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Lab Resource: Single Cell Line

Generation of human-induced pluripotent stem cell line from PBMC of healthy donor using integration-free Sendai virus technology

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A B S T R A C T

We developed a well-characterized human induced pluripotent stem cell (iPSC) line obtained from healthy individuals' peripheral blood mononuclear cells (PBMC). The PBMCs were primed and reprogrammed using a non-integrating sendai viral vector, and the iPSC lines demonstrated complete differentiation capacity. This line, YBLi004-A, is available and registered in the human pluripotent stem cell registry. The line's legitimacy was validated using pluripotent marker expression, in vitro differentiation into three germ layers (ectoderm, mesoderm, and endoderm), karyotyping, and STR analysis. This iPSC line could be used as a healthy control for studies involving disease-specific-iPSCs, e.g. drug toxicity and efficacy testing.

1. Resource table

Unique stem cell line identifier	YBLi004-A			
Alternative name of stem cell line	YBL/SH/2018/0062			
Institution	Yashraj Biotechnology, Navi Mumbai, India			
Contact person and email	Dr. Amit Khanna; amit.khanna@yashraj.com			
Types of cell lines	iPSC			
Date archived/stock date	21 March 2019			
Origin	Human			
Additional origin info required for human ESC or iPSC	iPSC Line name	Gender	Age	Ethnicity
	YBLi004-A	Female	35	Asian (Indian)
Disease status	Healthy			
Cell source	PBMC			
Clonality	Clonal			
Methods of reprograming	Non-integrating Sendai virus			
Key marker	Pluripotent stem cell markers: OCT4, NANOG, and SSEA4.			
Authentication	Identity and purity of line confirmed			
Cell line repository/bank	https://hpscreg.eu/cell-line/YBLi004-A			
Ethical statement	Institutional Ethics Committee (IEC), Yashraj Biotechnology Limited Institutional Committee for Stem Cell Research (IC-SCR), Yashraj Biotechnology Limited.			

2. Resource utility

This human iPSC line could serve as a cell resource as healthy control for studies involving patient-specific iPSCs and for studies on assessing the efficacy and toxicity of drug substances.

3. Resource details

The groundbreaking research conducted by Takahashi and Yamanaka in 2006 introduced induced pluripotent stem cell (iPSC) technology, revolutionizing our understanding of various diseases through disease modeling (Takahashi et al., 2007). Basu et al. (2016) noted that the genetic diversity of the Indian population is a result of numerous historical and cultural events. Unique nutritional and environmental characteristics of the Indian subcontinent further emphasize these distinctions. To study diseases in populations of Indian ethnicity, age and gender-matched control lines from healthy individuals are essential. These control lines with similar ethnicity help mitigate the impact of gene-environment interactions, enabling a more comprehensive investigation of pathophysiology. In our study, we utilized a non-integrating viral vector containing human OCT4, KLF4, SOX2, and C-MYC transcription factors to transduce peripheral blood mononuclear cells (PBMCs) (Chitrangi et al., 2023). The resulting healthy iPSC line exhibited typical embryonic stem cell morphology (Fig. 1A) and normal karyotypes (35, XX) at passage 7, as confirmed by G-banding karyotyping (Fig. 1F and Supplementary Fig. 1). Flow cytometry analysis

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demonstrated positive expression of OCT4A, NANOG, and SSEA-4 (Fig. 1B), while immunofluorescence staining confirmed the expression of OCT4A, NANOG, and SSEA-4 (Fig. 1C). The in vitro differentiation potential of the iPSC line was evaluated through embryoid body (EB) formation in suspension cultures (Fig. 1G). Quantitative real-time PCR (qRT-PCR) analysis showed the expression of representative markers from all three germ layers - ectoderm (OTX2), mesoderm (FOXA2), and endoderm (SOX17) - confirming their spontaneous differentiation potential (Fig. 1H). The non-integrating Sendai virus used for reprogramming was confirmed to be lost from the cells by semi-quantitative RT-PCR analysis after 10 passages (Fig. 1E). Short tandem repeat (STR) DNA profiling analysis revealed that the genotypes of these iPSC lines were 100 % matched with the source donor's PBMC, confirming the purity of the cell lines and indicating no cross-contamination from other cell lines (Fig. 1F).

4. Material and methods

4.1. Esthical statement

Ethical approval for this study was obtained from the Institutional Ethics Committee (IEC) and Institutional Committee for Stem Cell Research (IC-SCR) of Yashraj Biotechnology, Maharashtra, India. Healthy volunteers were enrolled in studies approved by IEC and IC-SCR under protocol #YBLBC17SB. Whole blood samples were collected by Aspira Pathlab. and Diagnostics (NABL certificate No. MC-2447). The project is also approved by the National Apex Committee for Stem Cell Research and Therapy (NAC-SCRT) with registration ID NAC-SCRT/134/20200209, and the Drugs Controller General of India (DCGI) with registration ID ECR/305/Indt/MH/2018.

4.2. Isolation and reprogramming of PBMC

Isolation and reprogramming of PBMCs for iPSC culture involved the collection of peripheral blood from a healthy individual aged 35 years (Female), following approval from the IEC and IC-SCR. PBMCs were

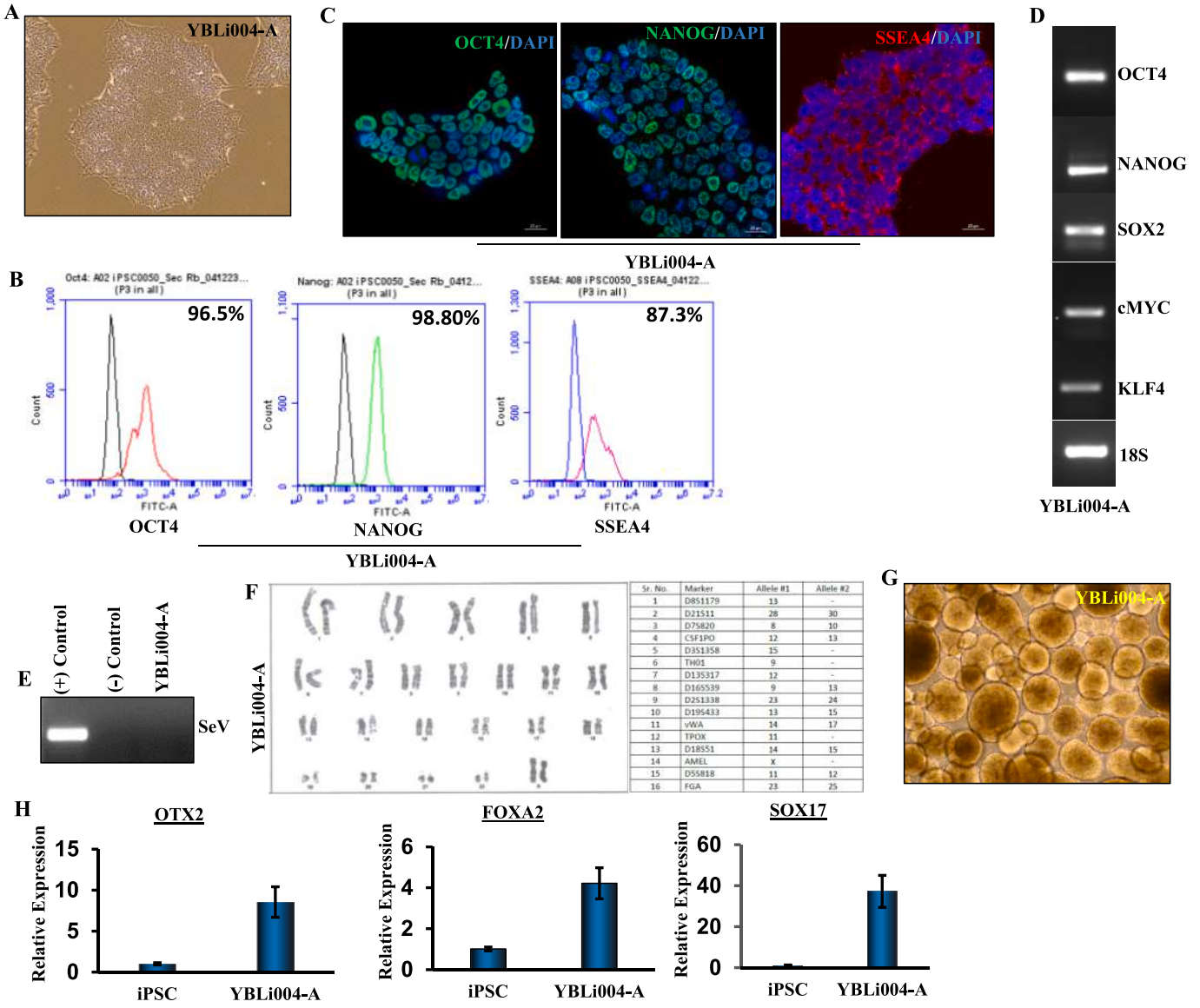

Fig. 1. (A) Phase contrast morphological representation of iPSC line; (B) FACS analysis for the pluripotent marker expression; (C) Immunocytochemistry for pluripotent marker expression; (D) Gene expression data for the pluripotent marker; (E) Gene expression data for virus clearance; (F) Karyotyping and STR analysis for genotyping; (G) morphological representation of EB formation; (H) qRT-PCR data for trilineage differentiations.

isolated from the blood using Ficoll-based density gradient centrifugation (Histopaque-1077, Sigma, 10771), cryopreserved in CryoStor CS10 (Stem Cell Technologies, 07930), and stored in liquid nitrogen until further use. PBMCs were thawed and reprogrammed using CytoTune™ 2.0 Sendai viruses (Thermofisher, A16517). Briefly, thawed PBMCs were seeded onto one well of a 6-well tissue culture dish in PBMC medium containing StemPro™-34 SFM Medium (Thermo Fisher, 10639–011) with cytokines (100 ng/mL SCF, 100 ng/mL FLT-3, 20 ng/mL IL-3, and 20 ng/mL IL-6). After 2 days, fresh complete StemPro™-34 medium containing cytokines was added without disturbing the cells. One day before transduction, 0.5 mL of medium was gently removed, and 1 mL of fresh complete StemPro™-34 medium containing cytokines was added without disturbing the cells. Cells were counted using a hemocytometer, and the volume of each virus was calculated to reach the required target MOI using the live cell count and the titer information as stated on the Certificate of Analysis (CoA) of the CytoTune™ 2.0 reprogramming vector.

The formula used for calculating the volume of virus (μL) was: MOI (CIU/cell) × number of cells / Titer of virus (CIU/mL) × 10^-3 (mL/μL).

Cells were harvested and seeded at the required density in wells of a 24-well plate (1.0 × 10^5 cells/well) in a minimal volume (~100 μl) for transduction. The calculated volumes of CytoTune™ 2.0 Sendai virus was added to 0.4 mL of pre-warmed StemPro™-34 medium containing cytokines and 4 μg/mL of Polybrene. The solution was thoroughly mixed by gently pipetting the mixture up and down, and this step was completed within 5 min. Cells were then incubated at 37 °C in a humidified atmosphere with 5 % CO2 overnight. The next day, the virus was removed by centrifuging the cells at 400 × g for 10 min, and the supernatant was aspirated and discarded. Cells were resuspended in 0.5 mL of complete StemPro™-34 Medium containing cytokines (as mentioned above) in the 24-well plate. Following transduction, the cells were cultured for 2 days at 37 °C in a humidified atmosphere with 5 % CO2 without any media change.

After two days, 1 × 10^4 to 1 × 10^5 live cells were seeded onto vitronectin-coated plates in complete StemPro™-34 medium without cytokines. Over the next 2–4 days, cells were maintained with half media changes of StemPro™-34 medium without cytokines. On the 4th day, cells were transitioned from StemPro™-34 medium to complete mTeSR™1 media by half media change. Subsequently, for the next 20 days, plated PBMCs were maintained in complete mTeSR™1 media. Clumps of reprogrammed cells became visible on day 8 after viral transduction. By day 20, cells formed reprogrammed cell clumps that were manually picked up and plated on vitronectin-coated plates. Colonies were expanded manually for 3–4 passages. Subsequent passaging was performed by enzymatic dissociation using StemPro Accutase and ReLeSR. All cell culture procedures were conducted at 37 °C in a humidified atmosphere containing 5 % CO2 in a BSL2 facility. See Table 1.

Table 1
Characterization and Validation.

Classification	Test	Result	Data
Morphology	Photography	Visual record of the line Normal	Fig. 1 panel A
Phenotype	Qualitative analysis: 1) Flowcytometry 2). Immunocytochemistry 3). Qualitative RT-PCR	1. Expression of pluripotency markers: OCT3/4, NANOG, SSEA4	Fig. 1 panel B
		2. Expression of pluripotency markers: OCT3/4, NANOG, SSEA4	Fig. 1 panel C
		2. Expression of pluripotency genes: OCT3/4, NANOG, SOX2, cMYC, KLF4; and clearance of vector against sendai virus as positive control	Fig. 1 panel D & E
Genotype	Karyotype (G-banding) and resolution	YBLi004-A:46XX, Band resolution: 500	Fig. 1 panel F
Identity	STR analysis	16 Loci tested, 100 % matched	Fig. 1 panel F Supplementary Fig. 1
Microbiology	Mycoplasma testing	By luminescence-Negative	Supplementary Table 1
Differentiation potential	Embryoid body formation	Expression of genes in embryoid body OTX2 (ectoderm), FOXA2 (mesoderm) and SOX17 (endoderm)	Fig. 1 panel H
Donor screening	HIV	Negative	Data available with authors

4.3. Immunocytochemistry

Cells were cultured on 8 well chamber slides, fixed with 4 % paraformaldehyde for 15 min, and permeabilized with 0.1 % Triton-X-100 for 10 min. After blocking with 1 % Bovine Serum Albumin, incubated in primary antibody against the pluripotency markers OCT4, NANOG and SSEA4 (Table 2) overnight at 4 °C. Next day, incubated with secondary antibody for 60 min at room temperature. Subsequently, cell nuclei were labelled with 4′, 6-Diamidin-2-phenylindo (DAPI, Invitrogen). Images were acquired with Carl Zeiss confocal microscope (LSM 780), and analysis was performed using the Zen 2012 acquisition software.

4.4. RNA isolation and Semi-Quantitative/quantitative PCR

For gene expression analysis, cells were collected, and total RNA was extracted using the RNeasy Mini Kit (Qiagen, 74104) following the manufacturer's protocol. The extracted RNA (1 μg) was used for cDNA synthesis with the iScript™ cDNA Synthesis Kit (Biorad, 1708891) following the manufacturer's instructions. For semi-quantitative PCR, GoTaq Green Master Mix (Promega, M7122) was used according to the manufacturer's protocol, and the resulting PCR products were visualized by running on a 1.5 % agarose gel. Real-time PCR amplification reactions were performed using the HOT FIREPol EvaGreen qPCR Mix Plus (ROX) Kit (Solis Biodyne, 082400001). The relative mRNA expression of each target gene was quantified by calculating Ct (threshold cycle) values and normalized to 18SrRNA levels. Each sample was analyzed in triplicate.

4.5. Flow cytometry analysis

Cell suspension was fixed with BD Cytofix and permeabilized using Perm/Wash buffer (BD Biosciences, 554723) and stained with antibodies (Table 2). 10, 000 events were acquired with BD Accuri™C6 Plus Flow Cytometer (BD Biosciences).

4.6. Detection of SeV genome and transgenes

After 10 passages, hiPSC lines were tested for SeV residues. PCR was performed using primers and instructions (Table 2) as recommended by the manufacturer. As a positive control, RNA was used from the reprogramming leftovers. Negative control RNA was obtained from the hiPSC line Gibco® Episomal hiPSC Line, (Gibco, A18945). Data included in Fig. 1E.

Table 2
Reagent details.

Target	Primer sequence	
Vector clearance		
SeV	Forward	GGATCACTAGGTGATATCGAGC
	Reverse	ACCAGACAAGAGTTTAAGAGATATGTATC
Other genes		
SOX2	Forward	CCAAGACGCTCATGAAGAAG
	Reverse	TGGTCATGGAGTTGTACTGC
cMYC	Forward	CCTGGTGCTCCATGAGGAGAC
	Reverse	CAGACTCTGACCTTTTGCCAGG
OCT3/4	Forward	CCTGAAGCAGAAGAGGATCACC
	Reverse	AAAGCGGCAGATGGTCGTTTGG
KLF4	Forward	CATCTCAAGGCACACCTGCGAA
	Reverse	TCGGTCGCATTTTTGGCACTGG
SOX17	Forward	ACGCTTTCATGGTGTGGGCTAAG
	Reverse	GTCAGCGCCTTCCACGACTTG
FOXA2	Forward	GGGGTAGTGCATCACCTGT
	Reverse	CCGTTCTCCATCAACAACCT
18sRNA	Forward	ACCCGTTGAACCCCATTCGTGA
	Reverse	GCCTCACTAAACCATCCAATCGG
OTX2	Forward	AGAACTCAGCCGCCTTCCT
	Reverse	AAACCATACCTGCACCCTCG
Nanog	Forward	AATGAAATCTAAGAGGTGGCAG
	Reverse	TCCTCTCCACAGTTATAGAAGG

Antibodies used for Immunostaining

Antibody	Dilution	Company	Catalog #	RRID
Rabbit Anti-Oct4	1:200	Cell signaling technology	2750	AB_823583
Mouse Anti-SSEA4	1:100	abcam	16,287	AB_778073
Rabbit Anti-Nanog	1:100	abcam	109,250	AB_10863442
Goat Anti-Rabbit IgG H&L (Alexa Fluor® 488)	1:2000	abcam	150,081	AB_2734747
Goat Anti-Mouse IgG H&L (Alexa Fluor® 647)	1:2000	abcam	150,075	AB_2752244

4.7. Karyotyping

Karyotyping was performed at passage 7 by GTG- banding analysis performed by Medgenome Labs Private Limited, Mumbai. Cells were treated with KaryoMAX® Colcemid™ Solution (ThermoFisher Scientific, 15212–012) overnight at 37 ∘C and thereafter processed following standard procedures in routine diagnostics. Data is included in Supplementary Fig. 1.

4.8. Short tandem repeat (STR) genotyping

STR typing was performed at passage 7 by Medgenome Labs Private Limited. 16 loci (including 13 core loci required for sample entry into CODIS) plus Amelogenin gender-determining marker were analyzed using the AmpFLSTR Identifier PCR amplification kit (ThermoFisher Scientific) Data included in Supplementary Fig. 1.

4.9. Embryoid body (EB) formation and pluripotency

iPSC colonies at passage 7 were non-enzymatically detached and transferred to low attachment six-well plates supplemented with mTeSRl plus media for 15 days with everyday media change. Suspension EBs on day 15 were collected and harvested to determine the trilineage differentiation potential by determining the three germ layers (ectoderm, mesoderm, and endoderm) marker gene expression by qRT-PCR (Table2). Data included in Fig. 1H.

4.10. Endotoxin, mycoplasma and bioburden testing

Spent media from optimally confluent iPSC cultures were collected after 48 h and tested using MycoAlert™ mycoplasma detection kit, (Lonza, LT07-318) as per the manufacturer's instructions, performed routinely. Bioburden testing was performed routinely by plating spent media on the Nutrient Agar Plate (Himedia, MP001)- for bacterial count and on the Sabouraud Dextrose Agar Plate (Himedia, MPH063)- for fungal count. Endotoxin test was performed routinely using Pierce LAL Chromogenic Endotoxin Quantitation Kit (Thermo fisher, 88282) as per the manufacturer's instruction.

CRediT authorship contribution statement

Jaganmay Sarkar: Conceptualization, Formal analysis, Investigation, Methodology, Supervision, Writing – original draft. **Shreya Dhepe:** Methodology. **Amrita Shivalkar:** Methodology. **Rutuja Kuhikar:** Data curation, Formal analysis. **Shruti More:** Project administration, Validation. **Vijay Bhaskar Reddy Konala:** Investigation, Supervision, Validation. **Paresh Bhanushali:** Conceptualization, Funding acquisition. **Amit Khanna:** Conceptualization, Supervision, Writing – review & editing.

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.

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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.2024.103402.

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