**Supplement 1. Detailed methods**

**Samples**

3 mm disks of dried whole bloodspots were received in 96 well plates (one blood spot per well).

**Genomic DNA isolation**

Genomic DNA was extracted from 3mm bloodspot punches in a 96 well plate using “Ready PCR DNA” (5 Prime, Gaithersberg, MD, USA). Room temperature steps were performed on the Biotec Precision Microplate Pipetting System (Winooski, VT, USA).

After room temperature incubation steps, the plates were covered with a plastic film and placed in a pre-warmed 99oC block in the Mastercycler gradient thermocycler (Eppendorf, Westbury, NY, USA) with a cool down step to 37oC, to minimize condensation on the film. Plates were spun down for 3-5 min. at 3000 RPM. DNA was transferred to 0.5 ml pre-labeled tubes with sample number. A second extraction was performed on the same plates using 30ul of elution buffer at 99oC. Purified DNA was stored at 4oC in plastic boxes labeled as first and second extraction respectively (**Supplemental Table 1**).

**Primers and Probes**

Probes for each allele specific assay were designed and purchased from TIB MolBiol (Adelphia, NJ, USA) or Roche (Indianapolis, IN, USA). The Roche probe is labeled on the 5' end (Fluorescein-SP), and the TIB probe has an internal label (XI). Primers were purchased from the Oligonucleotide and Peptide Synthesis Facility at the University of Minnesota (**Supplemental Table 2**).

Simple probes to detect the 167delT and 235delC alleles were designed complimentary to the deafness allele sequence. Simple probes to detect the 35delG and V37I alleles were designed complimentary to the wildtype sequence.

**PCR**

The PCR reaction was set up in the BioTek Microplate Pipetting System. For the amplification a 25ul *asymmetric* PCRreaction was set up as follows: 1xPCR Buffer II, 0.625 U Amplitaq DNA Polymerase (Applied Biosystems, Foster City, CA, USA ), 0.2mM dNTPs (Denville Scientific Incorporated, Metchen, NJ, USA) as well as the simple probes designed above (**Supplemental Table 3**).

The PCR amplification was performed in a Mastercycler Gradient cycler (Eppendorf, Westbury,NY,USA). It consists of a denaturation at 95 oC for 6 minutes, followed by amplification at 95 oC for 1 minute, corresponding annealing temperature for 1 minute (see table),and 72 oC for 1 minute. A final extension at 72 oC for 6 minutes was followed by a 4 oC cool down (**Supplemental Table 4**).

**Mutation Detection**

Mutation detection was performed using simple probe technology and the LightCycler 480 (Roche). The amplified samples in 96 well plates were spun down at 2000 rpm for 2-3 minutes before placing the plate in the LightCycler.

Melting curve analysis consisted of an initial denaturation step at 95oC for 30 seconds, annealing step at 45oC for 30 seconds and a slow heating step (0.1oC/s) to 85oC, with a final cool down at 4oC. Change in fluorescence over change in time was acquired continuously (10 acquisitions per degree) and Tm curve analysis was performed by the Light Cycler software **(Supplemental Figure 1)**.

**Sequencing**

*GJB2* is a gene encoded by two exons. Exon 2 is the only coding exon and harbors nearly all deafness alleles reported for this gene 23. Exon 1 is a non-coding exon with a low frequency of mutations 11. Primers were designed to sequence exon 2 in one reaction in a 96 well plate format. Primer F: 5'gattcctgtgttgtgtgcattcgt ; Primer R: 5' gggcaatgcgttaaactggc produce an amplicon of 745 basepairs. The PCR amplification was performed in a Mastercycler Gradient cycler. It consists of a denaturation at 95oC for 2 minutes, followed by amplification at 95oC for 30 seconds, 53oC for 30 seconds and 72oC for 1 minute (30 cycles). A final extension at 72oC for 4 minutes was followed by a 4oC cool down. Amplicons were sequenced at the Biomedical Genomics Center at the University of Minnesota on an ABI 3130 Genetic Analyzer.

**Supplemental Table 1**: Genomic DNA purification method

|  |  |  |  |
| --- | --- | --- | --- |
| Volume | Solution | Incubation Time | Temperature (0C) |
| 150 ul | Purification | 15 min | Room Temp. |
| 150 ul | Purification | 15 min | Room Temp. |
| 150 ul | Elution | 15 min | Room Temp. |
| 50 ul | Elution | 15 min | 99oC |

**Supplemental Table 2**. Primer and probe sets for *GJB2* allele specific assays

|  |  |  |  |
| --- | --- | --- | --- |
| Allele | Amplification primers | Simple Probe | Amplicon size (bp) |
| **c.35delG** | F: 5’-tttccagagcaaaccgc-3’  R: 5’-ctcctttgcagccacaac-3’ | Fluorescein-SPC\*-cgatcctggggggtgtgaacaaaC3 Blocker  (Roche) | 154 |
| **c.167delT** | F: 5’ggagatgagcaggccga  R: 5’-tcttcttctcatgtctccggta-3’ | gcaacaccc\_gcaXIgccag—PH  (TIB Molbiol) | 179 |
| **c.235delC** | F: 5’ggagatgagcaggccga  R: 5’-tcttcttctcatgtctccggta-3’ | ctatgggcc\_tgcaXIgctgat—PH  (TIB Molbiol) | 179 |
| **p.V37I** | F: 5’-cgcccagagtagaagatggat-3’  R: 5’-aagtcggcctgctcatctc-3’ | cctttgcagccXIacaacgaggat-PH  (TIB Molbiol) | 165 |

**Supplemental Table 3.** Amplification conditions

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  | MgCl2  (Applied Biosystems) | Forward Primer | Reverse Primer | Probe |
| **c.35delG** | 1.1 mM | 0.2 uM | 1.0 uM | 0.06uM |
| **c.167delT** | 4.0 mM | 0.2 uM | 0.6 uM | 0.032uM |
| **c.235delC** | 4.0 mM | 0.2 uM | 0.6 uM | 0.032uM |
| **p.V37I** | 3.0 mM | 0.4 uM | 0.2 uM | 0.08 uM |

**Supplemental Table 4**. Amplification annealing and cycles

|  |  |  |
| --- | --- | --- |
|  | Annealing temperature | Cycles of amplification |
| **c.35delG** | 48.8 oC | 40 |
| **c.167delT** | 65.5 oC | 50 |
| **c.235delC** | 65.5 oC | 50 |
| **p.V37I** | 55.1 oC | 40 |

**Supplemental Figure 1**. Representative Melt Curve analysis for the c.35delG allele in *GJB2* using the Lightcycler. Using sequence complementary to the wildtype sequence surrounding the 35delG mutation, a Simple probe with a fluorescent tag was designed. Upon amplification of a 154bp fragment of *GJB2*, exon 2, amplified DNA is melted and annealed to the probe and then heated. The melt curve shows a single peak (dark blue) at approximately 67o for wildtype, but melts at a lower temperature, 62o, when the guanine is deleted at position 35 (grey) (homozygous 35delG). A heterozygote for the c.35delG allele will have two melting peaks (black). The negative control (purple) is also shown.

**Supplemental Figure 1.**

