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Rapid RT–PCR Amplification from Limited Cell Numbers

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We describe a rapid and efficient RT–PCR method particularly suited to procedures involving limited cell and target gene copy numbers. Purified leukocytes and myeloid colonies derived from patients with chronic myelogenous leukemia (CML) in chronic phase were used for direct RT–PCR. Purified cells and colonies were lysed using a small quantity of DEPC-treated water containing RNasin as an RNA inhibitor. The untreated lysate was either used immediately for RT–PCR or frozen at −70°C for later use. By this method we were able to consistently amplify *bcr–abl* transcripts from as few as 10 cells. No noticeable difference was observed between products amplified from fresh and frozen samples.

When working with cultured cells or very small cell numbers from patient samples, it is often difficult to perform reliable reverse transcriptase–polymerase chain reaction (RT–PCR) using standard RNA purification methods.[1] The multiple manipulations and transfers required for this method, in combination with small initial target gene copy numbers, make it difficult to obtain sufficient RNA after purification for successful amplification. This is an even greater problem when it is necessary to amplify a control gene such as β-2 microglobulin to assess RNA integrity. In addition, the time and resources necessary for standard RNA extraction can be prohibitive when working with large numbers of samples. Shi et al.[2] describe a rapid method for direct RT–PCR from whole blood that eliminates formal RNA extraction. Although fast, this method is not ideally suited to use with small cell numbers, as it does not avoid the problem of template loss that can accompany sample transfers during RNA purification. We describe a simplified method for RT–PCR amplification from cells that eliminates any loss of RNA and provides rapid and efficient amplification from small cell numbers.

MATERIALS AND METHODS

Preparation of Total RNA from Cells

To each reaction tube containing cell samples, 10 μl of diethylpyrocarbonate (DEPC)-treated H_2O (all DEPC-treated water was heat-inactivated after overnight incubation) containing 2–4 units of RNasin (0.2–0.4 U/μl) (Promega) was added, mixed in the pipette tip to assist in lysis, and incubated for 20 min on ice. The lysate was then either frozen at −70°C or used directly as a template for the RT–PCR reaction as described below.

Primers

Oligonucleotide sequences for the *bcr–abl* transcript are shown in Table 1. Their relative positions are shown in Figure 1. The nested reaction yields either a 305- or a 234-bp product depending on expression of bcr exon 3 (Fig. 1).

RT–PCR

To the 10 μl of lysate was added 40 μl of the combined RT–PCR mixture given in final concentrations: 25 pmoles each of oligonucleotides CMLND and CMLNC, 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.1% Triton X-100, 200 μM dNTPs, 1.5 mM $MgCl_2$, 1.25 units of *Taq* polymerase (AmpliTaq, Perkin-Elmer Cetus), 10 units of RNasin, 7.5 units of AMV-RT (Boehringer Mannheim), and DEPC-treated H_2O to bring the final volume including sample to 50 μl. The reaction mixture was incubated for 30 min at 42°C, brought up to 95°C for 5 min, and amplified using 40 cycles of 94°C for 30 sec, 55°C for 1 min, and 72°C for 1 min followed by a final elongation at 72°C for 7 min on an Omnigene thermocycler (Hybaid Ltd). For the second reaction, 5 μl from the first amplification tube was transferred to a second reaction tube containing fresh PCR ingredients: 25 pmoles oligonucleotides CMLNA and CMLNB, 500 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.1% Triton X-100, 1 mM dNTPs, 1.5 mM $MgCl_2$, 1.5 units of *Taq* polymerase, and DEPC-treated H_2O up to a final volume of 50 μl. After mineral oil overlay, the tubes were placed on the thermocycler block once it reached 65°C

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TABLE 1 Oligonucleotide Sequences for *bcr-abl* RT–PCR

Primer	Sequence 5′ → 3′	Location
CMLNA	TGG AGC TGC AGA TGC TGA CCA ACT CG	M-*bcr*
CMLNB	ATC TCC ACT GGC CAC AAA ATC ATA CA	exon II *abl*
CMLNC	GAA GTG TTT CAG AAG CTT CTC C	M-*bcr*
CMLND	TGA TTA TAG CCT AAG ACC CGG A	exon II *abl*

to minimize nonspecific annealing occurring at lower temperatures. The samples were denatured at 95°C for 5 min, amplified using 40 cycles of 94°C for 30 sec, 55°C for 1 min, and 72°C for 1 min, followed by a final elongation at 72°C for 7 min. A dilution of 10^{-6} μg of K562 RNA mixed into 1 μg of total HL60 RNA and a second tube with water serving as the sample were used as positive and negative controls, respectively. Fifteen microliters of the final amplification product were run on a 2% ethidium-stained agarose gel and photographed.

Dilution Preparation

To test the sensitivity of the assay, a dilution series of decreasing numbers of chronic myelogenous leukemia (CML) leukocytes in phosphate-buffered saline (PBS) was prepared. Leukocytes were isolated from the marrow of a patient with CML in chronic phase using a 3% dextran density gradient and concentrated to 10^6 cells/ml for the initial dilution. From this initial stock, serial dilutions were made so that 2 μl of a dilution added to a reaction tube would contain the desired number of cells to be analyzed (0.01–500 cells). It is important when using this method to keep the amount of diluent to a minimum to ensure efficient lysis of the cells. Reaction tubes with cells were kept on ice for cell lysis.

Colony Preparation

We also tested this method on individual myeloid and erythroid colonies derived from standard clonogenic methylcellulose assays of CML bone marrow mononuclear cells. Individual colonies containing ~50–200 cells each were plucked from the cultures after 14 days, placed directly into microcentrifuge tubes, and placed on ice in preparation for cell lysis and rapid RT–PCR.

RESULTS

Figure 2 shows the results of typical leukocyte cell titration and colony experiments. Dilutions containing as few as 10 CML cells showed amplification of the *bcr–abl* transcript. In addition, consistent amplification was observed in 10 of 10 replicates each containing 10 CML cells. Samples containing a single cell showed a detection rate of 6 of 10. The uniformity of intensity of bands shown in Figure 2A is characteristic of nested PCR in our experience; however, well-to-well block variation can affect amplification efficiency. Individual colonies showed strong amplification with no detectable nonspecific amplification. Samples that were frozen and assayed 60 days after lysis showed no obvious difference in amplification from those that were assayed immediately.

DISCUSSION

Many RNA purification methods are associated with a number of disadvantages, particularly when working with small cell numbers. Perhaps most important is the level of uncertainty that is introduced by the inherent risk of template loss during multiple sample manipulations.[1,2] In addition, standard RNA purification methods can be time consuming when working with large sample numbers.

The rapid RT–PCR method used in this study offers the advantage of maintaining a high degree of sensitivity while avoiding false negatives caused by template loss. Because the RNA release step uses only RNasin to inhibit RNases, this method avoids the inhibition of the RT–PCR reaction by DEPC, which has been noted to be a problem in other quick RNA preparative methods.[3] The small amount of time necessary for RNA preparation and the ability to freeze samples for later analysis make rapid RT–PCR a desirable method for working with large sample numbers. This method has proved to be simple and reliable for work with individual colonies and for the am-

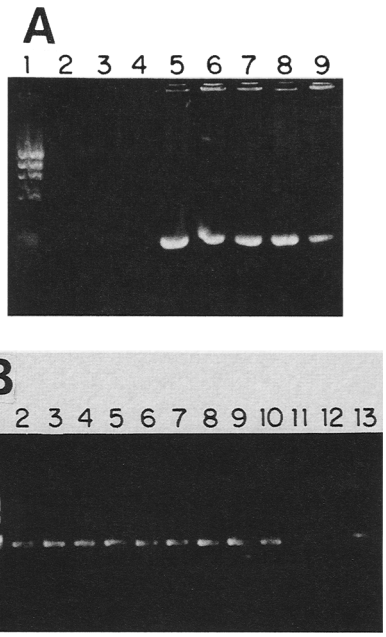

FIGURE 2 Amplification of *bcr–abl* mRNA. Ethidium-stained agarose gel with amplification products following rapid RT–PCR from (*A*) CML leukocyte dilution series. (Lane *1*) ϕX174 *Hae*III marker; (lane *2*) 0.01 cells; (lane *3*) 0.1 cells; (lane *4*) 1 cell; (lane *5*) 10 cells; (lane *6*) 50 cells; (lane *7*) 200 cells; (lane *8*) 500 cells; (lane *9*) 10^{-6} K562-positive control. (*B*) Individual myeloid and erythroid colonies derived from short-term methylcellulose culture of CML bone marrow mononuclear cells. (Lane *1*) ϕX174 *Hae*III; (lanes *2,3,5,6,9,10*) burst-forming unit–erythroid (BFU–E) colonies; (lanes *4,7,8*) Colony-forming unit–granulocyte/macrophage (CFU–GM); (lane *11*) HL60 negative; (lane *12*) H_2O negative control; (lane *13*) 10^{-6} K562-positive control.

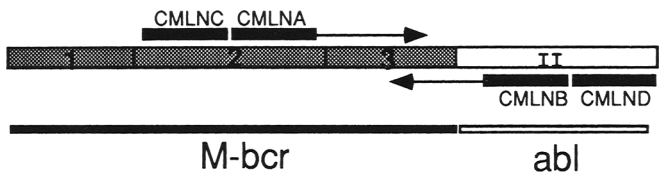

FIGURE 1 Relative positions of primers for detection of *bcr–abl* rearrangement.

plification of RNA when sample material is limited. Total time from beginning of RNA isolation to end of gel electrophoresis is 10 hr.

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