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Use of a Sensitive Fluorescent Intercalating Dye to Detect PCR Products of Low Copy Number and High Molecular Weight

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The ability to routinely and specifically amplify and detect PCR products ranging in size from <1 to >10 kb, regardless of target template sequence or structure, would facilitate several tasks in human genome research.[1,2] Generation of a wide size range of PCR products would potentially expedite isolation of uncloned DNA (gaps) represented in physical maps and provide a means for maintaining order and orientation of closely linked loci during analysis. Long-range PCR offers an alternative to isolation of genomic or cDNA clones from tissues or species where appropriate libraries are unavailable. This methodology also provides a powerful strategy to pursue directed transposon-based mapping and sequencing of templates of 100 kb or greater.[1–4] Further development of basic PCR technology has been necessary in order to make it feasible to use large DNA targets as primary templates for genome analysis. Application of these extended PCR capacities would potentially save time, materials, and cost.

The ability to use one set of general PCR conditions to amplify a wide size range of products, while maintaining stringent criteria for reproducible and specific DNA amplification, is essential for the ultimate utility of long-range PCR to genome research. Previously, we defined a single set of PCR reaction and thermal cycling conditions to produce specific and reproducible amplification of a range of PCR fragments extending up to 9.0 kb.[2] Our ability to generate large PCR products was attributed to the use of recombinant *Tth* DNA polymerase, the addition of 0.01% gelatin to PCR reaction mixtures, and the ability to increase the extension time in the later PCR cycles (auto segment extension). Equally as important, we found that the method of DNA detection was critical, not only for the ability to ascertain the presence of PCR products, but for the reproducible detection of low-copy-number, high-molecular-weight amplified fragments. Specifically, we found that the use of end-labeled fluorescent primers in a PCR reaction did not provide adequate sensitivity for detection of less abundant DNA products. Detection of these large amplification products is improved significantly, however, if the number of fluorescent units per DNA molecule is increased.[2] Either intercalation of a fluorescent agent or incorporation of fluorescently-labeled nucleotides into a PCR product will enhance sensitivity of visualization of DNA.

In a previous series of experiments, we detected PCR products using the inherent fluorescence of the DNA intercalating agent, ethidium bromide.[2] In the current studies, we describe a modified protocol, adapted for our specific detection requirements, involving the use of an alternative fluorescent intercalating reagent, the thiazole orange dimer, TOTO-1 [1,1′-(4,4′,7,7′-tetramethyl-4,7-diazaundecamethylene)-bis-4-[3-methyl-2,3-dihydro-(benzo-1,3-thiazole)-2-methylidene]-quinolinium tetraiodide].[5,6] The greater sensitivity of DNA detection achievable with TOTO-1, as compared with the use of conventional ethidium bromide staining of large PCR products, has enabled us to detect PCR products ranging in size from 1 to 12.2 kb. In addition, we have extended our studies to include the amplification of guanine + cytosine (G + C)-rich, high-molecular-weight DNA templates. In all cases, our PCR has resulted in the generation and detection of highly specific and reproducible amplification products.

MATERIALS AND METHODS

PCR Amplification—Templates and Primers

Several DNA templates were used in these studies. First, a transposon model system was used in these experiments, as described previously.[2,7,8] This system was kindly provided by D. Berg (Washington University, St. Louis, Mo). DNA templates were λ phage 138 clones carrying the *Escherichia coli lacZ* gene with a small amount of surrounding *E. coli* DNA.[7,8] A single Tn*5supF* transposon had been inserted randomly at a unique location within each identical cloned fragment,[7,8] as shown in Figure 1. The preparation of the PCR templates has been described previously.[2] Briefly, each representative phage, containing a

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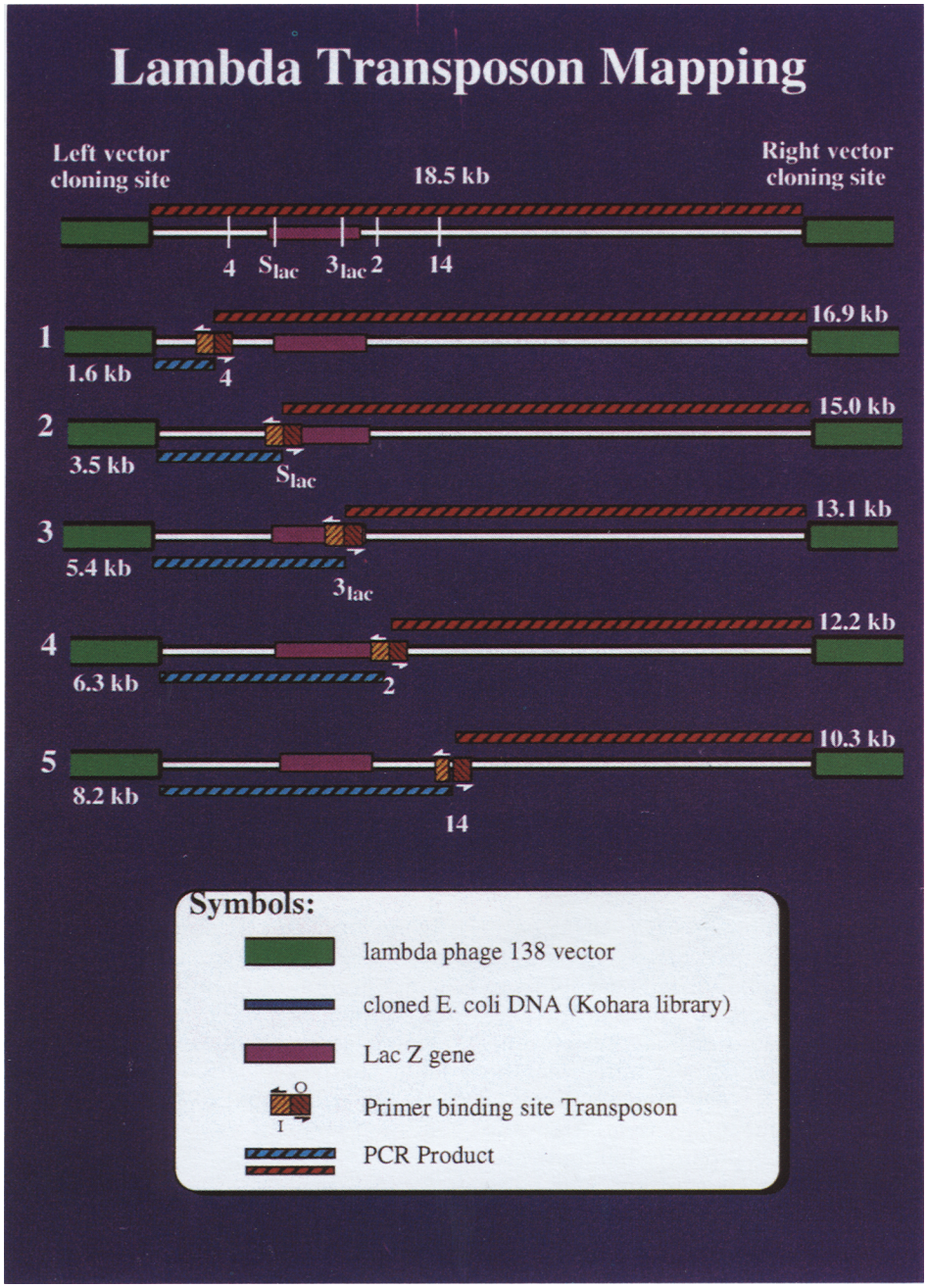

FIGURE 1 Mapping of transposon insertions. The model system used in these studies employs introduction of the transposon Tn*5supF* into random locations of individual phage λ 138 clones. Each phage clone carries the *E. coli lacZ* gene with a small amount of surrounding DNA.[7,8] Mapping of transposon insertions is carried out using primers specific for Tn*5supF* inside (I) and outside (O) ends and for λ DNA adjacent to the left (L) and right (R) cloning sites.[2]

unique transposon insertion site, was suspended in 50 μL of SM buffer[9] and stored at 4°C until use. Aliquots of 5 μl phage lysate (50–100 ng) were used for each PCR reaction. PCR was performed using primers that are complementary to Tn*5supF* inside (I) and outside (O) ends, and for the λ DNA adjacent to the left (L) and right (R) cloning sites.[2]

DNA templates with differing sequence and G + C content were used in these PCR studies. All of these templates were derived from randomly isolated *p*UC118 clones containing insert DNA from the G + C-rich interval between the glucose 6-phosphate dehydrogenase and color vision loci on human Xq28.[10] DNA sequence analysis of Xq28 has demonstrated that the region averages ~60–65% CpG residues, but is as high as 80% CpG in DNA-coding regions.[10] Clones containing inserts of 3.7, 7.8, and 8.1 kb were used in this experiment. PCR primers are complementary to the *p*UC118 vector sequences flanking the *Eco*RI cloning site. The primer pair sequences are as follows: upstream (U) 5′-AGGAAACAGCTATGACCATGATTAC-3′; downstream (D) 5′-CTAGGAGATCTCAGCTGGACG-3′.

Each PCR reaction contained 50 ng of DNA (vector plus insert). We had reported previously that if the melting temperature of each member of a primer pair differs significantly, nonspecific amplification products often result. This is apparent particularly when extension times are increased to achieve long-range PCR.[2] For this reason, the sequences of all primer pairs were adjusted so that the melting temperature of each oligonucleotide, in the presence of the 50 mM KCl PCR buffer, was within 1°C of each other. The software program Oligo (National Biosciences) was used for assistance in primer design.

PCR Reaction Components and Cycling Conditions

Reaction mixtures (final volume, 50 μl) were prepared in MicroAmp reaction tubes (Perkin-Elmer). The following components (at the specified final concentrations) were added to the tubes to form a lower reaction mixture before melting AmpliWax PCR Gems (Perkin-Elmer): 30 pmoles of primer I or O, or 30 pmoles of primer L or R for the transposon model system, or 30 pmoles of the U and D *p*UC118 primers to amplify DNA from human Xq28; 2.5 mM $MgCl_2$; 1.0 mM total dNTPs; 25 mM Tris-HCl (pH 8.9); and one AmpliWax PCR Gem in an initial volume of 13.5 μl. After melting the AmpliWax PCR Gem and allowing the thermolabile barrier to resolidify above the lower reaction mixture, the following components were added to form an upper layer in the reaction tubes: 1× r*Tth* PCR buffer (Perkin-Elmer); 2.5 units of r*Tth* DNA polymerase (Perkin-Elmer); 0.01% gelatin; and 5.0 μl of template DNA (phage plaque lysate or *p*UC118 plasmid DNA) in a volume of 36.5 μl. Samples were then placed in the GeneAmp PCR System 9600 (Perkin-Elmer) and amplified using the following cycling conditions: An ini-

tial 10 cycles, each consisting of 95°C for 10 sec; 30 sec at 58°C for the transposon system or 49°C for the *p*UC118 clones; and 72°C for 3 min. This was followed by 20 cycles, each consisting of 95°C for 10 sec; 30 sec at 58°C for the transposon system or 49°C for the *p*UC118 clones; and 72°C for 3 min, plus a 30-sec increment every cycle. Samples were stored at 4°C following PCR before analysis.

Detection of PCR Fragments Using TOTO-1

We have adapted the protocol for DNA detection using TOTO-1 from the original methodology described[5,6] to accommodate our particular detection requirements.[11] TOTO-1 (Molecular Probes, Inc.) was diluted to 0.1 mM in dimethylsulfoxide (DMSO) in light-protected vials and stored in 1-μl aliquots at −20°C. All laboratory manipulations were performed in a light-protected environment. A working 0.25 μM stock solution was prepared in sterile distilled H_2O. Four microliters of the working stock of TOTO-1 was aliquoted into individual microtiter wells, followed by the addition of 1 μl of PCR product.[5,6] We found that while 1 μl of the larger PCR products (10.3–12.2 kb) was necessary for DNA detection, the more abundant lower-molecular-weight PCR products had to be diluted as much as 1 : 150 in distilled H_2O. One microliter of the diluted sample was then added to the TOTO-1. The intercalation of TOTO-1 with DNA was performed at ambient temperature for 30 min.[5,6] Gel loading buffer (2×; Applied Biosystems) was added to the TOTO–PCR sample and loaded onto an 0.8% agarose gel. Fluorescent PCR products were detected using the Gene Scanner 362A Fluorescent Fragment Analyzer (Applied Biosystems).[12] The Gene Scanner 362A utilizes an argon-based laser system, which differs slightly from the two-color confocal laser-excited detection system used by Rye et al.[5] The blue channel on the Gene Scanner 362A, which has been traditionally reserved for detection of FAM (S-carboxy-fluorescein), was used in these studies for the fluorescent detection of TOTO-1. To correct for minor differences between the FAM and TOTO-1 fluorescence profiles, a TOTO-1 matrix was prepared using the directions of the manufacturer.[12] Samples were subjected to electrophoresis in the dark for 8 hr in 1× TBE buffer, at room temperature.

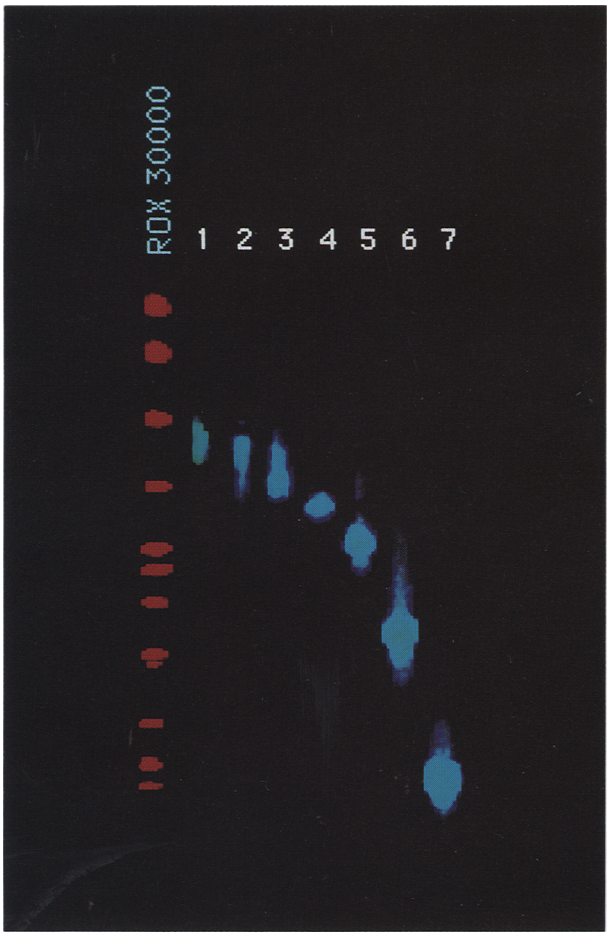

FIGURE 2 Lanes *1–7* contain PCR fragments of 12.2, 10.3, 8.2, 6.3, 5.4, 3.5, and 1.6 kb, respectively. Lanes *3–7* correspond to λ clones 1–5, shown in the schematic drawing in Fig. 1. In these PCR reactions, the L and I primers were used. Lanes *6* and *7* correspond to λ clones 5 and 4, respectively. For these reactions, the R and O primers were used. A 1 : 150 dilution was performed on samples in lanes *3–7* before reacting with the TOTO-1 dye and subjecting to electophoretic analysis. Note that despite the dilution of 1 : 150, the 1.6–8.2-kb fragments were overloaded. The amount of PCR product decreases with increasing fragment size, however; therefore, it was necessary to use 1 μl of the samples containing 10.3-kb and 12.2-kb PCR fragments. The ROX 3000 size standards (Applied Biosystems) were used in this experiment, as intercalation of DNA fragments (within this size range) with TOTO-1 does not significantly alter the mobility during electrophoretic separation.

RESULTS AND DISCUSSION

The use of TOTO-1 for DNA detection has enhanced our ability to visualize an array of PCR fragments of varying sizes and sequence. Using the conditions described above, we have detected PCR fragments ranging from 1.6 to 12.2 kb (Fig. 2). The use of ethidium bromide had not enabled routine detection of the 10.3- and 12.2-kb PCR products.[2] TOTO-1 DNA intercalation, however, allowed us to determine that our detection methods had been limiting previously and that DNA amplification was extending through 12.2 kb of DNA. In our system, yield of specific PCR products varied inversely with size of the amplification product.[2] For example, we estimated previously that amplification of the 3.5- and 5.4-kb targets yielded ~1–5 μg of DNA, the 6.0-kb target yielded ~1–2 μg, and the 8.2-kb fragment yielded ~0.5–1.0 μg.[2] In the current studies, we estimate yields of ~0.5–1.0 ng for the 10.3-kb product and ~0.2–0.5 ng for the 12.2-kb amplification fragment. Under the present set of conditions, detection of a PCR fragment >12.2

kb would be difficult, as it would be necessary to detect DNA present at femtomolar (fM) concentrations (i.e., 0.2 ng/50 μl = 4 pg/μg = 0.5 attomoles/μl = 500 fM, or ~10,000 molecules/μl). The high sensitivity of DNA detection achievable using TOTO-1 thus enabled us to distinguish between low PCR yield versus failure of the PCR reaction to amplify high-molecular-weight templates. As a result, we can now focus our efforts on development of methods to improve yield of high-molecular-weight PCR products, as well as to extend our ability to amplify fragments perhaps up to 25–50 kb.

We have also now examined large PCR templates from the highly G + C-rich region of the genome Xq28 (Fig. 3). The CpG enrichment of the 8.1-kb fragment, obtained by sequence data, is shown in Figure 4. We used the identical PCR reaction conditions as described for DNA amplification in our transposon model system,[2] with differing primer pairs and correspondingly different annealing temperatures. Synthesis specificity was high using the PCR conditions described. Yield of PCR product, however, was lower than expected, which necessitated the analysis of undiluted PCR products. Our studies involving the incorporation of novel fluorescent dUTP analogs[13] demonstrate the presence of the specific, fluorescence-labeled PCR product, but the existence of supercoiled DNA, is revealed using either TOTO-1 or ethidium bromide intercalation into these PCR samples. The supercoiled plasmid DNA is thus not readily available for amplification, thereby effectively reducing the number of target templates in these DNA preparations. This observation supports the view that the low yield is caused primarily by the presence of tightly supercoiled vector DNA, rather than by the G + C content of the target region.

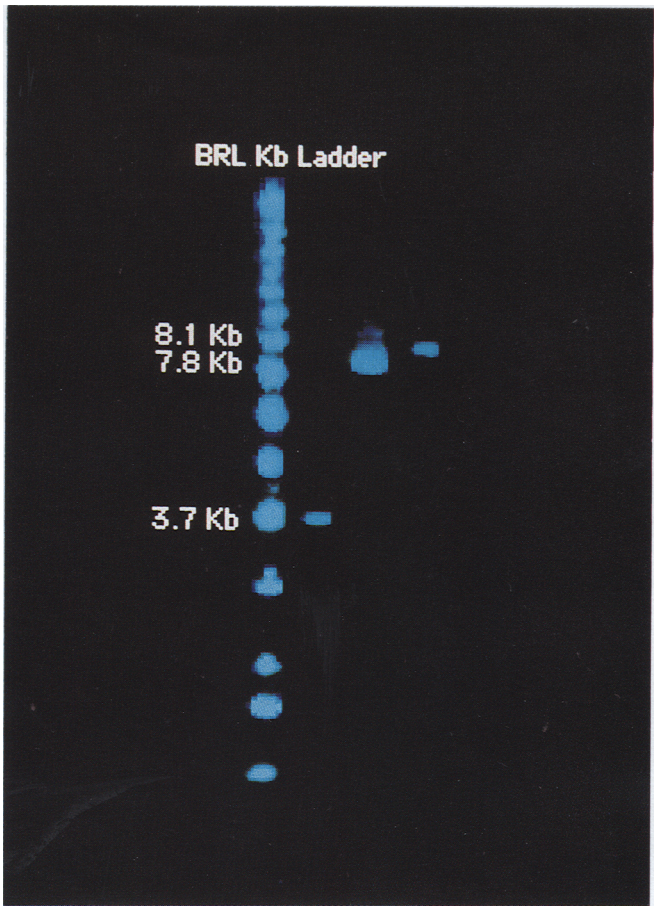

FIGURE 3 DNA templates with differing sequence and G + C content were used in these PCR studies. All of these templates were randomly isolated *p*UC118 clones containing insert DNA from the G + C-rich interval between the glucose 6-phosphate dehydrogenase and color vision loci on chromosome Xq28. Clones containing inserts of 3.7, 7.8, and 8.1 kb were used in this experiment. PCR primers are complementary to *p*UC118 vector sequences upstream (U) and downstream (D) of the *Eco*RI cloning site. TOTO-1 was added to 1 μl of each PCR mixture and was subsequently electrophoresed on an 0.8% agarose gel as described in the legend to Fig. 2. Fluorescence detection was achieved using the model 373A Fluorescent Fragment Analyzer (Applied Biosystems).

The use of TOTO-1 for detecting PCR products >100 bp is superior to ethidium bromide. TOTO-1 forms highly fluorescent complexes with double-stranded DNA but is virtually nonfluorescent in the absence of nucleic acid. Background fluorescence is therefore minimized. In contrast, because of its low binding constant to DNA, the use of ethidium bromide requires high concentrations of dye. Because unbound ethidium bromide fluoresces to some extent, a high background results. Staining DNA with TOTO-1 reduces exposure to hazardous chemicals in the gel or electrophoresis buffer, as only the amplified DNA contacts the dye.[5,6] Convenience, safety, nominal cost, and sensitivity of TOTO-1 for DNA detection are characteristics that make this dye a valuable tool for developing and evaluating conditions that enable reliable generation of specific PCR products covering a wide range of molecular weights.

As discussed in our earlier publications,[2–4] our approach toward generating the long PCR products that we are detecting with these novel dyes involves attempts to mimic, in vitro, conditions that enable cells to copy long stretches of DNA in vivo. Although not the primary subject of this report, we are continuing to obtain data suggesting, as noted previously,[2] that three variables in particular may be crucial to generating significantly longer PCR products (i.e., >25 kb) with acceptable specificity and reproducibility. First, we have already observed that increasing the extension times results in both enhanced yield of large fragments, such as the 10.3- and 12.2-kb targets analyzed in the present studies, as well as amplification of higher-molecular-weight products. Specificity and reproducibility of amplification are not routinely acceptable for our automation requirements, however, and both aspects need to be addressed further. Second, our preliminary data in-

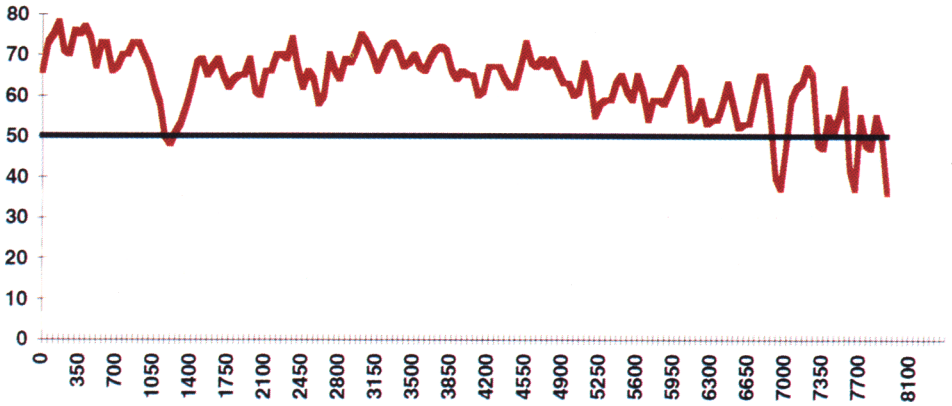

FIGURE 4 The CpG enrichment of the 8.1-kb fragment from Xq28 is shown schematically as the number of CpG residues per 100 nucleotides. The window size for this analysis was 100 nucleotides, with a 50-nucleotide offset. Nucleotide numbers are displayed on the horizontal axis, and percent of GpC content on the vertical axis.

dicate that alternative buffer systems prove more effective for amplifying larger target templates. The current buffer system was used as a starting point, for historical reasons, and has not been optimized fully for long PCR. Analysis of buffering capacity at the optimal extension temperature for r*Tth* DNA polymerase suggested to us that other buffers may yield superior results. Our preliminary data suggest that the use of a single, or combination, of buffers with pKa's falling within the neutral pH range at optimal enzyme extension temperatures (more similar to in vivo conditions) enhance our ability to obtain large PCR products. Finally, as first noted in our earlier publication,[2] it is possible that combinations of enzymes with different exonuclease activities may prove more useful in long-distance PCR as compared with r*Tth* DNA polymerase alone. Other promising improvements include the addition of a thermostable pyrophosphatase, as well as accessory proteins to the PCR reaction. Thus, we are continuing our efforts to analyze variables that might impact performance of long-distance PCR and hope to define conditions enabling routine and specific amplification of fragments ranging from <1.0 kb to well in excess of 25 kb.[13]

Finally, we are currently evaluating the use of alternative intercalating dyes, as well as incorporation of novel dNTP analogs,[14] to further enhance sensitivity of detection of small amounts of DNA. Assuming that we are able to adequately develop and refine technologies for long-range PCR and transposon-based mapping and sequencing, this approach should be well suited for complete automation enabling routine application. Moreover, it should be possible to scale PCR reactions down to 1–5 μl, providing additional savings in analysis costs. Such systems, however, will require highly sensitive methods of detection and assume that PCR products are highly specific. Although future technology development must therefore focus on stringent criteria for sensitivity, specificity, and reproducibility in PCR, the potential benefits to genome mapping and sequencing could be enormous.

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