Table 3: Experimental protocols

Table 3a: HFE amplification protocol for small amplicon melting analysis

DNA was extracted using a QIAamp DNA Blood Kit (QIAGEN, Inc., Valencia, CA), concentrated by ethanol precipitation and quantified by A260.

For high-resolution melting analysis, we used small amplicon melting with primers as close to the SNP as dimer and misprime constraints permit, as described in Liew et al. (2004) [(14)]. The PCR protocol followed here was modified slightly from the protocol described in (12). The amplicon was 40bp long. PCR was performed in a LightCycler with reagents commonly used in clinical laboratories. Ten microliter reaction mixtures consisted of 25ng of genomic DNA, 3 mo MgCl2, 1x LightCycler FastStart DNA Master Hybridization Probes master mix, 1x LCGreen Plus, 0.5 μM forward (CCAGCTGTTCGTGTTC-TATGAT) and reverse (CACACGGCGACTCTCAT) primers and 0.01U/μl Escherichia coli (E. coli) uracil N-glycosylase (UNG, Roche). The PCR was initiated with a 10 min hold at 50°C for contamination control by UNG and a 10 min hold at 95°C for activation of the polymerase. Rapid thermal cycling was performed between 85°C and the annealing temperature at a programmed transition rate of 20°C/s for 40 cycles. Samples were then rapidly heated to 94°C and cooled to 40°C followed by melting curve analysis between 60°C and 85°C to confirm the presence of amplicon.

Prior to analysis on the HR1, samples were again rapidly heated to 94°C and cooled to 40°C to promote heteroduplex formation.

Following amplification, an additional melting was performed to denature the perfectly complementary post-extension duplexes after which the temperature was rapidly decreased to re-anneal strands independent of the presence or absence of a single mismatched base-pair.

ble 3b: HFE amplification protocol for Temperature-Gradient Capillary Electrophoresis (TGCE) analy

The PCR protocol followed here was modified slightly from the protocol described in Bernard et al. (1998) [(15)]. The amplicon was 242bp long. PCR was performed in a Perkin Elmer 9700 block cycler with similar reagents to those used for amplification in the Light-Cycler. Ten microliter reaction mixtures consisted of 25ng of genomic DNA, 3 mM MgCl2, 1x LightCycler FastStart DNA Master Hybridization Probes master mix, 0.4 μM forward (CACATGGTTAAGGCCTGTTG) and reverse (GATCCCACCCTTTCAGACTC) primers and 0.01U/μl Escherichia coli (E. coli) uracil N-glycosylase (UNG, Roche). All samples were then overlayed with mineral oil to prevent evaporation. The PCR was initiated with a 10 min hold at 25°C for contamination control by UNG and a 6 min hold at 95°C for activation of the polymerase. Thermal cycling consisted of a 30s hold at 94°C, a 30s hold at 62°C and a 1min hold at 72°C for 40 cycles followed by a 7min hold at 72°C for final elongation.

Upon completion of these thermal cycles the samples were then heated to 95°C for 5min followed by a slow cool over approximately 60min to 25°C.

Table 3c: Analysis protocol for Temperature-Gradient Capillary Electrophoresis (TGCE)

The protocol followed here is similar to that described in Margraf et al. (2004) [16]. To prepare samples for TGCE analysis, PCR amplicons were transferred to 24 well TGCE trays and diluted 1:1 with 1xFastStart Taq polymerase PCR buffer (Roche). These samples were then overlayed with mineral oil and the trays loaded into the TGCE instrument. TGCE was performed on a commercial instrument (Reveal (TM-melting temperature or trademark?) mutation discovery system, reagents and Revelation software by SpectruMedix LLC, State College, PA) [17?]. DNA samples were injected electro-kinetically at 2 kV for 45 seconds, resulting in peak heights ranging from 5,000-40,000 intensity units with ethidium bromide staining. Optimal results were obtained when the temperature was ramped from 60-65°C over 21 minutes and data was acquired over 35 minutes. Sequential camera images were converted to plots of image frame number (time) versus intensity units (DNA concentration).