**Supplementary Table 1-A. PCR oligonucleotides and sequencing conditions for *NKX2-5*, *FOXE1,* and *TSHR*.**

**PCR and internal sequencing oligonucleotides**

|  |  |
| --- | --- |
| ***NKX2-5***  ***(NM\_004387.4 and NG\_013340.1 RefSeq-Gene)*** | **Sequence (5’ to3’)** |
| **NKX2.5-EX1-(F)-M13F** | **GTAAAACGACGGCCAGTGGCACCATGCAGGGAAGCTG** |
| **NKX2.5-EX1-R** | **CACCAGGCATCTTACATTCTGAAC** |
| **NKX2.5-EX2-(F)** | **CCACGAGGATCCCTTACCATTA** |
| **NKX2.5-EX2-(R)-M13F** | **GTAAAACGACGGCCAGTGGTCTCCGCAGGAGTGAATG** |
|  |  |
| ***FOXE1***  ***(NM\_004473.4 and NG\_011979.1 RefSeqGene)*** |  |
| **M13F-FOXE1-F1-F** | **GTAAAACGACGGCCAGTCTGAGCTCTCCGCAGAAGG** |
| **FOXE1-F1-R** | **CGCGGGGTAGTAGACTGGAG** |
| **M13F-FOXE1-F2-F** | **GTAAAACGACGGCCAGTCGCCGTCTATGCAGGCTAC** |
| **FOXE1-F2-R** | **GAACGTGTGAACAGCCGATG** |
|  |  |
| ***TSHR***  ***(NM\_000369.5 and NG\_009206.1 RefSeqGene)*** |  |
| **TSHR-EX1F** | **GAGGATGGAGAAATAGCCCCGAG** |
| **TSHR-M13F-EX1R** | **GTAAAACGACGGCCAGTCACTACTTCGGGCTGTTATTGAG** |
| **TSHR-M13F-EX2F** | **GTAAAACGACGGCCAGTTAAGGTGAATTATTAGAAAAGC** |
| **TSHR-EX2R** | **CTTGATAGAACACGTTTAGAGAA** |
| **TSHR-M13F-EX3F** | **GTAAAACGACGGCCAGTGCAGAATCCATGAGGGTTGT** |
| **TSHR-EX3R** | **AGAAACCAGCTCCCATTGCC** |
| **TSHR-M13F-EX4F** | **GTAAAACGACGGCCAGTACCCTGTGGCGTAAATGCATA** |
| **TSHR-EX4R** | **CCCGACCCAGGCTATACACCATT** |
| **TSHR-M13F-EX5F** | **GTAAAACGACGGCCAGTAGTTTGACTACAGGTTGTCTTC** |
| **TSHR-EX5R** | **GCTTTACTTATCTTCAACCTACC** |
| **TSHR-M13F-EX6F** | **GTAAAACGACGGCCAGTTATTGTGTCCTGTTATTTAAGTGCATA** |
| **TSHR-EX6R** | **GTACTCTATAGAGTATATATGATAAGG** |
| **TSHR-M13F-EX7F** | **GTAAAACGACGGCCAGTTGGGATACATATGTGGGACCTG** |
| **TSHR-EX7R** | **TGTTGGTCACACTAACTCTGG** |
| **TSHR-M13F-EX8F** | **GTAAAACGACGGCCAGTTGGTCACATTTTATTCTGATATTTGT** |
| **TSHR-EX8R** | **CTCCCCTTAATGTCTCCATTTATTCC** |
| **TSHR-EX8RSeq** | **ATATTCTTTTGTATGTCTTACTC** |
| **TSHR- EX9F** | **TATCCATCCCTCCTTAGACCAGAA** |
| **TSHR- M13F-EX9R** | **GTAAAACGACGGCCAGTACTGTGTCCCATGGATTTCCTGT** |
| **TSHR-M13F-EX10F** | **GTAAAACGACGGCCAGTTGGCACTGACTCTTTTCTGT** |
| **TSHR-EX10R** | **GTGTCATGGGATTGGAATGC** |
| **TSHR-EX10FSeq** | **ACTGTCTTTGCAAGCGAGTT** |
| **TSHR-EX10RSeq** | **GTCCATGGGCAGGCAGATAC** |

**Note: Underlined sequences indicate the attached universal M13F sequence.**

**Overview of PCR and Sanger sequencing conditions:**

All reactions were carried out in 30 l under the standard conditions recommended for HotStarTaq® DNA Polymerase ([www.qiagen.com/HB-0452](http://www.qiagen.com/HB-0452), QIAGEN GmbH, Hilden, GERMANY), with 0.1 M of each primer. The utilized annealing temperature was 60oC for most reactions, except for the *FOXE1* (63oC) and exon 10 *TSHR* (66oC) amplicons, which also used final concentrations of 1X and 0.5X, respectively, of Q-Solution 5X (QIAGEN GmbH, Hilden, GERMANY). A modified dNTP 10 mM mix containing 25% of deaza-dGTP and 75% of dGTP (ratio 1:3) was employed to achieve better sequence results for the *FOXE1* amplicons, which possess a high GC content (>70%). All PCR products were evaluated by agarose gel electrophoresis, subjected to further enzymatic purification (ExoSAP-IT® PCR Product Cleanup, Affymetrix, Inc., Santa Clara, CA, USA), and then unidirectionally sequenced with the universal M13F primer (5’-GTAAAACGACGGCCAGT-3’) and Big Dye® Terminator Cycle Sequencing chemistry (Life Technologies Corp.; performed at PSOMAGEN INC. Rockville, Maryland, USA). Genotypes were determined by direct visualization of each electropherogram after its annealing with reference sequences using Chromas Pro Version 1.7.7. (Technelysium Pty Ltd.) and with a Phred score >40. Ambiguous results were resolved by performing opposite-strand sequencing with the complementary primer (reverse or forward without the M13F sequence) or internal sequencing primer (TSHR-EX8RSeq, TSHR-EX10Fseq, and TSHR-EX10RSeq). Variant annotation compliant with Human Genome Variation Society nomenclature (<https://varnomen.hgvs.org/>) was performed using the Alamut® Visual version 2.14 software (SOPHiA GENETICS, Lausanne, Switzerland).

**Supplementary Table 1-B. Oligonucleotides and PCR conditions employed to search rare variants in healthy and ethnically matched controls by means of allele-specific PCR assays.**

|  |  |
| --- | --- |
| **GENE VARIANT** | **PRIMER NAME, SEQUENCE, AND OPTIMAL ANNEALING TEMPERATURE** |
| ***FOXE1* NM\_004473.3:**c.370G>C or p.(Gly124Arg) at forkhead domain.  [NP\_004464.2:p.(Gly124Arg)](https://variantvalidator.org/service/validate/) | **Gly124Arg-FOXE1-Fwd**  **5´-CTGGCTACCGTGAAGGAAGA-3´**  **WT-Gly124-FOXE1-R**  **5´-AGCGCCCAGTAGTTGCC-3´**  **MUT-Arg124-FOXE1-R**  **5´-GCGCCCAGTAGTTGCG-3´**  **ANNEALING TEMPERATURE: 61º C** |
| ***FOXE1* NM\_004473.3:**c.1004C>G or p.(Ala335Gly).  [NP\_004464.2:p.(Ala335Gly)](https://variantvalidator.org/service/validate/) | **WT-Ala335-FOXE1-F**  **5´-CGGCCAGTTCGGAGC-3´**  **MUT-Gly335-FOXE1-F**  **5´-CGGCCAGTTCGGAGG-3´**  **Ala335Gly-FOXE1-Rev**  **5´-AACGTGTGAACAGCCGATG-3´**  **ANNEALING TEMPERATURE: 64º C** |
| ***NKX2-5* NM\_004387.3:**c.355G>T or p.(Ala119Ser).  [NP\_004378.1:p.(Ala119Ser)](https://variantvalidator.org/service/validate/) | **NKX2-5-Fwd**  **5´-AGTGCACTTGGCAGAGTGAG-3´**  **WT-Ala119-NKX2-5-R**  **5´-TCTCCAGCTCCACCGC-3´**  **MUT-Ser119NKX2-5-R**  **5´-CTTCTCCAGCTCCACCGA-3´**  **ANNEALING TEMPERATURE: 64º C** |
| ***TSHR* NM\_000369.2:**c.352G>A or p.(Asp118Asn).  [NP\_000360.2:p.(Asp118Asn)](https://variantvalidator.org/service/validate/) [EXON 4]  ***TSHR* NM\_000369.2:**c.1264T>C or p.(Trp422Arg).  [NP\_000360.2:p.(Trp422Arg)](https://variantvalidator.org/service/validate/) [EXON 10] | **ARMS-TSHR-EX4R-NL**  **5´-GCTCTTTGAGGGCATCAGGGTC-3´**  **ARMS-TSHR-EX4R-NL-B**  **5´-GCTCTTTGAGGGCATCAGAGTC-3´ \***  **ARMS-TSHR-EX4R-D118N-B**  **5´-GCTCTTTGAGGGCATCAGAGTT-3´ \***  **ANNEALING TEMPERATURE: 65º C**  **ARMS-TSHR-EX10F**  **5´-GGGGACAGTGAAGACATGGTGT-3´**  **ARMS-TSHR-EX10R-NL-B**  **5´-GCCAGCAGACTAACGAGCCA-3´ \***  **ARMS-TSHR-EX10R-W422R-B**  **5´-GCCAGCAGACTAACGAGCCG-3´ \***  **ANNEALING TEMPERATURE: 65º C** |

\* Underlined nucleotides: Introduced mismatches to increase genotyping astringency of PCR assays.

**Overview of allele-specific PCR assays:**

All reactions were carried out in 30 l under the standard conditions recommended for HotStarTaq® DNA Polymerase ([www.qiagen.com/HB-0452](http://www.qiagen.com/HB-0452), QIAGEN GmbH, Hilden, GERMANY), with 0.1 M of each primer and 10-30 ng genomic DNA. Reactions were carried out with 25-30 PCR cycles, and the presence of amplicons was determined by agarose gel electrophoresis. All assays included a positive DNA control for each genotype (heterozygous patient and normal homozygous healthy control).