

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) (12). 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 mM MgCl₂, 1x LightCycler FastStart DNA Master Hybridization Probes master mix, 1x LCGreen Plus, 0.5 μ M forward (CCAGCTGTTTCGTGTTC-TATGAT) and reverse (CACACGGCGACTCTCAT) primers and 0.01U/ μ l 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.

Table 3b: HFE amplification protocol for Temperature-Gradient Capillary Electrophoresis (TGCE) analysis

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 LightCycler. Ten microliter reaction mixtures consisted of 25ng of genomic DNA, 3 mM MgCl₂, 1x LightCycler FastStart DNA Master Hybridization Probes master mix, 0.4 μ M forward (CACATGGTTAAGGCCTGTTG) and reverse (GATCCCACCCTTTCAGACTC) primers and 0.01U/ μ l Escherichia coli (E. coli) uracil N-glycosylase (UNG, Roche). All samples were then overlaid 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).