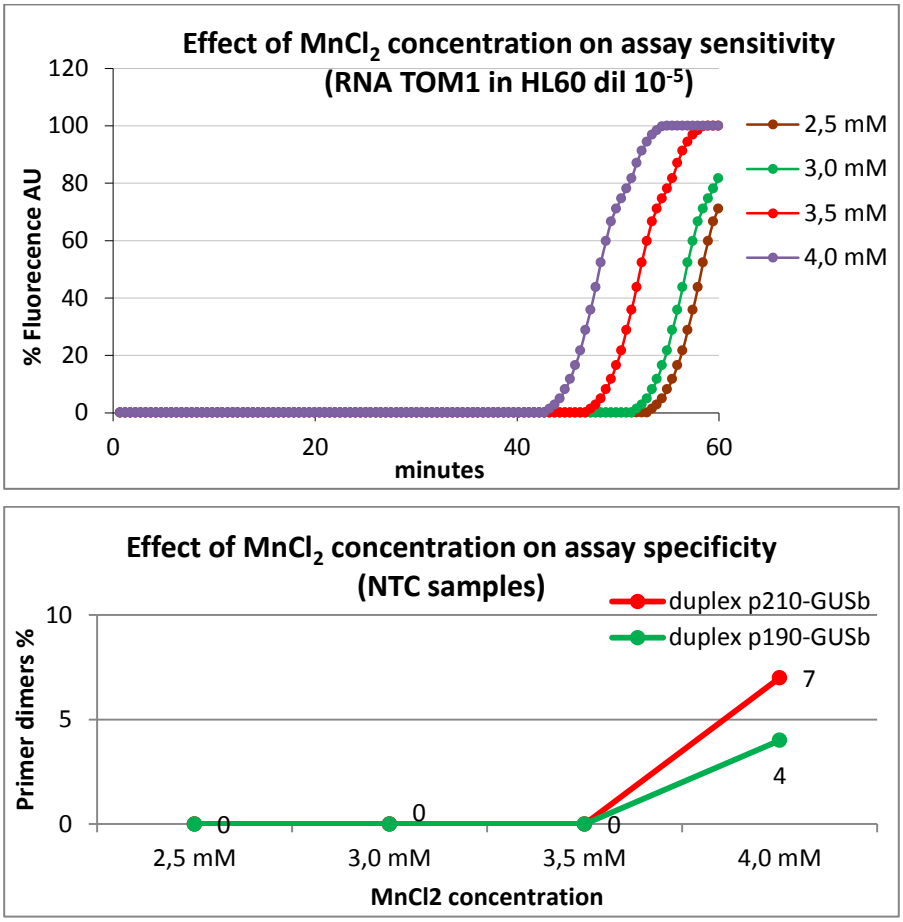


Figure 24: Effect of $MnCl_2$ concentration on the assay performance

p210 and p190 duplex assays were run on RNA TOM1 and K562 diluted in HL60 (dilution 10^{-5}) and NTC (water samples) using different $MnCl_2$ *concentrations. Increasing the* $MnCl_2$ *concentration we obtain an improvement on 10^{-5} RNA dilution detection. Regarding the specificity we can notice an increase in primer dimers formation at 4.0 mM.* $MnCl_2$ *concentration 3.5 mM was selected as optimal LAMP condition to allow a sensitive and specific amplification for both p210 and p190 targets.*

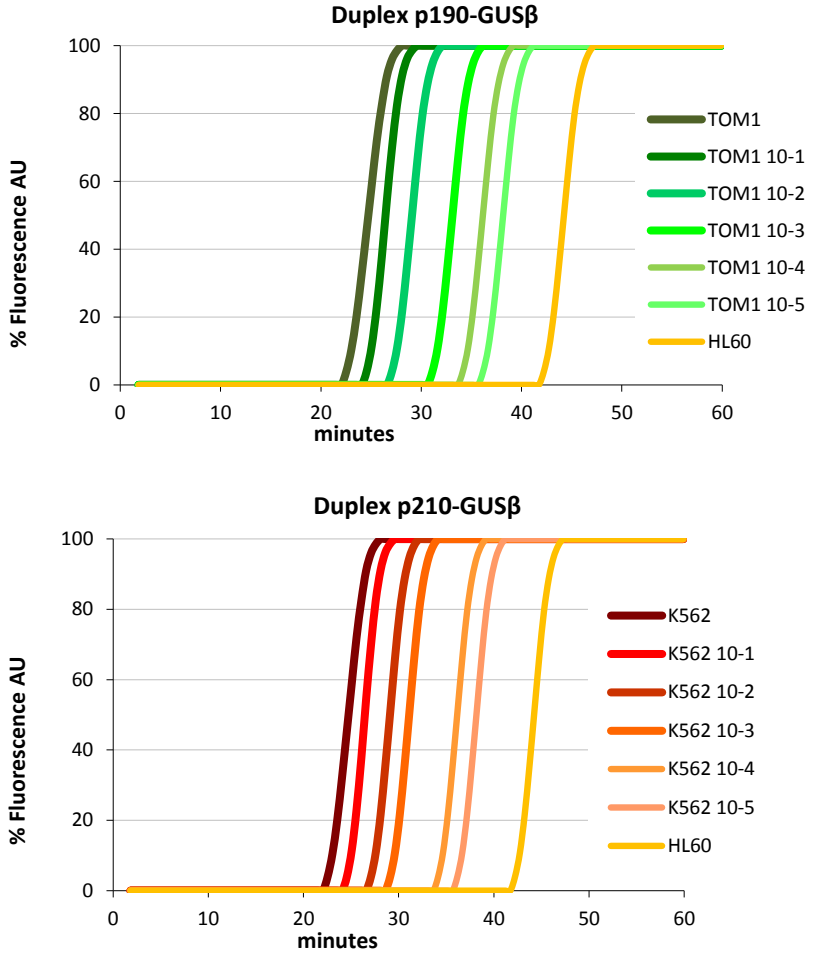


Figure 25 : p210 and p190 optimized duplex assays

Amplification plots of p190 and p210 duplex assay are shown. Serial dilutions of RNA positive for the specific transcript into RNA negative for the translocations (HL60) were used to optimize the reactions. The reactions were performed at 66°C for 60 minutes using 500 ng/rx of RNA. GUSβ amplification occurred only in negative samples (HL60) validating the result. The amplification of p190 occurred in

the specific 500nm channel (green), the amplification of p210 occurred in the 570nm channel (red) and the amplification of Internal control in the specific 530nm channel (yellow).

Once demonstrated the feasibility of a LAMP duplex assay we optimized a triplex system in which both target transcripts and the internal control can be amplified. The optimization was performed on serial dilutions of positive RNA into negative RNA (HL60), in order to obtain very sensitive assays able to detect the target transcripts down to a dilution of 10^{-5}, and on water and negative RNA samples to evaluate the specificity.

The optimization was mainly focused on primers concentration in order to found the correct formulation that allowed simultaneous p190 and p210 amplification. The final concentration of each single primer of the p210 and p190 sets was optimized to obtain a perfect balance between the two different amplification reactions. Moreover the IC concentration was recalibrated in order to avoid that a stronger Internal Control amplification cause a decrease in assay sensitivity.

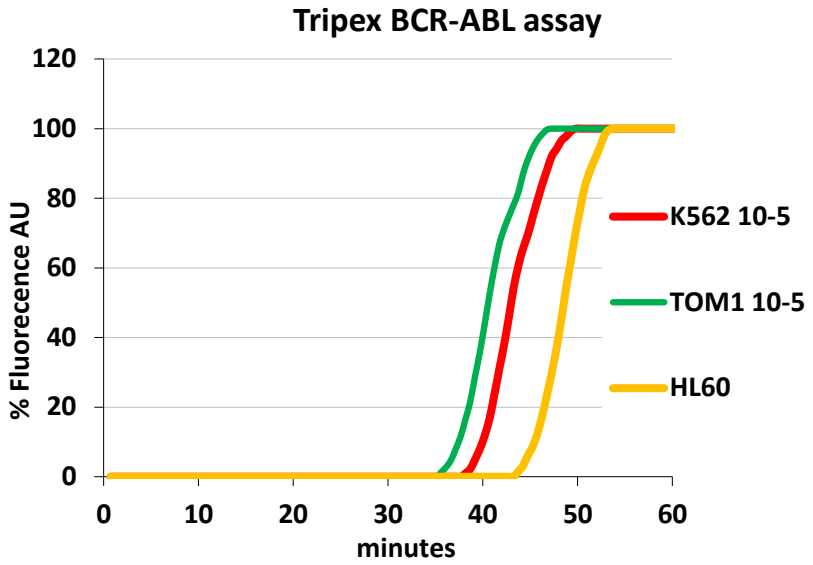


Figure 26: BCR-ABL optimized triplex assay

Amplification plot of BCR-ABL triplex assay is shown. Serial dilutions of RNA positive for the specific transcript into RNA negative for the translocations (HL60) were used to optimize the reaction. The reaction was performed at 66°C for 60 minutes using 500 ng/rx of RNA. In yellow GUSβ amplification, in red p210 amplification and in green p190 amplification.

6. Assay reaction controls implementation

The introduction of plasmids negative and positive controls are very important to guarantee the validity of the assay results. The correct reaction controls amplification in specific channels and at specific threshold times ensures that mix preparation and assay set up have been prepared properly, ensuring reliable results.

The negative control is a plasmid containing the sequence of interest of the GUSβ transcript whereas the dual positive control is a mix of two different plasmids, one specific for p190 and the other for p210. This strategy permits to control the amplification of the three different transcripts with the use of only two controls, incrementing the number of reactions dedicated to clinical samples loading.

Moreover, to avoid controls denaturation before adding them to the reaction, we performed several tests to find an optimal concentration that can be used directly.

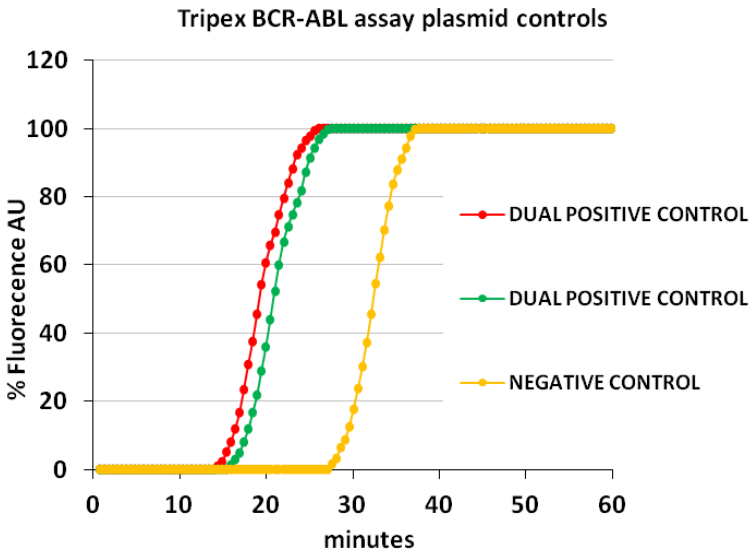


Figure 27: Plasmid controls amplification

Amplification plot of BCR-ABL triplex assay plasmid controls is shown. In the yellow channel (530 nm) occurs the negative control amplification (GUSβ plasmid). The dual positive control (a mix of p210 and p190 plasmids) occurs both in green (500 nm) and in red (570nm) channels. The amplification of the controls in the correct channel and at the correct threshold time ensures the reliability of the results.

7. Assay freeze-drying

To ensure greater product stability and allow the storage and transport in a practical and affordable way, the ideal format of the final assay is the lyophilized form. The lyophilization was made with the use of the Diasorin freezedryer Virtis-Advantage Plus FL.

Using the freeze-dried formulation we observed an amplification delay on all the tested targets. This reaction delay was due to an impairment of the enzyme efficiency during the freeze-drying process. To overcome this problem we needed to re-optimize the enzyme concentration in order to obtain the same liquid format performances.

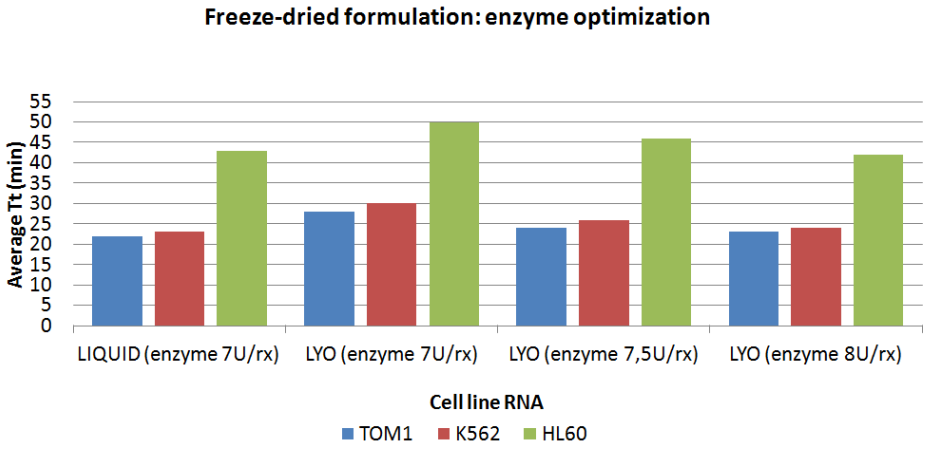


Figure 28: Freeze-dried formulation: enzyme optimization

In the graph are shown the average Tt values for the RNA extracted from different cell lines. The freeze-dried reaction was performed using different enzyme

concentrations. In the presence of 8U/rx of enzyme we can obtain performances similar to the liquid assay.

Moreover tests performed on water and negative RNA samples showed a decreased specificity of the freeze-dried formulation respect to the liquid one. This phenomena is due to the creation of primer dimer during the freezing step of the lyophilization process. To maintain the same high specificity level of the liquid formulation it was necessary the separation in a second cake of the labelled probes. The probes , interacting with the primers, contributed to the primer dimer formation causing a decrease in the assay specificity.

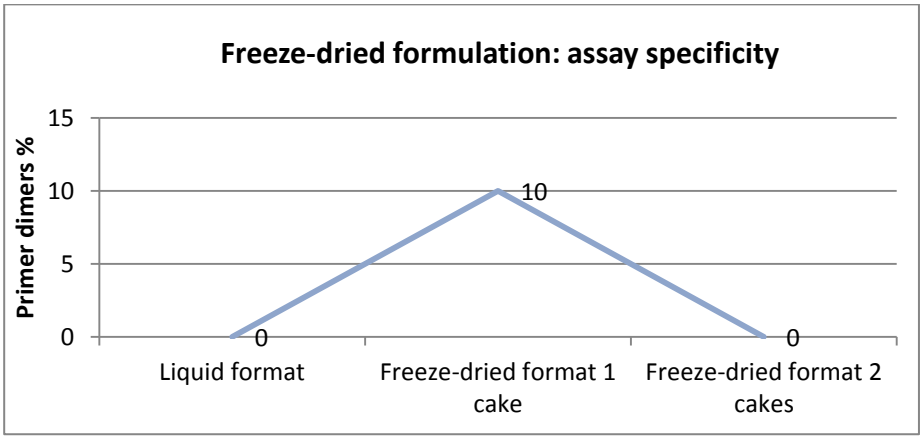


Figure 29: Freeze-dried formulation: assay specificity

In the graph are shown the primer dimers percentages obtained performing liquid format versus freeze-dried format 1 or 2 cakes on NTC samples. The introduction of two different cakes in the freeze-dried format permits to obtain the same specificity level of the liquid format.

The final freeze-dried assay consists of two different cakes: one containing dextran plus dNTPs, enzyme and primers, and the other one containing dextran and probes.

Once freeze dried, the cakes can be easily reconstituted adding the two remaining elements of the liquid reaction mix: buffer and manganese.

8. Final assay performances (freeze-dried formulation)

8.1. Analytical sensitivity and specificity

To assess the analytical sensitivity we performed the triplex LAMP assay using 500 ng/reaction of dilutions of positive RNA into negative RNA (pure RNA, 10^{-1},10^{-2},10^{-3},10^{-4}). The RNA was extracted from cell lines using the Qiagen RNeasy kit. We used K562 cell line positive for p210, TOM1 cell line positive for p190 and HL60 cell line negative for both the transcripts.

100 replicates for each dilution were tested and we obtained a sensitivity of 100% on 10^{-3} and of 87.5% on 10^{-4} for p210, and 100% on 10^{-3} and 92.5% on 10^{-4} for p190.

To assess the analytical specificity we tested the triplex LAMP assay using 500 ng/reaction of negative RNA deriving from 7 different cell lines (HL60,RS4-11,NB4,MV4,REH,KASUMI,697) and on water samples (NTC).

558 replicates of negative RNA and 382 replicates of water were tested. No amplification occurred in the water samples and only internal control amplification was observed using negative RNA showing an assay specificity of 100%.

These assay performances were also confirmed using phenol-chloroform extraction method, commonly used in the Hospitals.

In this case 40 replicates of 10^{-2},10^{-3},10^{-4} RNA/RNA dilution were tested for both the transcripts. We obtained a sensitivity of 100% on 10^{-3} and 72.5% on 10^{-4} for p210 and 100% on 10^{-3} and 92.5% on 10^{-4} for p190.

For the assessment of specificity we tested 240 replicates of negative RNA (HL60 and NB4 cell lines) and we observed a specificity of 100%.

		K562 10^{-2}			K562 10^{-3}			K562 10^{-4}		
Extraction Method	No. replicates	% Detection	Avg Tt	Std. Dev	% Detection	Avg Tt	Std. Dev	% Detection	Avg Tt	Std. Dev
Qiagen RNeasy	100	100	28.2	3.1	100	28.4	1.6	87.5	36.0	5.3
TRIzol®	40	100	26.7	1.2	100	30.1	2.6	72.5	40.0	6.9

		TOM1 10^{-2}			TOM1 10^{-3}			TOM1 10^{-4}		
Extraction Method	No. replicates	% Detection	Avg Tt	Std. Dev	% Detection	Avg Tt	Std. Dev	% Detection	Avg Tt	Std. Dev
Qiagen RNeasy	100	100	27.7	1.3	100	31.3	2.0	92.5	39.1	5.3
TRIzol®	40	100	26.2	0.8	100	29.8	2.2	92.5	37.9	6.1

Table 15: Triplex assay analytical sensitivity

Analytical sensitivity was evaluated on RNA/RNA dilutions (K562 in HL60 for p210 and TOM1 in HL60 for p190). The RNA extraction was performed using two different methods: Qiagen RNeasy kit and Phenol-Chloroform procedure. We obtained a sensitivity of 100% for 10^{-3} dilution for both p210 and p190.

			NEGATIVE RNA			NTC (water)	
Extraction Method	No. replicates	Cell line tested	% Detection	Avg Tt	Std. Dev	No. replicates	% Detection
Qiagen RNeasy	558	7	100	42.8	2.0	382	0
TRIzol®	240	2	100	41.4	2.5		

Table 16: Triplex assay analytical specificity

Analytical specificity was evaluated on negative RNA for the two transcripts and on NTC samples (water). Seven different cell lines were evaluated using Qiagen RNeasy extraction kit (HL60,RS4-11,NB4,MV4,REH,KASUMI,697) and 2 different cell lines were evaluated using phenol-chloroform procedure (HL60 and NB4). We observed the correct internal control amplification (530nm) in all the negative RNA tested and no amplification occurred in the water samples. The assay specificity is 100%.

8.2. Assay robustness

It is know that the PCR reaction can be frequently inhibited by several compounds.

To assess the robustness of the LAMP technology we tested with the BCR-ABL LAMP assay the most common inhibitors found in literature.

We considered both potential inhibitors carried over from cell isolation, such as haemoglobin, heparin, IgG, lactoferrin, ficoll and phenol, and potential contaminants carried over from RNA extraction process , such as genomic DNA, isopropanol, ethanol and guanidine isothiocyanate.

In the first case HL60 lysates were spiked with potential inhibitors and extracted using Qiagen RNeasy Mini kit whereas in second case potential inhibitors were spiked into HL60 RNA extracts. The results were compared with negative controls ,normal HL60 lysates and extracted HL60 RNA. This

study demonstrates that residual inhibitor from cell isolation causes the failure of OD ratios either A260/280 or A260/230 or both, but this does not cause a delay in HL60 RNA threshold time.

Potential Inhibitor	Concentration	Avg Tt	Std. Dev	A_{260}/A_{280}	A_{260}/A_{230}	Tt diff
Control	0	38.79	0.12	2.1	1.9	-1.94
HAEMOGLOBIN	0.18 mg/ml	40.73	0.96	2.1	1.7	
Control	0	38.19	0.39	2.1	1.9	-3.44
HEPARIN	17.5 USP/ml	41.63	0.67	2.1	1.3	
Control	0	39.04	0.28	2.1	1.9	-2.5
IgG	16 mg/ml	41.54	0.86	2.1	1.5	
Control	0	39.31	0.88	2.1	1.9	-1.38
LACTOFERRIN	1.5 µg/ml	40.69	0.57	2.1	1.7	
Control	0	40.95	1.12	1.8	1.9	0.22
FICOLL	5%	40.73	0.64	4.4	0.4	
Control	0	40.87	0.33	1.8	1.8	-0.48
PHENOL	0.20%	41.35	0.73	1.6	1.9	

Table 17: Potential inhibitors carried over from cell isolation

In the table are shown the threshold time values and the spectrophotometric absorbance ratio of HL60 RNA derived from a contaminated cell lysate. The results were compared with those obtained from normal lysates used as controls. Inhibitors carried over from cell isolation can affect RNA OD ratios but they have no impact on the assay performance in terms of amplification threshold time.

Regarding contaminants derived from extraction process guanidine isothiocyanate generated a low A260/230 ratio but also in this case HL60 RNA Threshold times were not affected. 5% Isopropanol and 5% ethanol did not affect the OD ratios but the threshold times were significantly delayed. These concentrations are really very high and can't be present in normally extracted RNA. A more realistic concentration of these two inhibitors was then tested (1%) giving optimal results.

Potential Inhibitor	Concentration	Avg Tt	Std. Dev	A_{260}/A_{280}	A_{260}/A_{230}	Tt diff
Control	0	39.02	0.36	1.8	1.9	-0.41
GENOMIC DNA	5%	39.43	0.60	2.0	1.8	
Control	0	40.48	2.42	1.8	1.9	-8.48
ISOPROPANOL	5%	48.96	1.02	1.8	2.0	
Control	0	40.07	0.26	1.8	1.8	-6.49
ETHANOL	5%	46.56	1.19	1.8	1.9	
Control	0	39.40	0.31	1.8	1.8	0.15
GUANIDINE ISOTHIOCYANATE	5 mM	39.25	0.61	1.9	0.3	

Potential Inhibitor	Concentration	Avg Tt	Std. Dev	A_{260}/A_{280}	A_{260}/A_{230}	Tt diff
Control	0	40.47	0.37	1.7	1.9	-1.93
ISOPROPANOL	1%	42.40	0.93	1.7	1.9	
Control	0	40.69	0.76	1.8	1.8	-0.84
ETHANOL	1%	41.52	0.77	1.8	1.7	

Table 18: Potential contaminants carried over from RNA extraction process

In the table are shown the threshold time values and the spectrophotometric absorbance ratio of HL60 RNA derived from a contaminated extraction process. The results were compared with those obtained from normal RNA used as controls. The presence of genomic DNA doesn't impact OD ratio or threshold time values. Guanidine isothiocyanate causes a non optimal A260/230 ratio whereas isopropanol and ethanol can affect the assay performance resulting in delayed threshold time values. Using more realistic isopropanol and ethanol contaminants concentration this inhibitory effect disappears.

Finally we assessed the robustness of the Q-LAMP in the presence of non optimal concentrations of the RNA samples. The assay was able to amplify correctly and at very similar Threshold times, RNA samples presenting concentration 10 fold lower than the optimal one, showing a very high robustness of the LAMP amplification method.

	SAMPLE	Avg Tt (min)
p190	TOM1 100 ng/µL	22.42
	TOM1 90 ng/µL	22.49
	TOM1 80 ng/µL	21.83
	TOM1 70 ng/µL	22.26
	TOM1 60 ng/µL	23.92
	TOM1 50 ng/µL	23.06
	TOM1 40 ng/µL	23.02
	TOM1 30 ng/µL	22.26
	TOM1 20 ng/µL	22.48
	TOM1 10 ng/µL	22.82
	TOM1 10-3 100 ng/µL	30.11
	TOM1 10-3 90 ng/µL	27.39
	TOM1 10-3 80 ng/µL	29.34
	TOM1 10-3 70 ng/µL	28.78
	TOM1 10-3 60 ng/µL	31.30
	TOM1 10-3 50 ng/µL	31.32
	TOM1 10-3 40 ng/µL	30.96
	TOM1 10-3 30 ng/µL	31.85
	TOM1 10-3 20 ng/µL	35.58
	TOM1 10-3 10 ng/µL	33.65

	SAMPLE	Avg Tt (min)
p210	K562 100 ng/µL	21.59
	K562 90 ng/µL	21.37
	K562 80 ng/µL	20.92
	K562 70 ng/µL	21.45
	K562 60 ng/µL	22.82
	K562 50 ng/µL	22.39
	K562 40 ng/µL	22.22
	K562 30 ng/µL	21.71
	K562 20 ng/µL	21.66
	K562 10 ng/µL	22.63
	K562 10-3 100 ng/µL	28.39
	K562 10-3 90 ng/µL	27.69
	K562 10-3 80 ng/µL	27.72
	K562 10-3 70 ng/µL	28.65
	K562 10-3 60 ng/µL	33.80
	K562 10-3 50 ng/µL	30.06
	K562 10-3 40 ng/µL	31.51
	K562 10-3 30 ng/µL	30.93
	K562 10-3 20 ng/µL	32.41
	K562 10-3 10 ng/µL	30.93

	SAMPLE	Avg Tt (min)
IC GUSβ	HL60 100 ng/µL	39.37
	HL60 90 ng/µL	39.42
	HL60 80 ng/µL	39.31
	HL60 70 ng/µL	39.64
	HL60 60 ng/µL	39.53
	HL60 50 ng/µL	39.40
	HL60 40 ng/µL	38.92
	HL60 30 ng/µL	38.69
	HL60 20 ng/µL	39.07
	HL60 10 ng/µL	39.24

Table 19: Assay robustness at non optimal RNA sample concentrations

In the table are shown the Threshold time values of the GUSβ, p190 and p210 transcripts amplification.

In yellow results of the Internal control amplification GUSβ on HL60 RNA. In green results regarding p190 amplification on both pure TOM1 RNA and TOM1 10-3 dilution. In red results regarding p210 amplification on both pure K562 RNA and K562 10-3 dilution. The results were obtained starting from an optimal RNA

concentration of 100 ng/uL to a ten fold lower concentration of 10 ng/uL for each target.

9. Clinical validation

To assess the clinical value of BCR-ABL LAMP assay we performed a validation on clinical samples.

9.1. Qiagen extraction method

30 p190 and 30 p210 positive onset clinical samples and 30 negative clinical samples obtained from healthy donors have been tested. The RNA was extracted from leucocytes present in bone marrow or peripheral blood using Qiagen RNeasy kit.

All the clinical samples have been previously analyzed with the reference method (RT-PCR method - Biomed Protocol)

A 100% of agreement with the PCR method was demonstrated, no false negative or false positive results were detected.

		Results by conventional RT-PCR (Biomed)			
		p190	p210	Negative	Tot
Results by RT-QLAMP	p190	**30**	-	-	30
	p210	-	**30**	-	30
	Negative	-	-	**30**	30
	Tot	30	30	30	**90**

Table 20: Diagnostic sensitivity and specificity using Qiagen RNeasy extraction kit

90 clinical samples have been tested obtaining a 100% concordant results with the reference method.

9.2. Phenol-Choloroform extraction method

To confirm these performances also using a different extraction method we tested 10 p190 and 10 p210 positive samples and 30 negative samples from healthy donors extracted using the phenol-chloroform method.

All the positive samples have been detected obtaining a 100% of agreement with the PCR method. The negative samples results were validated by the internal control amplification.

		Results by conventional RT-PCR (Biomed)			
		p190	p210	Negative	Tot
Results by RT-QLAMP	p190	**10**	-	-	10
	p210	-	**10**	-	10
	Negative	-	-	**30**	30
	Tot	10	10	30	**50**

Table 21: Diagnostic sensitivity and specificity using phenol-chloroform extraction method

50 clinical samples have been tested obtaining a 100% concordant results with the reference method.

10. Real-life evaluation

In order to demonstrate the consistency and superiority of the *Iam* BCR-ABL performance in a routine diagnostic laboratory environment, the assay prototype has been tested by laboratory technicians in different Hospitals: Hospital Papa Giovanni XXIII of Bergamo, Hospital Sant'Orsola of Bologna and Hospital Policlinico Tor Vergata of Rome.

100 p190 positive, 100 p210 positive and 100 negative onset samples have been tested in parallel with the reference home-brew method (Biomed Protocol). All cases resulted in concordant results with the home-brew method.

Only one sample was found to be discordant : negative using the PCR and p190 positive for the LAMP assay. The sample was tested using a third method, the quantitative Real Time PCR, resulting a very low p190 positive sample. This case proved the very high sensitivity of the LAMP method respect to the PCR.

		LAMP BCR-ABL assay status		3° method
Sample ID	Assigned Status by RT-PCR	Initial Test	Repeat Test	Quantitative PCR
64806*	NEG	P190	P190	P190
		C500 = 30.2	C500 = 27.88	positive

Table 22: Discordant result at Bergamo Hospital.

In the table is shown the discordant result resolution . The analysis with the third method proved the real positivity of the sample as detected by the LAMP test.

Moreover at hospital of Bologna three follow up samples, characterized by very low level of target transcripts, were correctly detected and diagnosed confirming the very high sensitivity of the method.

The samples presented a p210/ABL percentage ratio of 0.056%, 0.046% and 0.2% respectively, corresponding to 10^{-4} and 10^{-3} transcript dilutions.

Sample ID	Assigned Status by Real-time PCR	Assigned Status by LAMP
65475	P210 (0.056%)	P210 C570 = 22.3
65469	P210 (0.046%)	P210 C570 = 21.67
65448	P210 (0.2%)	P210 C570 = 25.43

Table 23: Follow up samples analysis

To assess the high sensitivity level of the assay we tested at hospital of Bologna three follow up samples characterized by a very low p210/ABL ratio percentage. In all the three cases we were able to detect and discriminate the presence of the p210 transcript.

All these data support the great reliability, sensitivity and robustness of the new diagnostic method.

DISCUSSION

A reciprocal translocation between chromosome 9 and chromosome 22 is responsible for the formation of the aberrant Philadelphia chromosome t(9;22). The result of the translocation is a juxtaposition of the ABL gene on chromosome 9 to the BCR gene on chromosome 22, thus creating the oncogenic fusion gene BCR-ABL (Morgan GJ et al., 1989).

The break point on chromosome 9 occurs at exon 2 of ABL, however the break points on chromosome 22 can differ in position on the BCR gene. The variable break points on chromosome 22 result in the creation of different BCR-ABL isoforms. The most frequent isoforms are the so called p210 Mbcr (Major breakpoint cluster region) and the p190 mbcr (minor breakpoint cluster region) followed by other rare isoforms like the p230 μbcr (Dongen et al., 1999).

The BCR-ABL fusion gene codes for an oncogenic protein with elevated tyrosine kinase activity, which is responsible for the neoplastic transformation observed in the Chronic Myeloid Leukemia (CML) (>95% of cases) and in Acute Lymphoblastic Leukemia (ALL) (30% of cases). BCR-ABL p210 is the hallmark for CML. In BCR-ABL positive ALL cases, the fusion gene can present both p190 (60% of cases) and p210 (40% of cases) isoforms (Cimino et al., 2006; Hughes et al., 2006).

Thanks to the availability of molecular drugs (Tyrosine Kinase Inhibitors), which are able to selectively inactivate the BCR-ABL chimeric protein, the management of Philadelphia positive leukemias is constantly improving over time (Mediterr J et al., 2014). The molecular detection of the BCR-ABL transcripts is mandatory in order to diagnose CML and Philadelphia Positive

ALL allowing implementation of the correct therapy (Dongen et al., 1999; Jabbour et al., 2014; Baccarani et al., 2013). Finally, the discrimination of the isoform, possible exclusively by molecular methods, facilitates the choice of the specific quantitative assay for the molecular monitoring during the treatment.

The molecular technique used for the routine diagnosis in Hospital laboratories is based on RT-PCR (Reverse Transcription-Polymerase Chain Reaction).

The procedure has been standardized by the European BIOMED-1 Concerted Action with participants of 14 laboratories in eight European countries (Dongen et al., 1999). It is a time consuming method consisting of several steps that must be performed by skilled personnel and in equipped laboratories. Moreover the risk of cross-contamination due to the multistep procedure and the absence of an internal control of the reaction may also lead to false positive or false negative results.

DiaSorin Q-LAMP is a rapid, highly specific, nucleic acid detection method, based upon loop mediated isothermal amplification (LAMP), which produces target amplification at uniform temperature (Notomi et al., 2000). The technique is emerging as an easy to perform and rapid tool for molecular diagnostics applications in clinical routine.

The aim of my PhD project is to set up a qualitative novel molecular method, based on an evolution of the Loop mediated isothermal Amplification (Q-LAMP) (Fu et al., 2011) for a rapid, one step detection of BCR-ABL p190 and p210 fusion transcripts.

The assay consists of a multiplex primer system. Primer sets specific for the amplification of p190 and p210 were designed together with specific labeled probes that allow the differentiation of the fusion transcripts. They consist of a common backward part, localized on exon 2 of ABL, and two different forward parts specific for the two different transcripts. The p190 forward primers are designed on exon 1 of BCR gene, whereas the p210 forward primers are localized on exon 13 of BRC gene in order to amplify both p210 forms (b2a2,b3a2). A dual positive control containing defined concentrations of both p190 and p210 target is included in the assay and used to validate individual runs.

In addition to the BCR-ABL primers, we have designed primers which bind to the GUSβ housekeeping transcript sequence present in extracted clinical samples and in the Negative Control (NC). Amplification of the endogenous control sequence generates a fluorescent signal at a different wavelength than p190 and p210 target amplifications. Correct amplification of the endogenous control sequence in negative samples verifies the efficiency of the extraction process, demonstrates RNA integrity and the absence of inhibitors, validating in this way the negative results. The amplification of the GUSβ transcript is delayed with respect to the target one. In this way we can avoid any competition during the amplification in order to detect also very low quantity of the target transcripts.

Initially the primer sets selection and the reaction conditions optimization were performed using plasmids in which was cloned the target specific sequence. Thanks to the use of a DNA polymerase presenting both stand displacement and reverse transcription activity we have implemented an

assay able to amplify the targets directly from RNA avoiding the necessity of a separated RT step.

The ability of the final assay to amplify both p190 and p210 transcripts and the internal control in one single tube reaction, directly from RNA, is an important feature that renders our assay competitive compared to the currently used protocol.

The assay has a very high sensitivity, evaluated on serial dilutions of positive cell line RNA into negative cell line RNA, and a great specificity of 100%, evaluated on both water samples and negative RNA from 7 different cell lines. Moreover the RNA for sensitivity and specificity tests was extracted using two different extraction methods, commonly used in diagnostic laboratories: Qiagen RNeasy kit and phenol-chloroform protocol. Using the Qiagen extraction method we obtained a sensitivity of 100% on 10^{-3} and of 87.5% on 10^{-4} for p210, and 100% on 10^{-3} and 92.5% on 10^{-4} for p190. Using phenol-chloroform protocol we obtained a sensitivity of 100% on 10^{-3} and 72.5% on 10^{-4} for p210 and 100% on 10^{-3} and 92.5% on 10^{-4} for p190.

To assess the robustness of the Q-LAMP technology we tested, using the BCR-ABL Q-LAMP assay, the most common PCR inhibitors found in literature. We considered both potential inhibitors carried over from cell isolation (such as haemoglobin, heparin, IgG, lactoferrin, ficoll and phenol), and potential contaminants carried over from RNA extraction process (such as genomic DNA, isopropanol, ethanol and guanidine isothiocyanate). This study demonstrates that residual inhibitors can cause the failure of OD ratios either A260/280 or A260/230 or both, but this does not cause a delay

in the assay threshold time confirming also in this case the superiority of our assay compared to PCR.

Moreover the assay was tested in the presence of non optimal RNA samples concentrations, starting from 100 ng/uL to a ten fold lower concentration of 10 ng/uL, showing in all the cases the ability to detect correctly and at very similar threshold times all the target transcripts (GUSβ, p190 and p210). These results confirmed the very high robustness of the LAMP amplification method.

The BCR-ABL Q-LAMP assay was successfully tested on negative and positive clinical samples (n= 140), extracted using Qiagen RNeasy kit or phenol-chloroform protocol, obtaining a 100% agreement with the reference RT-PCR method. These concordant results allowed to validate our new assay from a diagnostic point of view.

In order to demonstrate the consistency and advantage of the BCR-ABL Q-LAMP performance in a routine diagnostic laboratory environment, we collaborated with Hospital Papa Giovanni XIII of Bergamo, Hospital Sant'Orsola of Bologna and Hospital Policlinico Tor Vergata of Rome. The prototype of the assay was used by the Hospital technicians to diagnose new onset cases in parallel with the reference home-brew method. The results were very encouraging because, using our assay, the Hospitals could diagnose correctly the status of the samples in an easier and more rapid way (positive samples detected after 20 minutes instead of more than 3 hours). Remarkably BCR-ABL Q-LAMP has proven to be more sensitive than the PCR, around one Log, when tested on samples presenting a very low quantity of transcript.

In conclusion BCR-ABL Q-LAMP is a highly sensitive and specific assay for an easy, one step and rapid detection of the two major BCR-ABL transcripts (p190 and p210). The assay was validated on RNA clinical samples extracted using the methods commonly used by the Hospitals. The presence of an internal control guarantees the reliability of the results and, thanks to the Q-LAMP technology, the assay shows a very high robustness because it is insensitive to the most common PCR inhibitors. Moreover the final assay presents in a lyophilized format that confers a great product stability and allows the storage and transport of the kit in a practical and affordable way.
Finally, during the real-life evaluation of the assay, we obtained the real prove of the superior performances of our method respect to the standard one in order of simplicity of execution, rapidity of the test and sensitivity level. All these characteristics render BCR-ABL Q-LAMP feasible and convenient in routine diagnostic laboratories even in the absence of specialized personnel and equipped laboratories.
For these reasons this highly performing assay may represent a substantial improvement over the existing molecular diagnostic routine for the diagnosis of Chronic Myeloid Leukemia (CML) and Acute Lymphoblastic Leukemia (ALL).

Abelson HT et al. (1970) Cancer Res 30, 221332222

Aichberger KJ et al. (2011) Am J Hematol 86, 533–39

Allan NC et al. (1995) Lancet 345, 1392–97

Apperley JF (2007) Lancet Oncol 8, 1018–29

Apperley JF et al. (1986) Bone Marrow Transplant 1, 53–66

Baccarani et al. (2013) Blood 122(6)

Baccarani M et al. (2006) Blood 108, 1809–20

Baccarani M et al. (2009) J Clin Oncol 27, 6041–51

Bader P et al. (2009) J Clin Oncol 27, 377–84

Balduzzi A et al. (2005) Lancet 366, 635–42

Bassan R and Hoelzer D (2011) J Clin Oncol 29, 532–43

Beillard E et al. (2003) Leukemia 17, 2474-2486

Ben-Neriah Y et al. (1986) Cell 44, 5777586

Bernards A et al. (1987) Mol Cell Biol 7, 3231-3236

Bonifazi F et al. (2001) Blood 98, 3074–81

Borowitz MJ et al. (2008) Blood 111, 5477–85

Branford S et al. (2008) Blood 112, 3330–38

Campana D. (2012) Leukemia 19(4), 313-8

Ching-Hon Pui (2006) N Engl J Med. 354(2), 166-78

Cimino et al. (2006) Haematologica 91, 377-380

Conan-Doyle A (1882) Lancet 1882, 490

Conter V et al.(2010) Blood 115, 3206–14

Cortes JE et al. (2010) J Clin Oncol 28, 424–30

Cortes JE et al. (2010) J Clin Oncol 28, 424–30

Cortes JE et al. (2012) N Engl J Med 367, 2075–88

Cortes JE et al. (2013) N Engl J Med 369, 1783–96

Cross NCP et al. (2012) Leukemia 26, 2172–75

Daley GQ and Ben Neriah Y (1991) Adv. Cancer Res 57, 51 - 184

Deininger M et al. (2009) Blood 114, 462

Diekmann D et al. (1991) Nature 351, 400-402

Dieter Hoelzer et al. (2002) Hematology 11, 190–220

Diouf B et al. (2011) Nat Med 17, 1298–303

Dongen et al. (1999) Leukemia 13, 1901–1928

Dreyer ZE et al. (2011) J Clin Oncol 29, 214–22

Druker BJ et al. (2001) N Engl J Med 344, 1031–37

Druker BJ et al. (2006) N Engl J Med 355, 2408–17

Faderl S et al. (1999) N Engl J Med 341(3), 164-72

Franz WM et al. (1989) EMBO J 8, 1377147

Fu et al. (2011) Applied Biochemistry and Biotechnology 163(7), 845-50

Gleissner B et al. (2002) Blood 99(5), 1536-43

Goff SP et al. (1980) Cell 22, 7777785

Gokbuget N et al. (2012) Blood 120(9), 1868-76

Gorre ME et al. (2001) Science 293, 876–80

Groffen J et al. (1984) Cell 36, 93-9

Hansen JA et al. (1998) N Engl J Med 338, 962–68

Harms DO et al. (2003) Blood 102, 2736–40

Harrison CJ (2009) Haematologica 144, 147–56

Harvey RC et al. (2010) Blood 115, 5312–21

Hehlmann R et al. (1994) Blood 84, 4064–77

Hehlmann R et al. (2011) J Clin Oncol 29, 1634–42

Heisterkamp N et al. (1983) Nature 306, 239-242

Heisterkamp N et al. (1985) Nature 315, 758-761

Hiroto Inaba et al. (2013) Lancet 381, 9881

Hochhaus A et al. (2012) J Clin Oncol 30, 232-38

Hoelzer et al. (2002) Hematology 200(2), 162-92

Hughes et al. (2006) Blood 183(1)

Hughes T et al. (2006) Blood 108, 28–37

Hughes T et al. (2009) J Clin Oncol 27, 4204–10

Hughes TP et al. (2003) N Engl J Med 349, 1423–32

Hunger SP et al. (2012) J Clin Oncol 30(14), 1663-9

Jabbour E and Kantarjian H (2012) Am J Hematol 87, 1037–45

Jabbour et al. (2014) Am J Hematol. 89(5), 547-56

Kantarjian H et al. (2002) N Engl J Med 346, 645–52

Kim TD1 et al. (2013) Leukemia 27, 1316–21

Konopka JB and Witte ON (1985) Mol Cell Biol 5, 3116-3123

Larson RA et al. (2012) Leukemia 26, 2197–203

Leung W et al. (2011) Blood 118, 223–30

Mann G et al. (2010) Blood 116, 2644–50

Marks DI et al. (2010) Blood 116, 366–74

McWhirter JR and Wang JYJ (1993) EMBO J 12, 1533-1546

McWhirter JR and Wang JYJ (1991) Mol Cell Biol 1, 1553-1556

Mediterr J et al. (2014) Hematol Infect Dis 6(1), e2014005

Melo JV et al. (2003) Apperley Hematology 2003, 132-52

Montani D et al. (2012) Circulation 125, 2128–37

Morgan GJ et al. (1989) Recenti Prog Med. 80, 508-19

Muller AJ et al. (1991) Mol Cell Biol 11, 17855-17992

Müller MC et al. (2009) Blood 114, 4944–53

National Cancer Institute (2010) Cancer Genet Cytogenet. 200(2), 149-53

Notomi et al. (2000) Nucleic Acids Research 28(12)

Nowell PC and Hungerford DA (1960) Science 132, 1497-9

O'Brien SG et al. (2003) N Engl J Med 348, 994–1004

Pane F et al. (2002) Oncogene 21(56), 8652-67

Pavlu J et al. (2011) Blood 117, 755–63

Pendergast AM et al. (1991) Cell 66, 161-171

Pieters R et al. (2007)Lancet 370, 240–50

Porkka K et al. (2008)Blood 112, 1005–12

Preudhomme C et al. (2010) N Engl J Med 363, 2511–21

Pui CH and Howard SC (2008) Lancet Oncol 9, 257–68

Pui CH et al. (2008) Lancet 371, 1030–43

Pui CH et al. (2009) N Engl J Med 360, 2730–41

Pui CH et al. (2011) J Clin Oncol 29, 386–91

Ravandi F et al. (2010) Blood 116, 2070–7

Relling MV et al. (2011) Clin Pharmacol Ther 89, 387–91

Rosenberg N et al. (1988) Adv Virus Res 35, 39981

Schafer ES and Hunger SP (2011) Nat Rev Clin Oncol 8, 417–24

Schrappe M et al. (2011) Blood 118, 2077–84

Schrappe M et al. (2012) N Engl J Med 366, 1371–81

Schultz KR et al. (2009) J Clin Oncol 27, 5175–81

Shtivelman E et al. (1986) Cell 47, 277-284

Shtivelman ET et al. (1985) Nature 315, 550-554

Stam K et al. (1987) Mol Cell Biol 7, 1955-1960

Steegmann JL et al. (2012) Leuk Lymphoma 53, 2351–61

Szczepanski et al. (2002) Best Pract Res Clin Haematol. 15(1), 37-57

Talpaz M et al. (1986) N Engl J Med 314, 1065–69

Uppal G and Gong J (2015) J Clin Pathol 68(9), 680-4

Van Dongen JJM et al. (1999) Leukemia 13, 1901–1928.

White HE et al. (2010) Blood 116, e111–17

Witts LJ (1968) Br Med J 1, 201–08

Xu H et al. (2012) J Clin Oncol 30, 751–7

Yang JJ et al. (2011) Nat Genet 43, 237–41

THE ISOTHERMAL LOOP MEDIATED AMPLIFICATION (LAMP) METHOD IS LESS AFFECTED BY AMPLIFICATION REACTION INHIBITORS IN RESPECT TO PCR. Presented at SIES congress, Rome 2013

Tettamanzi V., Minnucci G., Amicarelli G., Pultrone C., D'Agostini E., Rossi V., Cazzaniga G., Colotta F.

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REAL-TIME MULTIPLEX MOLECULAR DETECTION OF BCR-ABL P190 AND P210 TRANSCRIPTS BY Q-RT-LAMP ON THE LIAISON IAM SEMI-AUTOMATIC INSTRUMENT.

Presented at EHA congress, Milan 2014

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DIASORIN QLAMP AMPLIFICATION TECHNOLOGY COUPLED WITH LIAISON IAM INSTRUMENT AS A RAPID AND RELIABLE DIAGNOSTIC SYSTEM FOR DETECTION OF MOLECULAR MARKERS ASSOCIATED TO LEUKEMIAS. Presented at AMP congress, National Harbor (MD) 2014

G. Amicarelli, E. D'Agostini, R. Mesturini, G. Rizzo, C. Pultrone, V. Tettamanzi, F. Rigo, F. Colotta and G. Minnucci.

DiaSorin Spa, Gerenzano (VA), Italy.

RAPID AND RELIABLE DETECTION OF BCR-ABL MAJOR AND MINOR TRANSCRIPTS BY THE ONE-STEP, ISOTHERMAL RT-LOOP MEDIATED AMPLIFICATION REACTION MONITORABLE IN REAL TIME ON THE LIAISON IAM INSTRUMENT.

Presented at SIE congress, Florence 2015

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SEMI-AUTOMATED MOLECULAR DETECTION OF BCR-ABL MAJOR AND MINOR TRANSCRIPTS BY ISOTHERMAL RT-LOOP MEDIATED REACTION ON THE LIAISON IAM INSTRUMENT

Presented at EHA congress, Vienna 2015

Elena D'Agostini 1, Giulia Minnucci 1, Giulia Amicarelli 1, Cinzia Pultrone 1, Veronica Tettamanzi 1, Chiara Boroni 2, Orietta Spinelli 2, Francesco Colotta 2, Alessandro Rambaldi 2

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Dottorato di Ricerca

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