

A universal procedure for primer labelling of amplicons

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ABSTRACT

Detection and visualisation of nucleic acids is integral to genome analyses. Exponential amplification procedures have provided the means for the manipulation of nucleic acid sequences, which were otherwise inaccessible. We describe the development and application of a universal method for the labelling of any PCR product using a single end-labelled primer. Amplification was performed in a single reaction with the resulting amplicon labelled to a high specific activity. The method was adapted to a wide range of PCRs and significantly reduced the expense of such analyses.

At present, there are several methods for the amplification of DNA and RNA. Constant to the myriad of protocols and applications of nucleic acid amplification technologies, however, is the labelling of amplified DNA sequences. Labelling of DNA fragments, for successive visualisation, is achieved using a variety of stains or via the incorporation of fluorescent, luminescent, radioactive or otherwise chemically modified nucleotides. Alternatively, the newly synthesised DNA is labelled as a result of incorporation of 5' end-labelled, specific oligonucleotide primers. Primer labelling, as opposed to direct incorporation, has been shown to be more useful for the specific labelling of DNA amplification products (1). In particular, the primer labelling of DNA amplification fragments is widely practised for the analysis of microsatellite and other tandemly repeated sequences of eukaryotic genomes. Automated fragment analysis, based on the detection of fluorescent dyes, enables the rapid screening of sequence polymorphisms at multiple genomic loci. Intrinsic to these analyses are the specific primers which flank variable repeats and other polymorphic sequences. The expense, however, of producing a new, fluorescently-tagged primer for each of the thousands of potentially informative loci is prohibitive. We have designed and developed a single, universal priming system for the highly specific labelling of DNA amplification products. In addition to the application to high resolution genome mapping, this method was applied to the sensitive and automated detection of other polymorphic sequences, including RFLPs.

The procedure we describe is based on a triplex-primed PCR, employing two genome, or template, specific primers and a third labelled, universal primer. The labelled primer could be designed to have a unique sequence, exhibiting insignificant homology to

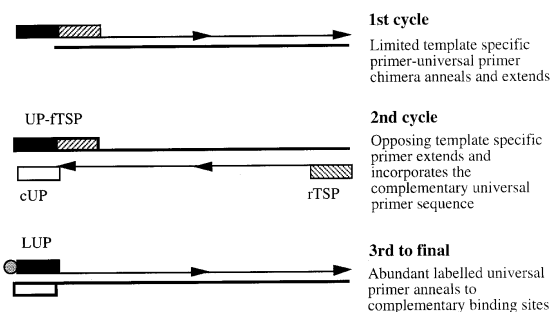


Figure 1. Schematic representation of the method for universal primer labelling of DNA amplification products. Thin lines are newly synthesised DNA while the thick lines depict template strands. Only one strand of the genomic DNA is shown in the first cycle. During the first cycle the universal primer sequence-template specific primer (UP-ftSP) is incorporated. The complementary sequence to the UPS (cUP) is synthesised during the second cycle via chain extension from the opposing template specific primer (rtSP). Successive cycles of PCR afford the annealing and incorporation of the labelled universal primer (LUP).

any sequence found in current DNA sequence databases (2). This design eliminated non-specific cross-reactivity with the target genome. PCR product labelling was achieved by including the labelled primer sequence as a chimeric extension on one of the two genome specific primers (Fig. 1). During the first cycles of DNA amplification the genome specific-labelling primer sequence chimera is incorporated into *de novo* synthesised DNA. The complementary sequence to this chimera being the template for the actual 5' end-labelled primer. High levels of labelled primer incorporation were achieved by limiting the quantity of chimeric primer to between 1/10th and 1/1000th that of the labelled oligonucleotide. Essentially, the chimeric primer was only required for the initial introduction of a priming site into the target genome sequence for the successive labelling reaction (Figs 1 and 2A).

To illustrate the feasibility of this method we have radioactively (Fig. 2B) and fluorescently (Fig. 3A–F) labelled a human (CA)_n repeat. An amplification product of the cyanobacterial phycocyanin gene was also labelled and the profile of partial restriction digest fragments determined (Fig. 3G). In addition, the phycocyanin gene was labelled via prokaryotic *in situ* PCR (data not shown; 3). Conditions for the described PCRs were not altered from previously described conditions (4) other than the addition of a chimeric primer at reduced concentrations and the third, labelled

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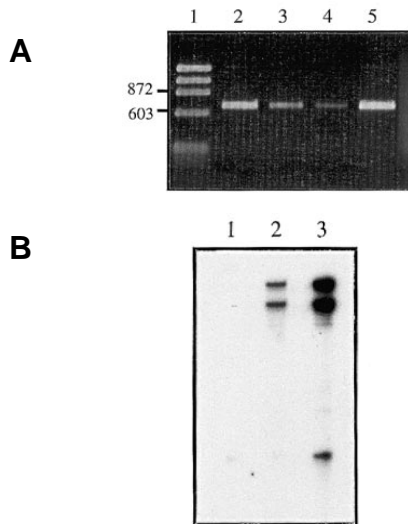


Figure 2. Effect of altering the ratio of template specific-labelling primer chimera to a universal labelling primer. (A) The quantity of amplified phycocyanin gene (30 cycles) from the cyanobacterium *Microcystis* NIES111 (4) using 10 pmol forward primer (PC β) with 1 pmol reverse chimera (PC α URP) and 10 pmol labelling primer (URP) (lane 2), 1 pmol PC α URP and no URP (lane 3), 0.1 pmol PC α URP and no URP (lane 4), and 0.1 pmol PC α URP and 10 pmol URP (lane 5). URP is the universal reverse sequencing primer labelled with TAMRA (ABI, Foster City, CA). Lane 1 is the molecular weight marker ϕ X174/*Hae*III (Boehringer Mannheim, Germany) and the 2% agarose gel in TAE was stained with ethidium bromide. (B) Incorporation of [α - 32 P]dATP end-labelled URP into the microsatellite locus D18S363 (GDB number 195027) from a heterozygous individual. Twenty pmol of forward primer (D18S363F) combined with 20 pmol (lane 1), 2 pmol (lane 2) and 0.2 pmol (lane 3) of the reverse chimeric primer (D18S363R-URP) and 20 pmol of end-labelled URP.

primer. Thermal cycling parameters were not altered in response to the increased T_m of the chimeric template-specific primer and non-specific amplification products were absent from the described applications.

Therefore, instead of obtaining a new fluorescently labelled set of primers for each new loci of interest, two unlabelled primers were synthesised, specific to the genome sequence, with one primer extended to contain the universal priming adapter sequence. Universal primers, as described here, may be any arbitrary sequence which is compatible with the PCR conditions employed and not being present within the template genome's DNA sequence. Labelling of the universal primer may also be accomplished using any of the currently available methods, including fluorescent, chemiluminescent, digoxigenin and radioactive nucleotide analogues. This protocol, unlike recently described advances in PCR product labelling (5), necessitates a single PCR with no interruptions to the cycling programme.

Paramount to the advantage of this design is the reduction in time required to obtain labelling primer sets, the cost of primer sets, and the efficient use of labelled primers where only a limited number of genotyping applications per locus may be required. It is envisaged that a suite of universal primers possessing altered sequence from those described and different fluorescent dye labels would allow multiplexing of amplification reactions. These combinations may be optimised for PCR annealing temperature ranges and for PCR product size considerations. In our hands a single fluorescently labelled primer has been used for microsatellite analysis in humans and canines, PCR-RFLP analysis of toxic

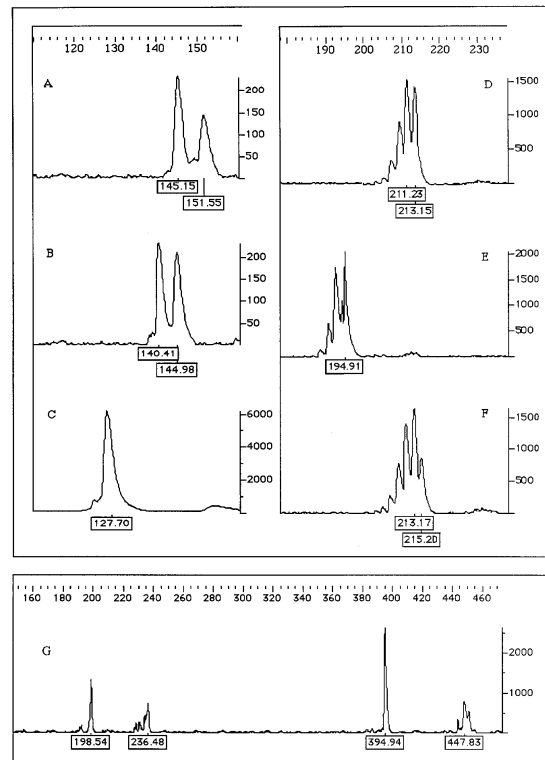


Figure 3. Automated electrophoresis and detection of universal primer labelled PCR fragments. (A), (B) and (C) Amplicons of the (CA) $_n$ dinucleotide repeat locus D18S363 (GDB number 195027). (D), (E) and (F) Amplicons from the same three individuals at the (CA) $_n$ dinucleotide repeat locus D7S435 (GDB number X54566). Primer ratio used was 1:10 (forward chimeric primer:reverse primer) with incorporation of the universal forward primer end-labelled with TAMRA (ABI). (G) Selective partial digest of the phycocyanin amplicon (4) from *Microcystis wesenbergii* NIES111 using the restriction enzyme *Hae*III. The amplicon was labelled by the described method with a chimeric:universal primer ratio of 1:10. The universal primer was labelled with TAMRA (ABI). Fragment sizes, in bp, are indicated for each peak.

cyanobacteria and *in situ* PCR in biofilms of heterotrophic bacteria. Further potential applications include the labelling of DNA generated by RAPD-, RFLP-, capture, and in-cell PCRs, as well as sequencing reactions.

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