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Multiplex PCR of *bcr-abl* Fusion Transcripts in Philadelphia Positive Acute Lymphoblastic Leukemia

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We describe a multiplex PCR assay for the detection of *bcr-abl* fusion mRNA in Philadelphia chromosome positive acute lymphoblastic leukemia (Ph+ ALL). The assay provides a quick method for screening p190 (e1:a2) and p210 (b2:a2 or b3:a2) *bcr-abl* mRNAs simultaneously. The assay proves to be highly sensitive with detection of as little as one positive *bcr-abl*-expressing cell in a background of 10^5 negative *bcr-abl* cells. Bone marrow and peripheral blood specimens from six patients were in total accordance when run by multiplex PCR and by the single primer PCR approach. The multiplex *bcr-abl* assay may prove to be highly useful for screening newly diagnosed patients with ALL for the *bcr-abl* fusion transcript and in following the course of disease during therapy.

Chronic myeloid leukemia (CML) and Philadelphia chromosome positive acute lymphoblastic leukemia (Ph+ ALL) are characterized at the molecular level by the *bcr-abl* fusion transcript, which can be detected by reverse transcription-polymerase chain reaction (RT-PCR).⁽¹⁾ In CML, the chimeric *bcr-abl* mRNA rises from the juxtaposition of *c-abl* exon 2 and either *bcr* exon 2 or exon 3 (b2:a2 or b3:a2), which code for the chimeric p210 protein. The identical molecular finding occurs in 30%–50% of patients with Ph+ ALL.⁽²⁾ The majority of Ph+ ALL patients have a more 5' *bcr* breakpoint within the first large *bcr* intron and express a fusion mRNA, e1:a2 (Fig. 1). This mRNA codes for the chimeric p190 protein. We have found that Ph+ ALL patients can uncommonly have both p210 and p190 transcripts.⁽³⁾ Furthermore, in very rare cases, CML patients have been described as possessing the e1:a2 molecular transcript.^(4,5) Therefore, multiplex PCR would be valuable in determining the types of molecular transcripts in patients through a single PCR reaction.

Bone marrow transplantation (BMT) is the only curative therapy for CML and is often the treatment of choice for Ph+ ALL.⁽⁶⁾ RT-PCR can be employed to monitor patients for the persistence of *bcr-abl* following BMT. RT-PCR of *bcr-abl* routinely detects *bcr-abl* transcripts down to a sensitivity of 10^{-5} or 10^{-6} . Follow-up of patients using this method, however, is both expensive and time-consuming. RT-PCR requires multiple steps, each step associated with a potential of contamination. The p210 and p190 *bcr-abl* mRNAs are detected in separate PCR reactions, and an additional amplification of a control gene is re-

quired to validate the integrity of target mRNA. For example, detection of the p210-type mRNAs (b2:a2 or b3:a2) is achieved in a single reaction along with an additional reaction for the amplification of a control gene, $\beta 2$ microglobulin. Similarly, the detection of *bcr-abl* in Ph+ ALL requires a separate reaction with p190-specific primers as well as a reaction for amplification of the p210 mRNA, and a $\beta 2$ microglobulin control.

To simplify this procedure we have developed a nested, multiplex RT-PCR assay in which three sets of external primers (for the detection of p190 and p210 *bcr-abl* transcripts and $\beta 2$ microglobulin) are mixed simultaneously in the initial PCR amplification and three sets of internal primer pairs are combined in a second nested amplification. In addition, by combining the reverse transcription and first-stage PCR step, we have further reduced the number of manipulations, maintained sensitivity, and minimized the risk of contamination.

MATERIALS AND METHODS

Preparation of Total RNA from Cultured Cells

Total RNA from cultured K562, HL60, and ALL-1 cells were extracted using guanidine hydrochloride. Poly(A)⁺ RNA was isolated through Northern blotting after being electrophoresed through formaldehyde denaturing gels.⁽⁷⁾

Patient Samples

Bone marrow and/or peripheral blood samples were obtained after informed

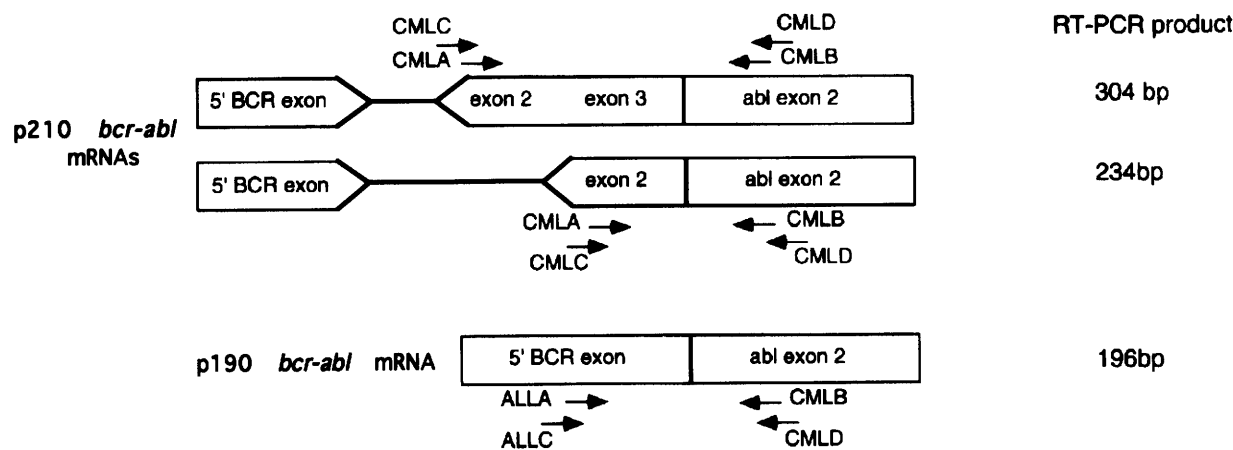


FIGURE 1 Schematic of *bcr-abl* transcript PCR amplification along with the p190, p210, and $\beta 2$ microglobulin primers. Because the same 3'-primers (*abl* primers) are used in the amplification of p190 and p210 *bcr-abl* mRNAs, CML B and CML D are written as both the 3' ALL primers as well as the 3' CML primers.

consent from six patients with CML or Ph+ ALL. These patients were enrolled in studies approved by the Internal Review Board at the Fred Hutchinson Cancer Research Center.

Preparation of Serial Dilutions

RNA dilutions were made with RNA prepared from K562 (a cell line positive for a b3:a2 p210 *bcr-abl* rearrangement), ALL-1 (a patient cell line positive for the e1:a2 p190 *bcr-abl* rearrangement), and HL60 cells (negative for any *bcr-abl* transcripts). Serial dilutions, from 10^{-1} to 10^{-6} concentrations, of both K562 and ALL-1 were combined with a background of 1 μ g of total HL60 RNA to serve as positive controls for the p210 (b3:a2) and p190 (e1:a2) *bcr-abl* mRNAs, respectively.

Preparation of Total RNA from Patient Samples

Leukocytes were separated from patient bone marrow and peripheral blood using a 3% dextran sulfate in $1\times$ phosphate-buffered saline (PBS) solution. Patient specimens were diluted with 20 ml of $1\times$ PBS and then again diluted with 20 ml of 3% dextran sulfate/ $1\times$ PBS solution. After 1 hr at room temperature, suspended leukocytes were transferred and further isolated by the use of salt lysis buffers (0.22% and 1.6% salt lysis buffers). Leukocytes were then washed twice with ice-cold PBS and lysed in a guanidinium solution. Total RNA was extracted

according to the acid guanidinium thiocyanate/phenol/chloroform method of Chomczynski and Sacchi.⁽⁸⁾ The RNA pellet was rehydrated with 40 μ l of diethylpyrocarbonate (DEPC)-treated water. Ten microliters was used directly for the coupled RT-PCR amplification step, and the remainder was stored at -80°C .

Primers

The relative positions of the oligonucleotides used in the nested, multiplex PCR of *bcr-abl*, and the size of PCR products are shown in Figure 1. Primer sequences are listed in Table 1.⁽⁹⁾

Coupled Reverse Transcription-First-round Amplification

Ten microliters of RNA was added to 40 μ l of the coupled reaction mixture. The

mixture contained the following component concentrations in a final volume of 50 μ l: 500 nM CMLD, 500 nM CML C, 170 nM ALL D, 170 nM ALL C, 250 nM 3'- $\beta 2$, 250 nM 5'- $\beta 2$, 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.1% Triton X-100, 200 μ M nucleotides, 1.5 mM MgCl₂, 4.0 units of RNasin (Promega), 6.25 units of AMVRT (Boehringer Mannheim), 1.25 units of Amplitaq polymerase (Perkin-Elmer Cetus), and DEPC-H₂O. Reaction tubes were kept on ice at all times to prevent nonspecific amplification. After mineral oil overlay, the reaction tubes were placed on the thermocycler block after the block reached 85°C . The reaction tubes were incubated for 30 min at 42°C , then 5 min at 95°C , followed by 40 cycles of 30 sec at 94°C , 1 min at 55°C , and 1 min at 72°C , followed by a final elongation of 7 min at 72°C on an Omnigene thermocycler (Hybaid Ltd).

TABLE 1 Primer Sequences

For p210 <i>bcr-abl</i> sequences	
CML A	5'-TGG AGC TGC AGA TGC TGA CCA ACT CG-3'
CML B	5'-ATC TCC ACT GGC CAC AAA ATC ATA CA-3'
CML C	5'-GAA GTG TTT CAG AAG CTT CTC C-3'
CML D	5'-TGA TTA TAG CCT AAG ACC CGG A-3'
For p190 <i>bcr-abl</i> sequences	
ALL A	5'-AGA TCT GGC CCA ACG ATG GCG AGG GC-3'
ALL B	5'-ATC TCC ACT GGC CAC AAA ATC ATA CA-3'
ALL C	5'-ACC ATC GTG GGC GTC CGC AAG A-3'
ALL D	5'-TGA TTA TAG CCT AAG ACC CGG A-3'
For $\beta 2$ microglobulin sequences	
3' $\beta 2$	5'-CCT CCA TGA TGC TGC TTA CAT GTC-3'
5' $\beta 2$	5'-ATG TCT CGC TCC GTG GCC TTA GCT-3'

Sequences of p190 and p210 were adopted from Maurer et al.⁽⁹⁾

Second-round Amplification

Five microliters of the coupled reverse transcription–first-round amplification mixture was transferred to a tube containing ingredients for a second round of amplification: 500 nM CML B, 500 nM CML A, 170 nM ALL B, 170 nM ALL A, 75 nM 3'- β 2, 75 nM 5'- β 2, 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.1% Triton X-100, 150 μ M nucleotides, 1.35 mM MgCl₂, 1.25 units of Amplitaq polymerase and DEPC-H₂O to a final volume of 50 μ l. Again, the reaction tubes were kept cold, overlaid with mineral oil, and placed on the thermocycler after the block reached 85°C. The second amplification consists of a 5-min denaturation at 95°C, 40 cycles at 94°C for 30 sec, 55°C for 1 min, 72°C for 1 min, and a final elongation at 72°C for 7 min.

Electrophoretic Analysis of PCR Products

Amplified products were run on a 2% agarose gel (GIBCO/BRL). The two p210 molecular rearrangements yield products that are 304 (b3:a2) and 234 (b2:a2) bp. The e1:a2 product is 196 bp and the β 2 PCR product is 370 bp (Figure 1).

Controls

For all nested–multiplex PCRs, four controls were used: a 10⁻⁶ positive p190 RNA control (the ALL-1 cell line), a 10⁻⁶ positive p210 RNA control (the K562 cell line), an HL60 negative control, and a water negative control. The 10⁻⁶ positive controls were made by mixing 1 pg of K562 RNA or ALL-1 RNA with 1 μ g of HL60 RNA.

RESULTS

The challenge in developing a multiplex PCR is optimizing the reaction so that all targets are amplified at a similar efficiency. Competition for common reagents (*Taq* polymerase and nucleotides) should be equalized during amplification. We found that an adjustment in primer concentrations was the most effective way to obtain similar amplification efficiencies over a broad range of target concentrations. In our optimized reaction the primer pairs for β 2, p210, and p190 are at a molar ratio of 1:2:0.7, respectively. No other adjustments of PCR reactants were necessary. Figure 2

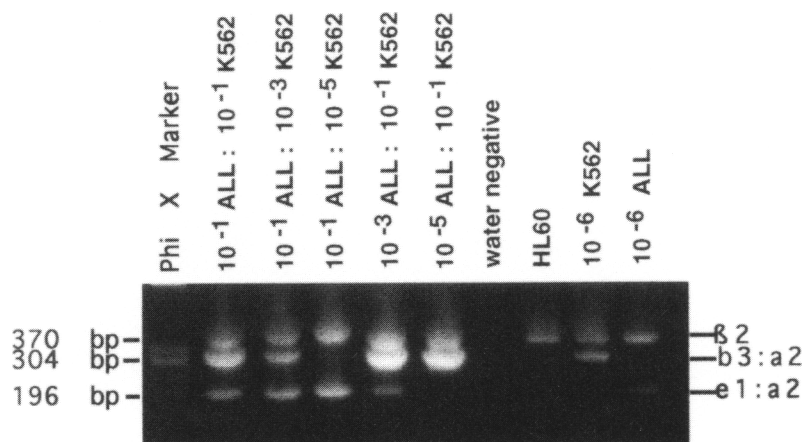


FIGURE 2 Optimization of multiplex PCR. Various primer concentrations were run on a dilution series in which varying concentrations of RNA from the K562 cell line (b3:a2 mRNA) and from the ALL-1 cell line (e1:a2 mRNA) were diluted into 1 μ g of HL60 RNA. The best equalized amplification of target RNA occurred when the molar ratio of β 2, p210, and p190 primers were 1:2:0.7, respectively. Amplification of both the p210 and p190 targets takes place when there is a two-log difference in concentration (lanes 3,5); however, at a four-log difference, the dominant target preferentially amplifies (lanes 4,6).

shows a PCR amplification of p210, p190, and β 2 gene targets using the optimized primer concentrations. Also demonstrated in Figure 2 is the fact that an equal amplification of p210 and p190 PCR products at a sensitivity of 10⁻³ is obtained when the concentration of one target RNA diminishes relative to the constant concentration of the other. However, when one target is in greater excess to another (e.g., when the concentration of the ALL-1 RNA is at a 10⁻¹ dilution compared with a 10⁻⁵ dilution of K562 RNA), the multiplex PCR favors the amplification of the target that is more abundant (Fig. 2, lanes 4,6).

Multiplex PCR was found to be sensitive enough to detect p210 and p190 targets in conditions of very low target copy number. Figure 3 shows a serial dilution of K562 and ALL-1 RNAs in a constant background of 1 μ g of HL60 RNA across a range of 10⁻¹ to 10⁻⁶ dilutions. Under the optimized primer concentrations, the p210 (b3:a2) and p190 (e1:a2) *bcr-abl* mRNAs can be detected at a sensitivity of 10⁻⁵. When a single fusion transcript is diluted in a background of 1 μ g of HL60, a sensitivity of 10⁻⁶ can be obtained (Fig. 3, lanes labeled 10⁻⁶ K562 and 10⁻⁶ ALL-1).

The accuracy of multiplex PCR was tested by comparing results of patient samples run by multiplex PCR to the same patient samples run by the single primer approach. Figure 4 shows nine

patient samples that were in accordance by both methods. In Figure 4a the results of multiplex PCR are shown, Figure 4b demonstrates the results of p190 amplification using p190 primers alone, and Figure 4c shows the results of p210 amplification using only p210-specific primers. Three of the samples were from three Ph+ ALL patients (8124, 6056, 8014), two were from a negative control Ph- ALL patient (8278), and the other four were from two CML patients (7291, 6318). CML patient 6318 has a p190 *bcr-abl* rearrangement by multiplex PCR and p190 single-primer PCR and is negative for p210 *bcr-abl* transcripts by multiplex PCR and p210 single-primer PCR.

DISCUSSION

Analysis of Ph+ ALL patients by PCR can be labor intensive because a large number of reaction tubes are needed to look for *bcr-abl* fusion transcripts. PCR screening of Ph+ ALL patients requires three sets of reaction tubes per patient specimen (β 2, p190, and p210 amplifications) because \leq 50% of adult Ph+ ALL patients harbor the p210 *bcr-abl* chimeric transcript. The knowledge of the type of *bcr-abl* transcript may have importance in prognosis and in following the patient for minimal residual disease (MRD) throughout the course of therapy. Recently we have described a trend for ALL patients with the p190 break-

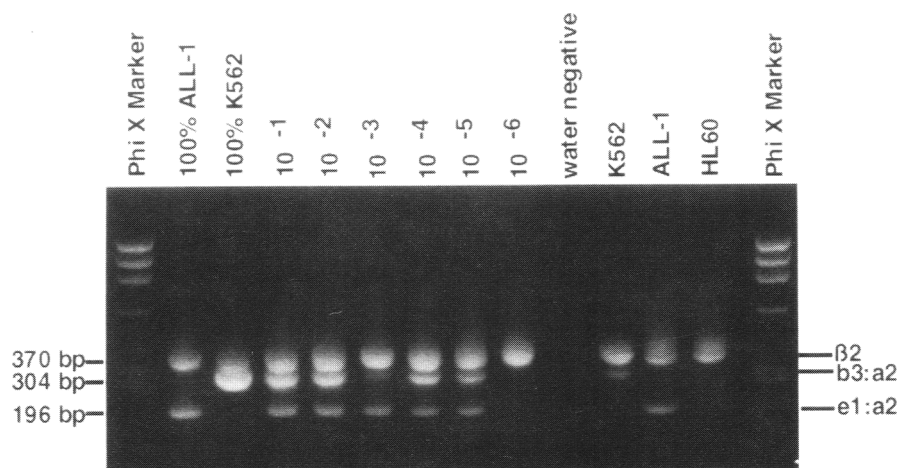


FIGURE 3 Sensitivity of the multiplex PCR. Serially diluted RNA from the K562 and ALL-1 cell lines were mixed with 1 μ g of HL60 RNA. Four positive control RNAs were used: 100% ALL-1, 100% K562, a 10^{-6} ALL-1 control, and a 10^{-6} K562 control. A nucleic acid negative reaction (water negative) and an HL60 RNA reaction were used as negative controls. Signals from the amplification of p210 and p190 *bcr-abl* rearrangement sequences can be detected down to the 10^{-5} dilution level.

point to have an inferior, disease-free survival compared with patients with the p210 transcript.⁽¹⁰⁾ The use of the multiplex PCR will allow us to test this hy-

pothesis on large numbers of patients in a cost-effective fashion.

An additional benefit of the described multiplex approach is derived by com-

paring the initial reverse transcription reaction with the first PCR ingredients, which further simplifies and shortens the procedure. Thus, taken as a whole, the multiplex PCR approach reduces time and effort and eliminates several opportunities for operator error and PCR contamination. In addition, the multiplex PCR saves valuable RNA needed for duplicate or triplicate PCRs that confirm the accuracy of PCR analysis. Instead of dividing an equal amount of RNA into two or three reaction tubes, a single aliquot of RNA is needed for the one multiplex PCR reaction tube.

The multiplex PCR assay introduced in this paper has a high degree of sensitivity and specificity similar to nested, single-primer PCR. Thus, it has potential for not only diagnostic evaluation of Ph+ leukemias but may also be used for sensitive monitoring of MRD throughout the course of treatment.

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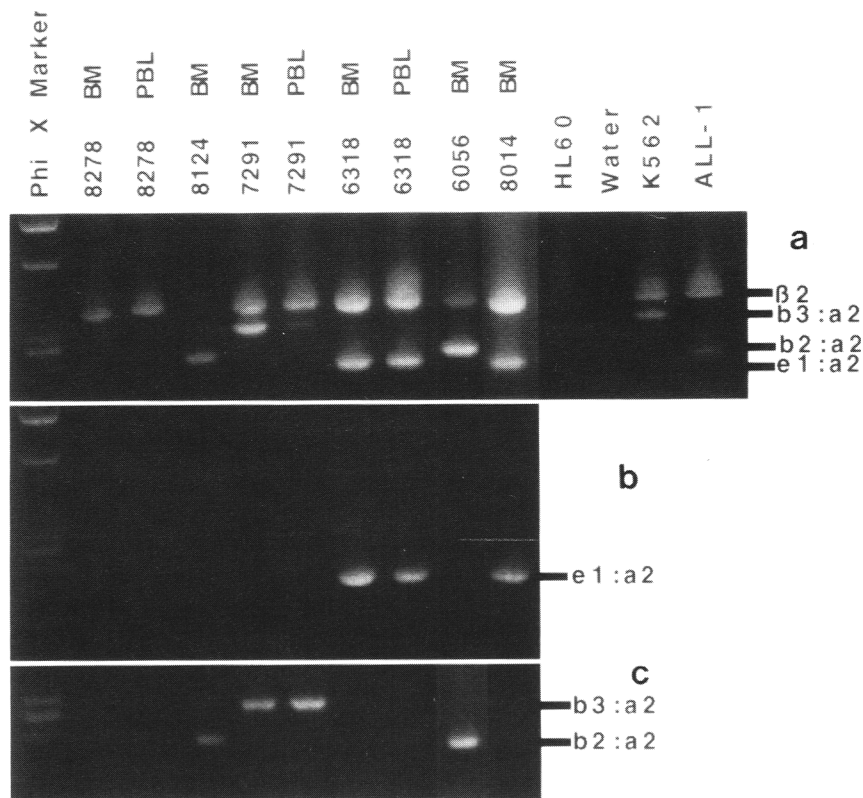


FIGURE 4 Patient series. A comparison of multiplex PCR to single-primer PCR on nine patient samples. UPN numbers and the specimen type [bone marrow (BM) or peripheral blood (PBL)] are designated. (a) Multiplex PCR; (b) single-primer p190 PCR; (c) single-primer p210 PCR. The controls shown at the right of a were from the multiplex PCR.

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