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A B S T R A C T

Primary Ciliary Dyskinesia (PCD) is a rare heterogeneous genetic disorder affecting motile cilia structure and function leading to lung disease. We generated induced pluripotent stem cells (iPSCs) from dermal fibroblasts of a female PCD patient carrying disease-causing variants in the CCDC40 gene. Reprogramming was performed with the human OSKM transcription factors using the Sendai-virus delivery system. The resulting transgene free iPSCs had normal karyotype, expressed pluripotency markers, could differentiate into the three germ layers in vivo and retained the disease-causing CCDC40 mutations. This iPSC line could be useful to model PCD disease and test gene therapy strategies.

Resource Table

Unique stem cell line identifier	UHOMi001-A
Alternative name(s) of stem cell line	iPCD02.30
Institution	Institute for Regenerative Medicine & Biotherapy (IRMB), Montpellier, FRANCE
Contact information of distributor	John De Vos john.devos@inserm.fr
Type of cell line	iPSC
Origin	human
Additional origin info	Age: 34 Sex: Female
Cell Source	Dermal fibroblasts
Clonality	Clonal
Method of reprogramming	hOCT4, hSOX2, hC-MYC, hKLF4 (CytoTune™-iPS 2.0 Sendai Reprogramming Kit - Invitrogen, Thermo Fisher Scientific Inc.)
Genetic Modification	YES
Type of Modification	Spontaneous mutation
Associated disease	Primary Ciliary Dyskinesia

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Gene/locus	Compound Heterozygous mutations the Coiled-Coil Domain Containing 40 Gene (CCDC40): c.1116_1117delCT (Exon 7) and c.3180 + 1G > A (Intron 19)
Method of modification	N/A
Name of transgene or resistance	N/A
Inducible/constitutive system	N/A
Date archived/stock date	2018-02-12
Cell line repository/bank	N/A
Ethical approval	The study was approved by the regional scientific ethical committee (CPP Sud Med IV) under the number ID-RCB: 2013-A00892–43/CILIPS, Promoter University Hospital Of Montpellier) and informed consent was obtained from the patient.

1. Resource utility

Primary Ciliary Dyskinesia (PCD) is a rare and heterogeneous genetic lung disease (Boaretto et al., 2016) associated with defective cilia motility (Knowles et al., 2013). Generation of a PCD IPS cell line is a promising model to study PCD since IPSC can be differentiated into airway epithelium.

2. Resource details

In this study, dermal fibroblasts were obtained from a 34-year-old woman with PCD with disease-causing variants in the *Coiled-Coil Domain Containing 40* (CCDC40) gene [c.1116_1117delCT (Exon 7) and c.3180 + 1G > A (Intron 19)]. Patient displayed chronic rhinitis, mild lower respiratory symptoms with bronchiectasis and infertility. To generate the UHOMi001-A, the four reprogramming factors OCT3/4, SOX2, KLF4, and C-MYC were transiently expressed in the fibroblasts using the integration-free Sendai virus gene-delivery method. UHOMi001-A had a normal morphology (Fig. 1, panel A, bright field microscopy; scale bar: 50 µm) and also had structurally and numerically normal diploid karyotype (46, XX), (Fig. 1, panel B). During culture, the iPSCs were tested for the absence/presence of Sendai virus vector which was analysed by PCR using Sendai virus vector (SeV) specific primers. After about 10 passages, the elimination of the reprogramming vector SeV was confirmed in the iPSC line using PCR, which was selected for further analysis (Fig. 1, panel H). The expression of the pluripotency genes OCT4, NANOG, SOX2 were assessed by qRT-PCR and the gene expression levels were similar to a characterized positive control human embryonic stem cell (hESC 291) used as a positive control genes (ΔΔCt method, Mean ± S.E.M) (Fig. 1, panel C). Expression of pluripotency markers was evidenced by immunocytochemistry staining, using antibodies against human OCT3/4, TRA-1-60, SOX2 and NANOG (Fig. 1, F scale bar 20 µm, X400) and by flow cytometry using the cell surface markers TRA-1-81 and SSEA4 (Fig. 1, panel E). Pluripotency was substantiated by the ability of UHOMi001-A to differentiate into the three germ layers in vivo, as confirmed by the formation of teratoma in an immunosuppressed mouse. Immunohistochemistry revealed in these teratomas the presence of neural rosettes (ectoderm layer), vimentin positive cells (mesoderm) and CDX2 positive cells (posterior gut endoderm marker) (Fig. 1, panel G). The UHOMi001-A iPS cell line identity with parental fibroblasts was confirmed by the presence of the disease-associated mutation in the CCDC40 gene by DNA sequencing (Fig. 1, panel D). DNA electrophoregram of 5'-Sanger sequencing showing the two mutations in CCDC40 gene [c.1116_1117delCT (Exon 7) and c.3180 + 1G > A (Intron 19)] present both in the iPSC cell line UHOMi001-A and the parental fibroblasts. Capital letters correspond to exonic nucleotides, lower case letters correspond to intronic nucleotides. Location of sequences variations are mentioned in red in the DNA sequence. Microsatellite analysis shows matching profiles of short tandem repeats (STR) amplified from a genomic DNA UHOMi001-A iPS cells and patient-derived dermal fibroblasts (matching of 18/18 loci: TAF9, D21S11, D21S1270, D13S634, D18S391, D13S742, AMXY, D21S1411N, D13S305, D21S213N, D18S1112N, D13S628, DYZ3, D18S535, D21S224, D21S370N, SRY and D18S386; available with authors). The cell line was also confirmed to be free of mycoplasma (Supplemental Table 1).

3. Materials and methods

3.1. Reprogramming of fibroblasts to iPSCs

Human dermal fibroblasts (hDFs) were cultured in Dulbecco's modified Eagle medium (DMEM)-GlutaMAX (Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (FBS) (Gibco, Life Technologies), 100 U/mL penicillin, 100 µg/mL streptomycin (Gibco, Life Technologies). The cells were transduced by Sendai-virus using the CytoTune®-iPS 2.0 Sendai Reprogramming Kit (Thermo Fisher Scientific), following manufacturer's instruction. The calculated volumes of each of the three CytoTune™ 2.0 Sendai-virus (SeV) was added to the cells using MOI of 10–10-6 (KOS MOI = 10, hc-Myc MOI = 10, hKlf4 MOI = 6). The colonies with an ES-like appearance were manually isolated based on morphology since Day 21.

3.2. Cell culture of iPSC cell line

UHOMi001-A cell line was cultured in feeder-free conditions on Matrigel (BD Biosciences) in mTeSR™1 medium (StemCell Technologies), or on Geltrex matrix (Thermo Fisher Scientific) in E8 medium (Thermo Fisher Scientific), according to the manufacturer's instruction. The cells were cultivated in at 37 °C in normoxic conditions and 5% CO_2 and picked manually. Then, they were later adapted by passaging them using EDTA dissociation (Versene Solution, Thermo Fisher Scientific) every week into clumps, using 1:20 split ratio.

3.3. Karyotyping

Karyotyping was performed at mechanic passage 15, on G and R-banded metaphases using standard procedures. At least 12 metaphases were examined per sample with an approximate resolution of 400–450 bands using a conventional microscope and IKAROS software (Metasystems). Karyotyping was performed using a service provider (Unit of Chromosomal Genetics, Department of Medical Genetics, Montpellier University Hospital).

3.4. Detection of disease-causing mutations in CCDC40 gene

The detection of mutations in CCDC40 gene was performed in the platform Montpellier University Hospital (Rare Genetic Diseases Laboratory). DNA extraction was performed using QIAamp DNA Mini Kit (Qiagen), following manufacturer's instructions. PCR MM2X (Promega) was performed with 1 µL of both forward and reverse

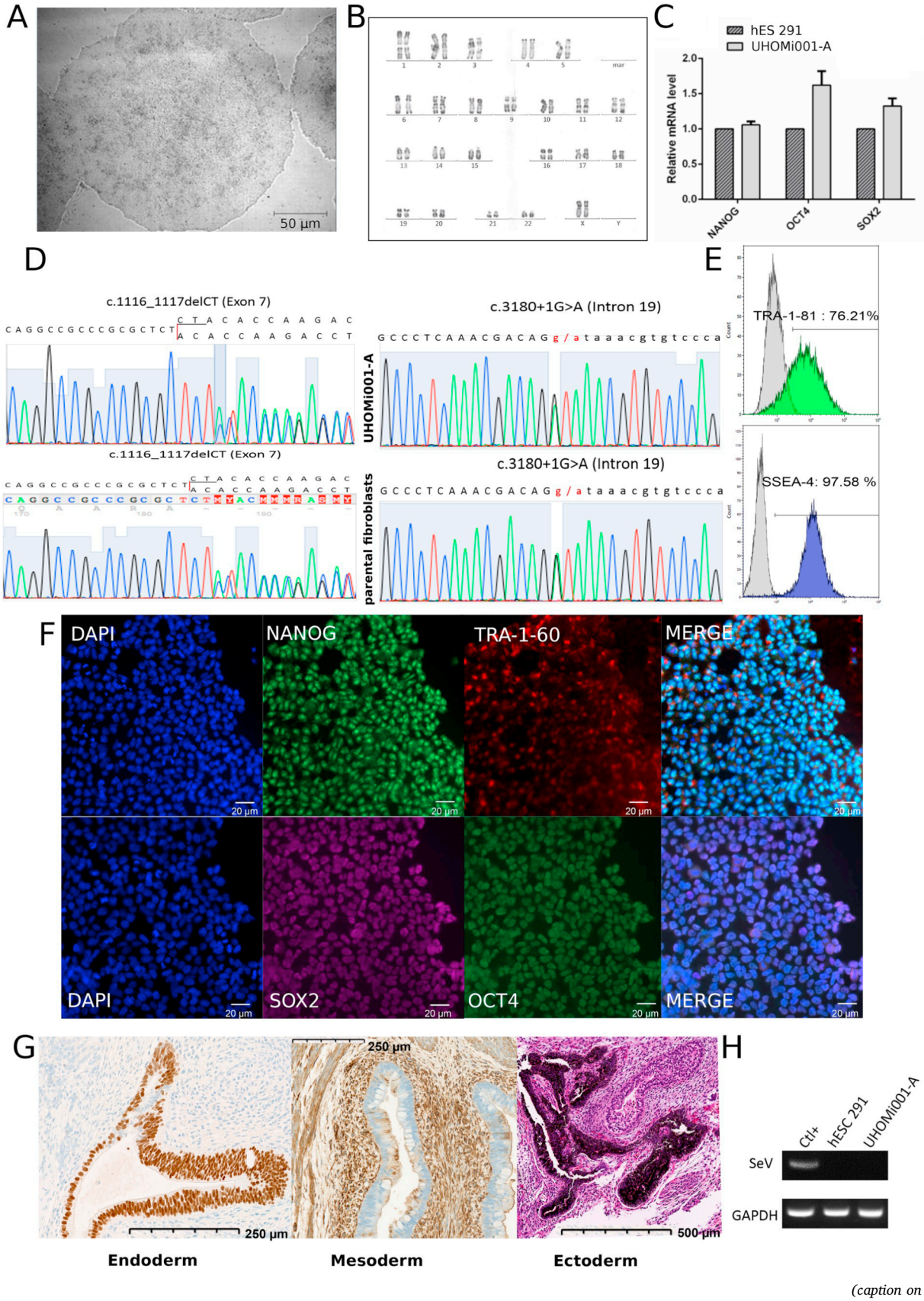

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Fig. 1. Characterization of the UHOMi001-A cell line. A. Morphology of iPS cell colonies as observed by bright field microscopy. Scale bar: 50 μm. B. Karyotyping. Representative karyotype (46 XX). C. Assessment of pluripotency of iPS cells by qRT-PCR of OCT3/4, NANOG and SOX2 genes (ΔΔCt method, Mean ± S.E.M). Human Embryonic Stem Cells line (hESC 291) was used as positive control. Gene expression levels were normalized to the housekeeping gene GAPDH using the following formula 100/2ΔΔCt where ΔΔCt = ΔCtsample − ΔCtpositive control. D. DNA electrophoregram of 5'-Sanger sequencing showing the two mutations in CCDC40 gene [c.1116_1117delCT (Exon 7) and c.3180 + 1G > A (Intron 19)] in the iPSC cell line UHOMi001-A and parental fibroblasts. Capital letters correspond to exonic nucleotides, lower case letters correspond to intronic nucleotides. Localisation of sequences variations are mentioned in red in the DNA sequence. Primers used are listed in Table 1. E. Expression of pluripotent stem cell markers TRA-1-81 and SSEA4 on the surface of UHOMi001-A iPS cells as measured by flow cytometry. F. Immunostaining for OCT4, NANOG, TRA-1-60 and SOX2 showing the iPS colonies uniformly expressing the markers. Nucleus is labelled with DAPI blue). X400, Scale bar: 20 μm. G. Immunochemistry of teratoma for the endodermal (CDX2 marker), mesodermal (vimentin marker) and ectodermal (pigmented neural tubes) germ layers. H. PCR showing the absence of Sendai-virus from the reprogrammed cells.

primers (10uM) and reacted during 35 cycles at Tm 60 °C using C1000 Thermal Cycler (Bio-Rad). Migration of the products was performed on 1.5% TAE agarose gel. Purification was performed with illustra™ ExoStar™ 1-Step (GEHealthcare Life Sciences). CCDC40 mutations were confirmed by Sanger sequencing of PCR amplicons Sanger sequencing was achieved using the BigDye® Terminator v1.1 Cycle Sequencing Kit on the Applied Biosystems 3130xl.

3.5. Quantitative real time polymerase chain reaction (qRT-PCR)

RNA isolation was performed using the RNAeasy mini kit (Qiagen) according to manufacturer's instructions. Reverse transcription (RT) was performed with 500 ng of RNA using Superscript II (ref. 18,064–014, Invitrogen). QRT-PCR were performed using the SYBR Green I Master kit (Roche Diagnostics). The amplification was run in a Light Cycler 480. Gene expression levels were normalized to the housekeeping gene Glyceraldehyde 3-Phosphate Dehydrogenase (GAPDH) using the following formula $100/2^{\Delta\Delta Ct}$ where ΔΔCt = ΔCtsample − ΔCtpositive control. Primers are listed in Table 1.

3.6. Teratoma formation

IPS cells were dissociated into small clumps with Versene Solution during 10 min at 37 °C. 1×10^6 cells were resuspended in a small volume of Matrigel (Corning, 354277) and transplanted subcutaneously in anesthetized NOD.CgPrkdcscidIl2rg tm1Wjl/SzJ (NSG) mice. Mice were sacrificed after seven to ten weeks. Teratomas were collected, fixed in 4% paraformaldehyde for 20 min and embedded in paraffin.

3.7. Immunohistochemistry

Paraffin-embedded teratoma sections were deparaffinized with xylene and rehydrated through alcohol gradient. Tissues were boiled for 20 min at temperature ranging from 95 to 100 °C for heat-induced antigen retrieval in Sodium Citrate Buffer pH 6.0 (Vector Labs). Slides were then taken out of water bath and allowed to cool in a vessel of tap water for 10 min. Sections were blocked during 15 min at room temperature (RT) in 2.5% Normal Horse Serum Blocking Solution (Vector Labs) and incubated with primary antibodies against CDX2 and Vimentin (Antibodies in Table 1), for 1 h at RT. Immunohistochemical staining was visualized using the avidin-biotin system (Dako, LSAB2 System-HRP) in combination with 3–3′-diaminobenzidine (DAB, Dako), according to manufacturer's instructions.

3.8. Immunofluorescence labelling

Cells were fixed in 4% PFA for 15 min at room temperature (RT) and washed 3 times in phosphate-buffered saline (PBS, Gibco). The cells were permeabilized with 0.5% Triton X-100 (Sigma Aldrich) for 15 min, blocked with 10% normal donkey serum (Sigma Aldrich) for 1 h at RT. Cells were incubated with the primary antibodies in PBS BSA 1% 0.1% Triton X-100 overnight at 4 °C. Primary antibodies are listed in Table 2. Secondary antibodies were used at the dilution of 1:2000 during one hour at RT. In addition, cell nuclei were stained with DAPI (Sigma Aldrich). Images of iPSCs were captured with Zeiss Axioimager Z1 Apotome.

3.9. Flow cytometry analysis

Cells were harvested using cell dissociation reagent (Versene Solution) and washed twice in PBS. Cells were then incubated 15 min at room temperature with a Live Cell/Dead Cell discriminator staining (Violet Zombie, Biolegend). IPS cells were incubated with a conjugated primary or the control isotype antibody for 30 min at 4 °C. After

Table 1
Characterization and validation.

Classification	Test	Result	Data
Morphology	Photography	Normal	Fig. 1 panel A
Phenotype	Immunocytochemistry	Expression of pluripotency markers: OCT4, NANOG, SOX2	Fig. 1 panel F
	Flow cytometry	Positive for antigen levels & cell surface markers. TRA-1-81: 76.21% SSEA-4: 97.58%	Fig. 1 panel E
Genotype	Karyotype (G-banding) and resolution	46XX, (G banding & R banding) Resolution 400–500	Fig. 1 panel B
Identity	Microsatellite PCR (mPCR)	not performed	N.A
	STR analysis	Specific 18 loci tested, 18/18 matched between iPS cell line and patient-derived dermal fibroblasts	available with the authors
Mutation analysis	Sequencing	Compound Heterozygous Variants in the Coiled-Coil Domain Containing 40 Gene (CCDC40): c.1116_1117delCT (Exon 7) and c.3180 + 1G > A (Intron 19)	Fig. 1 panel D
	Southern Blot OR WGS	N.A	N.A
Microbiology and virology	Mycoplasma	Mycoplasma testing by luminescence. Negative	Supplemental Table 1
Differentiation potential	Teratoma formation	Teratoma formation show the three germ layers formation (**ectoderm**: pigmented neural cells; **mesoderm**: vimentin marker; **endoderm**: posterior gut marker CDX2	Fig. 1 panel G
Donor screening (OPTIONAL)	HIV 1 + 2 Hepatitis B, Hepatitis C	Negative	not shown but available with the authors
Genotype additional info (OPTIONAL)	Blood group genotyping	N.A.	N.A
	HLA tissue typing	N.A	N.A

Table 2
Reagents details.

Antibodies used for immunocytochemistry/flow-cytometry			
	Antibody	**Dilution**	**Company Cat # and RRID**
Pluripotency Markers	Rabbit polyclonal anti SOX2	1:50	Santa Cruz Technology Cat# sc-20,088 RRID AB_2255358
	Rabbit Polyclonal anti OCT4	1:400	Santa Cruz Technology Cat# RRID AB_2167703
	Rabbit polyclonal anti NANOG	1:250	Abcam Cat# ab80892 RRID AB_2150114
	Mouse monoclonal anti TRA-1-60	1:500	Abcam Cat# ab16288 RRID AB_778563
	PerCP Mouse monoclonal SSEA4	1:250	Bd pharmingen Cat# 561565 RRID AB_10894210
	Mouse IgM, κ Alexa Fluor 488 anti-human TRA-1-81	1:100	Biolegend Cat# 330709 RRID AB_2561741
	Alexa Fluor 488 Mouse IgM, kappa Isotype Control	1:100	Biolegend Cat# 401617 RRID AB_1277908
	Mouse IgG3 PerCP Isotype Control (Clone 133,316) antibody	1:250	R&D Cat#C007C RRID AB_1207939
Differentiation Markers	Monoclonal mouse anti CDX2	1:500	Biogenex Cat# AM392, RRID AB_2650531
	Polyclonal goat anti Vimentin	1:1000	Santa Cruz Biotechnology Cat# sc-7557, RRID:AB_793998
Secondary antibodies	donkey anti mouse Alexa fluor 555	1:2000	Thermo Fisher Scientific Cat# A-31570, RRID:AB_2536180
	donkey anti rabbit Alexa fluor 488	1:2000	Thermo Fisher Scientific Cat# A-21206, RRID:AB_2535792

Primers		
	Target	Forward/Reverse primer (5′-3′)
Virus clearance (qPCR)	SeV product size: 181 bp	GGATCACTAGGTGATATCGAGC ACCAGACAAGAG TTTAAGAGATATGTATC
Pluripotency Markers (qPCR)	OCT4	GGGCTCTCCCATGCATTCAAAC CACCTTCCCTCCAACCAGTTGC
	NANOG	TGATTTGTGGGCCTGAAGAAA GAGGCATCTCAGCAGAAGACA
	SOX2	GGCCATTAACGGCACACTGCC TTACTCTCCTCTTTTGCACCCCTCC
House-Keeping Genes	GAPDH (qRT- PCR)	GACCTGACCTGCCGTCTAGAAA CCTGCTTCACCACCTTCTTGA
	GAPDH (PCR) product size: 300 bp	CTG GCG TCT TCA CCA CCA TGG CAT CAC GCC ACA GTT TCC CGG
Targeted mutation sequencing (Sanger Method)	CCDC40 Exon 7 product size: 328 bp	TCCTGGTGACCCTGTTTCTC TGGCCATGGTACATGGAGAC GTCCTCCCAGCCTGACTCT GCAGGACCTCTGTGTGAGTG
	IVS19 product size: 305 bp	

staining, cells were washed twice and analysed by flow cytometry using Beckman Coulter Gallios. Analysis were performed with Kaluza software.

3.10. Testing for Sendai virus clearance

Total RNA was isolated from iPS cells within the first week after transfection with Sendai virus and iPSCs after passage 10. RNA extraction and RT were performed as previously described (qRT-PCR section). PCR was performed using Q5 High-Fidelity DNA Polymerase with the primers listed in Table 1. The PCR mix was composed of cDNA (50 ng), the forward and reverse primers 1.5 µL at 10 µM for each, dNTPs (10 nM), Q5 buffer and Q5 DNA Pol5 (New England BioLabs). First, DNA was melted for 3 min at 95 °C, then 35 cycles (95 °C during 15 s, 55 °C for 15 s and 72 °C for 30 s), and final elongation was performed at 72 °C for 2 min using C100 Thermal Cycler (Bio-Rad). Products were analysed on 2.5% agarose gel, ran at 130 V electric field. Finally, the picture was acquired using ChemiDoc™MP Imaging System (Bio-Rad).

3.11. Short tandem repeat analysis (STR)

DNA extraction was performed as described above. STR analysis of generated iPSCs and parental fibroblasts was performed to detect 18 loci. Eighteen dinucleotide repeat were amplified by PCR in two multiplexes. Negative controls with no template DNA were included for each multiplex on every run. The amplified DNA samples were separated by capillary electrophoresis. Analysis was carried out with GeneMarker V2.6.7 (SoftGenetics).

3.12. Mycoplasma testing

Mycoplasma was tested on cell culture supernatants of UHOMi001-A iPS cells, using bioluminescence MycoAlert® Detection Kit (Lonza) according to manufacturer's instructions. Briefly, the ratio of Reading B to Reading A is used to determine whether a cell culture is contaminated by mycoplasma. Ratio < 0.9 means that the sample was negative for mycoplasma.

References

Boaretto, F., Snijders, D., Salvoro, C., Spalletta, A., Mostacciuolo, M.L., Collura, M., Cazzato, S., Girosi, D., Silvestri, M., Rossi, G.A., Barbato, A., Vazza, G., 2016. Diagnosis of primary Ciliary Dyskinesia by a targeted Next-Generation Sequencing Panel: molecular and clinical findings in Italian patients. J. Mol. Diagn. JMD 18, 912–922. https://doi.org/10.1016/j.jmoldx.2016.07.002.

Knowles, M.R., Daniels, L.A., Davis, S.D., Zariwala, M.A., Leigh, M.W., 2013. Primary ciliary dyskinesia. Recent advances in diagnostics, genetics, and characterization of clinical disease. Am. J. Respir. Crit. Care Med. 188, 913–922. https://doi.org/10.1164/rccm.201301-0059CI.