Usporedba osjetljivosti ugnježđene PCR i kvantitativne PCR u određivanju Bcr-Abl p210 prijepisa kronične mijeloične leukemije
Comparison of sensitivity of nested PCR and quantitative PCR in Bcr-Abl p210 transcript detection in chronic myelogenous leukemia

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Sažetak

Uvod: Za otkrivanje minimalne ostatne bolesti u bolesnika s kroničnom mijeloičnom leukemijom (KML) koji su postigli potpunu kliničku remisiju i potpun citogenetski odgovor može se primijeniti ugnježđena PCR (engl. *nested PCR*, nPCR) i kvantitativna PCR u stvarnom vremenu (engl. *quantitative real-time PCR*, qPCR). Cilj liječenja je postizanje molekularne remisije, pa postizanje visoke osjetljivosti molekularne pretrage ima presudnu ulogu i kliničku primjenu. Usporedili smo razinu osjetljivosti nPCR i qPCR u otkrivanju BCR-ABL p210 prijepisa u modelu razrjeđenja Bcr/Abl-pozitivnih stanica.

Materijal i metode: Za određivanje razine osjetljivosti načinjena su serijska razrjeđenja stanične linije K562 (Bcr-Abl pozitivne) sa staničnom linijom NB4 (Bcr-Abl negativnom) (raspon razrjeđenja pozitivnih stanica: 10-3-10-7). Izolirani uzorci RNA prepisani su u cDNA i testirani na p210 prijepis pomoću nPCR i qPCR. Objema metodama također su ispitani uzorci koštane srži i periferne krvi dvoje bolesnika s KML na terapiji imatinib-mesilatom.

Rezultati: U testu staničnog razrjeđenja nPCR je pokazala osjetljivost za otkrivanje Bcr-Abl pozitivnih stanica od 10-5, dok je qPCR pokazala osjetljivost od 10-6. Obje su metode otkrile p210 prijepise u uzorcima koštane srži bolesnika s KML. Međutim, qPCR je uz to otkrila prijepise i u uzorcima periferne krvi, no uz nižu razinu prijepisa u usporedbi s uzorcima koštane srži.

Zaključak: Iako se često navodi kako je nPCR za otprilike 1 log osjetljivija od qPCR, u našem pokusu s razrjeđenjem na biljeg pozitivne stanične linije K562 dokumentirali smo višu osjetljivost za standardiziranu metodu qPCR, koja je razvijena za potrebe Europskog programa za borbu protiv karcinoma.

Ključne riječi: nPCR, qPCR, osjetljivost, otkrivanje p210

Abstract

Background: For minimal residual disease detection in chronic myelogenous leukemia (CML) patients who have achieved complete clinical remission and complete cytogenetic response, nested PCR (nPCR) and quantitative real-time PCR (qPCR) can be used. Achieving of molecular remission is the goal of therapy, so it is of critical importance and clinical utility to obtain high sensitivity of molecular testing. We compared the level of sensitivity of nPCR and qPCR in the detection of BCR-ABL p210 transcripts in a Bcr/Abl-positive cell dilution model.

Materials and Methods: For determination of sensitivity level, serial dilutions of K562 cell line (Bcr-Abl-positive) in NB4 cell line (Bcr-Abl-negative) were made (range of dilution of positive cells: 10-3-10-7). Isolated RNA samples were transcribed into cDNA and tested for p210 transcript by nPCR and qPCR. Bone marrow and peripheral blood samples of two CML patients on imatinib mesylate therapy were also tested by both methods.

Results: In the cell dilution test, nPCR showed sensitivity for detecting Bcr-Abl positive cell of 10-5, and qPCR showed a sensitivity of 10-6. Both methods detected p210 transcripts in bone marrow samples of CML patients. However, qPCR also detected transcripts in peripheral blood samples, with a lower transcript level in comparison to bone marrow samples.

Conclusion: Although frequently quoted as nPCR being by approximately 1 log more sensitive than qPCR, in our marker-positive cell line K562 dilution experiment we documented higher sensitivity of the standardized Europe Against Cancer Program developed qPCR method.

Key words: nPCR, qPCR, sensitivity, p210 detection

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Uvod

Posljednjega desetljeća je velik broj studija pokazao kako otkrivanje vrlo niskih brojeva malignih stanica, tj. otkrivanje minimalne ostatne bolesti (MRD), značajno korelira s kliničkim ishodom kod mnogih hematoloških malignih bolesti (1). Kod većine bolesnika s novo dijagnosticiranom kroničnom mijeloičnom leukemijom (KML) može se terapijom imatinib mesilatom postići potpun citogenetski odgovor (CCR). Međutim, u dijela ovih bolesnika dolazi do recidiva zbog tvrdokorne populacije malignih stanica prisutne ispod granice otkrivanja standardnim tehnikama. To ukazuje na to da je cilj terapije postizanje molekularne remisije, pa je od presudne važnosti i za kliničku primjenu steći visoku osjetljivost molekularnih pretraga. Idealno bi tehnike koje se rabe za otkrivanje MRD trebale imati razinu osjetljivosti u rasponu od 10^{-5} do 10^{-6}, biti primjenjive u svih bolesnika s ovom bolešću, pružiti stanovitu kvantifikaciju biljega, biti brze, ne preskupe i lako standardizirane (2). Uz to, od velike je važnosti dobra međulaboratorijska ponovljivost i standardizacija nalaza.

Kod bolesnika s KML koji su postigli CCR prema citogenetici koštane srži i/ili FISH, ugnježđena PCR (nPCR) i kvantitativna PCR u stvarnom vremenu (qPCR) rabe se za otkrivanje BCR-ABL mRNA. Izvješća o osjetljivosti i uporabi nPCR i qPCR u motrenju razina prijepisa BCR/ABL nisu dosljedna (3,4).

U ovoj studiji usporedili smo razinu osjetljivosti nPCR i qPCR u otkrivanju BCR-ABL p210 prijepisa u kontroliranom pokusu s modelom razrjeđivanja BCR-ABL pozitivnih stanica. Isto tako smo usporedili i opisali dva pristupa u qPCR: apsolutnu i relativnu kvantifikaciju.

Materijali i metode

Razrjeđenja staničnih linija

Za određivanje razine osjetljivosti načinjena su serijska razrjeđenja stanične linije K562 (BCR-ABL pozitivne) sa staničnom linijom NB4 (BCR-ABL negativnom). Razrjeđenja pozitivnih stanica su bila deseterostruka, od 10^{-3} (jedna pozitivna stanica u 1000 negativnih) do 10^{-7} (jedna pozitivna stanica u 10 milijuna negativnih). Objema metodama ispitani su i uzorci koštane srži i periferne krvi dvoje bolesnika s KML na terapiji imatinib mesilatom.

Izolacija RNA i obrnuti prijepis

RNA je izolirana uz primjenu seta QIAamp RNA Blood Mini Kit (QIAGEN, Hilden, Njemačka) prema uputama proizvođača. Za sintezu cDNA upotrebljen je jedan mikrogram ukupne RNA uz primjenu seta GeneAmp Gold RNA PCR Core Kit (Applied BioSystems, New Jersey, SAD) prema uputama proizvođača.

Introduction

During the last decade, a large number of studies have shown that detection of very low numbers of malignant cells, i.e. detection of minimal residual disease (MRD), significantly correlates with the clinical outcome in many hematologic malignancies (1). For most patients with newly diagnosed chronic myelogenous leukemia (CML), a complete cytogenetic response (CCR) can be achieved with imatinib mesylate therapy. But because of persistent malignant cell population that is present below the limit of detection by standard techniques, a proportion of them will relapse. This suggests that obtaining a molecular remission should be the goal of therapy and it is of critical importance and clinical utility to obtain high sensitivity of molecular testing. Ideally, techniques used for MRD detection should have a sensitivity level in the 10^{-5} to 10^{-6} range, be applicable to all patients with the disease, provide some quantification of the target, be rapid, inexpensive and readily standardized (2). Also, good interlaboratory reproducibility and standardization of reporting are of critical importance.

In CML patients who have achieved CCR by bone marrow cytogenetics and/or FISH nested PCR (nPCR) and quantitative real-time PCR (qPCR) are used for detection of BCR-ABL mRNA. There are inconsistent reports on the sensitivity and usage of nPCR and qPCR in monitoring of BCR/ABL transcript levels (3,4).

In this study, we compared the level of sensitivity of nPCR and qPCR in detection of BCR-ABL p210 transcripts in a controlled experiment of BCR-ABL-positive cell dilution model. Also, we compared and discussed two approaches in qPCR: absolute and relative quantification.

Materials and Methods

Cell line dilutions

For determination of sensitivity level, serial dilutions of K562 cell line (BCR-ABL-positive) in NB4 cell line (BCR-ABL-negative) were made. Dilution of positive cells was performed in 10-fold steps, from 10^{-3} (one positive cell in 1000 negative) to 10^{-7} (one positive cell in 10 000 000 negative). Bone marrow and peripheral blood samples of two CML patients on imatinib mesylate therapy were also tested by both methods.

RNA isolation and reverse transcription

RNA was isolated using QIAamp RNA Blood Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. One microgram of total RNA was used for cDNA synthesis using GeneAmp Gold RNA PCR Core Kit (Applied BioSystems, New Jersey, USA) according to the manufacturer's protocol.

Otkrivanje BCR/ABL prijepisa p210

Test ugnježđene PCR proveden je prema protokolu BIOMED-1 (5). PCR je izvedena sa slijedećim početnicama: prvi korak: BCR-b1-A GAAGTGTTTCAGAAGCTTCTC C plus ABL-a3-B GTTTGGGCTTCACACCAT TCC, drugi korak: BCR-b2-C CAGATGCTGACCAACTCGTGT plus ABL-a3-D TTCCCCATTGTGATTATAGCCTA. Obje faze PCR provedene su u reakcijskom volumenu od 25 µL koji je sadržavao: 1x reakcijski pufer (50 mM KCl, 20 mM Tris HCl, pH 8,3), 1,5 mM MgCl2, 200 µM dNTP, 10 pmol svake početnice, 0,5 U Taq DNA polimeraze (Applied BioSystems, New Jersey, SAD) i 1 µL cDNA u prvoj fazi ili 1 µL PCR iz prve faze u drugoj fazi. PCR umnažanje provedeno je pod slijedećim uvjetima: početna faza od pet minuta kod 95 °C, tada 35 ciklusa od 95 °C kroz 30 sekunda, 65 °C kroz jednu minutu, 72 °C kroz jednu minutu, potom konačna faza ekstenzije kod 72 °C kroz 7 minuta. Proizvodi PCR analizirani su na 2%-tnom agaroznom gelu (Applied BioSystems, New Jersey, SAD) obojanom etidij-bromidom (Sigma Chemical Co., Deisenhofen, Njemačka).

Test QPCR proveden je prema protokolu EAC uz primjenu početnica i probi za tehnologiju TaqMan (6). Analiza PCR u stvarnom vremenu za *BCR-ABL* i unutarnji "kućepaziteljski" gen (engl. *housekeeping gene*) *ABL* provedena je uz primjenu sustava 7300 Real Time PCR System (Applied BioSystems, New Jersey, SAD) uz početne faze od po 2 minute kod 50 °C i 10 minuta kod 95 °C, potom 50 ciklusa od po 15 sekunda kod 95 °C i 1 minutu kod 60 °C. Primijenjena su dva pristupa kod qPCR: apsolutna kvantifikacija (omjeri broja kopija) i relativna kvantifikacija. Za apsolutnu kvantifikaciju upotrebljene su standardne plazmidne krivulje (Ipsogen, Luminy Biotech Enterprises, Marseilles, Francuska). Kako bi se poništile razlike između PCR u studiji relativne kvantifikacije, provedena je normalizacija ciljnoga gena (*BCR-ABL*) pomoću endogene kontrole (*ABL*). Sve su ekspresije izračunate uz primjenu programa 7300 System SDS Software RQ Study Application (Applied BioSystems, New Jersey, SAD).

Rezultati

U testu razrjeđenja BCR-ABL pozitivnih stanica nPCR je otkrila p210 prijepis u razrjeđenju 10^{-5} (slika 1.) u 3 neovisna pokusa. Kod pristupa apsolutne kvantifikacije omjer broja kopija izražen je kao kopije *BCR-ABL* za kopiju *ABL*. Kod pristupa relativne kvantifikacije rezultati su prikazani kao relativna ekspresija normaliziranog ciljnoga gena u razrijeđenim staničnim linijama i bolesničkim uzorcima u odnosu na normaliziranu ekspresiju ciljnoga gena u nerazrijeđenoj staničnoj liniji K562. Oba pristupa kod qPCR pokazala su osjetljivost od 10^{-6} (slika 2., tablica 1.). I ugnježđena i kvantitativna metoda otkrile su p210 prijepis u uzorcima koštane srži bolesnika s KML. Međutim, za razliku od nPCR, qPCR je također otkrila prijepise p210 u

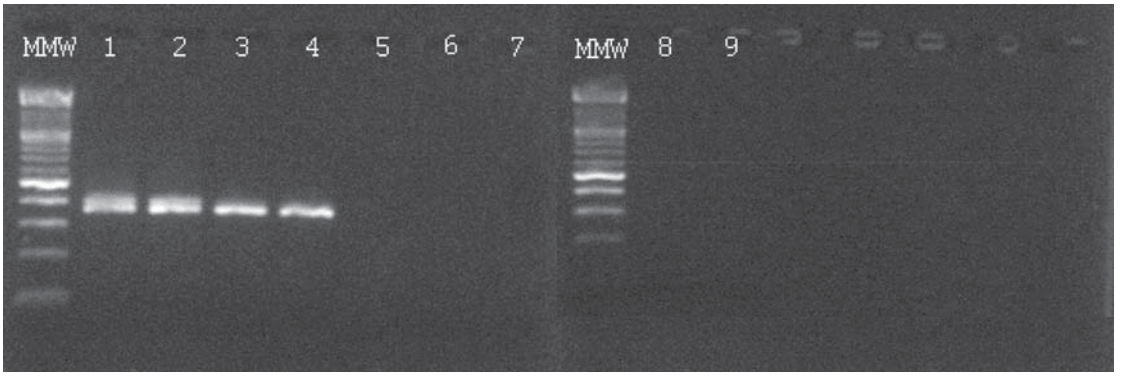
Detection of BCR/ABL p210 transcript

Nested PCR assay was performed according to BIOMED-1 protocol (5). PCR was performed with the following primers: first step: BCR-b1-A GAAGTGTTTCAGAAGCTTCTC C plus ABL-a3-B GTTTGGGCTTCACACCAT TCC, second step: BCR-b2-C CAGATGCTGACCAACTCGTGT plus ABL-a3-D TTCCCCATTGTGATTATAGCCTA. Both PCR steps were carried out in a 25 µL reaction volume containing: 1x reaction buffer (50 mM KCl, 20 mM Tris HCl, pH 8.3), 1.5 mM MgCl2, 200 µM dNTP, 10 pmol of each primer, 0.5 U of Taq DNA polymerase (Applied BioSystems, New Jersey, USA), and 1 µL of cDNA in the first step or 1 µL of first round PCR in the second step. The PCR amplifications were carried out under the following conditions: initial step of five minutes at 95 °C, then 35 cycles of 95 °C for 30 seconds, 65 °C for one minute, 72 °C for one minute, followed by a final extension step at 72 °C for 7 minutes. The PCR products were analyzed on 2% agarose gel (Applied BioSystems, New Jersey, USA) stained with ethidium bromide (Sigma Chemical Co., Deisenhofen, Germany).

QPCR assay was made according to EAC protocol using primers and probe for TaqMan technology (6). Real-time PCR analysis for *BCR-ABL* and internal housekeeping gene *ABL* was performed using 7300 Real Time PCR System (Applied BioSystems, New Jersey, USA) with the initial steps of 2 minutes at 50 °C and 10 minutes at 95 °C, followed by 50 cycles of 15 seconds at 95 °C and 1 minute at 60 °C. Two qPCR approaches were used: absolute quantification (copy number ratios) and relative quantification. For absolute quantification plasmid standard curves were used (Ipsogen, Luminy Biotech Enterprises, Marseilles, France). To compensate for inter-PCR variations in the relative quantification study, normalization of target gene (*BCR-ABL*) with an endogenous control (*ABL*) was performed. All expressions were calculated using 7300 System SDS Software RQ Study Application (Applied BioSystems, New Jersey, USA).

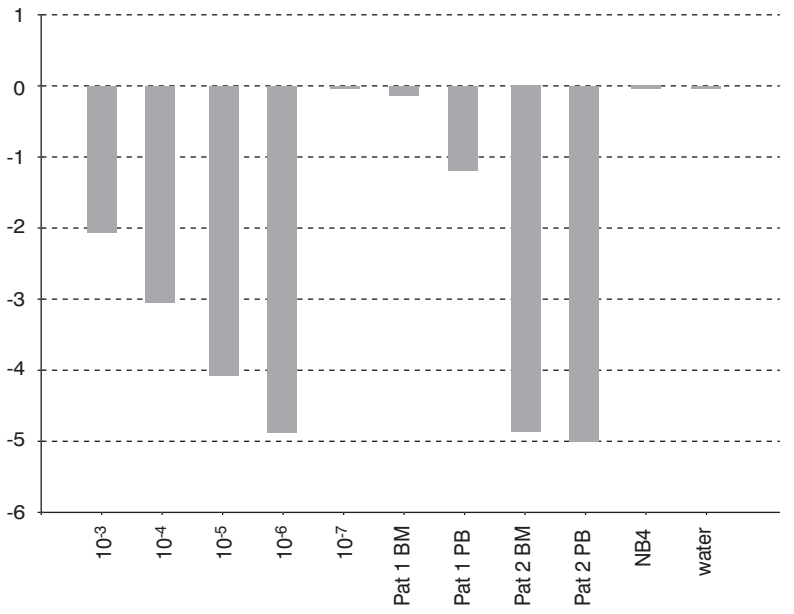
Results

In the BCR-ABL-positive cell dilution assay, nPCR detected p210 transcript in 10^{-5} dilution (Figure 1) in 3 independent experiments. In the absolute quantification approach, the copy number ratio was expressed as *BCR-ABL* copies *per* copy of *ABL*. In the relative quantification approach, results are shown as relative expressions of normalized target gene in diluted cell lines and patients samples against normalized expression of target gene in undiluted K562 cell line. Both qPCR approaches showed a sensitivity of 10^{-6} (Figure 2, Table 1). Both nested and quantitative methods detected p210 transcript in bone marrow samples of CML patients. However, contrary to nPCR, qPCR also detected p210 transcripts in peripheral blood samples of CML patients, irrespective of the approach used. Transcript levels



Slika 1. Agarozni gel obojan etidij-bromidom s rezultatima otkrivanja BCR-ABL p210 prijepisa u serijskim razrjeđenjima stanične linije K562 sa staničnom linijom NB4 i u dvoje bolesnika na terapiji imatinib mesilatom pomoću nPCR. MMW = biljeg molekularne težine; 1 = nerazrijeđena stanična linija K562; 2 = razrjeđenje 10^{-3}; 3 = razrjeđenje 10^{-4}; 4 = razrjeđenje 10^{-5}; 5 = razrjeđenje 10^{-6}; 6 = razrjeđenje 10^{-7}; 7 = stanična linija NB4; 8 i 9 = voda.

Figure 1 Ethidium bromide-stained agarose gel showing results of BCR-ABL p210 transcript detection in serial dilutions of K562 cell line in NB4 cell line and two patients on imatinib mesylate therapy using nPCR. MMW = molecular weight marker; 1 = undiluted K562; 2 = 10^{-3} dilution; 3 = 10^{-4} dilution; 4 = 10^{-5} dilution; 5 = 10^{-6} dilution; 6 = 10^{-7} dilution; 7 = NB4; 8 and 9 = water.



Slika 2. Otkrivanje BCR-ABL p210 prijepisa u serijskim razrjeđenjima stanične linije K562 sa staničnom linijom NB4 i u dvoje bolesnika na terapiji imatinib mesilatom pomoću qPCR uz primjenu tehnologije TaqMan (relativna kvantifikacija, kalibrator: nerazrijeđena stanična linija K562). BM = koštana srž; PB = periferna krv.

Figure 2 Detection of BCR-ABL p210 transcripts in serial dilutions of K562 cell line in NB4 cell line and two patients on imatinib mesylate therapy using qPCR with TaqMan technology (relative quantification, calibrator: undiluted cell line K562). BM = bone marrow; PB = peripheral blood.

uzorcima periferne krvi bolesnika s KML bez obzira na primijenjeni pristup. Razine prijepisa bile su niže u usporedbi s uzorcima koštane srži. Analiza uzoraka u 3 neovisna pokusa pokazala je kako su ovi testovi ponovljivi (srednja razlika triju mjerenja bila je 0,16% za apsolutnu kvantifikaciju i 0,18% za relativnu kvantifikaciju; standardna devijacija za apsolutnu kvantifikaciju bila je 2,1%, a za relativnu kvantifikaciju 2,0%).

were lower in comparison to bone marrow samples. Analysis of samples in 3 independent experiments demonstrated the assays to be reproducible (mean difference of 3 measurements 0.16% for absolute quantification, 0.18% for relative quantification; standard deviation for absolute quantification 2.1%, for relative quantification 2.0%).

Tablica 1. Omjer broja kopija BCR-ABL p210 prijepisa u serijskim razrjeđenjima stanične linije K562 sa staničnom linijom NB4 i kod dva bolesnika na terapiji imatinib mesilatom određeni korištenjem TaqMan tehnologije. Svijetlo sivo = u području minimalnog molekularnog odgovora, tamno sivo = u području značajnog molekularnog odgovora (prema referencama 7. i 8.), BM = koštana srž, PB = periferna krv.

Table 1 Copy number ratios of BCR-ABL p210 transcript determined in serial dilutions of cell line K562 in cell line NB4 and two patients on imatinib mesylate therapy using TaqMan technology. Light shadow = Minor Molecular Response range, dark shadow = Major Molecular Response range (according to refs. 7 and 8), BM = bone marrow, PB = peripheral blood.

Sample	Copy number ratio (BCR-ABL/ABL)
K562 undiluted	20.18
K562 10^{-3} dilution	0.020
K562 10^{-4} dilution	0.0017
K562 10^{-5} dilution	0.0002
K562 10^{-6} dilution	0.000016
K562 10^{-7} dilution	undetected
Pat 1 BM	0.8
Pat 1 PB	0.078
Pat 2 BM	0.00003
Pat 2 PB	0.00001
NB4 undiluted	undetected
water	undetected

Rasprava

Prema protokolu BIOMED-1 osjetljivost od 10^{-6} može se postići primjenom nPCR. Niža osjetljivost našega testa nPCR u usporedbi s protokolom BIOMED-1 može se pripisati pokusu s različitim načinom razrjeđenja. U protokolu BIOMED-1 je K562 RNA razrijeđena u HL60 RNA (RNA u RNA), dok su u našem pokusu razrjeđivanja stanice K562 razrijeđene u stanicama NB4 (stanice u stanicama). Na taj smo način eksperimentalne uvjete učinili sličnijima situaciji *in vivo*. Pred-PCR faze kao što su izolacija RNA i učinkovitost obrnutog prijepisa mogu također biti izvori niže osjetljivosti (9,10).

Obje metode postigle su prihvatljivu osjetljivost za otkrivanje MRD kod KML. Međutim, qPCR je pokazala veću osjetljivost nego nPCR. Osjetljivost koja se može dobiti analizom PCR ovisi o nekoliko parametara, koji uključuju broj ispitivanih stanica, ukupnu količinu ispitivane RNA i broj ciklusa PCR (11). Ove razlike između PCR mogu se nadoknaditi upotrebom normaliziranja ciljnoga gena endogenom kontrolom (*ABL, GADPH, B2M*). Uz višu osjetljivost, prednosti qPCR pred standardnom nPCR za otkrivanje prijepisa p210 uključuju skraćeno vrijeme od prijema uzorka do izdavanja nalaza, smanjenu mogućnost kontaminacije poslije PCR, smanjenu varijabilnost rezultata (prikuplja-

Discussion

According to BIOMED-1 protocol, a sensitivity of 10^{-6} can be achieved using nPCR. Lower sensitivity of our nPCR assay in comparison to BIOMED-1 protocol can be attributed to a different dilution experiment. In BIOMED-1 protocol K562 RNA was diluted into HL60 RNA (RNA in RNA), whereas in our dilution experiment K562 cells were diluted in NB4 cells (cells in cells). In that way, we made the experimental conditions more resembling *in vivo* situation. Also, the pre-PCR steps, as RNA extraction and efficiency of the reverse transcription can be the source of lower sensitivity (9,10).

Both methods reached acceptable sensitivity for detection of MRD in CML. However, qPCR showed higher sensitivity than nPCR. The sensitivity that can be obtained by PCR analysis is dependent on several parameters, which include the number of cells investigated, the total amount of RNA analyzed and the number of PCR cycles (11). These inter-PCR variations can be compensated by using normalization of target gene with an endogenous control (*ABL*, *GAPDH*, *B2M*). Besides higher sensitivity, advantages of qPCR over standard nPCR for p210 transcript detection include a decreased turnaround time, decreased chance for post-PCR contamination, decreased variability of re-

nje podataka obavlja se u eksponencijalnoj fazi reakcije PCR), visok protok i mogućnost dobivanja kvantitativnih rezultata. U našem pokusu su i metoda apsolutne kao i metoda relativne kvantifikacije pokazale jednaku osjetljivost. Mogući nedostatci metode apsolutne kvantifikacije su to što primjena plazmidnih standarda smanjuje broj jažica raspoloživih za bolesničke uzorke, i povećava opasnost od onečišćenja, te nešto viši troškovi. Relativna kvantifikacija zasniva se na relativnoj učinkovitosti umnažanja ciljnoga i kontrolnog gena, pa je preduvjet rutinsko uključenje prvog dijagnostičkog uzorka bolesnika. Na taj se način ovu metodu može primjenjivati za određivanje relativne razine MRD u usporedbi s dijagnostičkim uzorkom. Bez obzira rabi li se apsolutna ili relativna kvantifikacija, određivanje kretanja kvantitativnih brojeva ostatnih BCR-ABL pozitivnih stanica kroz određeno razdoblje pruža važne terapijske podatke u praćenju bolesnika s KML. U zaključku, utvrdili smo kako je qPCR primjerena, ponovljiva i pouzdana metoda za motrenje MRD nakon postizanja CCR kod kronične mijeloične leukemije.

sults (data collection occurs in the exponential phase of PCR reaction), high throughput, and possibility of obtaining quantitative results. In our experiment, both absolute and relative quantification methods showed equal sensitivity. Potential drawbacks of the absolute quantification method are that use of plasmid standards reduces the number of wells available for patient samples and increases the risk of contamination, also slightly increasing the costs. Relative quantification relies on the relative efficiencies of the target and control gene amplifications, so it is a prerequisite that the patient's first diagnostic sample is routinely included. In that way this method can be used for determination of the relative level of MRD in comparison to diagnostic sample.
Whether using absolute or relative quantification, the determination of the trend in the quantitative numbers of residual BCR-ABL-positive cells over a period of time provides important therapeutic information in the follow-up of CML patients. In conclusion, we found the qPCR to be an appropriate, reproducible and robust method for MRD monitoring after achieving CCR in chronic myelogenous leukemia.

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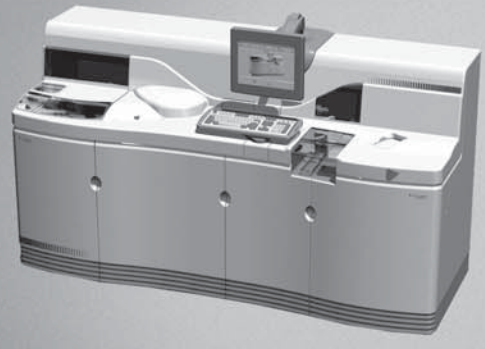

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