Detection of BCR-ABL Transcripts from the Philadelphia Translocation by Hybridization in Microtiter Wells and Time-Resolved Immunofluorometry

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Two hybridization assays have been developed to detect BCR-ABL mRNA transcripts arising from the Philadelphia translocation. Both assays use time-resolved immunofluorometric detection of polymerase chain reaction-amplified BCR-ABL mRNA sequences hybridized to specific probes. In configuration I, biotinylated amplified target is immobilized onto streptavidin-coated wells and hybridized to a probe labeled with the hapten digoxigenin. Hybrids are detected via an alkaline phosphatase-labeled antibody and fluorosalicylylphosphate as substrate. The fluorosalicylate produced forms highly fluorescent complexes with Tb^{3+}-EDTA. In configuration II, biotinylated probe is immobilized onto streptavidin-coated wells. PCR, performed in the presence of hapten-labeled deoxyribonucleotide, generates labeled product, which is hybridized to immobilized probe and quantified as above. BCR-ABL transcripts from one leukemic cell amidst mRNA from 500 000 normal granulocytes are detectable with signal/background ratios as high as 36.4 and 24.6 for configurations I and II, respectively. The respective CVs for the assays were 6.6–9.0% and 5.1–12.5%.

Indexing Terms: *leukemia/polymerase chain reaction/biotin–streptavidin interaction*

The Philadelphia translocation was first described in 1960 by Nowell and Hungerford, who, while examining cells from patients with chronic granulocytic leukemia, noticed the presence of "a minute chromosome" (*1*). In 1973, Rowley demonstrated that the "minute chromosome" was actually chromosome 22 with a shortened long (q) arm (*2*). Not until the mid 1980s, though, was the Philadelphia (Ph^1) chromosome specifically identified as the product of a reciprocal translocation involving most of the *ABL* protooncogene from chromosome 9 and a portion of the *BCR* gene from chromosome 22 [t(9;22)(q34;q11)] (*3–5*).[2] Thus, it took more than 20 years for a simple observation to flower into one of the best-understood and best-documented cases of a DNA abnormality being associated with malignancy.

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[2] Nonstandard abbreviations: CML, chronic myelogenous leukemia; PCR, polymerase chain reaction; SA, streptavidin; dNTP, deoxyribonucleoside triphosphate; Ph^1, Philadelphia chromosome; FBS, fetal bovine serum; RNase, ribonuclease; NP-40, Nonidet P-40; DIG, digoxigenin; DEPC, diethylpyrocarbonate; DMSO, dimethyl sulfoxide; FSAP, phosphate ester of 5′-fluorosalicylate; PBS, phosphate-buffered saline; DTT, dithiothreitol; and NHS-LC-Biotin, sulfosuccinimidyl 6-(biotinamido)hexanoate.

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Chronic myelogenous leukemia (CML), a myeloproliferative disorder arising from the neoplastic transformation of a pluripotent hematopoietic stem cell, is characterized at the molecular level by the presence of Ph^1: >95% of CML patients display the *BCR-ABL* translocation (*6, 7*). The breakpoint in chromosome 22 occurs consistently within a 5.8-kb region termed the breakpoint cluster region, whereas in chromosome 9 the breakpoint may occur at different sites within a >100-kb intron preceding *ABL* exon 2 (*3–5*). Because the breakpoints in the *BCR* and *ABL* genes may occur over a wide range of bases, several fused *BCR-ABL* combinations are possible at the DNA level. Transcription, however, consistently results in either of two possible chimeric 8.5-kb CML-specific mRNA transcripts, depending on whether *ABL* exon 2 fuses to *BCR* exon 2 (b_2a_2 transcript) or *BCR* exon 3 (b_3a_2 transcript) (*6, 8*).

Translation of these mRNA transcripts produces a 210-kDa protein, p210, which possesses increased tyrosine kinase activity relative to the 145-kDa normal ABL protein (*9, 10*). It is the presence of p210 that has been associated with the pathogenesis of CML (*11, 12*). The exact mechanism remains unknown.

Currently, diagnosis of CML relies on either cytogenetic analysis of bone marrow aspirates or the detection of specific *BCR* rearrangements by use of restriction enzymes, Southern transfers, and hybridization with specific ^{32}P-labeled DNA probes (*12, 13*). For cytogenetic studies to be of value, a high proportion of cells (~5%) must be positive for Ph^1. Detection via *BCR* rearrangement assays requires that 1–5% of the cells be positive for Ph^1 (*12, 13*). However, more-sensitive methods are required for monitoring patients undergoing treatment and for early detection of relapse. The use of the polymerase chain reaction (PCR) (*14, 15*) to amplify the CML-specific mRNA sequences, followed by Southern transfer and hybridization with ^{32}P-labeled probes, dramatically improved sensitivity such that one Ph^1-positive cell could be detected in the presence of 10^6 normal cells (*16*).

Time-resolved fluorometry with lanthanide chelates is already recognized as one of the most sensitive techniques used with immunoassays (*17*). Enzymatically amplified time-resolved fluorometric immunoassays with even higher sensitivities have been introduced (*18, 19*). In the present work, two hybridization assays that involve time-resolved immunofluorometry have been designed and applied to the detection of PCR-amplified CML-specific mRNA. In the first assay (configuration I), PCR is performed in the presence of a biotinylated primer so that the amplified product is

end-labeled with biotin. The product is then captured on streptavidin (SA)-coated microtiter wells and one strand is removed by treatment with NaOH. Subsequently, the single-stranded target remaining is hybridized with an oligonucleotide probe multiply-labeled with digoxigenin (DIG). The hybrids formed are detected with an alkaline phosphatase-labeled anti-DIG antibody, with the phosphate ester of 5'-fluorosalicylic acid used as the enzyme's substrate. The 5'-fluorosalicylate produced forms highly fluorescent ternary complexes with Tb^{3+}-EDTA in an alkaline solution. In the second assay (configuration II), the probe is first biotinylated at the 5'-end and then captured on SA-coated microtiter wells. The hapten is incorporated in the PCR product by performing the amplification in the presence of DIG-dUTP. The labeled product is then denatured and hybridized with the immobilized probe. The hybrids are detected as above.

Here we report our investigations of the characteristics of both assays and compare their performances. Although both methods were developed by using the Ph^1 translocation as a model, they may easily be adapted to other diseases for which a specific abnormality has been characterized at the molecular level.

Materials and Methods

Materials

The K562 cell line, which is positive for the b_3a_2 transcript from the Philadelphia chromosome, was obtained from the American Type Culture Collection (Rockville, MD) ATCC CCL 243. The polystyrene culture flasks (25 cm^2) were from Corning (Corning, NY). Fetal bovine serum (FBS), RPMI 1640 with L-glutamine, penicillin, and streptomycin used for cell culture were from Gibco Labs. Life Technologies (Gaithersburg, MD), as were Moloney murine leukemia virus reverse transcriptase and Polymorphoprep.

Deoxyribonucleoside triphosphates (dNTPs) and Sephadex G-25 columns (Nap-5) were from Pharmacia LKB (Montreal, Canada). The polystyrene magnetic beads (2.8 μm in diameter) coated with oligo$(dT)_{25}$ and the magnetic particle concentrator (MPC-E) were from Dynal (Great Neck, NY). Human placental ribonuclease (RNase) inhibitor, Nonidet P-40 (NP-40), blocking reagent (cat. no. 1096176), DIG-dUTP, terminal deoxynucleotidyl transferase, and the alkaline phosphatase-labeled polyclonal anti-DIG antibody from sheep (Fab fragments) were obtained from Boehringer Mannheim (Laval, Canada). The long-chain derivative of the *N*-hydroxysuccinimide ester of biotin [sulfosuccinimidyl 6-(biotinamido)hexanoate, or NHS-LC-Biotin] was from Pierce (Rockford, IL). Diethylpyrocarbonate (DEPC), dimethyl sulfoxide (DMSO), Tween 20, mineral oil, and SA were from Sigma (St. Louis, MO). Terbium chloride hexahydrate was obtained from Aldrich Chemical Co. (Milwaukee, WI). The phosphate ester of 5'-fluorosalicylic acid (FSAP) was from CyberFluor (Toronto, Canada); a 0.01 mol/L stock solution of FSAP was prepared by dissolving 11.8 mg of the compound in 0.01 mol/L NaOH and was kept refrigerated until use. Polystyrene Microlite 2 microtiter wells were from Dynatech Labs. (Chantilly, VA). Tris, NaCl, and all other general laboratory chemicals used in the preparation of buffers were from BDH (Toronto, Canada).

All oligonucleotides used in this work were synthesized by Biosynthesis (Lewisville, TX). A summary of their sequences, characteristics, and use is given in Table 1.

mRNA Isolation and cDNA Synthesis

mRNA was isolated from K562 cells by using magnetic polystyrene beads with covalently attached oligo$(dT)_{25}$ tails (*20*). K562 cells (10^6), grown in suspension in 25-cm^2 polystyrene culture flasks in media containing, per liter, 100 mL of FBS, 900 mL of RPMI 1640 with L-glutamine, 50 kIU of penicillin, and 50 mg of streptomycin, were washed three times with 400 μL of phosphate-buffered saline (PBS; 0.14 mol/L NaCl, 2.7 mmol/L KCl, 10 mmol/L sodium phosphate, 1.76 mmol/L potassium phosphate, pH 7.4). The cells were pelleted at 12 000*g* and then lysed on ice for 1 min in the presence of 28 units of RNase inhibitor in 100 μL of lysis buffer [per liter, 0.14 mol of NaCl, 1.5 mmol of $MgCl_2$, 10 mmol of Tris, pH 8.6, 5 mL of NP-40, and 1 mmol of dithiothreitol (DTT)]. The lysate was then centrifuged at 12 000*g* for 2 min to remove nuclei and cell debris. The supernatant was transferred to a

Table 1. Summary of oligonucleotides used as assay probes and PCR primers.

Oligo	Sequence	Size	Characteristics	Use
A	5'-GCTGAAGGGCTTTTGAACTCTGCTTA-3'[a]	26mer	Complementary to BCR-ABL mRNA (b_3a_2 transcript)	DIG-dUTP-tailed detection probe for assay I
B	5'-NH_2-GCTGAAGGGCTTTTGAACTCTGCTTA-3'[a]	26mer	Complementary to BCR-ABL mRNA (b_3a_2 transcript)	Labeled with biotin and used as capture probe for assay II
C	5'-TTTCAGAAGCTTCTCCCTGAC-3'	21mer	Homologous to *BCR* exon 2	5' primer for PCR 1
D	5'-CAGTGCAACGAAAAGGTTGGGGTC-3'	24mer	Complementary to *ABL* exon 2	3' primer for PCR 1
E	5'-NH_2-GGAGCTGCAGATGCTGACCAAC-3'[a]	22mer	Homologous to *BCR* exon 2	Labeled with biotin and used as 5' PCR 2 primer for assay I
F	5'-GGAGCTGCAGATGCTGACCAAC-3'[a]	22mer	Homologous to *BCR* exon 2	5' PCR 2 primer for assay II
G	5'-TCAGACCCTGAGGCTCAAAGTC-3'[a]	22mer	Complementary to *ABL* exon 2	3' primer for PCR 2

[a] Sequences as in ref. *16*.

microcentrifuge tube containing 200 μg of the magnetic beads suspended in 100 μL of binding buffer (1 mol/L LiCl, 20 mmol/L Tris, pH 7.5, 2 mmol/L EDTA, 4 g/L sodium dodecyl sulfate) and was incubated at room temperature for 5 min. Before addition of the cell lysate, the magnetic beads were conditioned by washing twice with 400 μL of PBS and once with 400 μL of binding buffer. After a 5-min incubation, in which the mRNA was allowed to hybridize with the oligo$(dT)_{25}$ tails, the beads were separated from solution with use of the MPC-E magnet. The supernatant was removed and the beads were washed at least three times with 400 μL of washing buffer (0.15 mol/L LiCl, 10 mmol/L Tris, pH 7.5, 2 mmol/L EDTA) to ensure that all other cellular RNA and protein had been removed. Lastly, to elute the mRNA from the solid phase, we resuspended the beads in 10 μL of 2 mmol/L EDTA and incubated at 65°C for 2 min. The beads were then separated from solution and the eluted mRNA was quickly added to 10 μL of reverse transcription reaction mixture and incubated for 1 h at 37°C. This reaction mixture consisted of 100 mmol/L Tris, pH 8.3, 150 mmol/L KCl, 6 mmol/L $MgCl_2$, 400 U of reverse transcriptase, 0.02 mol/L DTT, 2 mmol/L of each dNTP, 28 units of RNase inhibitor, and 1 μmol/L PCR 1–3′ primer (oligo D). After the reaction was complete, the tubes containing the mRNA–cDNA hybrids were heated to 95°C for 5 min to inactivate the reverse transcriptase.

For isolation of mRNA from normal cells, whole blood was collected into evacuated tubes containing EDTA. Polymorphonuclear granulocytes were then isolated by using Polymorphoprep according to the instructions in the product insert. The principle of density gradient centrifugation allows for separation of monocytes from granulocytes, with the erythrocytes being completely sedimented. Once isolated, the granulocytes were washed several times with PBS to remove all traces of the density gradient medium. The remainder of the mRNA extraction procedure was exactly as described for the K562 cells.

All solutions used in the isolation of mRNA and cDNA synthesis were prepared with DEPC-treated water to eliminate RNase activity. Furthermore, all pipets, pipet tips, mineral oil, and solutions were irradiated with ultraviolet light in a laminar flowhood for at least 1 h to destroy any contaminating DNA carryover from previous PCR experiments. Solutions that were irradiated were treated in glass containers, given reports that ultraviolet irradiation of solutions in polystyrene containers reduces the efficiency of PCR (*21*). Finally, a "no mRNA template" (negative control) was also included for each reverse transcription-PCR so that contamination could be monitored.

Oligonucleotides

Labeling oligonucleotides B and E with biotin. Oligo B, which was used as a probe in assay configuration II, and oligo E, used as a PCR primer in assay configuration I, were labeled with a 500:1 molar excess of NHS-LC-biotin at pH 9.1. We used the long-chain derivative of NHS-biotin to minimize steric hindrance in the subsequent binding of the biotinylated oligonucleotide to streptavidin (*22*). After adding 20 μL of 0.5 mol/L carbonate buffer, pH 9.1, to 100 μL of 0.1 mmol/L oligonucleotide solution, we added 120 μL of a 45 mmol/L solution of NHS-LC-biotin in an equivolume solution of DMSO and H_2O. The reaction was allowed to proceed for 2 h at room temperature. The labeled oligonucleotide was then diluted to 500 μL with 10 mmol/L sodium phosphate buffer, pH 6.8, and purified by size-exclusion chromatography on Sephadex G-25 disposable columns (Nap-5) according to the manufacturer's instructions. Three purifications were done, each time with a new column that had been equilibrated with 10 mL of the sodium phosphate buffer described above. The final concentration of the purified oligonucleotide was 3.3 μmol/L.

Tailing oligonucleotide A with DIG. Oligo A, which was used as a probe in assay configuration I, was tailed with DIG-dUTP/dATP by using terminal transferase (*23*). The tailing reaction was performed in a final volume of 20 μL of the following: 0.2 mol/L potassium cacodylate, 250 mmol/L Tris (pH 6.6), 0.25 g/L bovine serum albumin, 5 mmol/L $CoCl_2$, 0.05 mmol/L DIG-dUTP, 0.5 mmol/L dATP, 50 U of terminal transferase, and 5 μmol/L oligo A. The reaction mixture was then incubated at 37°C for 30 min. Purification was not required.

Polymerase Chain Reaction

PCR 1 was carried out in a total reaction volume of 100 μL, consisting of 50 mmol/L KCl, 10 mmol/L Tris (pH 9.0), 1 mL/L Triton X-100, 2.5 mmol/L $MgCl_2$, 0.5 μmol/L each of the 5′ and 3′ PCR 1 primers (oligos C and D), and 40 μmol/L of each dNTP. The sample volume added was 10 μL. About 100 μL of mineral oil was layered over each reaction mixture to prevent evaporation. PCR was initiated with the "hot start" method (*24*), and the 48-well Perkin-Elmer Cetus (Norwalk, CT) DNA Thermal Cycler was used. Reproducibility of identical samples was not affected by their position within the heating block. The PCR mixtures were first heated to 95°C for 5 min, during which time 2.5 U of *Thermus aquaticus* DNA polymerase (Taq polymerase) was added to each tube. PCR 1 was carried out for 25 cycles, each cycle being denaturation at 95°C for 30 s, primer annealing at 60°C for 30 s, and primer extension at 72°C for 1 min. Finally, the reaction mixtures were held at 72°C for 10 min to allow for product reannealing and were then cooled to 4°C. PCR 1 products were then diluted 50-fold in water, and 5 μL of the dilution was added to each PCR 2 reaction mixture for reamplification.

PCR 2 was performed in a final volume of 100 μL with all reagents and conditions identical to PCR 1 except that oligo G was used as the 3′ PCR 2 primer in both configurations; biotinylated oligo E and unlabeled oligo F were used as the 5′ PCR 2 primers in assay configurations I and II, respectively; and 30 μmol/L dTTP and 10 μmol/L DIG-dUTP were used in configu-

ration II instead of 40 μmol/L dTTP. PCR 2 was run for 30 cycles in configuration I and 25 cycles in configuration II.

To summarize, PCR 1 is identical for both configurations, whereas PCR 2 is designed to produce either a biotinylated amplified product (for assay configuration I) or a DIG-labeled product (for configuration II). The total time required for both PCR 1 and 2 is ~4 h.

Hybridization Assays

Configuration I: hybridization of DIG-tailed probe with immobilized target. Opaque polystyrene microtiter wells, which had been coated overnight with 100 μL of a 1.4 mg/L solution of SA in PBS, were washed three times with a solution of 50 mmol/L Tris, 0.15 mol/L NaCl, and 1 mL/L Tween 20, pH 7.5. PCR 2 product carrying biotin at its 5′-end was then diluted 10-fold in PBS containing 1 mL/L Tween 20, and 100 μL of this was pipetted into each well in duplicate. The microtiter wells were shaken in the Amersham Amerlite Shaker/Incubator (Oakville, Canada) at room temperature for 30 min and were then washed three times. Afterwards, 100 μL of a 0.2 mol/L NaOH solution was added, and the samples were incubated for 20 min with shaking at room temperature. The wells were then washed three times. Subsequently, 100 μL of 7.1 nmol/L DIG-tailed probe (oligo A) in blocking solution (10 g/L blocking reagent, 0.1 mol/L maleic acid, and 0.15 mol/L NaCl, pH 7.5) were pipetted into each well. Hybridization was carried out for 10 min at 42°C with shaking, after which time the wells were washed three times and 100 μL of the 750 U/L alkaline phosphatase-conjugated anti-DIG antibody diluted in wash buffer was added. After a 30-min incubation at room temperature, the wells were washed three times and 100 μL of the substrate (1 mmol/L FSAP, 1 mmol/L $MgCl_2$, 0.1 mol/L NaCl, and 0.1 mol/L Tris, pH 9.1) was added. After reaction for 30 min at room temperature, 100 μL of developing solution (0.4 mol/L NaOH, 2 mmol/L Tb^{3+}, 3 mmol/L EDTA, and 1 mol/L Tris) was added to each well and incubated for 1 min. The fluorescence produced was measured with the CyberFluor (Toronto, Canada) 615 Immunoanalyzer, a time-resolved fluorometer, at an excitation wavelength of 337 nm and an emission wavelength of 615 nm.

Configuration II: hybridization of DIG-labeled target with immobilized probe. After the SA-coated wells were washed three times with wash buffer, 100 μL of a 0.33 μmol/L biotinylated probe solution in blocking solution was added to each well and incubated for 30 min at room temperature. The wells were then washed three times, and 90 μL of blocking solution, preheated at 42°C, was pipetted into each well. PCR 2 products generated for this configuration were denatured by heating at 95°C for 5 min and immediately placed on ice. We then added in duplicate 10 μL of the PCR product to the 90 μL of blocking solution already present in the well. Hybridization proceeded for 30 min at 42°C, after which time we again washed the wells three times. The addition of the alkaline phosphatase-labeled anti-DIG antibody and FSAP substrate and the measurement of the fluorescence produced were exactly as described for configuration I above. Total assay time for both configurations I and II, not including PCR, was 2 h.

Results and Discussion

Assay configurations I (immobilized target) and II (immobilized probe) are illustrated in Fig. 1. To determine the maximum fluorescence achievable, we investigated diluting the PCR 2 product before adding it to the SA-coated wells. For configuration I (Fig. 2A), fluorescence increased as the amount of product added increased (i.e., at lower dilutions). We chose as optimum a 10-fold dilution of the PCR 2 product, which corresponds to 10 μL of the original PCR 2 mixture per well. Addition of higher quantities of product per well did not result in significant improvements of the signal because of saturating the SA binding sites.

For configuration II, the signal also increased with the amount of PCR 2 product added (Fig. 2B). Again, a 10-fold dilution was chosen as the optimum. At fivefold dilution, the fluorescence continued to increase, but the signals corresponding to 10 and 1000 cells became indistinguishable. We attribute this to the fact that, at higher target concentrations (greater number of cells combined with a low dilution of PCR product), competition arises between target reannealing and hybridization of the denatured target to immobilized probe.

Next, the time required for the hybridization reaction to occur at 42°C in both assays was studied (Fig. 3). In assay configuration I, a 10-min hybridization period generated the greatest signal-to-background ratio, whereas assay II required 30 min to achieve the best ratio. With longer incubation periods, the signal-to-background ratio decreased because of increased nonspecific binding of the labeled reactants to the solid phase. The reasoning behind the shorter hybridization time required for assay I is that the NaOH treatment

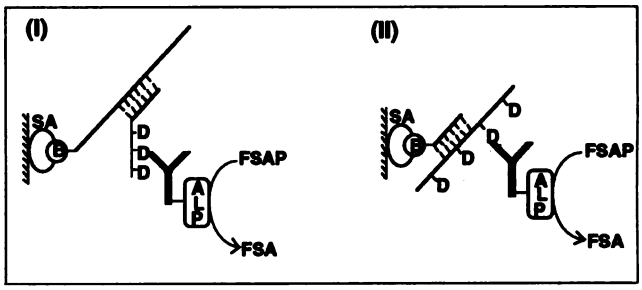

Fig. 1. Schematic diagrams of the proposed hybridization assays. *Configuration I* (immobilized target): Amplified product carrying biotin (B) at its 5′-end is first immobilized onto SA-coated microtiter wells. NaOH is used to wash away the complementary strand. A probe carrying multiple DIG (D) molecules at its 3′-end is then hybridized to the immobilized product. The hybrids are detected with an anti-DIG–alkaline phosphatase conjugate. Alkaline phosphatase hydrolyzes FSAP and the fluorosalicylate (FSA) produced forms fluorescent complexes with Tb^{3+}-EDTA. *Configuration II* (immobilized probe): A biotinylated probe is first immobilized onto SA-coated microtiter wells. Amplified product carrying multiple DIG molecules is denatured and hybridized to the immobilized probe. The addition of the anti-DIG–ALP conjugate and FSAP is the same as in *I*.

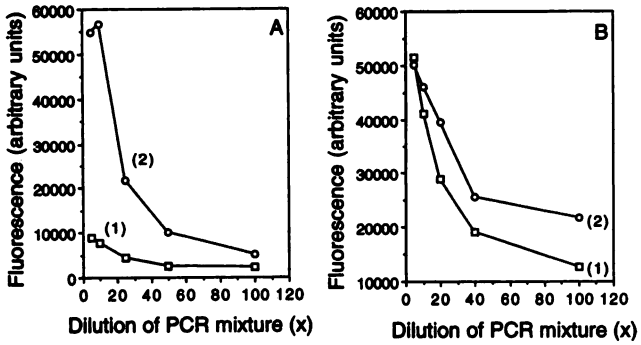

Fig. 2. Optimization of the dilution of (*A*) the biotinylated PCR 2 product used as target in assay configuration I and (*B*) the multiply-labeled PCR 2 product used as target in assay configuration II.

Lines 1 and 2 represent the PCR 2 product from mRNA corresponding to 100 and 10 000 K562 cells, respectively (*A*), or corresponding to 10 and 1000 K562 cells, respectively (*B*).

removes one strand of the immobilized target so that the probe hybridizes with a single-stranded target, whereas in configuration II there is competition between probe and complementary strand. NaOH treatment did not remove SA from the solid phase nor did it disrupt the biotin–SA interaction.

Figure 4A shows the effect of the concentration of the DIG-tailed probe on the signal-to-background ratios observed. Investigation of concentrations in the range of 0.4 to 14 nmol/L showed that the signal-to-background ratios were constant in the range of 1 to 10 nmol/L. We decided upon a final DIG-tailed probe concentration of 7.1 nmol/L and used this for further investigation for configuration I. Also, we conducted experiments to determine the optimal concentration of alkaline phosphatase-labeled anti-DIG antibody within the range 100–7500 U/L (Fig. 4B). The signal increased with the antibody concentration, but at high concentrations the signal-to-background ratio decreased because of the higher nonspecific binding of the labeled antibody to the solid phase. A final antibody concentration of 750 U/L was ultimately chosen as the optimum for both assays I and II.

To assess the reproducibility of both hybridization

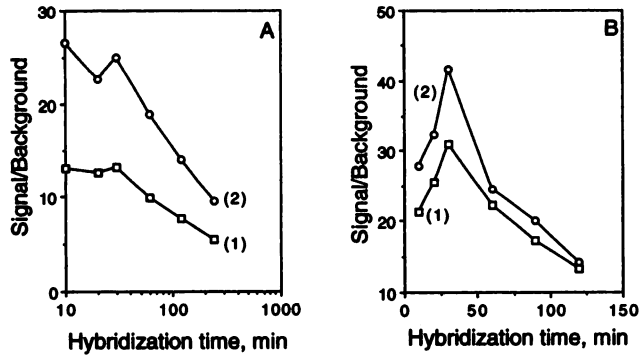

Fig. 3. Optimization of hybridization time for assay configurations I (*A*) and II (*B*).

The signal-to-background ratio is the ratio of fluorescence obtained in the presence and absence of target DNA (PCR 2 product). *Lines 1 and 2* represent 10-fold dilutions of PCR 2 product from mRNA corresponding to 100 and 10 000 leukemic cells, respectively (*A*) or corresponding to 10 and 1000 leukemic cells, respectively (*B*).

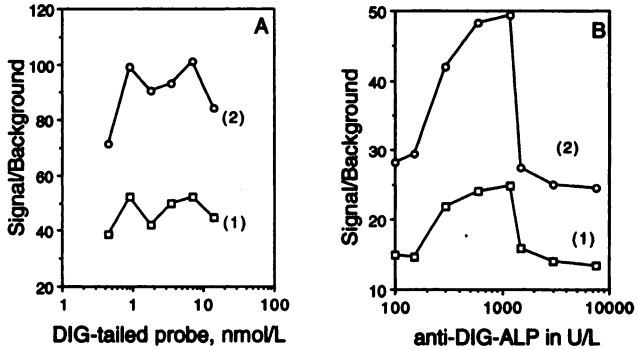

Fig. 4. Studies: (*A*) concentration of DIG-tailed probe used for hybridization assay I; (*B*) concentration of alkaline phosphatase-labeled anti-DIG antibody.

(*A*) The diluted probe was added to a 10-fold-diluted PCR 2 product immobilized on the SA-coated solid phase. (*B*) Optimization was carried out with a 10-fold-diluted biotinylated PCR 2 product hybridized to 0.71 pmol of DIG-tailed probe for 10 min at 42°C. Signal-to-background ratios as in Fig. 3. For both (*A*) and (*B*), PCR 2 product representative of mRNA corresponding to 100 (*line 1*) and 10 000 (*line 2*) leukemic cells was used.

assays, we determined CVs for PCR 2 products generated from mRNA representative of 1, 10, and 1000 K562 cells in the presence of 500 000 normal cells. For nine replicates, configuration I produced CVs of 6.6%, 9.0%, and 7.7%, and configuration II gave CVs of 7.6%, 12.5%, and 5.1% for 1, 10, and 1000 K562 cells, respectively.

To assess the sensitivity and specificity of the proposed assays, we analyzed mixtures of mRNA from Ph^1-positive cells (K562 cells) and normal granulocytes. In Fig. 5, the signal-to-background ratio plotted vs the BCR-ABL mRNA corresponding to various numbers of K562 cells in the presence of 500 000 normal granulocytes demonstrates that one K562 cell is easily detectable, with signal-to-background ratios of 36.4 and 24.6 for assay configurations I and II, respectively. Thus, both assays offer at least 10-fold greater sensitivity (lower detection limit) than a recently reported assay for amplified mRNA that does not involve hybridization but rather detects PCR products double-

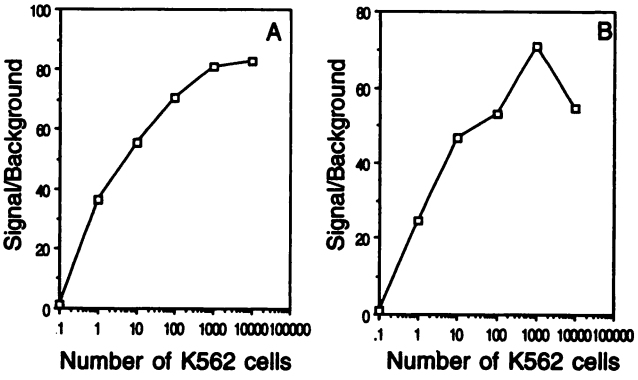

Fig. 5. PCR 2 products representative of mRNA from 0.1 to 10 000 K562 cells in the presence of mRNA from 500 000 normal granulocytes, as analyzed with (*A*) assay configuration I (immobilized PCR 2 product) or (*B*) assay configuration II (immobilized probe).

In assays I and II, mRNA from one leukemic cell was easily detected, with signal-to-background ratios of 36.4 and 24.6, respectively. The background is defined as the fluorescence obtained in the presence of 500 000 normal cells with no K562 cells present.

labeled with biotin and DIG (*25*). Because only one-tenth of the original PCR mixture was applied to each well, the signal represents amplified mRNA sequences from less than one cell.

The quantitative nature of the proposed assays is evident from the fact that the signal increases with the number of K562 cells. The curve for configuration I (Fig. 5A) begins to plateau, indicating that the solid phase has become saturated with the product. In configuration II, however (Fig. 5B), we observe that at the high end, the signal-to-background ratio drops, most probably because of a displacement phenomenon in which the hybridized target is being drawn back to its complementary strand because of the greater stability imposed by a greater number of hydrogen bonds.

Comparing the two assay configurations, we find that assay configuration I is advantageous in terms of a shorter hybridization time. Configuration II seems to lend itself better to simultaneous detection of various DNA abnormalities; e.g., microtiter wells could be commercially prepared with various probes already immobilized, thus requiring only the addition of PCR product. In configuration I, simultaneous detection would first require immobilization of the target sequence followed by the addition of various labeled probes. The use of SA-coated wells offers the additional advantage of a universal solid phase that may be used for a variety of biotinylated probes.

Several factors contribute to the success of both assay configurations. The first is that only diagnostically significant mRNA is amplified by PCR because reverse transcription using a specific 3′ primer (oligo D) complementary to *ABL* exon 2 limits the types of cDNA that are produced. The use of nested PCR further minimizes nonspecific amplifications, so that a highly enriched PCR 2 product results. Previous experiments have shown (*25*) that, when only one round of PCR is performed, some nonspecific fragments are present at 400 bp, along with the specific product. Moreover, the yield of PCR decreases as the number of normal cells present in the mixture increases. This is attributed to the fact that the PCR primers, unavoidably, anneal to the normal ABL mRNAs (which initially are present in excess), as well as to the BCR-ABL chimeric transcripts. In contrast, when amplifying nucleic acid sequences from, e.g., infectious agents, the primers are directed specifically to those sequences and are not related to the host DNA or RNA.

To achieve the lowest detection limit, we performed nested PCR, which does not significantly compromise the assay's practicality. To justify the use of nested PCR, we performed experiments in which mRNA corresponding to 100 and 1000 leukemic cells in the presence of mRNA from 500 000 normal cells was amplified with a single PCR of 30 cycles and assayed by the configuration I assay. The signal-to-background ratios obtained were 1.4 and 9.5 for 100 and 1000 leukemic cells, respectively, compared with a ratio of 36.4 obtained for mRNA representative of only 1 leukemic cell and amplified with nested PCR.

The present work introduces, for the first time, the enzymatically amplified time-resolved fluorometry to microtiter well-based hybridization assays. Although the number of BCR-ABL mRNA copies per K562 cell is not known, the sensitivity and specificity achieved are high and make the proposed assays suitable for early detection of relapse. Furthermore, hybridization within microtiter wells greatly reduces turnaround times by eliminating time-consuming, labor-intensive procedures such as electrophoresis, Southern transfer, and membrane hybridization with radioactive probes. Thus, automated analyzers already in use for immunoassays may also be used for the detection of DNA abnormalities. Besides achieving greater sensitivity, the assays presented here are advantageous over our previously reported assay (*25*) in that hybridization with specific probes allows for confirmation of expression of the BCR-ABL mRNA transcript (b_3a_2 in this case). Furthermore, the current work represents a generalized method that may easily be adapted for the detection of point mutations, deletions, translocations, and other genetic aberrations known to cause a specific disease. Clinical evaluation of the proposed assays is currently in progress.

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