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Lab Resource: Multiple Cell Lines

# Generation of two isogenic human induced pluripotent stem cell lines from a 15 year-old female patient with MERRF syndrome and A8344G mutation of mitochondrial DNA

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A B S T R A C T

MERRF syndrome is predominantly caused by A8344G mutation in the mitochondrial DNA (mtDNA), affecting *MT-TK* gene, which impairs the mitochondrial electron transport chain function. Here, we report the generation of two isogenic induced pluripotent stem cell (iPSC) lines, TVGH-iPSC-MRF-M$^{low}$ and TVGH-iPSC-MRF-M$^{high}$, from the skin fibroblasts of a female MERRF patient harboring mtDNA A8344G mutation by using retrovirus transduction system. Both cell lines share the same genetic background except containing different proportions of mtDNA with the A8344G mutation. Both cell lines exhibited the pluripotency and capacity to differentiate into three germ layers.

Resource table.

| | |
|---|---|
| Unique stem cell lines identifier | TVGHi005-A, TVGHi006-A |
| Alternative names of stem cell lines | TVGH-iPSC-MRF-M$^{low}$ (TVGHi005-A)<br>TVGH-iPSC-MRF-M$^{high}$ (TVGHi006-A) |
| Institution | Department of Medical Research, Taipei Veterans General Hospital, Taipei, Taiwan |
| Contact information of distributor | Shih-Hwa Chiou, shchiou@vghtpe.gov.tw |
| Type of cell lines | iPSC |
| Origin | Human |
| Cell Source | Skin fibroblasts |
| Clonality | Clonal |
| Method of reprogramming | Retrovirus |
| Multiline rationale | Isogenic clones |
| Gene modification | No |
| Type of modification | N/A |
| Associated disease | MERRF syndrome |
| Gene/locus | MT-TK/base pairs 8295 to 8364 on mitochondrial DNA |
| Method of modification | N/A |
| Name of transgene or resistance | N/A |
| Inducible/constitutive system | N/A |
| Date archived/stock date | 30 March 2017 |
| Cell line repository/ bank | Department of Medical Research, Taipei Veterans General Hospital, Taipei, Taiwan |
| Ethical approval | Approvals from the Taipei Veterans General Hospital Institutional Review Board (2016-09- 019C) were obtained. |

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## Resource utility

The MERRF syndrome-specific iPSC lines can be utilized to generate disease-specific differentiated tissues for *in vitro* disease modelling, drug discovery and personalized medicine.

## Resource details

Mitochondrial diseases are a group of metabolic disorders caused by the defects of the mitochondrial respiratory chain function (Wu et al., 2010). Myoclonic epilepsy with ragged red fibers (MERRF) syndrome is a maternally inherited mitochondrial encephalomyopathy associated with the mitochondrial DNA (mtDNA) A8344G mutation that affects *MT-TK* gene encoding mitochondrial tRNA-Lysine (Ma et al., 2005). MERRF syndrome is characterized by myoclonic epilepsy, generalized seizures, ataxia, and ragged red fibers observed on the modified Gomori trichrome stained muscle biopsy samples (Shoffner et al., 1990). The lack of a cellular disease model complicates the understanding of the genetic and molecular mechanisms underlying pathophysiology of MERRF syndrome. Therefore, we reprogrammed skin fibroblasts obtained from a 15 year-old Taiwanese female patient with poor learning, myoclonus and epilepsy into induced pluripotent stem cells (iPSCs) by using retroviral vectors encoding OCT4, SOX2, KLF4, and GLIS1. A previous study demonstrated that reprogramming of cultured cells derived from a patient with mitochondrial encephalomyopathy, lactic acidosis with stroke-like episodes (MELAS) syndrome harboring the A3243G mtDNA mutation generated iPSC clones characterized by different proportions of the mtDNA mutation (Folmes et al., 2013). Similarly, in this study, we generated two isogenic iPSC clones, one characterized by low proportion of the A8344G mtDNA mutation (1%, TVGH-iPSC-MRF-$M^{low}$) and another characterized by high proportion of the A8344G mutation (76%, TVGH-iPSC-MRF-$M^{high}$), as was demonstrated by pyrosequencing (Fig. 1A and Table 1). In addition, to evaluate the genetic stability of the iPSCs, G-banding karyotype analysis was performed, and it was revealed that both cell lines exhibited normal chromosomal structure (Fig. 1B). Whereas both cell lines had reprogramming vectors integrated into their genome at passage 20, as was shown by genomic DNA PCR (Fig. 1C, left panel), their expression was silenced, as was demonstrated by RT-PCR (Fig. 1C, right panel). STR analysis of 16 loci revealed 100% match between TVGH-iPSC-MRF-$M^{low}$ cells and parental fibroblasts, however, there was one-repeat mismatch between TVGH-iPSC-MRF-$M^{high}$ and parental cells at D21S11 locus. We also observed human embryonic stem cells-like morphology of the iPSC lines (Fig. 1F, scale bar = 50 μm). To further characterize the iPSCs, we examined their pluripotency and differentiation capacity. RT-PCR and real-time PCR analysis demonstrated high expression levels of mRNAs encoding stemness markers (OCT4, SOX2, DPPA4, and NANOG), as compared to the original fibroblasts, and comparable to the levels in H9 hESCs (Fig. 1D & E). Moreover, we also confirmed the protein expression of OCT4, NANOG, TRA-1-60, and TRA-1-81 by immunofluorescence staining (Fig. 1G, scale bar = 200 μm). Next, *in vitro* and *in vivo* differentiation capacity into three germ layers was examined by embryoid body (EB) and teratoma formation assays. As shown in Fig. 1H, both iPSC lines could be differentiated into EBs expressing mesoderm (alpha smooth muscle, α-SMA), ectoderm (neurofilament200, NF200), and endoderm (alpha fetoprotein, AFP) markers (scale bar = 100 μm). Similarly, immunohistochemistry examination of iPSC-derived teratomas also showed expression of markers of three germ layers: α-SMA (mesoderm), AFP (endoderm), and GFAP (ectoderm) (Fig. 1I, scale bar = 50 μm and Table 2). Finally, in our previous study we have demonstrated that iPSC lines described here, as well as cardiomyocytes derived from them, exhibit impaired mitochondrial function and elevated levels of reactive oxygen species, which validates their applicability for disease modelling (Chou et al., 2016).

## Materials and methods

### *Generation of human induced pluripotent stem cells*

The skin fibroblasts of a 15 year-old female patient with MERRF syndrome were reprogrammed into iPSCs by transducing with retroviral vectors encoding OCT4, SOX2, KLF4, and GLIS1. In brief, the plasmids pMXs-OCT4, pMXs-SOX2, pMXs-KLF4, and pMXs-GLIS1 (Addgene) were separately packaged into retroviral particles by using the packaging cell line AmphoPack-293. Retroviral transduction was performed twice at one day interval. On day 7 post transduction, $5 \times 10^4$ infected cells were seeded onto a 10-cm dish pre-coated with inactivated mouse embryonic fibroblast (MEF) feeder cells and incubated at 37 °C, 5% $CO_2$. On the next day, the medium was replaced with hES medium (DMEM/F12 supplemented with 20% KnockOut Serum Replacement (Gibco), 1× MEM Non-Essential Amino Acids Solution (Gibco), 1% GlutaMAX-I (Gibco), 0.1 mM β-mercaptoethanol, (Gibco), 1% Penicillin-Streptomycin (Gibco) and 10 ng/ml bFGF (Gibco)) and the medium was changed every day afterwards. 28 days later, the emergent colonies were dislodged by treatment with Collagenase Type IV (Gibco) at 37 °C for 15 min in the presence of 10 μM Y-27632 (Sigma-Aldrich) and transferred to a fresh feeder for characterization and expansion. The iPSCs were cultured on feeder-coated dish and passaged every 5 days in hES medium with split ratios 1:2–1:4.

### *DNA and RNA extraction and PCR*

Genomic DNA was isolated using QIAamp DNA Mini Kit (QIAGEN) following the manufacturer's instructions. The total cellular RNA was purified using TRIzol reagent (Thermo Fisher Scientific), and 5 μg was used to synthesize cDNA using SuperScript III Reverse Transcriptase (Thermo Fisher Scientific) according to the protocol of the manufacturer. PCR was performed using the Taq DNA Polymerase Master Mix (Ampliqon). The following cycling conditions were used: initial denaturation at 95 °C for 3 min, followed by 30 cycles of amplification (95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s) and a final extension at 72 °C for 5 min. Quantitative PCR was performed using the SYBR Green PCR Master Mix (Thermo Fisher Scientific) and StepOnePlus Real-Time PCR System (Thermo Fisher Scientific). The nucleotide sequences of the gene-specific primers used in this study are listed in Table 3.

### *Immunofluorescence and immunohistochemistry staining*

Immunofluorescence and immunohistochemistry staining was performed with the primary antibodies against the stemness and three germ layer markers, which are listed in Table 3. In brief, the cells were fixed in 4% (w/v) paraformaldehyde for 10 min at room temperature (RT), and then treated with 1% NP-40 for 10 min at RT for additional permeabilization. Blocking of non-specific binding was performed by incubating the cells in 3% (w/v) bovine serum albumin (Sigma-Aldrich) and 5% (v/v) FBS for 30 min at RT. Subsequently, the cells were incubated with primary antibodies for 16 h at 4 °C, and then incubated with the matching fluorochrome-conjugated secondary antibodies (after 1:200 dilution) for 2 h at RT. The nuclei were stained with DAPI and slides were visualized on the Olympus IX71 inverted fluorescence microscope. For immunohistochemistry staining of teratomas, the slides were deparaffinised in xylene and rehydrated in Target Retrieval Solution (Dako) according to the manufacturer's instructions. Sections of the slides were blocked with 10% FBS for 1 h at RT and incubated with the indicated primary antibodies listed in Table 3 for 1 h at RT. DAB substrate solution (Dako) was applied to the sections of the slides to reveal the colour of antibody staining. Briefly, the slides were incubated with DAB for 5 min at 37 °C and then rinsed with water followed by covering with mounting solution (VectaMount, Vector Laboratories).

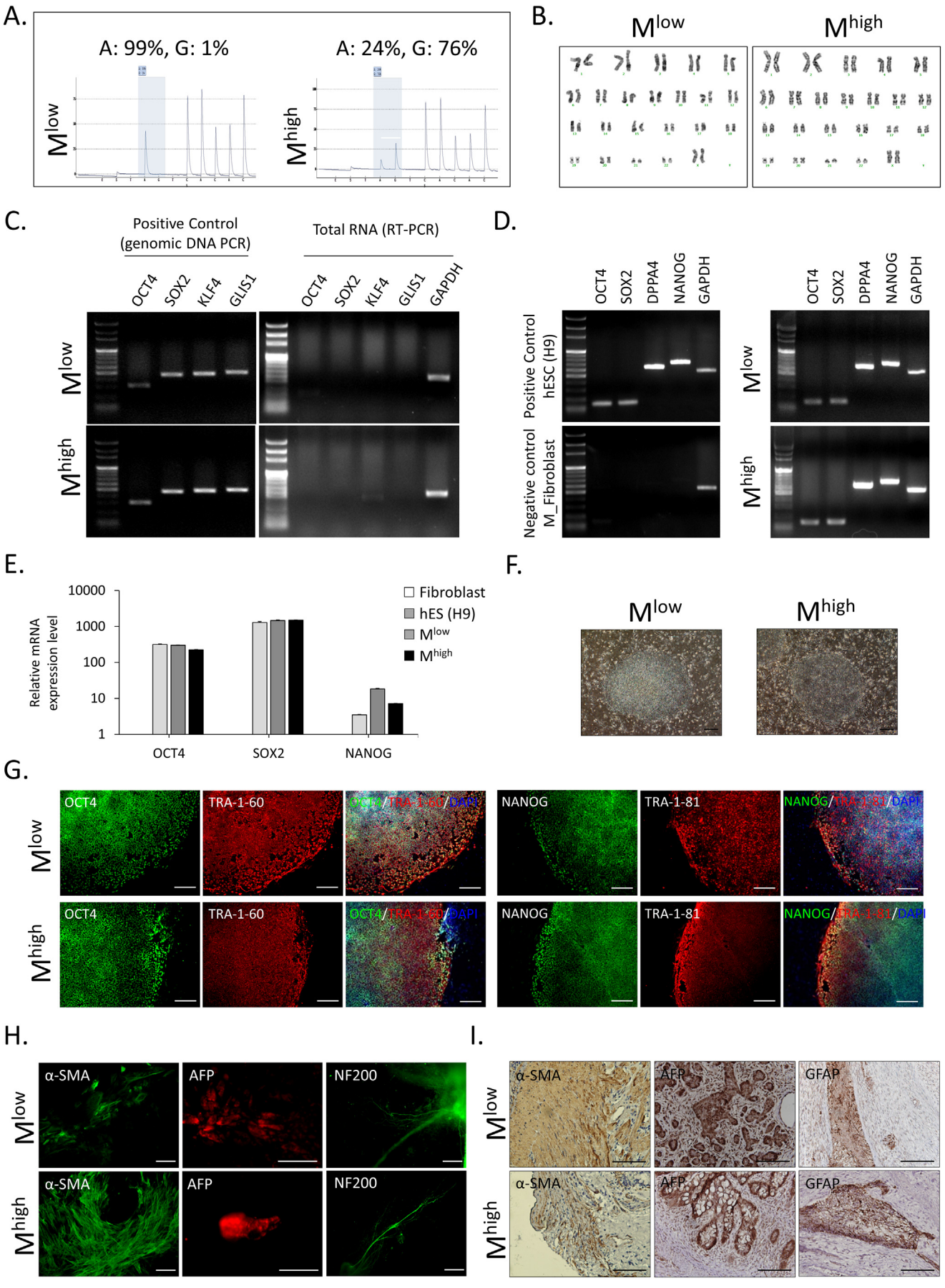


**Fig. 1.** Characterization of TVGH-iPSC-MRF-M$^{low}$ and TVGH-iPSC-MRTVGH-iPSC-MRF-M$^{high}$ cell lines.

**Table 1**
Summary of cell lines.

| iPSC line names | Abbreviation in figures | Gender | Age (year) | Ethnicity | Genotype of locus | Disease |
|---|---|---|---|---|---|---|
| TVGH-iPSC-MRF-Ml (TVGHi005-A) | $M^{low}$ | Female | 15 | Taiwanese | mtDNA A8344G 1% | MERRF syndrome |
| TVGH-iPSC-MRF-Mh (TVGHi006-A) | $M^{high}$ | Female | 15 | Taiwanese | mtDNA A8344G 76% | MERRF syndrome |

*In vitro differentiation*

For embryoid body (EB) formation assay, iPSCs were detached with Accutase solution in the presence of 10 μM Y-27632 and seeded into ultralow attachment 6-well plates (Corning) in DMEM/F12 supplemented with 20% FBS, 0.1 mM NEAA, 1 mM GlutaMax-1, 0.1 mM 2-mercaptoethanol, 50 U/ml penicillin and 50 mg/ml streptomycin. After 7 days, EBs were transferred onto 0.1% gelatin-coated plates in DMEM/F12 supplemented with 20% FBS, 0.1 mM NEAA, 1 mM GlutaMax-1, 0.1 mM 2-mercaptoethanol, 50 U/ml penicillin and 50 mg/ml streptomycin and cultivated for another 14 days. The markers of 3 germ layers, mesoderm (alpha smooth muscle, α-SMA), ectoderm (neurofilament200, NF200), and endoderm (alpha fetoprotein, AFP), were detected by immunostaining as described in the previous section.

*In vivo differentiation*

For teratoma formation assay, $1 \times 10^6$ of iPSCs were harvested with Accutase solution and were implanted into testis of a NOD/SCID mouse. Eight weeks after implantation, the teratomas were harvested, fixed with 10% formaldehyde, embedded in paraffin and immunostained for three germ layer markers: α-SMA (mesoderm), AFP (endoderm) and GFAP (ectoderm).

*Karyotyping*

Karyotyping was performed by Sofiva Genomics Co, Taipei, Taiwan. In brief, the passage 17 cells were grown to 80% confluence in a 25 T flask, and treated with 10 μg/ml Colcemid at 37 °C for 1 h. The cells were then dissociated into single cells by trypsin, and treated with a hypotonic solution (0.075 M KCl) at 37 °C for 10 min. The cells were fixed with methanol:glacial acetic acid (3:1) and analysed for G-banding with 20 metaphase spreads being counted.

*STR analysis*

For STR-PCR, DNA from iPSCs and the original fibroblasts was extracted and purified by PureLink Genomic DNA Mini Kit (Thermo Fisher Scientific). AmpFLSTR Identifiler PCR Amplification Kit (Thermo Fisher Scientific) was used to determine the genetic signature based on multiplex analysis of 15 loci (D8S1179, D21S11, D7S820, CSF1PO, D3S1358, TH01, D13S317, D16S539, D2S1338, D19S433, vWA, TPOX, D18S51, D5S818, FGA) and the Amelogenin gender-determination marker.

*Mycoplasma detection*

For detecting mycoplasma contamination, iPSCs were cultured for at least 72 h, then the culture medium was collected and analysed according to the manufacturer's protocol of Mycoplasma PCR Detection Kit (ABM Inc.).

*Pyrosequencing assay*

The proportion of mtDNA with A8344G mutation was determined by Mission Biotech Corporation. Briefly, the biotin-labelled PCR products were isolated by Streptavidin Sepharose High Performance beads (GE Healthcare) and sequenced by using the PyroMark Q24 System (QIAGEN). Quantitative analysis was done using the PyroMark Q24 software. The nucleotide sequences of the primers used for

**Table 2**
Characterization and validation of the iPSCs.

| Classification | Test | Results | Data |
|---|---|---|---|
| Morphology | Photography | Normal morphology | Fig. 1F |
| Phenotype | Qualitative analysis: immunocytochemistry | Positive for staining/expression of pluripotency markers: OCT4, NANOG, TRA-1-60, and TRA-1-81. | Fig. 1G |
| | Quantitative analysis: RT-qPCR | Stemness gene expression level compared to hESCs. $M^{low}$, OCT4: 94.6%, SOX2: 113.5%, NANOG: 141.2%. $M^{high}$: OCT4: 96.3%, SOX2: 115.8%, NANOG: 106.3%. | Fig. 1E |
| Genotype | Karyotype (G-banding) and resolution | 46XX, Resolution: 640 × 480 | Fig. 1B |
| Identity | Microsatellite PCR (mPCR) or STR analysis | Not performed | N/A |
| | | Perfect match at 16 sites (D8S1179, D2S1338, D21S11, D19S433, D7S820, vWA, CSF1PO, TPOX, D3S1358, D18S51, TH01, Amelogenin, D13S317, D5S818, D16S539, FGA) between TVGH-iPSC-MRF-$M^{low}$ and parental cells; one-repeat mismatch between TVGH-iPSC-MRF-$M^{high}$ and parental cells at D21S11. | STR analysis |
| Mutation analysis (IF APPLICABLE) | Pyrosequencing analysis | mtDNA A8344G mutation confirmed | Fig. 1A |
| | Southern blot or WGS | Not performed | N/A |
| Microbiology and virology | Mycoplasma | Mycoplasma testing by PCR. Negative. | Supplementary Fig. 1 |
| Differentiation potential | Embryoid body formation and teratoma formation | Embryoid bodies: smooth muscle actin (SMA) for mesoderm, α-fetoprotein (AFP) for endoderm, and NF200 for ectoderm. Teratoma formation: SMA for mesoderm, AFP for endoderm, and GFAP for ectoderm. | Fig. 1H and I |
| Donor screening (OPTIONAL) | HIV 1 + 2 Hepatitis B, Hepatitis C | N/A | N/A |
| Genotype additional info (OPTIONAL) | Blood group genotyping | N/A | N/A |
| | HLA tissue typing | N/A | N/A |

**Table 3**
Reagents details.

| Antibodies used for immunocytochemistry/flow-cytometry | | | |
|---|---|---|---|
| | Antibody | Dilution | Company Cat # and RRID |
| Pluripotency marker | Rabbit anti-OCT4 (IgG) | 1:200 | Cell Signaling Technology Cat# 2840, RRID: AB_2167691 |
| Pluripotency marker | Rabbit anti-NANOG (IgG) | 1:200 | Cell Signaling Technology Cat# 3580, RRID: AB_2150399 |
| Pluripotency marker | Mouse anti-TRA-1-60 (IgM) | 1:100 | Millipore Cat# MAB4360, RRID:AB_2119183 |
| Pluripotency marker | Mouse anti-TRA-1-81 (IgM) | 1:200 | Cell Signaling Technology Cat# 4745P, RRID:AB_10829904 |
| Differentiation marker | Mouse anti-SMA (IgG2a) | 1:200 | Thermo Fisher Scientific Cat# A25531, RRID: AB_2651005 |
| Differentiation marker | Mouse anti-AFP (IgG2a) | 1:100 | Cell Signaling Technology Cat# 3903S, RRID:AB_2224073 |
| Differentiation marker | Rabbit anti-neurofilament200 (NF200) (IgG1) | 1:200 | Millipore Cat# AB1982, RRID:AB_2313731 |
| Differentiation marker | Mouse anti-GFAP (IgG1) | 1:200 | Cell Signaling Technology Cat# 3670, RRID:AB_561049 |
| Secondary antibody | Alexa Fluor 488 Goat anti-Mouse IgG | 1:250 | Thermo Fisher Scientific Cat# A-11001, RRID:AB_2534069 |
| Secondary antibody | Alexa Fluor 488 Goat anti-Rabbit IgG | 1:250 | Molecular Probes Cat# A-11008, RRID:AB_143165 |
| Secondary antibody | Alexa Fluor 594 Goat anti-Mouse IgG | 1:250 | Thermo Fisher Scientific Cat# A-11005, RRID:AB_2534073 |
| Secondary antibody | Alexa Fluor 594 Goat anti-Rabbit IgG | 1:250 | Thermo Fisher Scientific Cat# A-11037, RRID:AB_2534095 |
| Secondary antibody | Alexa Fluor 594 Goat anti-Mouse IgM | 1:200 | Thermo Fisher Scientific Cat# A-21044, RRID:AB_2535713 |

| Primers | | |
|---|---|---|
| | Target | Forward/reverse primers (5′ → 3′) |
| Transduced plasmid (RT-PCR) | OCT4 Plasmid/305 bp | F: CCCCAGGGCCCCATTTTGGTACC<br>R: TTATCGTCGACCACTGTGCTG |
| Transduced plasmid (RT-PCR) | SOX2 Plasmid/462 bp | F: GGCACCCCTGGCATGGCTCTTGGCTC<br>R: TTATCGTCGACCACTGTGCTG |
| Transduced plasmid (RT-PCR) | KLF4 Plasmid/484 bp | F: ACGATCGTGGCCCCGGAAAAGGACC<br>R: TTATCGTCGACCACTGTGCTG |
| Transduced plasmid (RT-PCR) | GLIS1 plasmid/501 bp | F: GGCAGTTTCCACTCCATCCA<br>R: TTATCGTCGACCACTGTGCTG |
| Pluripotency marker (RT-PCR & RT-qPCR) | OCT4/144 bp | F: GACAGGGGGAGGGGAGGAGCTAGG<br>R: CTTCCCTCCAACCAGTTGCCCCAAAC |
| Pluripotency marker (RT-PCR & RT-qPCR) | SOX2/151 bp | F: GGGAAATGGGAGGGGTGCAAAAGAGG<br>R: TTGCGTGAGTGTGGATGGGATTGGTG |
| Pluripotency marker (RT-PCR) | DPPA4/408 bp | F: GGAGCCGCCTGCCCTGGAAAATTC<br>R: TTTTTCCTGATATTCTATTCCCAT |
| Pluripotency marker (RT-PCR) | NANOG/391 bp | F: CAGCCCTGATTCTTCCACCAGTCCC<br>R: TGGAAGGTTCCCAGTCGGGTTCACC |
| House-keeping gene (RT-PCR) | GAPDH/547 bp | F: GTATCGTGGAAGGACTCATGACC<br>R: TCTCTTCCTCTTGTGCTCTTGCT |
| Pluripotency marker (RT-qPCR) | NANOG/126 bp | F: GGGATTGGGAGGCTTTGCT<br>R: GCACAACCAACAAATTAGGGGA |
| Targeted mutation amplification | mtDNA/292 bp | F: CATGCCCATCGTCCTAGAAT<br>R: Biotin-TTTTATGGGCTTTGGTGAGG |
| Targeted mutation sequencing | mtDNA 8344 | TAAGTTAAAGATTAAGAGA |

pyrosequencing are listed in Table 3.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scr.2018.05.011.

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