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Sex determination by low stringency PCR (LS-PCR)

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There are many situations, such as the detection of an infectious agent or sex determination, when PCR is used to obtain an all or none response. In these cases an internal control is required so if faced with a negative result, one can still be sure that the PCR conditions were perfect. For instance when amplifying Y-chromosome derived sequences in humans, the control normally consists of simultaneous amplification of a locus on the X-chromosome (1) or on an autosome (2). However, such multiplex amplifications have the inherent problem that each primer pair has its own specific conditions of hybridization and thus control is not perfect. Indeed, occasionally one can, even in normal males, observe amplification of the control locus and not the Y-chromosome locus. We wish to describe a simple solution to this problem, low-stringency PCR (LS-PCR). The essence of LS-PCR is that amplification is performed using specific primers but under low stringency conditions such as those routinely utilized when undertaking RAPD (Random Amplified Polymorphic DNA, 3). We have found that despite the low stringency conditions, the specific PCR product is still readily detectable but that additional non-specific products are now also amplified that serve as a perfect internal control.

The approach is illustrated here with two situations, sex determination in man and in the parasitic trematode *Schistosoma mansoni* (Figure 1). In the former case, the primers amplify the ZFY locus on the human Y-chromosome (1) resulting in a single 340 bp amplification product when male DNA is used and in the absence of detectable amplification products with female DNA when stringent amplification conditions are used (Lanes A and B). When the amplification programme is altered to LS-PCR conditions, the same male specific band of amplification is apparent but is now set against a background of RAPD products (Lanes C to F). Female DNA appears to direct the synthesis of all the amplification products except the ZFY locus. To date we have not detected polymorphisms or other sex linked loci amongst the low stringency products which are thus identical in all samples.

The situation with schistosome sex determination is quite different in that it is the female that is heterogametic (ZW as opposed to the male ZZ) and the primers used amplify a highly reiterated set of tandem repeats on the W chromosome (4). Specific amplification results in an intense band of 450 bp, when using female DNA, together with a series of higher molecular weight products that presumably result from degenerate regions in the repeats. There are no detectable products when male DNA

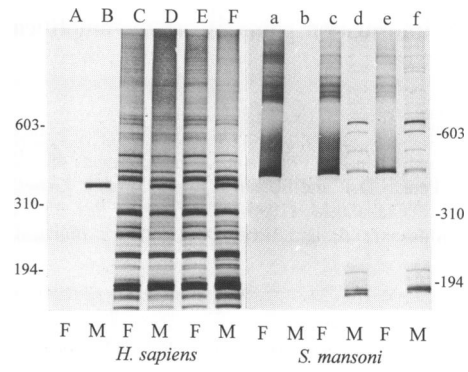

Figure 1. Human DNA from either normal males (B, D and F) or normal females (A, C and E) was amplified using the primers 5'-ATTTGTTCTAAGTCGCCATATTCTCT and 5'-CATACAGCTGAAGCTTGTAGACACACT, as previously described for the amplification of ZFY (1), in a reaction volume of 50 μl containing 50 ng of template, 5 mM $MgCl_2$, 250 μM dNTPs, 50 mM KCl, 0.1% Triton X-100, 10 mM Tris–HCl, pH 8.5, 50 pmoles of each primer and 1 unit of Taq polymerase (Promega). For specific amplification (A and B) the reaction mixture was submitted to 35 cycles of denaturation at 94°C for 45 secs, annealing at 63°C for 1 min and extension at 72°C for 30 secs. Five μl of the final product was applied to the gel. For low stringency PCR (C to F), the same reaction mixture was used in conjunction with the following temperature profile: an initial denaturation at 95°C for 5 min, two cycles of 30°C for 2 mins for annealing, 72°C for 1 min for extension and 30 secs at 95°C for denaturation followed by 33 cycles where the annealing step was altered to 40°C. In the final cycle the extension step was for 5 mins. In this case, 10 μl of the final reaction mixture was applied to the gel. Schistosome DNA prepared from male (b, d and f) and female (a, c and e) cercariae shed from snails infected from single miracidia, and thus essentially cloned, was amplified using the primers 5'-GTGAAATTCTTCCTTCACAC and 5'-GACATTCAACTCAATGTTCG for the amplification of the repetitive female specific sequence W1 (3). For specific amplification (a and b), a 10 μl reaction mixture consisting of 1.0 ng of template, 1.5 mM $MgCl_2$, 200 μM of each dNTP, 50 mM KCl, 10 mM Tris–HCl, pH 8.5, 6.4 pmoles of each primer and 0.8 units of Taq DNA polymerase (Cenbiot RS, Brazil) was submitted to the following temperature profile: 95°C for 3 mins for denaturation followed by 25 cycles of 51°C for 1 min for annealing, 72°C for 3 mins for extension and 92°C for 1 min for denaturation. In the final cycle, the extension was continued for 5 mins. For low stringency PCR (c to f) the same reaction mix was used with the temperature profile detailed above for use with human DNA. For analysis of the schistosome amplification products, 5 μl of the final reaction mix was applied to the gel. Electrophoresis was undertaken using 7% polyacrylamide gels. Following separation the gels were fixed with 10% ethanol/0.5% acetic acid for 20 mins and DNA bands revealed by staining with 0.2% silver nitrate for 30 mins and reduction with 0.75 M NaOH/0.1 M formaldehyde for 10 mins as previously described (2). The molecular weight markers on the left apply to the amplifications of human DNA and those on the right to the amplifications of schistosome DNA.

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is used under high stringency conditions. In this instance, alteration to LS-PCR conditions does not result in a significant alteration in the amplification pattern obtained with the female DNA. We interpret this result as being due to the highly repetitive nature of the target sequence which effectively competes with other amplification products resulting in their subamplification and lack of detectability on the gel. The male DNA, on the other hand, now produces a relatively complex pattern of amplification products which contrasts significantly with that obtained with female DNA. Again we have not found significant polymorphism in this pattern which we take as a male specific pattern for the purposes of parasite sex determination in the laboratory.

We are now routinely using LS-PCR for sex determination and are exploring its application to other situations such as the diagnosis of infectious agents where it is useful to simultaneously amplify both specific and arbitrarily primed DNA fragments either for the purposes of generating a control or for extending the amount of information gained from the amplification.

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