Dry-reagent disposable dipstick test for visual screening of seven leukemia-related chromosomal translocations

Despina P. Kalogianni¹, Vasiliki Bravou², Theodore K. Christopoulos¹,³,*,
Penelope C. Ioannou⁴ and Nicholas C. Zoumbos²

¹Department of Chemistry, University of Patras, Patras 26500, Greece, ²Hematology Division, Department of Internal Medicine, University of Patras Medical School, Patras, Greece 26500, ³Foundation for Research and Technology Hellas, Institute of Chemical Engineering and High Temperature Chemical Processes, (FORTH/ICE-HT), P.O. Box 1414, Patras 26504, Greece and ⁴Department of Chemistry, University of Athens, Athens 15771, Greece

Received August 13, 2006; Revised November 9, 2006; Accepted December 1, 2006

ABSTRACT

We report the first dry-reagent, disposable, dipstick test for molecular screening of seven chromosomal translocations associated with acute and chronic leukemia. The dipstick assay offers about 10 times higher detectability than agarose gel electrophoresis and, contrary to electrophoresis, allows confirmation of the sequence of the polymerase chain reaction (PCR) product by hybridization within a few minutes without the need of instrumentation. Biotinylated amplified DNA is hybridized with a dA-tailed probe and applied to the strip, which contains oligo(dT)-conjugated gold nanoparticles in dry form. Upon immersion of the strip in the appropriate buffer, the solution migrates and the hybrids are captured by immobilized streptavidin at the test zone generating a characteristic red line. The excess nanoparticles are captured by oligo(dA) strands immobilized at the control zone of the strip producing a second red line. We studied the: t(9;22)(q34;q11), t(15;17)(q22;q21), t(11;17)(q23;q21), t(5;17)(q32;q21), t(11;17)(q13;q21), t(8,21)(q22;q22) and inv(16)(p13;q22) that generate the BCR-ABL, PML-RARα, PLZF-RARα, NPM-RARα, NuMA-RARα, AML1-ETO and CBFβ-MYH11 fusion genes, respectively. A single K562 cell was detectable amidst 10⁶ normal leukocytes. A dipstick test was developed for actin, as a reference gene. The dipstick assay with appropriate probes can be used for identification of the fusion transcripts involved in the translocation.

INTRODUCTION

Chromosomal translocations have a primary role in the pathogenesis of leukemia because they affect specific genes encoding transcription factors or other cell-cycle regulators. The structural and functional characterization of these rearrangements has provided valuable insight into the mechanisms of malignant transformation of the hematopoietic cells (1–3). Moreover, the fusion genes that are formed as a result of the translocations constitute important tumor markers whose detection and/or quantification assist in the diagnosis, prognosis, monitoring the response to treatment and detection of minimal residual disease (4–6). The chimeric proteins that are encoded by the fusion genes in the leukemic cells could in principle serve as tumor markers but there is a lack of suitable antibodies for the development of immunoassays (7).

Conventional cytogenetics and fluorescence in situ hybridization (FISH) are used widely for the detection of chromosomal rearrangements albeit their sensitivity is limited to 1–5% of leukemic cells in the total cell population (1,4,5). To date the highest detectability is achieved by methods that are based on the exponential amplification of translocation-specific nucleic acid sequences, e.g. via the polymerase chain reaction (PCR). Molecular studies of chromosomal rearrangements have shown that the breakpoints in various patients are spread over a large segment of genomic DNA, which is difficult to amplify by PCR on a routine basis. The resulting fusion mRNA transcripts, however, are the same in most patients and consequently RNA is preferred as a starting template for the molecular assays of chromosomal translocations (1–6). PCR primers are designed to hybridize at opposite sides of the junction region so that exponential amplification occurs only when the fusion sequence is present in the sample.

*To whom correspondence should be addressed. Tel: +30 2610 996022; Fax: +30 2610 997118; E-mail: tkc@chemistry.upatras.gr

© 2007 The Author(s).
This is an Open Access article distributed under the terms of the Creative Commons Attribution Non-Commercial License (http://creativecommons.org/licenses/by-nc/2.0/uk/) which permits unrestricted non-commercial use, distribution, and reproduction in any medium, provided the original work is properly cited.
Currently, the most widely used method for the detection of PCR products entails separation by agarose gel electrophoresis followed by ethidium bromide staining. Hybridization assays that are performed in microtiter wells have been proposed for the post-PCR detection of amplified products because they are easily automatable (8,9). However, they require specialized instrumentation and multiple pipetting, incubation and washing steps in order to capture the amplified sequence, hybridize with a specific probe, remove the excess of probe, add the appropriate substrate and read the generated signal. Alternatively, flow cytometry can be used for post-PCR detection of amplification products that are fluorescently labeled or have been subjected to an oligonucleotide ligation reaction and are captured on polystyrene beads (10,11). Flow cytometry is suitable for the development of multiplex assays but requires expensive instrumentation. On the other hand, real-time PCR allows continuous monitoring of the amplified fragments during PCR by a homogeneous fluorometric hybridization assay and is used widely for quantification of the fusion transcripts. Real-time PCR, however, requires highly specialized, expensive equipment along with costly reagents (1,4,5).

In this work, we report the first dry-reagent, disposable, dipstick test for the molecular screening of chromosomal translocations. The test allows visual detection and confirmation of PCR-amplified leukemia-specific transcripts by hybridization within minutes. The test is simple and does not require special instrumentation. A total of seven well-defined chromosomal rearrangements were selected. The Philadelphia translocation, t(9;22)(q34;q11), which is present in >95% of patients with chronic myeloid leukemia (CML), involves the movement of most of the ABL (Abelson murine leukemia) protooncogene from chromosome 9 to the BCR (breakpoint cluster region) gene on chromosome 22, thus resulting in the BCR-ABL fusion gene (12). The t(15;17)(q22;q21) translocation, which is the diagnostic hallmark of acute promyelocytic leukemia (APL), joins the PML (promyelocytic leukemia) gene on chromosome 15 with the retinoic acid receptor alpha gene (RARα) on 17q to produce the PML-RARα fusion gene (1,3,13). Three other chromosomal translocations, associated with acute myeloid leukemia (AML), disrupt the RARα gene, namely, the t(11;17)(q23;q21) that involves the PLZF (promyelocytic leukemia zinc finger) gene, the t(5;17)(q32;q21) implicating the NPM (nucleophosmin) gene, and the t(11;17)(q13;q21) that involves the gene encoding the nuclear mitotic apparatus protein (NuMA). The generated fusion transcripts are PLZF-RARα, PML-RARα and NuMA-RARα, respectively (1,3,13). Also two other common translocations with a significant prognostic value for AML were detected: The t(8;21)(q22;q22), that fuses the acute myeloid leukemia 1 (AML1) gene with the ETO (eight twenty one) gene (generating the AML1-ETO fusion gene) and the inv(16)(p13;q22) that joins the CBFβ (core binding factor beta) gene on 16q22 and the MYH11 (myosin smooth muscle heavy chain) gene on chromosome 16p13 to produce the CBFβ-MYH11 fusion (14,15).

**MATERIALS AND METHODS**

**Instrumentation**

PCR amplification was performed in a thermal cycler from MJ Research (Waltham, MA). A digital camera, Kodak DC 120, and the Gel Analyzer software for DNA documentation were purchased from Kodak (New York, NY).

**Materials**

Agarose, deoxynucleoside triphosphates (dNTPs) and DNA molecular weight markers (φ x 174 DNA, Hae III digest) were obtained by HT Biotechnology (Cambridge, UK). Ethidium Bromide was purchased from Research Organics (Cleveland, OH). TRizol Reagent and SuperScript II reverse transcriptase were obtained from Invitrogen (CarlsBad, CA). Tag DNA polymerase was purchased from Hytest (Turku, Finland), Promega (Madison, WI) and Finzymes (Espoo, Finland). Ribonuclease inhibitor (RNasin) was from Promega (Madison, WI). Terminal deoxynucleotidyl transferase (TdT) was purchased from MBI Fermentas (Vilnius, Lithuania). All common reagents were from Sigma (St. Louis, MO). The dry-reagent DNA strip was manufactured as described earlier (16).

Plasmids pSG5-PLZF-RARα, pSG5-NPM-RARα and pSG5-NuMA-RARα containing the PLZF-RARα, the NPM-RARα and the NuMA-RARα complete fusion c-DNAs at the EcoRI site of the vector pSG5 were kindly provided by Dr Jonathan Licht (Division of Hematology-Oncology, Mount Sinai School of Medicine, NY) (17,18). Samples for BCR-ABL, PML-RARα, AML1-ETO and CBFβ-MYH11 were collected at the Division of Hematology, University Hospital, Rio, Patras, Greece. The NB4 cell line (ACC 207) carrying the PML-RARα translocation was obtained from DSMZ (Braunschweig, Germany).

The primers and probes used in this study were synthesized by Invitrogen. Primer sequences for BCR-ABL (8,9), PML-RARα, NPM-RARα (19), PLZF-RARα (20) NuMA-RARα (21), CBFβ-MYH11 (22) and AML1-ETO (22) are listed in Table 1. Actin was amplified by using 5′-Biotin-ACAATGAGCTGCGTGTGGCT-3′ and 5′-TCTCCCTAAATGTCACGCACGA-3′ as upstream and downstream primer, respectively. The oligonucleotide probes used for the detection of amplified DNA were as follows: BCR-ABL, 5′-GCTGAAGGGCTTTGAACTC TGGTAA-3′ (b2-a2) (8,9), 5′-ATCTTGAGGTGGAGCG AGTACATT-3′ (b3) and 5′-GCTGAAGGGCTTTCC TATTTGATG-3′ (b2-a2) (8,9); PML-RARα, 5′-ATCTT GATGACCTTCTGGGACT-3′, PLZF-RARα, 5′-GTACGTCTTCATCCACGTGCAGC-3′, NPM-RARα, 5′-GCCCTGTAATTGCCTGTGCT-3′, NuMA-RARα, 5′-GCCTGAGTTGCATACTCGGGA GA-3′, AML1-ETO, 5′-TCT CAGTACCGTATGAGTTCGA GGTTTCTC-3′, CBFβ-MYH11, 5′-CAGCTCATGG ACCTC-3′ and Actin, 5′-TCTCCAAATATGCATCTGG GTCATCT-3′ (23).
Table 1. Sequences of the primers used in nested PCR (PCR I and PCR II)

| Fusion genes | Primers (5’ → 3’) | Primers (PCR II) (5’ → 3’) |
|--------------|--------------------|---------------------------|
| BCR-ABL      | A1 TTTCAGAAGGCT    | A3 GGAGCTGTCGA             |
|              | TCTCCCTGAC         | GATGCCTGACCAAC            |
|              | A2 CAGTGC          | A4 TCAAGCCTC              |
|              | AACGAAAAA          | GAGGCTCAAAGTC            |
|              | GGTGTTGGG GTC     |                           |
| PML-RARa     | B1 AGCTGATCCGC     | B3 CAACAGACAGC            |
|              | GAGCCGGTG          | CCGAAAGGAAGAAT            |
|              | B2 TGGATG          | B4 ATAGTTGTTAG            |
|              | CGCAGGGCGG         | CTCGAGGAGCTTGC            |
|              | GGAGAAAGC          |                           |
| PLZF-RARa    | C1 TCCAGAGGGA      | C2 TCGAGCTCTCTGA          |
|              | GCTGTTCAAGC        | TAACAGAG                 |
|              | R1 TCTTCTG         | R2 GGCGTGCACCC            |
|              | GATGCTGC           | ATAGTGTTG                |
|              | GGGGG             |                           |
| NPM-RARa     | D1 GGAAGATTCGAT    | D2 TCGGTGTCGAAA           |
|              | GGACATGGG          | AAGGCC                   |
|              | R1 TCTTCTG         | R2 GGCGTGCACCC            |
|              | GATGCTGC           | ATAGTGTTG                |
|              | GGGGG             |                           |
| NuMA-RARa    | E1 AGACCTGGGCAA    | E2 TTTCAGACGAG            |
|              | ATCCAGGG           | CGGTCTGC                 |
|              | R1 TCTTCTG         | R2 GGCGTGCACCC            |
|              | GATGCTGC           | ATAGTGTTG                |
|              | GGGGG             |                           |
| AML1-ETO     | F1 CTACCAGCGCA     | F3 ATGACCTCAGGTT          |
|              | TGAAAGACC          | TGTGGCTGC                 |
|              | F2 AGAGGA          | F4 TGAAGCTGGCTT           |
|              | AGGCCCAT           | GGAGGCTCT                 |
|              | TGCTGAA           |                           |
| CBFβ-MYH11   | G1 GCAGGGCAGGTT    | G3 GGGGCTGTCTGG           |
|              | TATTAGGGG          | AGTTTGATG                 |
|              | G2 TCTCTT          | G4 CTTAGCGCGCTGC          |
|              | CTCCTCATT          | ATGTT                    |
|              | CTGCTC            |                           |

*All the upstream primers used for PCR II are biotinylated at the 5’ end.

RNA extraction

The total mononuclear cells from patient samples were isolated by Ficoll density gradient centrifugation using the Biocoll separating solution from Biochrom (Berlin, Germany). The total RNA was isolated from about 10^6 cells by using the TRIzol reagent.

Reverse transcription (RT)

Reverse transcription reactions were carried out in a total volume of 20 μl containing 50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM MgCl2, 10 mM dithiothreitol, 0.5 mM each of the dNTPs, 0.5 μM downstream external primer, 40 U Rnasin, 200 U reverse transcriptase and 5 μl (1–5μg) total RNA. The reactions mixtures were incubated for 1h at 42°C and then held for 15 min at 70°C.

Polymerase chain reaction (PCR)

Two rounds of PCR (nested PCR) were performed for each chromosomal translocation. All amplifications were carried out in a total volume of 50 μl in the presence of 67 mM Tris-HCl, pH 8.8, 16.6 mM (NH4)2SO4, and 0.1 ml/l Tween-20. The upstream primers used for the second round of PCR (PCR II) were biotinylated at the 5’ end. The other components of the reaction mixtures along with cycling conditions are given in the next paragraphs.

**BCR-ABL.** PCR I was carried out by using 0.5 μM each of the primers A1 and A2 (upstream and downstream primer, respectively), 1.5 mM MgCl2, 40 μM each of the dNTPs, 1.25 U thermostable DNA polymerase and 1 μl of the reverse transcription (RT) mixture. The program of thermal cycling included: 5 min at 95°C followed by 20 cycles of 95°C for 30s, 65°C for 30s and 72°C for 1 min. At the end of the cycling, the mixture was held at 72°C for 10 min. A 1-μl aliquot was used for PCR II (35 cycles), which was performed exactly as PCR I using oligos A3 and A4 as upstream and downstream primers, respectively.

**PML-RARa.** PCR I was carried out by using 0.5 μM each of the primers B1 and B2, 1.5 mM MgCl2, 200 μM each of the dNTPs and 2.5 U DNA polymerase. The RT mixture was treated for 20 min, at 42°C, with 2 U of RNase H and 2 μl of a 3-fold dilution was used for amplification. The cycling steps were: 3 min at 95°C followed by 35 cycles of 94°C for 45s, 65°C for 45s and 72°C for 1 min, and a final

**Patient samples**

Bone marrow or peripheral blood samples from 22 patients with a diagnosis of Ph1 CML, based on cytology, histology, cytogenetic and molecular analysis, were used for the detection of BCR-ABL transcripts. The clinical information, including disease phase, type of therapy, hematological, cytogenetic and molecular remission status are presented in Table 2. Complete hematologic response was defined according to conventional criteria: (1) bone marrow with <5% blasts (2) peripheral blood leukocyte count <10000 per μl without peripheral blasts, promyelocytes or myelocytes (3) disappearance of all signs and symptoms related to CML (4) normalization for at least 4 weeks. Cytogenetic response was defined by prevalence of Ph1 metaphases: 0%, complete; 1–35%, partial; 35–65%, minor; 65–95%, minimal; greater than 95%, none. For patients 2, 3, 5 and 8, two samples were obtained at different time points in the course of the disease. The study also included two patients with essential thrombocytopenia which were used as negative controls. Two patient samples carrying the b2-a2 BCR-ABL transcript variant were kindly provided by Dr D. Mavrogianni, (Laiko Hospital, University of Athens Medical School, Athens, Greece).
### Table 2. Characteristics of chronic myelogenous leukemia patients

| Sample No | Patient | Age/Sex | Time point of analysis | Disease phase at diagnosis/hematologic response to treatment | Cytogenetic analysis | PCR |
|-----------|---------|---------|------------------------|------------------------------------------------------------|----------------------|-----|
| 1         | 1       | 68/M    | At diagnosis           | Chronic phase CML                                           | 100% Ph<sup>+</sup> metaphases | +   |
| 2         | 2       | 60/F    | During therapy with HU | No response                                                 | none CR-100% Ph<sup>+</sup> metaphases | +   |
| 3         | 2       | 62/F    | During therapy with imatinib | No response                                               | NA                    |     |
| 4         | 3       | 71/M    | During therapy with imatinib | CHN                                                      | Minimal CR-86% Ph<sup>+</sup> metaphases | +   |
| 5         | 3       | 73/M    | During therapy with imatinib | CHN                                                      | Complete CR-0% Ph<sup>+</sup> metaphases | +   |
| 6         | 4       | 51/M    | No therapy             | CHN                                                      | Partial CR-15% Ph<sup>+</sup> metaphases | +   |
| 7         | 5       | 64/F    | During therapy with imatinib | CHN                                                      | Complete CR-0% Ph<sup>+</sup> metaphases |     |
| 8         | 5       | 62/F    | During therapy with IFN/Ara-C | No response                                               | Minimal CR-85% Ph<sup>+</sup> metaphases | +   |
| 9         | 6       | 31/F    | During therapy with imatinib | CHN                                                      | NA                    |     |
| 10        | 7       | 24/M    | During therapy with imatinib | CHN                                                      | Minor CR-55% Ph<sup>+</sup> metaphases | +   |
| 11        | 8       | 63/F    | During therapy with HU  | CHN                                                      | NA                    |     |
| 12        | 8       | 64/F    | During therapy with imatinib | CHN                                                      | NA                    |     |
| 13        | 9       | 69/F    | During therapy with imatinib | CHN                                                      | Partial CR-15% Ph<sup>+</sup> metaphases | +   |
| 14        | 10      | 69/M    | During therapy with IFN | Blast crisis                                               | NA                    |     |
| 15        | 11      | 22/M    | After allogeneic BMT   | CHN                                                      | Complete CR-0% Ph<sup>+</sup> metaphases | +   |
| 16        | 12      | 68/F    | No therapy             | CHN                                                      | NA                    |     |
| 17        | 13      | 51/F    | At diagnosis           | Chronic phase CML                                           | 100% Ph<sup>+</sup> metaphases | +   |
| 18        | 14      | 49/F    | At diagnosis           | Chronic phase CML                                           | 100% Ph<sup>+</sup> metaphases | +   |
| 19        | 15      | 74/M    | During therapy with HU  | No response                                                | NA                    |     |
| 20        | 16      | 48/F    | During therapy with imatinib/IFN | No response                                               | None CR-100% Ph<sup>+</sup> metaphases | +   |
| 21        | 17      | 63/F    | During therapy with IFN | CHN                                                      | NA                    |     |
| 22        | 18      | 31/F    | During therapy with imatinib | CHN                                                      | Complete CR-0% Ph<sup>+</sup> metaphases | +   |

*Age refers to the time of obtaining the sample.

CHR: complete hematologic response, CR: cytogenetic response, HU: hydroxyurea, IFN: interferon-a, BMT: bone marrow transplantation, NA: not available.

extension step at 72°C for 10 min. A 2-μl aliquot of the product was added to the PCR II mixture, which was performed with primers B3 and B4 and 1 mM MgCl<sub>2</sub>. The cycling parameters for PCR II were: 5 min at 95°C followed by 35 cycles of 94°C for 30 s, 65°C for 30 s and 72°C for 50 s and a final step at 72°C for 10 min.

**AML1-ETO.** Primers F1 and F2 (0.5 μM of each) were used for PCR I along with 1.5 mM MgCl<sub>2</sub>, 200 μM each of the dNTPs, 2.5 U DNA polymerase and 1 μl of the RT mixture. A 1-μl aliquot of the product was used for PCR II, which was performed with primers F3 and F4. The PCR conditions included 30 s at 95°C followed by 35 cycles for PCR I (30 cycles for PCR II) at 94°C for 30 s, 65°C for 1 min and 72°C for 1 min. At the end of the cycling, the mixture was held at 72°C for 10 min.

**CBFI-MYH11.** Primers G1 and G2 (0.5 μM of each) were used for PCR I along with 2.5 mM MgCl<sub>2</sub>, 50 μM each of the dNTPs, 1.25 U DNA polymerase and 1 μl of the RT mixture. A 2-μl aliquot of the product was used for PCR II, which was performed with primers G3 and G4. The thermal cycling conditions for both rounds of PCR were: 1 min at 95°C followed by 35 cycles of 94°C for 30 s, 65°C for 1 min and 72°C for 1 min. At the end of the cycling, the mixture was held at 72°C for 10 min.

**PLZF-RARA and NPM-RARA.** Oligos C1 and D1 (0.2 μM of each) were used as upstream primers for PCR I of PLZF-RARA and NPM-RARA, respectively, whereas the oligo R1 was used as a downstream primer for both amplifications. The reaction mixture contained 1.5 mM MgCl<sub>2</sub>, 200 μM each of the dNTPs and 2.5 U DNA polymerase. A 1-μl volume of the product was transferred into the PCR II mixture. Oligos C2 and D2 served as upstream primers of PLZF-RARA and NPM-RARA, respectively, and R2 was the downstream primer. The cycling program for both PCR rounds comprised 5 min at 95°C followed by 35 cycles of 94°C for 30 s, 55°C for 1 min and 72°C for 1 min. A final extension step for 10 min at 72°C was performed.

**NuMA-RARA.** PCR I was performed by using 0.5 μM each of the primers E1 and R1, 1.5 mM MgCl<sub>2</sub>, 50 μM each of the dNTPs and 1.25 U DNA polymerase. PCR II was carried out with primers E2 and R2 and 1 μl of a 10-fold dilution of the PCR I product. The PCR conditions were as follows. Five minutes at 94°C followed by 20 cycles (for PCR I) or 25 cycles (for PCR II) of 94°C for 30 s, 65°C for 30 s (68°C for 30 s for PCR II) and 72°C for 1 min. At the end of the cycling, the mixture was held at 72°C for 10 min.

**Actin.** The PCR mixture contained 10 mM Tris-HCl, pH 8.8, 50 mM KCl and 1 μl/l Triton X-100, 2.5 mM MgCl<sub>2</sub>, 50 μM each of the dNTPs, 0.5 μM each of the primers, 1.25 U DNA polymerase and 2 μl of a 10-fold dilution of the RT mixture. The PCR conditions were as follows. Five minutes at 94°C followed by 35 cycles of 95°C for 30 s, 60°C for 30 s and 72°C for 1 min. At the end of the cycling, the mixture was held at 72°C for 10 min.

Negative controls (containing water instead of target) were included in each series of reverse transcription and amplification reactions to confirm the absence of contamination. The PCR products were first detected by 2% agarose gel electrophoresis and ethidium bromide staining.
Tailing of oligonucleotide probes with dATP

Oligonucleotide probes specific for the junction corresponding to each chromosomal translocation were tailed with dATP using terminal deoxynucleotidyl transferase (TdT). The tailing reactions were carried out in a final volume of 20 μl containing 0.2 M potassium cacodylate, 25 mM Tris (pH 7.2), 0.1 ml/l Triton X-100, 1 mM CoCl₂, 2 mM dATP, 400 pmol probe and 30 U of TdT. The mixture was incubated at 37°C for 1 h and the reaction was stopped by adding 2 μl of 0.5 M EDTA, pH 8.0. Methylmaleimide was also added at the end of the incubation time at a final concentration of 2.5 mM.

Dipstick test for rapid visual detection of translocation-specific amplified sequences

The dry-reagent strip (4 mm × 70 mm) consisted of an immersion pad, a conjugation pad, a laminated membrane and an absorbent pad assembled on a plastic adhesive backing that provides rigidity. The four parts were positioned in such a way that their ends overlapped in order to ensure continuous flow (by capillary action) of the developing solution from the wicking pad up to the absorbent pad. Gold nanoparticles (40 nm diameter) functionalized with poly(dT) strands, as described previously (16), were placed on the conjugate pad and allowed to dry at room temperature. Streptavidin (27 pmol) was immobilized by physical adsorption on the test zone of the strip. Similarly, poly(dA) strands (1.2 pmol) were immobilized on the control zone of the strip.

Following PCR amplification, the biotinylated products were mixed with 1 pmol of the tailed oligonucleotide probe and the volume was brought to 5 μl with 67 mM Tris-HCl, pH 8.8, 16.6 mM (NH₄)₂SO₄ and 0.1 ml/l Tween-20. The mixture was then denatured by adding 5 μl of 0.4 M NaOH and incubated for 10 min at room temperature. The mixture was neutralized by the addition of 5 μl of 0.4 M HCl and 5 μl of 0.4 M Tris-HCl, pH 7.5 and allowed to hybridize for 7 min at 37°C. A 10-μl aliquot of the solution was applied to the conjugate pad of the strip and the latter was immersed into the appropriate developing solution (40 g/l glycerol and 10 g/l SDS in phosphate-buffered saline, pH 7.4). The formation of a red line in the test zone of the strip signifies the presence of a translocation-specific amplified sequence in the sample. A red line is always formed at the control zone of the strip to confirm the proper functioning of the test. Thus, a result is positive when a red line is observed both in the test zone and control zone, whereas in the absence of the translocation, a red line is formed only in the control zone.

RESULTS AND DISCUSSION

Assay principle

The proposed method for molecular diagnosis of several leukemia-related chromosomal translocations involves reverse transcription, amplification of the related junction sequences by nested PCR (PCR I and PCR II) and detection of the products by the dry-reagent dipstick assay. PCR II products were biotinylated at the one end by using an upstream primer labeled with biotin at the 5’ end. The sizes of PCR II products were 200 bp for BCR-ABL b3-a2 transcript, 124 bp for BCR-ABL b2-a2, 366 bp for PML-RARα, 267 bp for PLZF-RARα, 423 bp for NPM-RARα, 315 bp for NuMA-RARα, 260 bp for AML1-ETO and 271 bp for CBFβ-MYH11. The integrity of extracted total RNA was confirmed by RT-PCR (one round) of actin mRNA, as a reference gene. The PCR product of actin was 372 bp. A similar dry-reagent dipstick assay was also developed for the detection of actin amplification product.

A schematic illustration of the dry-reagent dipstick test is presented in Figure 1. The assay entails rapid hybridization of the amplified fragments (target DNA), in solution, with a dATP-tailed oligonucleotide probe, application to the sample loading area on the conjugate pad of the strip and immersion of the strip.

Figure 1. Left panel: Illustration of the principle of the dry-reagent disposable dipstick test for visual detection of chromosomal translocations. Right panel: Scanning electron microscopy (SEM) images of the conjugate pad and the membrane of the strip. IP = immersion pad; CP = conjugation pad; M = membrane; AP = absorbent pad; TZ = test zone; CZ = control zone; SA = streptavidin; B = biotin.
(via the immersion pad) in the developing solution. The developing solution migrates to the opposite end of the strip, by capillary forces, and causes rehybridization of the poly(dT)-functionalized gold nanoparticles (Au NP), which are then connected to the probe via hybridization of the poly(dT) strands with the poly(dA) tail of the probe. As the solution passes through the test zone of the strip, the hybrids are captured by immobilized streptavidin, thus resulting in accumulation of Au NP which is detected visually as a characteristic red line. The red color of the Au NP is due to the plasmon resonance peak at 520 nm (24,25). The excess of poly(dT)-Au NP are captured by immobilized poly(dA) strands at the control zone of the strip, giving a second red line. In the absence of the translocation, no red line is observed at the test zone. A red line, however, is always formed at the control zone to confirm the proper functioning of the strip. Consequently, a sample is positive for a certain translocation when a red line is observed both in the test and the control zone. Scanning electron microscope (SEM) images of the conjugate pad (consisting of glass fibers) and the polyethersulfone membrane of the strip containing the Au NP are also presented in Figure 1.

The assay configuration described above is a heterogeneous hybridization assay. The conventional way of performing this type of hybridization assays involves pipetting the sample on a streptavidin-coated solid phase (e.g. microtitration wells), incubation, washing the solid phase, addition of an appropriately labeled detection probe, incubation, washing out the excess of labeled probe and measuring the signal of the probe that is bound to the solid phase. The strip-based assay offers the following significant advantages: (a) It eliminates the multiple pipetting and washing steps required for addition of reagents and separation of the bound from the free labeled (not hybridized) probe. Indeed, the continuous migration of the developing solution along the strip allows rapid reagent mixing, hybridization, binding to the solid phase and efficient removal of the excess of labeled probe. (b) It provides an appropriate amount of Au NP-labeled probe in dry form as an integral part of the strip, thus eliminating the need for reconstitution of reagents and dilution of the stock solution.

The detectability of the dipstick assay was assessed, for each chromosomal translocation and for actin, by preparing solutions containing various concentrations of amplified DNA. The DNA concentration of each stock solution was determined by agarose gel electrophoresis and ethidium bromide staining followed by densitometric analysis of the picture of the gel taken by a digital camera. The $\phi \times 174$ DNA markers were used as standards for the quantification. Afterwards, serial dilutions of the stock were prepared (using 67 mM Tris-HCl, pH 8.8, 16.6 mM (NH$_4$)$_2$SO$_4$, and 0.1 ml/l Tween-20) and 10-μl aliquots containing from 0 to 400 fmol target DNA were assayed by the strip.

**BCR-ABL translocation**

The results for the BCR-ABL translocation are presented in Figure 2. As low as 0.4 fmol of the amplified target DNA was detectable employing the strip method. The detectability of the dipstick assay was assessed, for each chromosomal translocation and for actin, by preparing solutions containing various concentrations of amplified DNA. The DNA concentration of each stock solution was determined by agarose gel electrophoresis and ethidium bromide staining followed by densitometric analysis of the picture of the gel taken by a digital camera. The $\phi \times 174$ DNA markers were used as standards for the quantification. Afterwards, serial dilutions of the stock were prepared (using 67 mM Tris-HCl, pH 8.8, 16.6 mM (NH$_4$)$_2$SO$_4$, and 0.1 ml/l Tween-20) and 10-μl aliquots containing from 0 to 400 fmol target DNA were assayed by the strip.

**Figure 2.** Upper left panel: Dry-reagent dipstick test for the detection of amplified DNA from BCR-ABL translocation. Upper right panel: Dry-reagent dipstick test for the detection of amplified DNA from the reference gene, actin. Lower panel: Dry-reagent dipstick test for the detection of total RNA from a Philadelphia chromosome positive cell line (K562 cells). The amounts of RNA, in pg, are as follows. 1:0.1; 2:0.5; 3:2.56; 4:12.8; 5:64; 6:320; 7:1.6 × 10³; 8:8 × 10³; 9:4 × 10⁴; 10:2 × 10⁵; 11:10⁶; 12:5 × 10⁶. The results obtained by agarose (2%) gel electrophoresis and ethidium bromide staining are also presented for direct comparison. M = DNA markers; N = negative, i.e. addition of water instead of RNA in the RT mixture.
DNA can be detected by visual observation. Furthermore, the intensity of the red line in the test zone of the strip increases as the amount of target DNA increases up to 100 fmol (10 nM). At 400 fmol target DNA (40 nM), the intensity becomes lower than the line intensity at 100 fmol DNA. This is attributed to the competition between reannealing of the two strands of target DNA and probe/target hybridization. A constant amount of 1 pmol poly(dA)-tailed probe was used throughout this work. The amount of probe was estimated experimentally and is determined by the number of poly(dT) strands on the surface of Au NP that are available for hybridization. The strip-based assay was compared directly (same amount of product) with agarose gel electrophoresis and ethidium bromide staining. The electropherograms are also presented in Figure 2. The lowest amount of amplified DNA detectable by electrophoresis was 6.4 fmol. Thus, the strip test for BCR-ABL amplified DNA provides 16 times better detectability than electrophoresis.

Data pertaining to the detection of the amplification product from the reference gene, actin, are also presented in Figure 2. As low as 1.6 fmol of actin amplified DNA can be easily detected by visual observation, whereas 25 fmol DNA are detectable by electrophoresis.

The sensitivity of the proposed assay for BCR-ABL, including reverse transcription, nested PCR and strip-based detection, was estimated as follows. Total RNA was isolated from Philadelphia chromosome-positive cells (K562 cells) and diluted (serial 5-fold dilutions) using 1 g/l of yeast tRNA as a diluent. Solutions containing 0.1 pg to 5 x 10^6 pg total RNA from Ph1-positive cells were subjected to RT, nested PCR and dipstick assay as described in the experimental section. The results are presented in Figure 2. For comparison, 5 μl of the amplified products were electrophoresed in 2% agarose gel and detected by staining with ethidium bromide (Figure 2). We were able to detect as low as 0.1 pg of total RNA by the strip assay whereas 2.56 pg RNA were barely detectable by electrophoresis. Again, the intensity of the red line in the test zone is related to the RNA level of the sample.

The overall sensitivity and specificity of the proposed method were also assessed by spiking experiments, in which normal leukocytes were spiked with K562 cells covering a range of 0–10^6 K562 cells in a total population of 10^6 cells. Following RNA isolation and reverse transcription, a 2-μl aliquot was used for PCR I and 1 μl of the product was used for PCR II. Subsequently, a 5-μl aliquot of amplified DNA was hybridized with the specific probe (b3-a2) in a total volume of 20 and 10 μl of the mixture was applied to the strip. The results are presented in Figure 3A. It is observed that the proposed method is able to detect a single K562 cell amidst 10^6 normal leukocytes, whereas 100 K562 cells were barely detectable by agarose gel electrophoresis and ethidium bromide staining. In parallel, a 2-μl aliquot from the RT mixture was used for a single PCR of 40 cycles. The cycling conditions were exactly as described in the experimental section for the first round of nested PCR. A 5-μl aliquot of amplified DNA was then hybridized with the specific probe and half of the mixture was applied to the strip. A band in the test zone of the strip was observed for 10 K562 cells in the presence of 10^6 normal cells (Figure 3A).

The clinical evaluation of the proposed method was carried out by using 22 samples from patients with the b3-a2 transcript and 5 samples containing the b2-a2 transcript. Two patients with essential thrombocythemia were also included as negative controls. The Ph1 chromosome-positive patients were undergoing treatment. All blood samples were subjected to the preparation steps described in the experimental section, for isolation of leukocytes, purification of mRNA, cDNA synthesis, nested PCR and detection by the strip. Amplification products were also analyzed by agarose gel electrophoresis. The results are presented in Figure 3B and C. It is observed that the strip test compares well with the electrophoresis results. Sample No. 7 was found negative both by electrophoresis and the strip test. Figure 4 shows typical results of the dipstick assay for the reference gene actin.

Two major types of mRNA are formed as a result of the Philadelphia translocation. Either BCR exon 3 or BCR exon 2 is joined to ABL exon 2 to generate the b3-a2 or the b2-a2 fusion transcript, respectively. Because the two pairs of primers used in nested PCR bind to BCR exon 2 and ABL exon 2, amplification product is produced regardless of the type of the transcript. We first detect transcripts b3-a2 and b2-a2 by using probes b3-a2 and b2-a2, respectively. However, because these probes share a common sequence (specific to ABL exon 2) we observed significant cross-hybridization with the two transcripts. In order to identify, unambiguously, the type of the transcript involved, we designed a probe specific for BCR exon 3 (probe b3). The PCR products of the samples that were found positive with probes b3-a2 or b2-a2, were subjected to another dipstick assay using probe b3. Samples that contain the b3-a2 transcript give a positive result with the b3 probe, whereas samples that contain the b2-a2 transcript give no signal. Figure 4 shows typical results of the dipstick assay (probe b3) for samples containing b3-a2 or b2-a2 transcripts.

### PML-RARα and AML1-ETO

The detection of the translocation PML-RARα was based on the analysis of the cell line NB4, one patient positive for the translocation and a patient negative for the translocation. The results are shown in Figure 5. A faint red band appears in the test zone of the strip at 0.4 fmol of amplified DNA (40 pM) and the intensity increases with the amount of loaded DNA, up to 400 fmol. The corresponding electropherogram shows a faint zone at 6.4 fmol of amplified DNA. For the detection of the translocation AML1-ETO, we were based on two patients (Figure 5). A red line is clearly detectable at the 0.4 fmol target DNA whose intensity increases with the amount of target DNA, up to 100 fmol. The signal is lower at 400 fmol DNA due to competition between reannealing of target strands and probe hybridization, as described above for BCR-ABL. The detectability by electrophoresis was 6.4 fmol.
The RNA for the analysis of CBFβ-MYH11 was received from a patient who was positive for the translocation. The lowest amount of amplified DNA that is detectable by visual inspection of the strip is 1.6 fmol (160 pM) (Figure 6). The plasmids pSG5-PLZF-RARa, pSG5-NPM-RARa and pSG5-NuMA-RARa were used for the analysis of PLZF-RARa, NPM-RARa and NuMA-RARa, respectively. Results pertaining to the detectability of the strip assay are presented in Figure 6. In all three translocations, the strip could detect easily 1.6 fmol (160 pM) of amplified DNA.

Specificity of the dipstick hybridization assay

The specificity of the proposed method is based both on the PCR step and the dipstick hybridization assay. Because the primer binding sites flank the junction sequence, amplification product is generated only if the translocation is present. Furthermore, the dipstick test allows confirmation of the amplified sequence by hybridization. We performed a series of experiments in order to study the specificity of the dipstick assay. Amplification products (50 fmol) from the seven translocations were hybridized, on a one-to-one basis, with each of the probes and the mixture was applied to the strip. It is observed (see Figure 3).
Reproducibility

The reproducibility of the strip assay was assessed by assaying 6.4 and 50 fmol target DNA (PCR products) five times (Figure 8). The strips were then scanned (desktop scanner ScanJet 4300C, Hewlett Packard) and the intensity of the test zone was estimated densitometrically.

The coefficient of variation (CV) (relative standard deviation) of the intensity was 7.6 and 4.9%, respectively. To determine the overall reproducibility of the test, three samples containing 12.8 pg, 1.6 × 10^3 pg and 10^6 pg RNA from Ph1 cells were subjected, in quadruplicate, to reverse transcription, nested PCR and strip assay (Figure 8). The test zones of the strips were analyzed by scanning and densitometry as above and the CV were found to be 29, 10.7 and 8.5%, respectively. Finally, we assessed the reproducibility of the detection of 1 K562 cell in the

Figure 7) that each probe is specific for its cognate target sequence.
presence of $10^6$ normal cells. The CV after scanning and densitometry of the test zones of the strips was 22% ($n=4$).

Conclusions

In recent years, several clinical investigations have constantly demonstrated the importance of molecular tests in the management of acute and chronic leukemia patients. PCR is by far the most sensitive method for diagnosis, classification of risk groups and assessment of minimal residual disease during follow up. The detection of the type of translocation by a qualitative PCR assay is sufficient for the diagnosis and correct assignment of risk group. The gold standard for screening of these translocations is cytogenetics and PCR combined with agarose gel electrophoresis. For this reason, throughout this work, we compare the proposed dipstick assay directly to agarose gel electrophoresis. However, the evaluation of minimal residual disease is more challenging. A negative PCR result (absence of the fusion transcript in the sample) usually predicts a favorable outcome. On the other hand, if the screening test is positive (i.e. residual leukemic cells are present), this is not necessarily associated with a relapse. In this case, a quantitative PCR assay for the determination of the concentration of fusion transcripts over a period of time is a more reliable indicator of the progression of the disease (26,27). The proposed dipstick assay is not a quantitative test but rather a rapid and cost-effective visual screening test before a decision for quantification is made. Nevertheless, we have analyzed five BCR-ABL samples by real-time PCR and the dipstick assay. The strips were then scanned and the intensity of the test zone was estimated densitometrically. The real-time PCR (Light cycler) values were 3.2, 173, 224, 992 and 7400, using glucose-6-phosphate dehydrogenase (G6PD) as a reference gene. The dipstick assay gave 11, 133, 459, 394, 560, respectively. These results confirm that the dipstick assay should be used only for screening purposes.

The nested PCR-dipstick method can detect 1 K562 cell whereas the single PCR-dipstick assay detects 10 K562 cells (see Figure 3). It is well established in the literature (28–30) that the sensitivity of real-time PCR is limited to about 10 or 100 K562 cells amidst $10^6$ normal cells. Therefore, the sensitivity of the single PCR-dipstick test is similar to that of real-time PCR. Furthermore, the combination of nested PCR with the dipstick assay (although more laborious) provides a 10-fold improvement in sensitivity. This difference in sensitivity is
important for clinical purposes because recent reports (31–34) state that complete remission in CML should be defined as negativity by nested PCR for BCR-ABL due to its superior sensitivity compared to real-time PCR. In recent studies for monitoring minimal residual disease, negative real-time PCR results need to be confirmed by nested PCR (31–34).

A comparison of the single round PCR-dipstick method with the real-time PCR (two methods of similar sensitivity) in terms of cost and practicality reveals the following: (a) The dipstick method can be performed simply by using a classical PCR instrument that constitutes standard equipment of every molecular diagnosis laboratory. On the contrary, the investment cost for a real-time PCR instrument is over 10 times higher than classical PCR. (b) The cost of the probes used in real-time PCR (e.g. Taqman and FRET probes) is at least 7 times higher than the probes used in the proposed method. (c) The dry-reagent dipstick format eliminates the solution preparation steps, it is very simple to perform and minimizes the training of qualified personnel.

We estimated that the cost of the dipstick assay is less than 2 Euros. The dipstick assay offers about 10 times higher detectability than agarose gel electrophoresis and ethidium bromide staining and, contrary to electrophoresis, allows confirmation of the sequence of the PCR product by hybridization within a few minutes without the need of instrumentation. The dipstick assay requires PCR products that are biotinylated at the 5’ end (during PCR) along with a poly(dA)-tailed probe specific for the junction sequence. The dry-reagent disposable strip format greatly simplifies the assay because it eliminates the multiple incubation and washing steps performed in current hybridization assays.

ACKNOWLEDGMENTS
This work was supported by a University-Industry collaboration grant (EPAN) funded by the General Secretariat of Research and Technology (GRST) of Greece and by Medicin Hellas S.A. (Gerasas, Greece 15344). We are grateful to Dr V. Drakopoulos (FORTH/ICE-HT) for carrying out the SEM analysis of the strip. We also express our gratitude to Dr J. Licht for providing the plasmids pSG5-PLZF-RARa, pSG5-NPM-RARa and pSG5-NuMA-RARa and Dr P. Ziros (University of Patras, Medical School) for growing the plasmids. We thank Dr A. Mougiou (University of Patras, Medical School) for making available to us the real-time PCR results along with the corresponding total RNA, and Dr D. Mavrogianni (Laikon Hospital, University of Athens Medical School) for providing samples containing the b2-a2 transcript.

REFERENCES
1. Bench,A.J., Erber,W.N. and Scott,M.A. (2005) Molecular genetic analysis of haematological malignancies. I. Acute leukemias and myeloproliferative disorders. Clin. Lab. Haem., 27, 148–171.

2. Crisan,D. (2000) Molecular mechanisms in myelodysplastic syndromes and implications for evolution to acute leukemias. Clin. Lab. Med., 20, 49–69.

3. Pandolfi,P.P. (2001) Oncogenes and tumor suppressors in the molecular pathogenesis of acute promyelocytic leukemia. Hum. Mol. Genet., 10, 769–775.

4. Liu Yin,J.A. (2002) Minimal residual disease in acute myeloid leukemia. Best Pract. Res. Clin. Haematol., 15, 119–135.

5. Liu Yin,J.A. (1999) Detection of minimal residual disease in acute myeloid leukemia: methodologies, clinical and biological significance. Br. J. Haematol., 106, 578–590.

6. Burmeister,T. and Thiel,E. (2001) Molecular genetics in acute and chronic leukemias. J. Cancer Res. Clin. Oncol., 127, 80–90.

7. Falini,B. and Mason,D.Y. (2002) Proteins encoded by genes involved in chromosomal alterations in lymphoma and leukemia: clinical value of their detection by immunocytochemistry. Blood, 99, 409–426.

8. Bortolin,S. and Christopoulos,T.K. (1994) Time-resolved immunofluorometric determination of specific mRNA sequences amplified by the polymerase chain reaction. Anal. Chem., 66, 4302–4307.

9. Bortolin,S. and Christopoulos,T.K. (1995) Detection of BCR-ABL transcripts from the philadelphia translocation by hybridization in microtiter wells and time-resolved immunofluorometry. Clin. Chem., 41, 693–699.

10. Zhang,Q.Y., Garner,K. and Viswanatha,D.S. (2002) Rapid detection of leukemia-associated translocation fusion genes using a novel combined RT-PCR and flow-cytometric method. Leukemia, 16, 144–149.

11. Chen,I.M., Chakerian,A., Combs,D., Garner,K. and Viswanatha,D.S. (2004) Post-PCR multiplex fluorescent ligation detection assay and flow cytometry for rapid detection of gene-specific translocations in leukemia. Am. J. Clin. Pathol., 122, 783–793.

12. Nashed,A.L., Rao,K.W. and Galley,M.L. (2003) Clinical applications of BCR-ABL molecular testing in acute leukemia. J. Mol. Diagn., 5, 63–72.

13. Zhang,T., Xiong,H., Kan,L.-X., Zhang,C.-K., Jiao,X.-F., Fu,G., Zhang,Q.-H., Lu,L., Tong,J.-H., Gu,B.-W. et al (1999) Genomic sequence, structural organization, molecular evolution, and aberrant rearrangement of promyelocytic leukemia zinc finger gene. Proc. Natl. Acad. Sci. U.S.A., 96, 11422–11427.

14. Yin,J.A. and Frost,L. (2003) Monitoring AML1-ETO and CBFBeta-MYH11 transcripts in acute myeloid leukemia. Curr. Oncol. Rep., 5, 399–404.

15. Shigesada,K., van de Sluis,B. and Liu,P.P. (2004) Mechanism of leukemogenesis by the inv(16) chimeric gene CBFB/PEBP2B-MYH11. Oncogene, 23, 4297–4307.

16. Glynou,K., Ioannou,P.C., Christopoulos,T.K. and Syriopoulou,V. (2003) Oligonucleotide-functionalized gold nanoparticles as probes in a dry-reagent strip biosensor for DNA analysis by hybridization. Anal. Chem., 75, 4155–4160.

17. Li,J.Y., English,M.A., Ball,H.J., Yeyati,P.L., Waxman,S. and Licht,J.D. (1997) Sequence-specific DNA binding and transcriptional regulation by the promyelocytic leukemia zinc finger protein. J. Biol. Chem., 272, 22447–22455.

18. Licht,J.D., Shankovich,R., English,M.A., Melnick,A., Li,J.Y., Reddy,J.C., Dong,S., Chen,S.J., Zelent,A. and Waxman,S. (1996) Reduced and altered DNA-binding and transcriptional properties of the PLZF-retinoic acid receptor-alpha chimera generated in the t(11;17)-associated acute promyelocytic leukemia. Oncogene, 12, 723–36.

19. Grimmwade,D., Biondi,A., Mazziconacci,M.J., Hagemeijer,J., Berger,R., Neat,M., Howe,K., Bastugne,N., Jansen,J., Radford-Weiss,I. et al (2000) Characterization of acute promyelocytic leukemia cases lacking the classic t(15;17): results of the European Working Party. Blood, 99, 1297–1307.

20. Jansen,J.H. de Ridder,M.C., Geertsera,W.M.C., Erpelinc,C.A., van Lom,K., Smit,F.M.E., Slaier,R., vd Reijden,B.A., de Greef,G.E., Sonneveld,P. et al (1999) Complete remission of t(11;17): Positive acute promyelocytic leukemia induced by all-trans retinoic acid and granulocyte colony-stimulating factor. Blood, 94, 39–45.

21. Wells,R.A., Catzavelos,C. and Kamel-Reid,S. (1997) Fusion of PML-RARA, PLZF-RARA and NuMA-RARA transcripts in APL. Leukemia, 11, 11422–11427.
22. Van Dongen, J.J.M., Macintyre, E.A., Gabert, J.A., Delabesse, E., Rossi, V., Saglio, G., Gottardi, E., Rambaldi, A., Dotti, G., Griesinger, F. et al. (1999) Standardized RT-PCR analysis of fusion gene transcripts from chromosome aberrations in acute leukemia for detection of minimal residual disease. *Leukemia*, 13, 1901–1928.

23. Galvan, B. and Christopoulos, T.K. (1997) Quantitative reverse transcriptase-polymerase chain reaction for prostate-specific antigen mRNA. *Clin. Biochem.*, 30, 391–397.

24. Weller, M.G. (2000) Immunochromatographic techniques—a critical review. *Fresenius J. Anal. Chem.*, 366, 635–645.

25. Hayat, M.A. (1989) Colloidal gold: Principles, methods and applications. Academic press, CA. Academic press, CA.

26. Schnittger, S., Weisser, M., Schoch, C., Hiddemann, W., Haferlach, T. and Kern, W. (2003) New score predicting for prognosis in PML-RARA+, AML1-ETO+, or CBFB-MYH11+ acute myeloid leukemia based on quantification of fusion transcripts. *Blood*, 102, 2746–2755.

27. Bortolin, S. and Christopoulos, T.K. (1996) Quantitative RT-PCR combined with time-resolved fluorometry for determination of BCR-ABL mRNA. *Clin. Chim. Acta*, 42, 1924–1929.

28. Schüller, F. and Dölken, G. (2006) Detection and monitoring of minimal residual disease by quantitative real-time PCR. *Clin. Chim. Acta*, 363, 147–156.

29. Press, R.D., Love, Z., Tronnes, A.A., Yang, R., Tran, T., Mongou-Tchokote, S., Mori, M., Mauro, M.J., Deininger, M.W. and Druker, B.J. (2006) BCR-ABL mRNA levels at and after the time of a complete cytogenetic response (CCR) predict the duration of CCR in imatinib mesylate-treated patients with CML. *Blood*, 107, 4250–4256.

30. Neumann, F., Herold, C., Hildebrandt, B., Kobbe, G., Aivado, M., Rong, A., Free, M., Rössig, R., Fenk, R., Schneider, P. et al. (2003) Quantitative real-time reverse-transcription polymerase chain reaction for diagnosis of BCR-ABL positive leukemias and molecular monitoring following allogeneic stem cell transplantation. *Eur. J. Haematol.*, 70, 1–10.

31. Elmaagacli, A.H., Beelen, D.W., Opalka, B., Seebker, S. and Schaefer, U.W. (2000) The amount of BCR-ABL fusion transcripts detected by the real-time quantitative polymerase chain reaction method in patients with Philadelphia chromosome positive chronic myeloid leukemia correlates with the disease stage. *Ann. Hematol.*, 79, 424–431.

32. Deininger, M.W. (2005) Management of early stage disease. *Hematology*, I, 174–182.

33. Thomas, D.A., Faderl, S., Cortes, J., O’Brien, S., Giles, F.J., Kornblau, S.M., Garcia-Manero, G., Keating, M.J., Andreeff, M. and Jeha, S. (2004) Treatment of Philadelphia chromosome-positive acute lymphocytic leukemia with hyper-CVAD and imatinib mesylate. *Blood*, 103, 4396–4407.

34. Guo, J.Q., Lin, H., Kantarjian, H., Talpaz, M., Champlin, R., Andreeff, M., Glassman, A. and Arlinhaus, R.B. (2002) Comparison of competitive-nested PCR and real-time PCR in detecting BCR-ABL fusion transcripts in chronic myeloid leukemia patients. *Leukemia*, 16, 2447–2453.