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APPLIED MOLECULAR BIOLOGY

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Characterization of Russian Universal Kits for Real-Time PCR: Application to Molecular Oncodiagnosis

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Abstract—Russian real-time PCR (Q-PCR) kits and reference foreign kits were experimentally compared using serial dilutions (30–300,000 copies) of a plasmid containing a cDNA fragment of fusion *PML-RARα* (DNA standard). The Russian RialitiTM kit and ABI TaqMan PCR core reagent kit were similar in the efficiency of amplification (1.919 and 1.927, respectively) and in the coefficient of correlation obtained for the standard calibration plots (0.999 and 0.996, respectively). The RialitiTM kit was used to estimate the copy number of *PML-RARα* and *ABL* (internal control) in cDNA specimens obtained from peripheral blood and bone marrow of patients with acute promyelocytic leukemia and the chromosome translocation t(15;17), which generates chimeric *PML-RARα*. The *PML-RARα* copy number per μg total RNA ranged from 58,600 to 831,500 before therapy. After 3.5 months of chemotherapy, the patient showed no signs of the minimal residual disease: *PML-RARα* was undetectable by Q-PCR, which agreed with clinical data. Studies with the example of *PML-RARα* demonstrated the suitability of the RialitiTM kit for oncodiagnosis.

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Key words: quantitative real-time PCR, fusion gene transcripts, chimeric *PML-RARα*, translocation, Q-PCR Universal Ready Mix, acute promyelocytic leukemia, minimal residual disease

INTRODUCTION

Modern oncohematology has come to employ not only classical cytogenetic, cytochemical, and immunophenotypic methods, but also new molecular biological techniques such as amplification of nucleic acids in the polymerase or ligase chain reaction (PCR, LCR), DNA sequencing, and hybridization of DNA to microarrays [1–5].

The new approaches allow simultaneous comparison of thousands of potential oncomarker genes and selection of the most important markers for diagnosing tumors, estimating the efficiency of therapy, and predicting the recurrence of a disease.

Real-time PCR (Q-PCR) is advantageous for solving such problems: copies of oncomarker genes can be detected and quantified immediately in test samples owing to the automated recording of the accumulation of specific amplification products during PCR [6, 7]. Computer analysis, a lack of postamplification manipulations, and standardization of results exclude the possibility of subjective interpretation, minimize the risk of contamination and consequent false-positive results, simplify gene diagnosis, and save diagnosis time [8]. Q-PCR is highly sensitive and detects one leukemic cell among 10^4–10^5 normal cells; i.e., its sensitivity is approximately ten times higher than that of other common techniques such as immunophenotyping, PCR, and reverse transcription PCR (RT–PCR) [9, 12].

In 2003, an international protocol standardizing real-time RT–PCR was developed within the framework of the Europe Against Cancer Program for diagnosing acute myeloblast leukemia (AML) and, in particular, acute promyelocytic leukemia (APL) [9]. The protocol contains recommendations with respect to the use of an ABI Prism 7700 instrument (Applied Biosystems, United States) and a commercial universal reagent kit with a TaqMan probe for assessing the minimal residual disease. Since foreign Q-PCR kits are rather expensive, it is desirable to develop and test Russian universal reagent kits to construct new sensitive and specific diagnostic systems based on Q-PCR for analyzing oncomarkers in various tumor diseases.

In this study, we comparatively tested Russian universal Q-PCR kits and showed that the kits are similar in main characteristics to their foreign analogs. The suitability of the Russian kits for oncodiagnosis by Q-PCR was demonstrated with the example of chimeric *PML-RARα*, an APL marker.

Table 1. Gene fragments cloned in pGEM-T

Gene	Chromosomal location	Primer sequences	Amplicon size, bp
Isoform L of chimeric *PML-RARα*	t(15;17)(q22;q21)	PML-C1 TCAAGATGGAGTCTGAGGAGG/ ENR962 GCTTGTAGATGCGGGGTAGAG	271
Isoform S of chimeric *PML-RARα*	t(15;17)(q22;q21)	PML-A1 CAGTGTACGCCTTCTCCATCA/ ENR962 GCTTGTAGATGCGGGGTAGAG	376
ABL, internal control	9q34	ABL-F CCTTCAGCGGCCAGTAGC/ ABL-R GGACACAGGCCCATGGTAC	318

EXPERIMENTAL

Specimens. We used 15 cDNA specimens from patients with verified APL in different stages of anticancer therapy. The cDNA preparations were obtained in the Laboratory of Cytogenetics, Institute of Carcinogenesis. Bone marrow (BM) aspirates (1–3 ml) were collected via sternal puncture; venous blood (3–5 ml) was drawn from the antecubital vein.

Cytogenetic examination of peripheral blood (PB) and BM cells was performed after short-time (24–28 h) culturing by the standard method. The karyotypes and chromosome abnormalities of PB and BM cells were identified according to the International System for Human Cytogenetic Nomenclature [13].

PB mononuclear cells were obtained by centrifugation through a Ficoll–Hypaque gradient (Pharmacia–Biotech, United Kingdom) and immediately used to isolate RNA.

Total RNA was isolated with the TRI reagent (Sigma, United States), which was used at 1 ml per 10^6–10^7 cells as recommended by the manufacturer. RNA concentration was estimated spectrophotometrically at 260 and 280 nm. RNA (0.5–2.5 μg) was dissolved in deionized water, treated with DEPC, and used to synthesize the first cDNA strand with M-MLV reverse transcriptase (Promega, United States) and arbitrary hexamer primers.

The cDNA quality was checked by amplifying a 276-bp fragment of *ABL* with primers A2 (5'-ttcagcggccagtagcatctgactt-3') and CA3 (5'-tgttgactggcgtgatgtagttgcttgg-3'). The sensitivity of RT–PCR was estimated according to the standard BIOMED-1 protocol by diluting the RNA of NB4 cells carrying translocation t(15:17) with the RNA of Kasumi-1 cells. The sensitivity was 10^4 chimeric molecules [1, 17].

The standard genetic-engineering methods were used [18].

Cloning of cDNA. The cDNAs of *PML-RARα* isoform S (bcr 1), *PML-RARα* isoform L (bcr 3), and *ABL* were synthesized by reverse transcription from total RNAs of the patients, amplified by PCR, and cloned in pGEM-T (Promega, United States) (Table 1).

The lack of nucleotide substitutions in the cloned fragments was verified by sequencing with an ABI Prism BigDye Terminator kit v. 3.1 (Applied Biosystems) [20]. The reaction products were analyzed using an ABI Prism 3100 Avant automated sequencer. The recombinant plasmids were linearized with *Shp*I and *Pst*I (SibEnzyme, Russia), which were selected using the Lasergene package (DNA Star, United States). Plasmid DNA concentration was measured with a GENESYS 2 spectrophotometer (Thermo Spectronic, United States).

Reagents. To clone the PCR products, we used the following: pGEM-T (Promega); *Escherichia coli* Z85; bacterial culture media (Difco, United States); reagents from Serva (Germany), Gibco–Invitrogen (United States), and Amersham–Pharmacia–Biotech (United Kingdom); gene-engineering enzymes from SibEnzyme and Boehringer–Mannheim (Germany); and M-MLV reverse transcriptase from Promega and Invitrogen (United States).

Universal Q-PCR kits. We used a TaqMan PCR core reagent kit (Applied Biosystems), JumpStart ready mix (Sigma), a kit from Sintol (Russia), and a RialitiTM kit (Biochip-IMB, Russia). The 2.5× Q-PCR mixture from the RialitiTM kit contained all main components, including the enzyme (but not primers, a probe, or DNA).

The reaction mixture contained reagents of the universal kits under study, 250 nM each of the specific primers, 200 nM TaqMan probe, and 5 μl of plasmid DNA or cDNA.

Q-PCR. The L and S isoforms of *PML-RARα* were amplified from cDNA with the primers and TaqMan probes (Table 2) recommended by the Europe Against Cancer Program [9]. Oligonucleotides were synthesized by Sintol. The *ABL* cDNA was used as an internal positive control. Each experiment was performed in triplicate.

The fluorescence intensity of the reporter dye FAM were normalized with respect to that of the passive reference dye ROX. The reaction was run on an ABI Prism sequence detection system (Applied Biosystems) and included incubation at 95°C for 10 min and 40 cycles of incubation at 95°C for 15 s and 60°C for

Table 2. Primers and probes used to detect *PML-RARα* and *ABL* by Q-PCR

Gene	Oligonucleotide sequence			Amplicon size, bp
	direct primer	reverse primer	TaqMan probe	
Isoform L of chimeric *PML-RARα*	ENF903 TCTTCCTGCCCAA CAGCAA	ENR962 GCTTGTAGATGC GGGGTAGAG	ENP942 Fam-AGTGCCCAGCCCTC CCTCGC-Tamra	127
Isoform S of chimeric *PML-RARα*	ENF905 CCGATGGCTTCG ACGAGTT			145
ABL	ENF1003 TGGAGATAACAC TCTAAGCATAACT AAAGGT	ENR1063 GATGTAGTTGCTT GGGACCCA	ENPr1043 Fam-CCATTTTTGGTTTG GGCTTCACACCATT-Tamra	123

Table 3. Comparison of Q-PCR reagent kits

Kit, manufacturer	Delta Rn (Reporter/Rox) 30–300000 copies of *PML-RARα*	Slope of the standard plot	Amplification efficiency, $E = 10^{-1/\text{slope}}$	Amplification efficiency, $E = (1–10^{1/\text{slope}}) \times 100\%$	Coefficient of correlation
TaqMan PCR core reagent kit, Applied Biosystems, United States	0.3 (<1)–1.6	–3.51	1.927	92.7	0.996
JumpStart Taq ready mix, Sigma, United States	1 (=1)–2.8	–3.40	1.968	96.8	0.993
Q-PCR kit, Sintol, Russia	0.6 (<1)–2.4	–3.40	1.968	96.8	0.995
RialitiTM, Biochip-IMB, Russia	1.2 (>1)–4	–3.53	1.919	91.9	0.999

1 min. We compared the results obtained in one plate, with the same reagents and DNA standards, and in one instrument.

Estimation of the copy number was performed by absolute quantitative analysis. Standard calibration plots were obtained with serial tenfold dilutions of a DNA standard (30, 300, 3000, 30,000, and 300,000 copies). A threshold, that is, the minimal fluorescence intensity corresponding to the beginning of the exponential phase in all reactions carried out with a given primer pair, was selected using the control program of the ABI Prism 7000 SDS v. 1.0 program.

RESULTS AND DISCUSSION

Comparison of Q-PCR Universal Kits

In the European Union, the TaqMan PCR core reagent kit (Applied Biosystems) has been recommended for detecting minimal amounts of leukemia cells by Q-PCR [9]; this makes it possible to select original regimes of anticancer therapy and to predict the disease progression in APL. Russian companies have only recently come to produce Q-PCR universal reagent kits. The sensitivity and reliability have not been compared so far between Russian kits and their analogs produced abroad by leading specialized companies. Using the available universal primers and TaqMan probes for Q-PCR (Table 2), we compared the Russian universal kits and reference analogs using as an example the detection of chimeric *PML-RARα*.

The kits were tested with the cloned fragments of the S and L isoforms of *PML-RARα* (DNA standards) and with cDNA specimens obtained from patients carrying a chromosome abnormality (karyotype 46XY t(15;17)). Two identical serial dilutions of one plasmid DNA were prepared simultaneously in one plate; in the instrument, one series was specified as standard and the other as unknown. The instrument had an individual detector for each reagent kit. Experiments were carried out by three researchers; the results were similar.

The main parameters compared for the kits were the efficiency of amplification (the slope of the calibration plot), the coefficient of correlation obtained for the observed dependence (R^2), the signal intensity normalized with respect to the ROX passive control (ΔRn), the coefficient of variation of the resulting estimates (CV), and the sensitivity (the minimal number of DNA copies detected in the reaction). The results are summarized in Table 3.

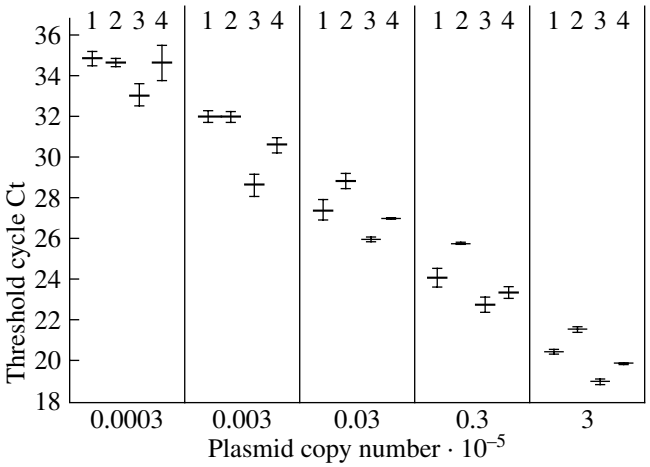

Fig. 1. Threshold cycle and CV at different copy numbers of the DNA standard for the universal reagent kits: (1) TaqMan PCR core reagent kit, Applied Biosystems; (2) JumpStart ready mix, Sigma; (3) Q-PCR kit, Sintol; and (4) RialitiTM, Biochip-IMB.

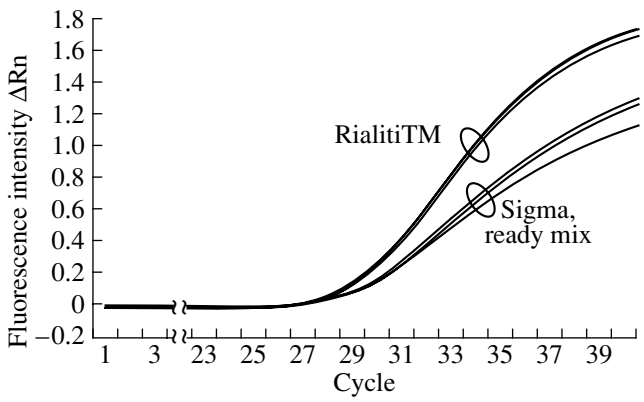

Fig. 2. Q-PCR kinetic curves obtained in parallel tests with the RialitiTM kit (upper bundle) and the JumpStart ready mix (lower bundle) for *PML-RARα* used at 3000 copies per reaction.

The Russian kits were as good as the foreign analogs in the parameters tested (Table 3). The coefficient of correlation R^2 (≈1), which reflects the quality of the calibration plot, was no less than 0.993 with all Q-PCR kits. The RialitiTM kit was superior to the other kits in R^2 (0.999). The TaqMan PCR core reagent kit had $R^2 = 0.996$.

The efficiency of PCR is theoretically equal to 2 and is computed as the slope of the calibration plot. The number of copies of a gene is estimated from exponential synthesis of the amplification product, during which the amplicon concentration doubles in every cycle. Real estimates are always lower than 2, which should be taken into account in quantitative PCR-based assays. The efficiency of PCR decreases when thermostable DNA polymerase is inhibited by the end product, the primer concentration drops, or the template is denatured incompletely. In addition, the efficiency depends on the primer design, amplification conditions, etc. [21–23]. We estimated the efficiency of amplification at 1.919 for the RialitiTM kit and at 1.927 for the TaqMan PCR core reagent kit. The Sintol kit ranked first in this parameter (1.96).

The slope of the standard calibration plot was –3.53 ($r^2 = 0.996$) for the TaqMan PCR core reagent kit and –3.51 ($r^2 = 0.999$) for the RialitiTM kit (Table 3). The accuracy of estimating the absolute copy number of a target gene depends first and foremost on the efficiency of the amplifying test and standard samples and the identity of PCR conditions. Estimates of template copy number may vary more than tenfold because of different contents of admixtures in DNA preparations, pipetting errors, and the dependence of the activity of some commercial thermostable polymerases on the reaction components (ROX, GC content, and template size) [24].

To calculate the CV of estimates obtained in the range of 30–300,000 copies of cloned *PML-RARα*, we used the standard calibration curve and compared the observed mean values and mean deviations with the known copy numbers. The threshold cycle (Ct) was determined from the threshold fluorescence intensity, which was 0.05–0.1 units in our experiments. The results obtained with serial dilutions of plasmid DNA and several reagent kits are shown in Fig. 1.

The CV ranged from 3.3% to 43.5% with the RialitiTM kit and from 7.8% to 33.0% with the Sintol kit (Fig. 1). The JumpStart ready mix and TaqMan PCR core reagent kit were characterized by the lowest standard deviations in the case of samples with a low copy number; for these two kits, the CV values in detecting 30 copies of the control plasmids were 12.3% (JumpStart ready mix) and 23.5% (TaqMan PCR core reagent kit).

In the case of the Russian kits, the CV obtained at a lower copy number was greater (33.0–43.5%) than in detecting 3000 copies (3.3%). The difference in Ct among the kits probably reflects the difference in *Taq* polymerase activity, which determines the threshold cycle and the sensitivity of PCR [25]. The best threshold cycle was observed with the Sintol kit, suggesting the highest level of sensitivity.

The kinetics of PCR was studied for the four kits (Fig. 2). The fluorescence intensity–amplification cycle kinetic curves were sigmoid. The chimeric *PML-RARα* fragment was used at 3000 copies per sample. The threshold was exceeded after 28 cycles; the exponential phase was between cycles 28 and 33. The fluorescence intensity (the reporter signal normalized with respect to the ROX reference signal and reflecting the activity of thermostable polymerase)

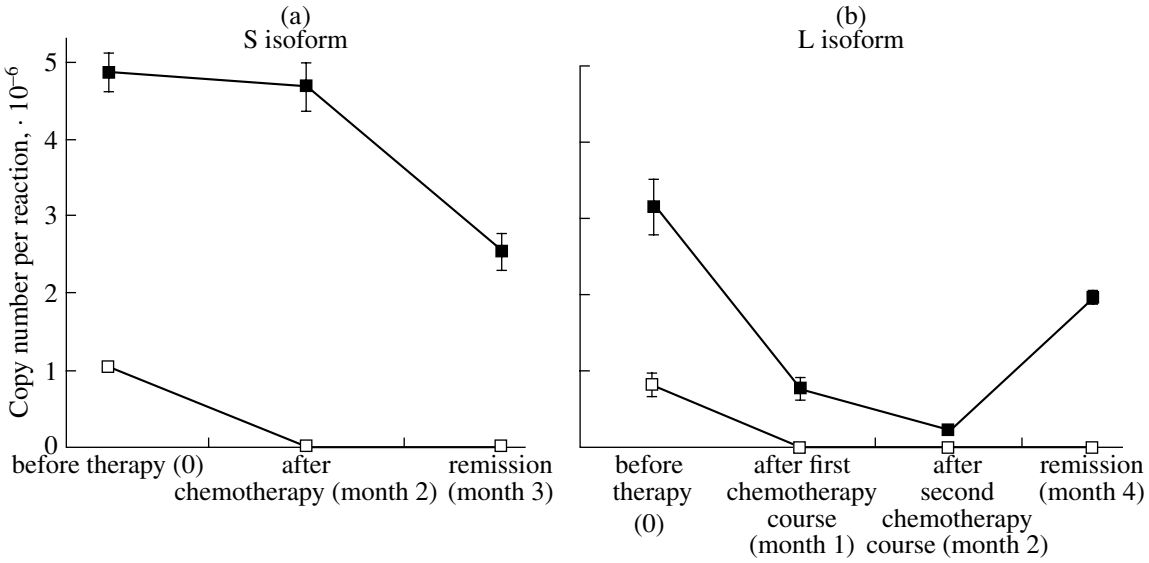

Fig. 3. Changes in *PML-RARα* expression in APL patients during therapy. (a) Patient S., 11 years, male, 88% blasts in BM, karyotype 46XY t(15;17). (b) Patient T., 24 years, male, 15% blasts in PB, karyotype 46XY t(15;17). Mean copy number per μg total RNA was estimated for (–□–) the fusion gene and (–■–) control *ABL*.

was approximately 1.5 times lower with the JumpStart ready mix than with the RialitiTM kit. Thus, the reagent concentrations are closer to optimal in the RialitiTM kit than in the JumpStart ready mix. The reaction curve was flattened after 39 cycles.

Taken together, the above results demonstrate that the Russian Q-PCR kits are as good as their foreign analogs in the main parameters, and even surpass them in some parameters. The results of analyzing serial dilutions of the plasmids with the Russian kits were highly reproducible.

Testing a Q-PCR Universal Kit with Clinical Samples

Molecular genetic diagnosis of APL is clinically important for evaluating the efficacy of therapy. Recent studies have shown that quantitation of tumor cells in PB and BM predicts the progress and recurrence of the disease. The most informative index characterizing the risk of recurrence is the dynamics of the minimal residual disease, which is due to tumor cells surviving antitumor therapy. Evaluation of the minimal residual disease in APL by means of Q-PCR consists in quantitating a specific marker of leukemia cells. The marker is one of the three (L, S, and V) isoforms of chimeric *PML-RARα* which results from chromosome translocation t(15;17)(q22;q21) and is absent in healthy people. Detection of transcripts of this fusion gene confirms the diagnosis and determines the strategy of APL treatment [10, 11, 14].

We used the RialitiTM kit to estimate the absolute copy number of *PML-RARα* in test cDNA specimens obtained from patients with the karyotype 46XY t(15;17) at different stages of therapy (Fig. 3).

The copy number of the *PML-RARα* isoforms L and S was estimated in the 15 cDNA specimens obtained from the PB and BM cells of two patients, T. (24 years) and S. (11 years). We detected the S isoform in patient S. and the L isoform in patient T.

Although *ABL* is commonly used as an internal positive control to check the level of gene expression, the copy number of the *ABL* cDNA varied in our specimens. This was probably explained by partial degradation of RNA during isolation, storage, and transportation (Fig. 3). Since the genes of interest may also be affected, the level of their expression should be compared with an endogenous control for every cDNA specimen.

Before treatment, the mean *PML-RARα* copy number was 831,500 copies per μg PB RNA and 785,800 copies per μg BM RNA in the first patient. The other patient had 1,040,300 and 58,600 copies per μg RNA in PB and BM, respectively. After one month of chemotherapy, transcripts of the fusion gene were detectable only in BM. The *PML-RARα* copy number decreased by a factor of 25–1800 to 415 and 2295 copies per μg RNA in patients T. and S., respectively. After 3.5 months of therapy, molecular remission was observed: marker *PML-RARα* was undetectable in PB and BM by Q-PCR, while the endogenous control gene was detected (i.e., the reaction was not inhibited). Our results showed that chemotherapy had been effective and had reduced the number of cells with the abnormal karyotype below the detection threshold (30 copies in the reaction). This finding agreed with

clinical data that both patients were in remission and needed no additional chemotherapy.

Thus, the Russian universal kits allow reliable detection and quantitation of a specific marker in specimens, which makes it possible to select individual regimens of therapy, prevent recurrence of the disease, and monitor the minimal residual disease. The efficiency of amplification, the coefficient of correlation, the CV, and the sensitivity did not considerably differ between the Russian and foreign kits. The results obtained for the example *PML-RARα* gene testify again to the suitability of Q-PCR for oncodiagnosis.

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REFERENCES

1. Van Dongen J.J., Macintyre E.A., Gabert J.A., et al. 1999. Standardized RT-PCR analysis of fusion gene transcripts from chromosome aberrations in acute leukemia for detection of minimal residual disease. Report of the BIOMED-1 Concerted Action: Investigation of minimal residual disease in acute leukemia. *Leukemia*. **13**, 1901–1928.
2. Shin L.Y., Kuo M.Ch., Liang D.Ch., Huang C.F., et al. 2003. Internal tandem duplication and Asp 835. Mutations of the FMS-like tyrosine kinase3 (*FLT3*) gene in acute promyelocytic leukemia. *Cancer.* **98**, 1206–1216.
3. Walker G.T., Little M.C., Nadeau J.G., et al. 1992. Isothermal in vitro amplification of DNA by a restriction enzyme/DNA polymerase system. *Proc. Natl. Acad. Sci. USA*. **89**, 392–396.
4. Chantratita W., Pongtanapisit W., Piroj W., Srichunrasmi C., Seesuai S. 2004. Development and comparison of the real-time amplification based methods—NASBA-Beacon, RT-PCR taqman and RT-PCR hybridization probe assays—for the qualitative detection of sars coronavirus. *Southeast Asian J. Trop. Med. Public. Health*. **35**, 623–629.
5. Romano J.W., Williams K.J., Shurtliff R.N., et al. 1997. NASBA technology: Isothermal RNA amplification in qualitative and quantitative diagnostics. *Immunol. Invest*. **26**, 15–28.
6. Heid C.A., Stevens J., Livak K. J., Williams P.M. 1996. Real time quantitative PCR. *Genome Res*. **6**, 986–994.
7. Holland P.M., Abramson R.D., Watson R., Gelfand D.H. 1991. Detection of specific polymerase chain reaction product by utilizing the 5'–3' exonuclease activity of *Thermus aquaticus* DNA polymerase. *Proc. Natl. Acad. Sci. USA*. **88,** 7276–7280.
8. Manzenyuk O.Yu., Moskalets O.V., Burdakova Yu.A., Suchkov S.V. 2002. Molecular typing in clinical diagnosis. *Biopreparaty.* **3**, 16–18.
9. Gabert J., Beillard E., van der Velden V.H., et al. 2003. Standardization and quality control studies of "real time" quantitative reverse transcriptase polymerase chain reaction of fusion gene transcripts for residual disease detection in leukemia: A Europe Against Cancer Program. *Leukemia*. **17**, 2318–2357.
10. Cassinat B., Zassadowski F., Balitrand N., Barbey C., Rain J.D., Fenaux P., Degos L., Vidaud M., Chomienne C. 2000. Quantification of minimal residual disease in acute promyelocytic leukemia patients with t(15;17) translocation using real-time RT-PCR. *Leukemia*. **14**, 324–328.
11. Boeckx N., Willemse M.J., Szczepanski T., van der Velden V.H.J., Langerak A.W., Vandekerckhove P., van Dongen J.J.M. 2002. Fusion gene transcripts and Ig/TCR gene rearrangements are complementary but infrequent targets for PCR-based detection of minimal residual disease in acute myeloid leukemia. *Leukemia*. **16**, 368–375.
12. Kondo M., Kudo K., Kimura H., et al. 2000. Real time quantitative reverse transcription polymerase chain reaction for the detection of AML-MTG8 fusion transcripts in t (8;21)—positive acute myelogenous leukemia. *Leukemia Res*. **24**, 951–956.
13. Mitelman F. (Ed). 1991. *ISCN: Guidelines for Cancer Cytogenetics, Supplement to an International System for Human Cytogenetic Nomenclature*. Basel, Switzerland: Karger.
14. Tobal K., Saunders M.J., Grey M.R., Yin J.A. 1995. Persistence of RAR alpha-PML fusion mRNA detected by reverse transcriptase polymerase chain reaction in patients in long-term remission of acute promyelocytic leukemia. *Br. J. Haematol*. **90**, 615–618.
15. Lowenberg B., Downing J.R., Burnett A. 1999. Acute myeloid leukemia. *N. Engl. J. Med*. **341**, 1051–1062.
16. Jurlander J., Caligiuri M.A., Ruutu T., et al. 1996. Persistence of the AML1/ETO fusion transcripts in patients treated with allogenic bone marrow transplantation for t(8; 21) leukemia. *Blood*. **88**, 2183–2191.
17. Miamoto T., Nagafuji K., Akashi K., et al. 1996. Persistence of multipotent progenitors expressing AML1/ETO transcripts in long-term remission patients with t(8; 21) acute myelogenous leukemia. *Blood*. **87**, 4789–4796.
18. Cross N.C., Hughes T.P., Lin F., et al. 1993. Minimal residual disease after allogenic bone marrow transplantation for chronic myeloid leukemia in first chronic phase. Correlation with acute graft-versus-host disease and relapse. *Br. J. Haematol*. **84**, 67–70.
19. Maniatis, T., Fritsch, E.F., Sambrook, J. 1982. *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor, N.Y.: Cold Spring Harbor Lab. Press.

20. Sanger F., Niclen S., Coulson A.R. 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA*. **74**, 5463–5467.

21. Pfaffl M.W. 2001. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res*. **29**, e45.

22. Ramakers C., Ruijter J.M., Deprez R.H. and Moorman A.F. 2003. Assumption-free analysis of quantitative real-time polymerase chain reaction (PCR) data. *Neurosci. Lett*. **339**, 62–66.

23. Peirson S.N., Butler J.N., Foster R.G. 2003. Experimental validation of novel and conventional approaches to quantitative real-time PCR data analysis. *Nucleic Acids Res*. **31**, e73.

24. Azeri B., Xing W., Sorge J.A., Hogrefe H.H. 2003. Amplification efficiency of thermostable DNA polymerases. *Anal. Biochem*. **321**, 226–235.

25. Wolf P., Grage H., Hagberg O., Radstromom P. 2004. Impact of DNA polymerases and the buffer systems on quantitative real time PCR. *J. Clin. Microbiol*. **42**, 408–411.

26. Swillens S., Goffard J.C., Marechal Y., de Kerchove d'Exaerde A., el Housni H. 2004. Instant evaluation of the absolute initial number of cDNA copies from a single real-time PCR curve. *Nucleic Acids Res*. **32**, e56.