Vectorette PCR isolation of microsatellite repeat sequences using anchored dinucleotide repeat primers

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ABSTRACT

We have developed a vectorette PCR approach to provide an improved method for isolation of microsatellite repeats. The modified procedure relies on PCR amplification using a vectorette-specific primer in combination with one of a panel of anchored dinucleotide repeat primers. The target DNA to be screened for microsatellite sequences can be from YAC, P1, cosmids, bacteriophage or plasmid clones. We have used this technique to isolate novel, polymorphic microsatellite repeats from clones containing the amelogenin gene (AMGX) located on human chromosome Xp22.3.

Despite the large numbers of human microsatellite markers now available, the identification and isolation of additional, novel, highly polymorphic DNA markers remains important. Such markers are needed for the refinement of genetic linkage maps, gene–disease association studies, pre-natal diagnosis using linked markers and loss of heterozygosity analyses of paired normal and tumour DNA samples. Polymorphic markers are also required for the construction of genetic maps for economically important animal and plant species. At present, identification and isolation of polymorphic microsatellite sequences involves screening of cloned DNA fragments with radioactively labelled di-, tri- or tetra-nucleotide repeat oligonucleotide probes. Positively hybridising clones are then isolated and DNA extracted and purified for sequence analysis. Oligonucleotide primers flanking the microsatellite sequence are designed and used for amplification of the repeat. This approach is time-consuming, particularly when large numbers of clones have to be plated, screened, isolated and sequenced.

We have developed a vectorette PCR approach to provide an improved method for isolation of microsatellite repeats. Vectorette PCR is a linker-specific amplification technique originally used for the isolation of terminus-specific sequences from large cloned DNA inserts (1). The modified procedure relies on PCR amplification using a vectorette-specific primer in combination with one of a panel of 12 anchored dinucleotide repeat primers: d(AC)₁₀ C, d(AC)₁₀ G, d(AC)₁₀ T, d(CA)₁₀ A, d(CA)₁₀ G, d(CA)₁₀ T, d(GT)₁₀ A, d(GT)₁₀ C, d(GT)₁₀ T, d(TG)₁₀ A, d(TG)₁₀ C and d(TG)₁₀ G. The target DNA to be screened for microsatellite sequences can be from YAC, P1, cosmids, bacteriophage or plasmid clones. An outline of the technique is presented in Figure 1.

This approach generates DNA sequence from one side flanking the microsatellite repeat. A reverse primer is designed from this sequence, which in combination with the universal vectorette primer, is used to re-amplify the vectorette libraries. The reverse primer will prime back through the repeat and permit sequencing of flanking DNA on the other side of the microsatellite repeat. This will then allow a pair of primers flanking the microsatellite to be generated for routine PCR analysis. We tested the ability of this technique to isolate novel, polymorphic dinucleotide repeat sequences from vectorette libraries made from plasmid, cosmids and yeast artificial chromosome (YAC) clones. The clones used in the study contained part (plasmid) or all (cosmid and YAC clones) of the amelogenin gene (AMGX) sequence, which maps to human chromosome Xp22.3, as determined by PCR analysis using exon specific primers (2).

Six separate vectorette libraries were constructed using 50 ng–1 μg cloned DNA (depending on target DNA complexity) restriction enzyme digested for 1 h at 37°C in 50 μl 1× reaction buffer containing 20 U of each of one of the following enzymes: Alu, BglII, DpnII, EcoRV, RsaI or Sau3A. A range of different restriction enzymes was used so as to increase the chance that the restriction fragment containing the microsatellite repeat was of an amplifiable size. Three pico-nucleos (5 μl) of the appropriate vectorette unit (1) were ligated to the restriction fragments in a total volume of 10 μl containing 1 μl 100 mM ATP, 1 μl 100 mM DTT and 1 U T4 DNA ligase. Ligation reactions were incubated at 20°C for 60 min followed by 37°C for 30 min. The whole process was repeated three times. This is necessary to re-digest any target DNA fragments which have ligated to each other and not to vectorette units. If a 4 bp cutting enzyme is used whose site is reformed on ligation (e.g. Sau3A) the restriction digest must be heat denatured (70°C/10 min) before ligation. Following ligation, 100 μl water was added to the vectorette library. Since yeast genomic DNA also contains microsatellite repeats that will co-amplify with human DNA during PCR, it is preferable to isolate the individual YAC band from a low melting point agarose pulsed-field gel prior to vectorette library construction. Following pulsed-field gel electrophoresis, the YAC band (~430 kb) containing the amelogenin gene was identified by ethidium bromide staining and excised from the gel. The agarose gel slice containing the YAC band was placed in a 1.5 ml microfuge tube and equilibrated overnight in 1× restriction enzyme buffer. The buffer was removed and the agarose gel slice melted by heating at 65°C. DNA (30 μl) was used for restriction enzyme digestion in a 50 μl volume as described above.

Microsatellite repeats were amplified by PCR using the universal vectorette primer (dCGAATCGTAAACCGTGTACGGAATCG) in combination with each one of the 12 anchored dinucleotide

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repeat primers. PCRs were performed in 30 µl 1× reaction buffer containing 3 µl vectorette library, 1.5 mM MgCl₂, 150 µM dNTPs, 10 pmol each primer and 1 U Taq DNA polymerase. Reaction mixtures were denatured at 96°C for 2 min, followed by 35 cycles of amplification consisting of: 96°C/20 s, 54°C/20 s, 72°C/45 s. PCR products were fractionated in a 2% NuSieve (FMC) agarose gel stained with ethidium bromide.

As expected, the number of PCR products observed increased with the complexity of the target DNA (Fig. 2). When a single band was observed (e.g. from a plasmid clone, Fig. 2a), PCR products were purified on a Sephadex G-50 column and ethanol precipitated. PCR products were sequenced using a 33P-labelled vectorette sequencing primer (dCGCTGTCCTCTCCTT) according to the manufacturer’s instructions. When multiple PCR products were observed (e.g. from cosmid and Y AC clones, Fig. 2b and c), individual PCR bands were excised from 0.5× TAE/1.5% NuSieve agarose gels and DNA purified by phenol/chloroform/IAA extraction and ethanol precipitation. PCR products were then sequenced as described above.

A total of six separate microsatellite sequences were identified (Table 1). Four were simple (TG:AC) tandem repeats and two were more complex repeats. Repeat 6 was the only sequence identified in both the plasmid and cosmid clones, although upon further investigation this repeat proved to be non-polymorphic. However, repeats 2 and 3 were both polymorphic with heterozygosity values of 0.56 and 0.76. Full data relating to these polymorphisms, which have been assigned locus symbols DXS9712 and DXS9713, has been submitted to the Genome Data Base.

![Figure 1](image-url) Schematic representation of PCR isolation of microsatellite repeat sequences using an anchored repeat primer.

![Figure 2](image-url) Microsatellite repeat-containing PCR products generated from (a) plasmid vectorette library (DpnII); (b) cosmid vectorette library (DpnII); (c) YAC vectorette library (DpnII) using universal vectorette primer and 1. d(AC)₁₀ 2. d(TG)₁₀ 3. d(AC)₁₀ 4. d(CA)₁₀ 5. d(TG)₁₀ 6. d(TG)₁₀ 7. d(AC)₁₀ 8. d(TG)₁₀ 9. d(TG)₁₀ 10. d(TG)₁₀ 11. d(TG)₁₀ 12. d(TG)₁₀.

We have used vectorette PCR successfully to isolate six novel, microsatellite repeats from clones containing the amelogenin gene (AMGX) located on human chromosome Xp22.3. This technique provides a simple and rapid method for identifying microsatellite repeats and obtaining flanking DNA sequences for oligonucleotide primer synthesis. The procedure does not require hybridisation of radioactive probes, or further rounds of subcloning and can also be adapted for high throughput sequencing on an ABI 370 DNA Sequencer (ABI Systems) if a nested vectorette sequencing primer is fluorescently labelled. In principle, a similar approach also could be used with anchored tri- and tetra-nucleotide primers. Tri- and tetra-nucleotide repeats generally possess greater PCR stability than dinucleotide repeats, thereby facilitating genotype scoring.

REFERENCES