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Advancements in Molecular Diagnostics: A Comprehensive Multiplex RT-PCR Approach for Detecting BCR-ABL Fusion Transcripts in Hematological Disorders

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Abstract: ***The Philadelphia chromosome was the first to be discovered as a chromosomal abnormality in haematological illnesses. The recommended method for detecting BCR-ABL in CML and ALL is RT-PCR. The three most commonly identified mRNA transcripts are e1a2 in ALL, e13a2 and e14a2 in CML and ALL. It can detect fusion genes in preserved cell pellets from individuals expressing any subtype of the major bcr (M-bcr) e13a2, e14a2, e13a3 and e14a3, the minor bcr (m-bcr) e1a2 and e1a3, the micro bcr (µ-bcr) e19a2 and e19a3, and the nano bcr (n-bcr) e6a2 and e6a3. The Multiplexed Real Time Polymerase Chain Reaction (RT-PCR) assay was used for testing. Agarose gel electrophoresis was used to examine the major (e14a2, e13a2) and minor (e1a2) transcripts. The outcomes were anticipated using known samples of internal control, as well as positive and negative controls. The Multiplex RT-PCR assay is a clinically useful, efficient, and quick method for detecting BCR-ABL fusion transcripts. We report a new, quick, and dependable multiplex PCR method for detecting typical and atypical BCR-ABL transcripts.***

Keywords: BCR-ABL fusion proteins, Chronic myeloid leukemia, Acute lymphoblastic leukemia, Multiplex-PCR. Leukemia

1. Introduction

The Philadelphia chromosome was the first recurrent chromosomal aberration to be identified in a hematologic disease. The discovery of BCR and ABL gene on 22q11 and 9q34 respectively along with the emergence of PCR made the detection of BCR-ABL fusion gene easy through RT-PCR making it a gold standard in diagnosis [1]. CML is associated with a cytogenetic disorder known as Philadelphia chromosome (Ph). This aberrant chromosomal translation gives raise to expression of various types of BCR-ABL transcripts in which most cases involve ABL exon 2 fused with the BCR gene [2]. Reverse transcription polymerase chain reaction (RT-PCR) for detection of each individual fusion transcript is impractical and time consuming [3]. However, multiplex PCR is becoming the most preferred method for the diagnosis of Ph+ leukaemia's. The advantages offered by this method, when compared with other conventional diagnostic methods, include its high sensitivity and specificity, as well as being quick and relatively cost-effective [4].

The portion of BCR that is preserved in the BCR-ABL1 fusion gene depends on the location of the three-breakpoint cluster region (BCR). Breaks in the major break point cluster region (M-BCR) give rise to e13a2 or e14a2 fusion mRNA and p190 BCR-ABL1 protein that is typically found in CML, whereas breaks in the minor break point (m-BCR) cluster region generate an e1a2 fusion mRNA and p190 BCR-ABL1 protein, which is typical of Ph+ acute lymphoblastic leukemia [5].The present RT-PCR techniques for the diagnosis of BCR-ABL1 are designed and optimized for detecting the major (M-bcr) and minor (m-bcr) transcripts [6]. Advancements in RT-PCR techniques, such as multiplex RT-PCR makes the detection of BCR-ABL1 variants effortless in patients with CML. BCR-ABL1 oncogene obtained as the molecular consequence of the chromosomal translocation encodes the chimeric BCR-ABL1 protein with constitutive kinase activity [7].

The fusion gene and the resultant abnormal protein that is a result of a reciprocal translocation between BCR and ABL genes is one of the driving molecular abnormalities in certain conditions including CML and ALL [8]. These fusion genes can be of various different types based on the site of break and fusion on each gene which also results in different molecular weights of the resultant protein [9]. The rare cases of break point in CML patients include m-BCR and µ-BCR. As to m-BCR break point, breakdown after exon 1 results in formation of ela2 fusion, and this fused mRNA is translated into 190 kDprotein [9]. Another rare break point is (µ- BCR) that due to breakdown at exon19 leads to e19a2 fusion which is then translated into 230kD protein [10]. This type of break point is more common in chronic neutrophilic leukemia (CNL).

Most current RT-PCR methods for detecting BCR-ABL1 designed and optimized for detecting M-bcr and m-bcr transcripts [11]. It an effort to improve the relevance of RT-PCR techniques, several methods including the multiplex

RT-PCR have been developed to detect the BCR-ABL transcripts in a single round multiplex with most prevalent transcripts (e1a2, e13a2, e14a2) and developed a novel multiplex PCR. This study was carried to determine the BCR-ABL1 fusion transcripts variants in CML and ALL patients and is currently administered based on the molecular diagnosis of the BCR-ABL 1 fusion gene.

2. Materials and methods

2.1. Collection of patient samples

In the BCR ABL study, the stored pellet was taken which has already processed with 19 CML and ALL patients with their consent after approval from the institutional human ethics committee (IEC No: EC/NEW/INST/2022/2627) Neuberg Anand Reference Laboratory, Bangalore, India during the month of May 2022. The 19 samples were subjected to quantitative analysis before being collected for the study, the results of the 19 samples are as follows 13 CML (p210), 1 ALL (p190) and 5 negative samples were found. Based on the patient's unique BCR and ABL copy numbers, the quantitative assay proved the positive samples with an IS%. The factors of inclusion criteria in this study involves the availability of pellet with sufficient cells, preserved integrity in the samples which was assessed by visualizing the control band in the PCR, and non-haemolysed samples. Exclusion criteria in this study are not applicable because only 19 samples were available for the project. The sample size of about 19 was calculated as this satisfies the recommended number for validation and pilot study. The samples were initially processed for diagnosis using a commercial kit, and saved pellets were used in our investigation for multiplex PCR.

2.2. Chemicals reagents and kits

Ammonium Chloride (NICE, Kerala, India);Sodium Bicarbonate (NICE, Kerala, India);Ethylene Diamine Tetra Acetic acid (Fisher Scientific, Mumbai, India);Qiagen RNA Blood mini kit (Qiagen, Germany);RNA Later (Sigma Life science, Germany); Platinum hot star mastermix (Thermo fisher, USA);High capacity cDNA Reverse transcription kit (Thermofisher, USA); BCRABL Primers (Barcode BioScience, Banglore, India);Agarose Bioreagent, Low EEO (Sigma Lifescience, Germany); Ethidium Bromide (Himedia. Mumbai, India); 100bp ladder (Thermo fisher, US); Blue Juice Gel loading dye (Thermo fisher, USA). Which involves the principle of oligonucleotide hydrolysis principle that allows higher specificity and sensitivity.

2.3 RNA extraction and cDNA synthesis

Total RNA was routinely isolated from stored pellets using the Qiagen RNA blood mini kit (QIAamp® RNA Blood Mini Handbook, February 2021). For the reverse transcription reaction, the Qubit assay (Thermo Fisher) was used to control the amount and quality of RNA. cDNA is produced using the High-capacity cDNA Reverse Transcription kit (Thermo fisher scientific) (LOT No: 01280980). cDNA reaction containing 2µl of 10X RT Buffer, 0.8µl of 25XdNTP Mix (100mM), 2µl of 10X RT Random Primers, 1.0µl of MultiScribe^{TM} Reverse Transcriptase, 4.2µl of Nuclease free water, 10µl of RNA (1µg)in a total reaction volume of 20µl for 25°C (10 min), 37°C (120 min), 85°C (5 min) and hold for 4ºC according to the manufacturer's recommendations. The PCR is carried out with 5µl of cDNA, which is equivalent to 150ng. RNA.

2.4 Principles and rationale of primer design

Our PCR primers designing was done based on certain principles for both typical and atypical BCR-ABL transcripts.: (1) detection of the three most predominant transcripts e13a2, e14a2, and e1a2 at least as reliably as previous methods, (2) quality of PCR results, (3) Integrity of stored samples, (4) Identification of unusual transcript types, (5) usage of internal control to accurately identify negative results, (6) all BCR-ABL PCR products must be smaller compared to the internal control product.

2.5 ABL Primer

ABL primer was designed accordingly it binds to ABL exon 2 facilitating the detection of fusion with exon 2 (a2) or exon 3 (a3).

2.6 BCR primers

A specific BCR primer binds to exon 1 (e1) for the detection of minor transcripts and another specific primer binds to exon 12 (e12) for detection of Major transcripts. The primers designed for M-bcr and m-bcr also detects micro (µ-bcr) exon 19 and nano (ν-bcr) exon 6 transcripts respectively. However, the resulting PCR products are very large, since all intervening exons between the breakpoint regions have to be bridged (Fig 2).

2.7 Internal control

Following the concept of Cross and co-workers (1993), involving an internal control PCR from the normal BCR gene is introduced [14], but the primers were chosen to produce a comparatively smaller control PCR product of size 718bp rather than 808bp.

2.8 Primer selection

Primer sequences are enlisted in (Table1), and their locations on BCR and ABL genes are shown in (Fig2). The primer sequences were adapted from Burmeister *et al.,* [1].

Table 1: Oligonucleotide primer sequences.

PRIMER	SEQUENCE (5'-3')	LOCATION	ORIENTATION
ABL-3	CCATTGTGATTATAGCCTAAGACCCGGAG	ABL exon 3	-
BCR-1	CTCCAGCGAGGAGGACTTCTCCT	BCR exon 1	+
BCR-6	CCTGAGAGCCAGAAGCAACAAAGATGCC	BCR exon 6	+
BCR-12	AGAACATCCGGGAGCAGCAGAAGAA	BCR exon 12	+
BCR-19	ACTGAAGGCAGCCTTCGACGTC	BCR exon 19	+
BCR-R	ATGTCCGTGGCCACACCGGACAC	BCR exon 19	-

2.9 PCR method

All the primers were obtained from Barcode Bioscience, Bengaluru. Individual primers had a concentration of about 100nM in 50µl reaction mixture. Multiplex mix contains30µl Platinum Hot star Master Mix, 2µl of primers (BCR-1, BCR-6, BCR-12, BCR-19, ABL-3, BCR-R) and 3µl of grade water. PCR program was used in Proflex thermal cycler: 15minutes at 95°C to activate the hot star polymerase according to the manufacturer's recommendations, 40cycles:95°C for 10seconds, 63°C for 20seconds, and 72°C for 30seconds followed by extension step at 72°C for 10 minutes [1].

2.10 Gel electrophoresis

After PCR, Aliquots of the ampliconsis electrophoretically separated in a 1.5% agarose gel in TAE buffer. The results are seen after gel has been run and the gel is viewed under ultraviolet light. The gel is always run with a 100bp ladder to see the location and size of the bands. The p210 (e14a2, e13a2), p190 (e1a2) in 19 samples with particular breakpoints fragments were analysed. The segregation of fragments between 100bp and 700bp was ensured by optimal electrophoresis runtime [1].

3. Results

The molecular analysis was performed with multiplex RT-PCR system which was first optimized by detecting the different fusion transcripts (e13a2, e14a2, e1a2). The multiplex RT-PCR primer combinations enabled for the simultaneous identification of typical and atypical BCR-ABL transcript types in one experiment. To properly interpret negative results, the internal control reaction should be taken into consideration. All three translocations were successfully amplified, and the PCR products were discriminated by their different fragment sizes upon gel electrophoresis, and the internal control product should be smaller than all BCR-ABL PCR product shown in figure 1.

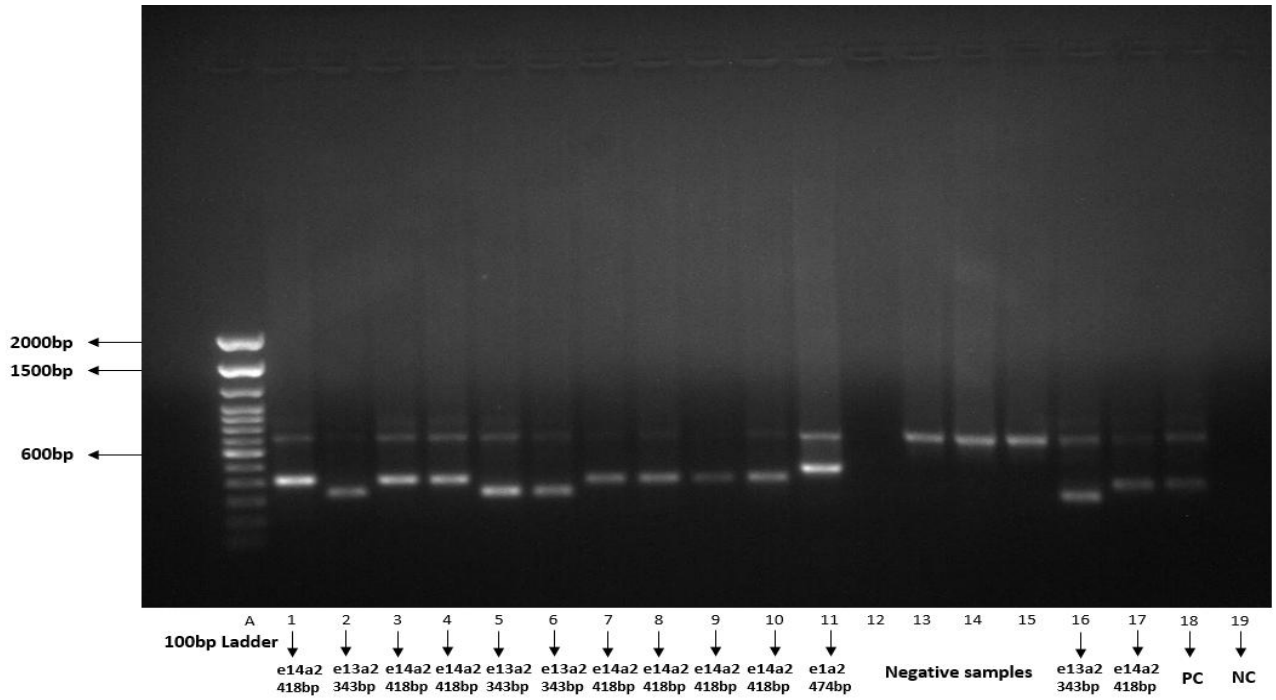
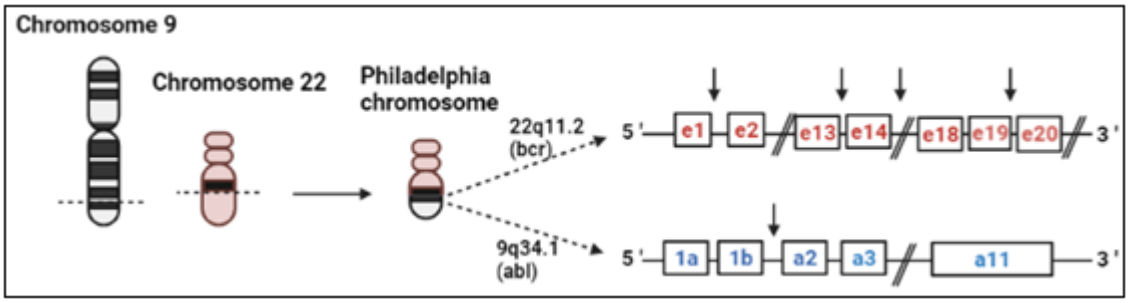


Figure 1: Multiplex RT PCR results. Lanes 1-10 represent bands from positive sample (Major detected) e13a2 and e14a2. Lane 11 is minor transcript e1a2 (p190) and lanes 12-15 is negative pellets showing BCR internal control band. Lanes 16, 17 major transcript. Lane 18 represent as positive control and Lane 19 represent as negative control. Lane A is 100bp ladder. The finding was established with the use of multiplex RT-PCR on 18 CML samples and one ALL sample. Our findings were consistent with prior diagnosis performed with other methods, such as a commercial PCR kit.

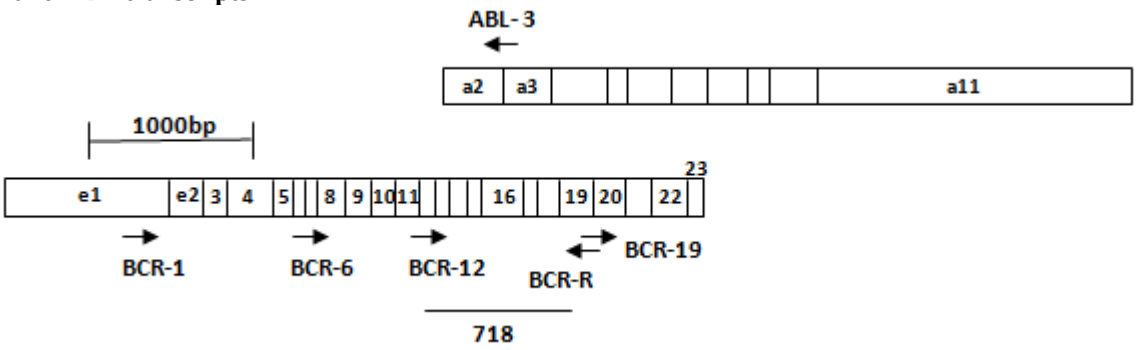
The expected bands of Major transcripts 418bp (e14a2), 343bp (e13a2) and Minor transcripts 474bp (e1a2). The quality of RNA and efficiency of cDNA synthesis were analysed by amplification of BCR gene as an Internal control. The amplified product (718bp) from BCR gene was the only band detected in BCR-ABL. The negative results

are absence of this bands indicated that does not have CML or ALL. The positive and negative controls are used for references of detection. The BCR and ABL breakpoint regions and resulting fusion transcripts splicing effects given by (Fig 2)

(A) BCR and ABL gene with break point regions



(B) BCR and ABL transcripts



(C) Fusion transcripts of BCR-ABL

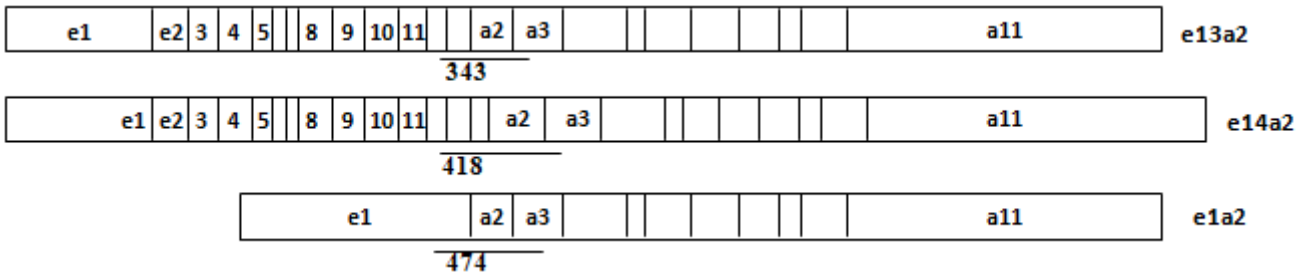


Figure 2: (A) Illustration of t (9;22) (q34;q11) translocation creating Philadelphia chromosome along with representation of breakpoint location between BCR and ABL1 genes. (B)ABL and BCR mRNAs transcripts along with location of primers and the 718bp control band. (C) BCR-ABL mRNA fusion transcripts with PCR products.
Note: By using BioRender, A, B, C images have been created.

We were able to detect Major P210 transcripts such as e14a2 and e13a2 as well as Minor 190 transcripts such as e1a2 in 19 patients using multiplex RT-PCR. The majority of patients displayed P210 BCR-ABL transcripts and less of P190 BCR-ABL transcripts. While the smaller PCR products are only partially generated in BCR-ABL positive instances, the control band may be faint or non-existent. Control, on the other hand, is required to correctly interpret BCR-ABL negative situations.

4. Discussion

Previously, multiplex RT-PCR has been used to characterize single or small groups of translocations occurring in different leukemic cells. These reports concluded that, compared to single RT-PCR reactions, multiplex RT-PCR systems allow rapid, specific, less expensive, integrity of stored samples and less time-consuming ensuring quality detection of most recurrent fusion transcripts in patients with leukaemia. Our aim in this study was to optimize such a multiplex RT-PCR system to screen simultaneously for the most predominantly occurring BCR-ABL fusion transcripts associated with CML and ALL cases. This exercise is to standardise and verify a published protocol for identification of BCR-ABL transcript type with a focus on maximising the resultant data and minimising cost when compared to the existing PCR method.

In CML or ALL, the most common BCR-ABL transcripts are e13a2, e14a2, and e1a2. For the purpose of simplicity, we shall refer to those three transcripts as "typical." It should be noted, however, that e1a2 is only prevalent in ALL, but uncommon and unusual in CML. This set of primers allows the simultaneous amplification of all described molecular variants of the BCR-ABL gene along with un-translocated BCR gene as an internal control. However, this study, increases the quality of PCR to analyse the BCR-ABL common transcripts from stored cell pellets. These primers

are specific for the e13a2, e14a2 and e1a2 fusion transcripts as well as the rare e19a2 variant, which is not detected by many of the previously described systems and commercially available kits.

In recent years, real-time PCR technology has been introduced into diagnostic medical applications. In molecular oncology, this technology made it possible to quantify the tumour burden and monitor the response to therapy. However, the parallel use of other molecular techniques such as FISH and cytogenetics is still recommended.

When compared with real-time PCR and other techniques, the main advantages of a multiplex PCR system, is its superior quality of PCR and low cost for commercial application and integrity of stored samples. This helps our system most suitable for early diagnosis of Ph+ leukaemia. Even when compared to other diagnostic labs that utilize commercial kit for the major, minor and micro transcripts detection, rather than multiplexing our lab has done research to simultaneously detect major and minor transcripts in a single tube reaction.

The presently available primers detect only three transcripts so for best quality results we suggest the use of HPLC purified PCR primers to obtain ideal primer quality, a hot star polymerase for clear results and optimal gel electrophoresis run time to get a clear distinguish between 50bp and 700bp products [1].

Our PCR was primary diagnosis which is unsuitable for evaluating minimal residual disease (MRD). The sensitivity of the PCR has deliberately not been maximized since it always carries the risk of false-positive results caused by contamination [1]. Also, we recommend to assess MRD by standardized methods. The limited sample size of about 19 is the limitation of this study since only 19patient's sample was available this satisfies the recommended number for validation and pilot study. Large scale study requires the use of above 50 patient samples. Multiplex RT-PCR reaction is useful and rapid detection method for identification of BCR-ABL1 variants. The occurrence of these transcripts associated with CML and ALL can assist in both assessment of prognosis and treatment of disease.

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References

[1] Burmeister, T., & Reinhardt, R. (2008). A multiplex PCR for improved detection of typical and atypical BCR–ABL fusion transcripts. *Leukemia research*, *32* (4), 579-585.

[2] Sastre, D. A., Argaraña, C. E., Heller, V. B., Gallo, M., Fernández, E. N., & Rodríguez, C. M. (2007). An analysis of multiplex-PCR in the detection of BCR-ABL transcripts in hematological disorders. *Genetics and Molecular Biology*, *30*, 520-523.

[3] Pakakasama, S., Kajanachumpol, S., Kanjanapongkul, S., Sirachainan, N., Meekaewkunchorn, A., Ningsanond, V., &Hongeng, S. (2008). Simple multiplex RT-PCR for identifying common fusion transcripts in childhood acute leukemia. *International journal of laboratory hematology*, *30* (4), 286-291.

[4] Akawi, N. A., Ismail, S. I., Abbadi, A. A., Al-Tarawneh, M. S., & Al-Khateeb, M. S. (2006). Highly Sensitive Multiplex RT-PCR System for the Detection of all Common BCR-ABL Transcripts Associated with Different Leukemias. *Jordan Medical Journal*, *40* (2), 96-104.

[5] Zhen, C., & Wang, Y. L. (2013). Molecular monitoring of chronic myeloid leukemia: international standardization of BCR-ABL1 quantitation. *The Journal of Molecular Diagnostics*, *15* (5), 556-564.

[6] Chasseriau, J., Rivet, J., Bilan, F., Chomel, J. C., Guilhot, F., Bourmeyster, N., &Kitzis, A. (2004). Characterization of the different BCR-ABL transcripts with a single multiplex RT-PCR. *The Journal of molecular diagnostics*, *6* (4), 343-347.

[7] del Pilar Moreno, M., Cortinas, M. N., Bonomi, R., Cardeza, A., & del Rosario Uriarte, M. (2001). A novel BCR-ABL fusion transcript (e15a2) in 2 patients with atypical chronic myeloproliferative syndrome. *Blood, The Journal of the American Society of Hematology*, *97* (11), 3668-3669.

[8] Nachi, M., Kihel, I., Entasoltane, B., Brahimi, M., Yafour, N., Guella, D., ... &Bekadja, M. A. (2020). Impact of the major BCR-ABL1 transcript type on clinical and biological parameters and molecular response in patients with chronic myeloid leukemia. *Hematology/Oncology and Stem Cell Therapy*.

[9] Burmeister, T., Maurer, J., Aivado, M., Elmaagacli, A. H., Grünebach, F., Held, K. R., ... & Thiel, E. (2000). Quality assurance in RT-PCR-based BCR/ABL diagnostics–results of an interlaboratory test and a standardization approach. *Leukemia*, *14* (10), 1850-1856.

[10] Chootawiriyasakul, K., Sirijerachai, C., &Chansung, K. (2019). Frequency of BCR-ABL Fusion Transcript Types with Chronic Myeloid Leukemiaby Multiplex PCR in Srinagarind Hospital, Khon Kaen Thailand. *J Med-Clin Res & Rev*, *3* (4), 1-4.

[11] Limsuwanachot, N., Siriboonpiputtana, T., Karntisawiwat, K., Chareonsirisuthigul, T.Chuncharunee, S., &Rerkamnuaychoke, B. (2016). Multiplex RT-PCR Assay for detection of common fusion transcripts in acute lymphoblastic leukemia and chronic myeloid leukemia cases. *Asian Pacific Journal of Cancer Prevention*, *17* (2), 677-684.

[12] Quintás-Cardama, A., & Cortes, J. (2009). Molecular biology of bcr-abl1–positive chronic myeloid leukemia. *Blood, The Journal of the American Society of Hematology*, *113* (8), 1619-1630.

[13] Burger, A., Konopleva, M. D., & Kantarjian, M. D. (2015). Blood First Edition Paper, prepublished online March 20, 2015; DOI 10.1182/blood-2014-10-605808.
[14] Cross, N. C., Melo, J. V., Feng, L., & Goldman, J. M. (1994). An optimized multiplex polymerase chain reaction (PCR) for detection of BCR-ABL fusion mRNAs in haematological disorders. *Leukemia*, *8* (1), 186-189.
[15] Bennour, A., Ouahchi, I., Moez, M., Elloumi, M., Khelif, A., Saad, A., &Sennana, H. (2012). Comprehensive analysis of BCR/ABL variants in chronic myeloid leukemia patients using multiplex RT-PCR. *Clinical Laboratory*, *58* (5), 433.
[16] Hassan, R., Ramli, M., Abdullah, W. Z., Mustaffa, R., Ghazali, S., Ankathil, R., ... & Baba, A. A. (2008). One-step multiplex RT-PCR for detection of BCR/ABL gene in Malay patients with chronic myeloid leukaemia. *Asia Pacific Journal of Molecular Biology and Biotechnology*, *16*, 41-44.
[17] Lyu, X., Yang, J., Wang, X., Hu, J., Liu, B., Zhao, Y., ... & Song, Y. (2016). A novel BCR-ABL1 fusion gene identified by next-generation sequencing in chronic myeloid leukemia. *Molecular Cytogenetics*, *9* (1), 1-7.
[18] Qin, Y. Z., & Huang, X. J. (2016). Molecular detection of BCR-ABL in chronic myeloid leukemia. *Chronic Myeloid Leukemia: Methods and Protocols*, 1-15.

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