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OPTIMIZATION OF MULTIPLEX RT-PCR FOR LEUKEMIA TYPING AND SUBTYPING

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Abstract
Molecular typization and subtypization of leukemia by RT-PCR, is important due to monitoring of minimal residual disease (MRD), during and after treatement of patients with acute leukemia. In our experiments of PCR optimization, we used BIO-RAD procedure for multiplex RT-PCR screening and split-out PCR for leukemia subtypization. This method is adapted or optimized for Perkin Elmer Gene Amp 9600 thermal cycler and Qiagen HotStar Taq DNA polymerase as well.According to manufacturer instructions, using of some other PCR engines require optimization of corresponding PCR parameters. We used Perkin Elmer 2400 thermal cycler and Hot Star Taq DNA polymerase. Total RNA was extracted from whole blood specimens, obtained from Clinics of hematology-KCU Sarajevo, taken from patients with acute leukemia, transferred to cDNA, and amplificated in PE 2400. Detection of PCR products is performed by 1,5% agarose gel electrophoresis. Obtained optimal PCR parameters were: annealing temperature - 65°C, number of cycles for both, first round PCR amplification and nested PCR amplification was 30, final concetration of MgCl in reaction mixture was 3,28 mM. Instead recommended amount of cDNA (5 µl) and Hot Star Taq DNA polymerase (0,4 µl) we used amounts of 10 µl and 1 µl
By using of this optimized PCR protocol we detected genetic aberration inv(16)(p13; q22) and by using of split out PCR, subtype G(192 bp electrophoretic band)(CBFB / MYH11 fusion genes).
Key words: leukemia, typization/subtypization, fusion gene transcripts, multiplex RT-PCR screening, split-out multiplex PCR.

Introduction

Detection of fusion gene transcripts, as specific markers and prognostic factors of different leukemia types/subtypes, is very useful approach in MRD (minimal residual disease) monitoring of patients with acute leukemia, during and after chemotherapy treatment. The sensitivity of this PCR analysis of break-point fusion regions method together with other two methods (flow cytometric immunophenotyping and PCR analysis of patient-specific functional regions of rearranged Ig and T cell receptor genes), is at least 10^{-3} (one leukemic cell between 10^3 normal cells).

Up to now, it was detected nine well defined chromosome aberrations, caused by fusion of different genes in the cases of acute leukaemia (Palisquardet al., 1998; Rabbits, 1994.).

- t(1:19)(q23;p13)	E2A/PBX1 fusion genes
- t(4;11)(q21;q23)	MLL/AF4
- t(8;21)(q22;q22)	AML1/ETO
- t(9;22)(q34;q11)	BCR/ABL (two types)
- t(12;21)(p13;q22)	TEL/AML1
- t(15;17)(q22;q21)	PKL/RARA
- inv(16)(p13;q22)	CBFB/MYH11
- del(1p32)	SIL/TAL1

All of these above mentioned genes, involved in production of fusion gene transcripts, are detected in the period from 1993 to 1996. According to these data, experts in the field of molecular biology, were able to designed PCR primers for their amplification and detection. The firm BIO-RAD designed own protocol for qualitative multiplex reverse transcription – polymerase chain reaction (RT-PCR), adapted for screening of 28 different gene translocations and detection for more than 80 breakpoints or mRNA splice variants, as specific markers of particular leukemia types/subtypes. This procedure is also optimized for PE 9600 thermal cycle and using of some other engine require corresponding PCR parameters optimization (Bio-Rad handbook, 2004.; Armitage,2004.; Cox and Sinclair, 1997.).

In our experiments of PCR optimization we used PE 2400 thermal cycler and recommended HotStar Taq DNA polymerase.

Material and Methods

HotStarTaq DNA polymerase(Qiagen)– has been developed to provide hot-start PCR for purpose of higher specificity, minimizing of non-specific amplification products, primer-dimers formation and contamination risks associated with conventional hot-start PCR methods. It is actually modified form of the recombination 94 kDa Taq polymerase, in inactivate state, with no activity at ambient temperature. Using of this enzyme in multiplex PCR, such as BIO-RAD multiplex RT-PCR, prevents the formation of misprimed products and primer-dimers at low temperatures, high PCR specificity and increasing of specific PCR products yield (Qiagen HotStarTaq DNA polymerase handbook, 2002.).

RNA extraction – During 1 to 2 hours after taking of whole blood specimens, from Clinics of haematology - KCU Sarajevo, total RNA was extracted by using of QIAamp RNA Blood Mini Kit (Qiagen cat. no. 52304). The lysis procedures of eritrocytes and leucocytes by using of EL and RLT buffers, is performed by incubation at 4°C and centrifugation at 2000 rpm. The pellet is resuspended in 500 µl EL buffer and than in 350 µl RLT buffer, as well. Disollved pellet is transferred into Qiagen shrederer spin columns (pink color) and centrifugated at 14000 rpm for two minutes. These spin columns were discarded and lysate from collection tubes were transferred into new uncolored spin columns with specific membrane for RNA addition. After centrifugation at 14000 rpm for three minutes, added RNA was resolved in 50 µl Rnase free water and store at - 20°C to next using. In this conditions, extracted RNA remain stability for more than one year(QIAamp RNA Blood Mini handbook, 1999.).

cDNA synthesis – Each specimen must have negative control (10 µl DEPC treated water and 4 µl cDNA mix). In the process of reverse transcription, both 0,2 ml PCR tubes (specimen and negative control) contained 11 µl cDNA synthesis mix (5xMMLV-RT buffer, 100 mM DTT, 10 mM dNTPs mix and MMLV reverse transcriptase), 10 µl extracted RNA and 4 µl BIO-RAD cDNA primer mix(total volume 25 µl). After incubation at 37°C for 45 min., reverse transcriptase was inactivated by incubation at 95°C for 5 min.cDNA was stored at -20°C (Cross et al., 1994.).

PCR amplification – For both, BIO-RAD RT-PCR screening and split-out PCR typing/ subtyping of leukemia, the same cycling programmes for first round PCR amplification and Nested PCR amplification were recommended and optimized for PE 9600 thermal cycle:

First round PCR amplification

15 min.	95°C		x1
30 sec.	95°C		
30 sec.	58°C		x25
1,5 min.		72°	
hold		4°C	x1

Nested PCR amplification

15 min.	95°C	x1
30 sec.	95°C	
30 sec.	58°C	x20
1,5 min.	72°C	
10 min	72°C	x1
hold	4°C	

According to standards of PCR optimisation, we modified annealing temperatures and number of cycles from these programs. We also modified $MgCl_2$ molarity, amount of cDNA and HotStar Taq DNA polymerase.

Detection of PCR products by agarose gel electrophoresis

PCR product are separated by electrophoresis in 1,5% agarose gel, at least 10 centimeters long, in 1xTBE buffer. The gel containing 0,5µg/ml of ethidium bromide. We added in the gel 14 µl of specimen (loading buffer and PCR products). After electrophoresis, the gels were examined by UV transilluminator.

Results and Discussion

After optimization of multiplex RT-PCR (BIO RAD), (adapted to thermal cycle PE 9600),by using of our PE 2400 PCR engine we obtained optimal results after analysis of electrophoretic bands by UV transilluminator under following conditions:

- annealing temperature – 65 C°
- number of cycles - 30 (for first PCR amplification and nested PCR amplification)
- $MgCl_2$ – final molarity 3,28 mM.
- 1 µl HotStar Taq DNA polymerase, instead standard amount of 0,4 µl per PCR reaction
- 10 µl cDNA, instead amount of 5 µl in first PCR master mix (10 x PCR buffer,$MgCl_2$,dNTPS mix, Hot Star Taq DNA polymerase and RNAse free water)

An internal control fragments of 911 bp, must be visible in all electrophoretic specimen lines. It means good integrity of RNA sample and the presence of PCR inhibitors.

One specimen showed genetic aberration inv (16) (p13; q22) by RT- PCR screening and often using of split- out M6 PCR primers (M6A, M6B, M6C, M6D and M6E), we detected 192 bp electrophoretic band, which means subtype CBFB/MYH11 – G.

An deletion on 16 chromosome was initially reported in 1982.This pericentric inversion is associated whit acute leukemia. There are 10 subtypes (A,B,C,D,E,F,G,H,I,J) or variants of CBFB/MYH11 fusion genes, which are generally associated with relatively good prognosis (Van Dongen et al., 1999; Liu et al., 1995; Claxton et al., 1994.).

Multiplex PCR is a demanding technique that requires extensive optimization of Taq polymerase, amount $MgCl_2$, additional reagents and PCR primers.

The cycling parameters such as annealing temperatures and number amplification cycles need to be changed. Hot Star Taq DNA polymerase has been developed to provide hot-star PCR for higher specify. It is activated by initial step (15 min. at 95 C°)(Cross et al., 1994.).

In our optimization experiments we used combination of this enzyme and corresponding PCR buffer (Qiagen). On this way, we obtained optimal results without any contamination, missprimed products and non-specific amplification products, as well.

By using of BIO-RAD multiplex RT-PCR for typing / subtyping leukemia, it is possible to detect new elektrophoretic bands as a result of new specific genes translocation.

For further characterisation of this genetic aberration is necessary to perform investigation by DNA sequencing (Springall et al., 1998.; Van der Reijden et al.,1995.).

Apstrakt: Molekularna tipizacija i subtipizacija leukemije korištenjem multiplex RT-PRC metoda, vezana je zbog praćenja minimuma rezidualne bolesti tokom i nakon tretmana pacijenata sa akutnom leukemijom. U ovom radu prezentirani su rezultati optimizacije ovoga metoda (proizvođač firma BIORD) dizajniranog za screening (RT-PCR) i subtipizaciju leukemije (split-out RT-PCR). Naime, ova procedura je prilagođena za PrkinEmlerGeneAmp 9600 PCR aparat i Qiagen HotStarTaq DNA polimerazu, te korištenje

drugih PCR aparata podrazumjeva optimizaciju procedure po osnovu odgovarajućih parametara, a u cilju dobijanja optimalnih rezultata. Za PCR amplifikaciju korišten je PE 2400 PCR aparat, kao i neophodna HotStarTaq DNA polimeraza. Ukupna RNA ekstrahovana je iz uzoraka cijele krvi dostavljenih od stručnjaka Klinike za hematologiju KCU – Sarajevo, uzete od pacijenata oboljelih od akutne leukemije. Nakon transfera RNA u cDNA, amplifikacije u PE 2400 pomoću odgovarajućeg seta primera (multiplex PCR), detekcije elektroforetskih separiranih amplificiranih PCR produkata u 1,5% agaroznom gelu sa etidium bromidom, optimalni rezultati su dobijeni korištenjem sljedećih optimizacijskih parametara :

- annealing temperature 65°C
- broj amplifikacijskih ciklusa za prvu i nested PCR amplifikaciju bio je 30
- finalni molaritet $MgCl_2$ – 3,28mM
- umjesto standardnih količina cDNA (5µl) i HotStarTaq DNA polimeraze (0,4µl), optimalni rezultati su dobijeni korištenjem 10 µl cDNA i 1 µl enzima.

Korištenjem ovakvih PCR parametara , detektovana je inv (16) hromosomska aberacija, a subtipiziranjem sa split-out PCR-om određen je subtip G (192 bp ele-kroforetska traka) što je upućivalo na CBFB/MYH1 fuziju gena.

Ključne riječi: leukemija, tipizacija/subtipizacija, fuzionisani genski transkripti multiplex RT-PCR screening i split-out RT-PCR.

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