



## Lab Resource: Multiple Cell Lines

## Generation of three induced pluripotent stem cell lines from a patient with Usher syndrome caused by biallelic c.949C > A and c.1256G > T mutations in the *USH2A* gene



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## A B S T R A C T

Mutations in the *USH2A* gene are the most common cause of Usher syndrome and autosomal recessive non-syndromic retinitis pigmentosa. Here, we describe the generation of three induced pluripotent stem cell lines from dermal fibroblasts derived from a patient carrying biallelic c.949C > A and c.1256G > T variants in the *USH2A* gene, using episomal reprogramming plasmids expressing *OCT4*, *SOX2*, *KLF4*, *MYCL*, *LIN28*, mir302/367 and shRNA targeting *TP53*. All three lines expressed pluripotency markers, displayed unaltered karyotypes as well as trilineage differentiation potential, and were negative for reprogramming episomes and mycoplasma.

## 1. Resource table

(continued)

Unique stem cell lines identifier	LEIi014-A LEIi014-B LEIi014-C	Cell Source	Dermal fibroblasts
Alternative names of stem cell lines	1012ips9 1012ips10 1012ips11	Clonality	Clonal
Institution	Lions Eye Institute	Method of reprogramming	Episomal plasmids
Contact information of distributor	Samuel McLenahan: <a href="mailto:smclenahan@lei.org.au">smclenahan@lei.org.au</a> Fred K. Chen: <a href="mailto:fredchen@lei.org.au">fredchen@lei.org.au</a>	Multiline rationale	Isogenic clones
Type of cell lines	iPSC	Gene modification	Yes
Origin	Human	Type of modification	Inherited mutations
		Associated disease	Usher syndrome type 2
		Gene/locus	<i>USH2A</i> /1q41
		Method of modification	N/A
		Name of transgene or resistance	N/A
			N/A

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<sup>1</sup> These authors contributed equally.

(continued)

Inducible/constitutive system	
Date archived/stock date	16/07/2018
Cell line repository/bank	<a href="https://hpscreg.eu/cell-line/LEIi014-A">https://hpscreg.eu/cell-line/LEIi014-A</a> <a href="https://hpscreg.eu/cell-line/LEIi014-B">https://hpscreg.eu/cell-line/LEIi014-B</a> <a href="https://hpscreg.eu/cell-line/LEIi014-C">https://hpscreg.eu/cell-line/LEIi014-C</a>
Ethical approval	University of Western Australia Human Research Ethics Committee RA/4/1/7916

## 2. Resource utility

Usher syndrome is the most common cause of deaf-blindness. Here, three iPSC lines were generated from a patient with Usher syndrome type 2 (USH2). Together with our previously published lines, LEIi0010-A and -B, these sibling lines provide a useful resource for investigating the effects of *USH2A* mutations in patient-derived cells.

## 3. Resource details

Usher syndrome is the most common disorder affecting both vision and hearing, accounting for approximately 5% of congenital deafness and 18% of retinitis pigmentosa (RP) cases worldwide (Millan et al., 2011; Toms et al., 2015). Mutations in the *USH2A* gene are the most common cause of Usher syndrome and autosomal recessive non-syndromic retinitis pigmentosa (Millan et al., 2011; McGee et al., 2010; Seyedahmadi et al., 2004). In this report, we present the generation and characterization of three iPSC lines from a patient with USH2 caused by compound heterozygous mutations in *USH2A*.

The female patient presented at the age 13 with mild congenital neurosensory hearing loss and retinal features consistent with retinitis pigmentosa. Two known pathogenic variants were identified in the *USH2A* gene (NM\_206933.2), located in exon 6 (c.949C > A p.(=, Tyr318Cysfs\*17)) and exon 7 (c.1256G > T p.(Cys419Phe)). We previously reported the generation of two iPSC lines from this patient's sister, who carries the same biallelic *USH2A* variants and displayed a similar disease phenotype (McLenaghan et al., 2019).

Dermal fibroblasts were obtained from a skin biopsy sample and reprogrammed into iPSC using episomal plasmids expressing *OCT4*, *SOX2*, *KLF4*, *MYCL*, *LIN28*, *TP53* shRNA, and the miR-302/367 cluster. Three clonal iPSC lines (LEIi014-A, LEIi014-B and LEIi014-C) were picked and expanded for further characterization (Fig. 1, Tables 1 and 2). All three lines formed iPSC colonies comprised of tightly packed small cells with high nucleus-to-cytoplasm ratios, typical of pluripotent stem cells (Fig. 1A). Expression of the pluripotency markers *OCT4*, *NANOG*, *SOX2* and *TRA-1-81* was demonstrated by immunostaining (Fig. 1A). All three lines were shown to carry the c.949C > A and c.1256G > T *USH2A* variants by Sanger sequencing (Fig. 1B). Digital karyotyping was performed on each line using the Illumina HumanCoreExome-24 Beadchip assay. Genome-wide copy number variation (CNV) analysis demonstrated both lines were female and had a normal diploid karyotype (46, XX). Analysis of the B allele frequencies (upper panels) and LogR ratios (lower panels) of 500,000 single nucleotide polymorphisms located across the genome confirmed the absence of chromosomal rearrangements induced by reprogramming (Fig. 1C). *OCT4*, *NANOG*, *C-MYC* and *KLF4* mRNA was expressed at similar levels in the three patient iPSC lines and a control human iPSC line (Fig. 1D). Upon spontaneous differentiation as embryoid bodies, all three iPSC lines showed induction of ectoderm (*PAX6*, *OTX1*), mesoderm (*DCN*, *IGF2*) and endoderm (*GATA2*, *SOX7*) genes and downregulation of *OCT4* expression, demonstrating the potential for differentiation into the three major developmental lineages (Fig. 1E). Genotyping of 16 microsatellite markers confirmed all three iPSC lines were derived from the patient's fibroblasts (Supp Fig. 1A). All three lines were negative for reprogramming episomes (Supp Fig. 1B) and mycoplasma (Supp Fig. 1C).

## 4. Materials and methods

### 4.1. Ethics

Genetic analysis was performed by the Australian Inherited Retinal Disease Registry and DNA Bank, with approval from the Sir Charles Gairdner Hospital Human Research Ethics Committee (2001–053). The generation of iPSC lines from patient fibroblasts was approved by the University of Western Australia Human Research Ethics Committee (RA/4/1/7916). Written consent was obtained from the patient and all procedures were carried out in accordance with the requirements of the National Health & Medical Research Council of Australia (*National Statement on Ethical Conduct in Human Research (2007, updated 2018)* and the Declaration of Helsinki.

### 4.2. Cell culture

Dermal fibroblasts were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal calf serum (ThermoFisher, Waltham, Massachusetts, United States). Fibroblasts were passaged once per week using a split ratio of 1:5. For passaging, fibroblast cultures were rinsed in phosphate buffered saline (PBS) then dissociated by incubation with TrypLE (ThermoFisher) for 5 min at 37 °C. An equal volume of culture media was then added to the suspension and cells were pelleted by centrifugation (200g for 5 min). Fibroblasts were then resuspended in culture media and seeded into new flasks.

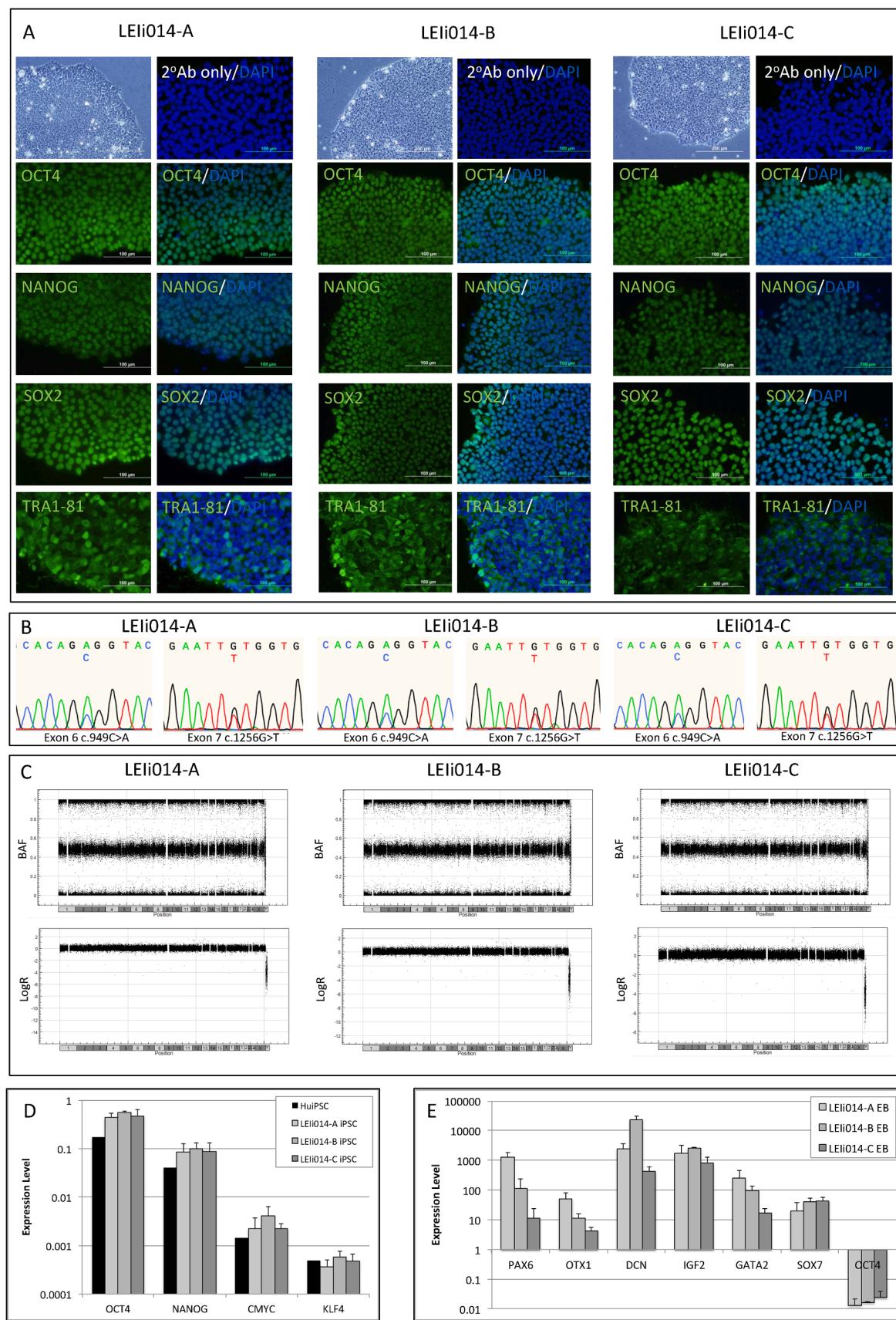
Human iPSC lines were cultured on Geltrex (ThermoFisher) coated culture plates in mTesR1 media (StemCell Technologies, Vancouver, Canada) and were passaged every 4–5 days using a split ratio of 1:3–1:5. For passaging, culture media was removed and replaced with EDTA solution (0.5 mM EDTA in PBS) and incubated at room temperature for 4–5 min. The EDTA solution was then replaced with mTesR1 media and iPSCs were harvested as floating cell clusters by mechanical dissociation before seeding onto new Geltrex-coated 6-well plates. Both fibroblasts and iPSC cultures were cultured at 37 °C with 5% CO<sub>2</sub>. A commercial iPSC line (ThermoFisher, Cat#A18945, HuiPSC) was used as a control.

### 4.3. Reprogramming

Patient fibroblasts were reprogrammed using the Episomal iPSC Reprogramming Plasmid kit (SC900A-1, System Biosciences, Palo Alto, California, United States), according to the manufacturer's instructions. Approximately 1 × 10<sup>6</sup> fibroblast cells were electroporated using the NEON electroporation system (3 × 10 ms pulses at 1650 V in a 100 μl tip) and plated into 6 wells of a 6-well plate containing DMEM supplemented with 10% fetal calf serum. On day 7 after electroporation, the media was changed to TeSR-E8 (StemCell Technologies). Individual iPSC colonies were picked for clonal expansion after 4 weeks. After 10–15 passages, iPSCs were cultured in mTesR1 media (StemCell Technologies).

### 4.4. Trilineage differentiation

To induce spontaneous differentiation, iPSCs were cultured as embryoid bodies (EB) for two weeks. Patient iPSCs were prepared as cell cluster suspensions (see above) and seeded into 6-well suspension culture plates containing mTesR1 media and 10 μM Y27632 (Abcam, Cambridge, United Kingdom). After 3 days of culture, media was changed to DMEM/F12 supplemented with 20% knockout serum replacement, antibiotic-antimycotic (ThermoFisher) and MEM non-essential amino acid solution (StemCell Technologies). At Day 7, EB were transferred to Geltrex-coated adherent plates and maintained for another 7 days in the same media. Media were changed daily and RNA was harvested for qPCR analysis at Day 14.



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**Fig. 1.** A) LEli014-A, LEli014-B and LEli014-C displayed typical iPSC colony morphology in culture. Expression of OCT4, NANOG, SOX2 and TRA-1-81 (green signal) was demonstrated by immunostaining analysis. Cell nuclei were labelled with DAPI (blue signal). B) Targeted Sanger sequencing demonstrated the presence of the c.949C > A and c.1256G > T variants in the *USH2A* gene in all three clonal iPSC lines. C) Digital karyotyping results for the LEli014-A, LEli014-B and LEli014-C iPSC lines confirmed the absence of reprogramming induced genomic alterations. D) Quantitative RT-PCR analysis demonstrated *OCT4*, *NANOG*, *C-MYC* and *KLF4* were expressed at similar levels in LEli014-A, LEli014-B and LEli014-C (grey bars), and a control human iPSC line (black bars, HuiPSC). Expression of each marker was normalized to *GAPDH* expression. E) Embryoid bodies derived from LEli014-A, LEli014-B and LEli014-C iPSC showed upregulation of ectoderm (*PAX6*, *OTX1*), mesoderm (*DCN1*, *IGF2*) and endoderm (*GATA2*, *SOX7*) markers, and downregulation of *OCT4* expression, compared with undifferentiated iPSC. Expression of each marker was normalized to *GAPDH* expression and expressed as fold-change compared with undifferentiated iPSC.

**Table 1**  
Summary of lines.

iPSC line names	Abbreviation in figures	Gender	Age	Ethnicity	Genotype of locus	Disease
LEli014-A	1012ips9	Female	15 years old	Caucasian	<i>USH2A</i> c.[949C > A]; [1256G > T]	Usher syndrome type 2
LEli014-B	1012ips10	Female	15 years old	Caucasian	<i>USH2A</i> c.[949C > A]; [1256G > T]	Usher syndrome type 2
LEli014-C	1012ips11	Female	15 years old	Caucasian	<i>USH2A</i> c.[949C > A]; [1256G > T]	Usher syndrome type 2

**Table 2**  
Characterization and validation.

Classification	Test	Result	Data
Morphology	Photography	Normal iPSC morphology	<a href="#">Fig. 1A</a>
Phenotype	Qualitative analysis (Immunocytochemistry)	Positive for expression of pluripotency markers: <i>OCT4</i> , <i>NANOG</i> , <i>SOX2</i> , <i>TRA-1-81</i>	<a href="#">Fig. 1A</a>
	Quantitative analysis (RT-qPCR)	Similar expression of <i>OCT4</i> , <i>NANOG</i> , <i>C-MYC</i> and <i>KLF4</i> in patient lines compared with control line	<a href="#">Fig. 1D</a>
Genotype	Karyotype (Illumina Beadchip) and resolution	46, XX Resolution 0.5 Mb	<a href="#">Fig. 1C</a>
Identity	STR analysis	DNA Profiling by STR analysis performed Matched at 16 loci	<a href="#">Supp. Fig. 1A</a> Submitted in archive with journal
Mutation analysis (IF APPLICABLE)	Sequencing	Compound heterozygous for c.949C > A and c.1256G > T in <i>USH2A</i>	<a href="#">Fig. 1B</a>
Microbiology and virology	Southern Blot OR WGS Mycoplasma	N/A Mycoplasma testing by PCR assay: Negative	N/A <a href="#">Supp. Fig. 1C</a>
Differentiation potential	Embryoid body formation	Upregulation of markers of ectoderm ( <i>PAX6</i> , <i>OTX1</i> ), mesoderm ( <i>DCN</i> , <i>IGF2</i> ) and endoderm ( <i>GATA2</i> , <i>SOX7</i> )	<a href="#">Fig. 1E</a>
Donor screening (OPTIONAL)	HIV 1 + 2 Hepatitis B, Hepatitis C	N/A	N/A
Genotype additional info (OPTIONAL)	Blood group genotyping HLA tissue typing	N/A N/A	N/A N/A

#### 4.5. Quantitative PCR analysis

Total RNA was isolated from cell culture samples using TRIzol reagent (ThermoFisher). cDNA was synthesized using the RT<sup>2</sup> First Strand Kit (Qiagen) and qPCR was performed using the CFX Connect Real-Time System (Bio-Rad Laboratories, Hercules, California, United States) with the PowerSYBR Green PCR Master Mix (Applied Biosystems, Foster City, California, United States). Data was analyzed using the  $\Delta\text{C}_T$  method. Gene expression values were expressed as fold-changes compared with the *GAPDH* housekeeping gene ([Fig. 1D](#)) or with expression levels in undifferentiated iPSCs ([Fig. 1E](#)). Mean expression values and standard deviations were calculated from three independently cultured iPSC or EB cDNA samples. Primers used in qPCR assays are listed in [Table 3](#).

#### 4.6. Immunostaining analysis

Patient iPSCs were cultured on Geltrex-coated coverslips for 5 days, then fixed by incubation with 4% paraformaldehyde for 15 min at 37 °C. Fixed iPSCs were washed with PBS, and permeabilized using 0.3% Triton X-100 in PBS for 15 min. Coverslips were then incubated with blocking buffer (5% normal goat serum in PBS containing 0.3% Triton X-100) for 1 h at room temperature, followed by primary antibodies (diluted in blocking buffer) at 4 °C overnight. Coverslips were washed three times in PBS followed by incubation with blocking buffer containing secondary antibodies and DAPI (1 µg/mL) for 2 h at room temperature in the dark. The antibodies used and their respective dilutions are listed in [Table 3](#). Coverslips were then washed three times in PBS and mounted onto slides. Cells were imaged using the Olympus BX60 fluorescence microscope equipped with DP-Controller 3.1.1.267 acquisition software (Olympus Corporation, Tokyo, Japan) and merged using ImageJ software (version 1.43).

#### 4.7. Digital karyotyping and microsatellite analysis

To identify genomic insertions or deletions (excluding balanced translocations), digital karyotyping was performed by the Australian Genome Research Facility, (AGRF, Perth, Western Australia), using the Infinium HumanCytoSNP-12 Beadchip SNP array (Illumina, San Diego, California, United States). CNV analysis was performed using GenomeStudio 2.0 software with the CNVpartition 2.0 plugin (Illumina). Microsatellite analysis was performed by the AGRF using the Promega Powerplex 16H system (Promega, Madison, Wisconsin, United States).

#### 4.8. Polymerase chain reaction assays

Genomic DNA was isolated from iPSCs and fibroblasts using the FlexiGene DNA Kit (Qiagen, Hilden, Germany). The Lookout

**Table 3**  
Reagents details.

Antibodies used for immunocytochemistry/flow-cytometry			
	Antibody	Dilution	Company Cat # and RRID
Pluripotency Markers	Mouse anti-OCT4	1:200	Santa Cruz Biotechnology Cat# sc-5279, RRID: AB_628051
	Rabbit anti-NANOG	1:100	Abcam Cat# ab21624, RRID:AB_446437
	Rabbit anti-SOX2	1:200	Thermo Fisher Scientific Cat# 48-1400, RRID: AB_2533841
	Mouse anti-TRA-1-81	1:100	Stem Cell Technologies Cat# 60,065 CE, RRID: AB_1089240
Secondary antibodies	Goat anti-mouse IgG Alexa Fluor 488	1:500	Thermo Fisher Scientific, Cat# A28175, RRID: AB_2536161
Primers			
	Target	Forward/Reverse primer (5'-3')	
Episomal Plasmids PCR Pluripotency Markers (qPCR)	Episomal Plasmids	AGGTCCCTCGAAGAGGGTCA/ TTCCAACCGAGAAAGGTGTT	
	OCT4	CCTGAAGCAGAACAGGATCAC/ AAAGGGCAAGATGGTCGTTGG	
	NANOG	CTCCAACATCTGAACCTCAGC/ CGTCACACATTGCTATTCTCG	
	C-MYC	CCTGGTGCTCCATGAGGAGAC/ CAGACTCTGACCTTTGCCAGG	
Ectoderm Markers (qPCR)	KLF4	KLFA CATCTCAAGGCACACCTCGGAA/ TCGTCGCATTGGACTCGG	
	PAX6	CTGAGGAATCAGAGAACAGGC/ ATGGAGCCAAGATGTAAGGAGG	
	OTX1	CTACCTGACATCTTCATGCCG/ GGAGAGGACTCTCTTGGCTG	
Mesoderm Markers (qPCR)	DCN	AGAGTACCTGGCTGGCTGG/ GTGGGCAGAACGTCACTTGAT	
	IGF2	AGACGTACTGTGCTACCCC/ TGCTCCAGGTGTCAATTG	
Endoderm Markers (qPCR)	GATA2	CTGTCGCAACGCCGTGT/ GTTCCGAGTCTGGATCCCTT	
	SOX7	TCGACGCCCTGGATCACT/ CTGGGAGACCGGAACATGC	
House-Keeping Genes (qPCR)	GAPDH	GTCTCCCTGACTTCAACAGCG/ ACCACCCCTGTGCTGTAGCCAA	
Targeted mutation sequencing	USH2A c.949C > A	TGACATTCTTGTAAACGACTCC/ AAGTTTGTGGCATTTGTTGAA	
	USH2A c.1256G > T	ATGTTGCTTTTACACAGGGCT/ AGCCACACTAAAACAGTTCTTGAA	

Mycoplasma PCR Detection Kit (Sigma-Aldrich, St. Louis, Missouri, United States) was used for mycoplasma detection according to the

manufacturer's recommendations. For detection of reprogramming plasmids in iPSCs, PCR reactions with primers targeting episomal plasmid sequences were performed, with early stage (p0) iPSCs used as a positive control. For sequencing of the c.949C > A and c.1256G > T variants, primers pairs targeting the affected exons were used to amplify genomic DNA using the Q5 High Fidelity 2X Master Mix (New England BioLabs, Ipswich, Massachusetts, United States), followed by amplicon purification using the Wizard Gel and PCR Clean-Up System (Promega) and Sanger sequencing (Australian Genome Research Facility). Primer sequences are listed in Table 3.

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## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.scr.2020.102129>.

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