Benchmarks

individuals. The entire coding region of *BRCA1* was evaluated using one PCR product for each exon and nine PCR products for exon 11. Primers for exon 11 were designed to produce PCR products greater than or equal to about 550 bp in length with a 100-bp overlap. Keeping products below this length makes gel analysis much easier.

For 10 individuals, there were 37 polymorphisms detected in the coding region and 10 detected in the intron regions. CEL I produced mismatch-specific cuts for all 47 polymorphisms with no false positives or false negatives, confirmed by DNA sequencing of all samples.

One hundred individuals were evaluated for a 487-bp PCR product, which is a section of exon 11 of *BRCA1*, on one gel using multiplexing PCR products. Five PCR products were mixed per CEL I reaction and analyzed in one gel lane (100 individuals = 20 reactions total on one gel). Three different polymorphisms were detected in this region (2196 G>A, 2201 C>T and 2430 C>T). Representative electropherograms for this data are shown (Figure 1). This test provides evidence that it is practical to detect mismatches in mixtures of five samples, although the determination of which one of the five samples is positive will require further analysis of individual samples. This test also allows the detection of multiple sequence variants in one PCR product. The sequence we use as an example is a highly polymorphic region of the *BRCA1* gene and contains multiple polymorphisms in the DNA pool. If the mutation or polymorphism being investigated is relatively rare, then this multiplexed analysis will allow its discovery about five times faster than using individual DNA analysis.

This test demonstrates the ability of CEL I to detect more than one polymorphism in a PCR product, a quality not present in most mutation detection assays. It also shows that CEL I can detect two polymorphisms that are five nucleotides apart from one another (i.e., polymorphisms 2201 and 2196, panels D and E from Figure 1).

In summary, we have presented the optimized conditions for the CEL I mutation detection assay. As seen from the two studies reported here, this assay is accurate, reliable and easy to set up.

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Modified Primers Facilitate Rapid Screening of 16S rRNA Gene Libraries

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A frequently used approach for studying the diversity of bacteria in natural environments involves PCR amplification of heterogeneous 16S rRNA genes from DNA template extracted directly from environmental samples (4). The mixed PCR products are separated by cloning. In this process, they are ligated to a suitable vector, frequently a plasmid TA-vector (e.g., pGEM®-T easy; Promega, Southampton, UK) or a blunt-ended vector. As a consequence of this ligation, the amplified gene may be inserted into the vector in one of two orientations, either forward or reverse, relative to the orientation of the multiple cloning site.

When screening this gene library to determine which clones contain unique 16S rRNA genes, it is common practice to use RFLP analysis of a PCR product derived from the unique vector sites that flank the 16S rRNA insert (3). The advantage of this approach is that it minimizes any risk of amplifying the hosts' 16S rRNA gene. Because many restriction sites are not symmetrically distributed across the 16S rRNA gene, two possible PCR products may be produced when a 16S rRNA gene is inserted into a vector and then amplified from sites in the vector. Thus, it is not possible from this RFLP analysis to easily distinguish between identical 16S rRNA gene sequences inserted in both orientations since they produce different RFLP patterns. As a result, the cost and time necessary for RFLP screening of large libraries increases.

To increase the efficacy of the culture-independent method, we have designed a set of PCR primers that overcome this problem of insert orientation. Universal 16S rRNA primers (6) were modified to include extra, non-16S rRNA sequence at the 5' ends of the original 63f and 1387r, with numbering based on the *E. coli* 16S rRNA gene (2). We ensured that there were no priming sites similar to the extra sequences for

Benchmarks

the PCR in either the *E. coli* genome (1) or any bacterial 16S rRNA genes in either the Ribosomal Database Project (RDB) (5) and EMBL (Release 60, September 1999) (8). The modified primers, 63f-mod (5'-CGTGCTCGT-GCTCGTGCTCARGCCTAAYACA-TGCAAGTC-3') and 1387r-mod (5'-C-AGCGACAGCGACAGCGAGGGC-GGWGTGTACAAGRC-3') were used for the initial amplification of the 16S rRNA gene from environmental DNA samples. These were ligated to an appropriate plasmid vector (either TA or blunt). (In both primers, the bold type denotes modifications.) After transformation of competent cells, the gene library was screened by colony PCR using 63f-ext (5'-CGTGC TCGTGCT-CGTGCT-3') and 1387r-ext (5'-CAGC-GACAGCGACAGCGA-3'), which annealed to the unique extra sequences flanking the 16S rRNA universal sites and thus gave the same PCR product from identical 16S rDNA templates, irrespective of the orientation of the inserted DNA (Figure 1).

The PCR conditions for the primers were optimized using the Opti-Prime PCR optimization kit (Stratagene, Am-

sterdam, Zuidoost, The Netherlands). This resulted in buffer system 3 and 2 [both consisting of 25 mM KCl, 3.5 mM Mg²⁺ (pH 8.3) and 75 mM KCl, 1.5 mM Mg²⁺ (pH 8.3), respectively] being the best buffers for 63f-mod/1387r-mod and 63f-ext/1387r-ext respectively. Bovine serum albumin (final concentration 0.9 mg/mL) was added to PCRs in which the 63f-mod/1387r-mod primer sets were included. Both sets of primers were amplified using either a PTC-100™ (MJ Research, Waltham, MA, USA) or Biotech Primus 96 (MWG, Ebersberg, Germany) thermal cycler, programmed to perform a touchdown program of 94°C for 2 min followed by 14 cycles at 92°C for 20 s, 70°C for 30 s (minus 1°C/cycle) and 75°C for 30 s, followed by 20 cycles at 92°C for 20 s, 56°C for 30 s and 75°C for 30 s.

This modification to the methodology has been successfully used in our laboratory to screen 16S rRNA gene libraries from activated sludge samples (Figure 1). Analysis of the 16S rRNA gene libraries constructed with these primers showed that there was high bacterial diversity in the libraries and little bias was introduced by modification of

the primers (data not shown). By adopting this methodological change, the orientation of the PCR product does not affect the subsequent RFLP analysis and makes screening of the libraries more cost effective.

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Figure 1. An example of the pattern produced by RFLP analysis of PCR products. The 16S rRNA products (produced using 63f-mod/1387r-mod) were derived from genomic DNA isolated from activated sludge and cloned into pGEM-T easy and a gene library constructed in *E. coli* XL1-Blue by standard methods (7). The orientation of the ligated DNA was determined by sequencing. Two clones were chosen that contained the 16S rRNA gene in either orientation. These clones were used to amplify the insert from either the unique M13 sites in the vector or from 63f-ext/1387r-ext. The amplified products were purified using a PCR purification kit (Qiagen, Valencia, CA, USA) and restricted with *Rsa*I (5 U at 37°C for 1 h). The DNA fragments were separated on a 2% w/v agarose gel (7) and visualized by staining with ethidium bromide and illumination with UV light. Lanes 1 and 6: 1 kb-plus ladder (Life Technologies, Rockville, MD, USA); lane 2: restricted 16S rRNA PCR product, in the forward orientation, derived from M13 sites in pGEM-T easy; lane 3: restricted 16S rRNA PCR product, in the reverse orientation, derived from M13 sites in pGEM-T easy; lane 4: restricted 16S rRNA PCR product in the forward orientation, derived from 63f-ext/1387r-ext; lane 5: restricted 16S rRNA PCR product in the reverse orientation, derived from 63f-ext/1387r-ext.