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Lab resource: Stem Cell Line

# Generation of a human iPSC line GIBHi002-A-2 with a dual-reporter for NKX2-5 using TALENs

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A B S T R A C T

The human transcription factor NKX2-5 plays an important role in cardiac formation and development, and thus it can be used for isolation of cardiomyocytes (CMs) differentiated from human pluripotent stem cells (hPSCs). Here, we knocked-in enhanced GFP (eGFP) and Pac (a puromycin resistant gene; Puro$^R$) into the exon 1 coding region of NKX2-5 from a human iPSC line iPSN0003 using TALENs. The generated GIBHi002-A-2 enables us to monitor and optimize cardiac differentiation procedures via the cardiac progenitor cells (CPCs), as well as to isolate iPSC-derived CMs for drug screening.

Resource Table:

| | |
|---|---|
| Unique stem cell line identifier | GIBHi002-A-2 |
| Alternative name(s) of stem cell line | NKX2-5 $^{GFP\text{-}Puro/wt}$ iPSC |
| Institution | Cell Inspire Biotechnology (CIB), Shenzhen, China; Department of Medicine, the University of Hong Kong (HKU), Hong Kong SAR, China |
| Contact information of distributor | Jiayin Yang; jyyang@cib.cc |
| Type of cell line | iPSC |
| Origin | Human |
| Additional origin info | Age: 24<br>Sex: female<br>Ethnicity if known: Caucasian |
| Cell Source | Epithelia cells from urine |
| Clonality | Clonal |
| Method of reprogramming | Retroviral Yamanaka factors |
| Genetic Modification | Yes |
| Type of Modification | Targeted knock-in |
| Associated disease | N/A |
| Gene/locus | NKX2-5, 5q35.1 |
| Method of modification | TALENs |
| Name of transgene or resistance | eGFP and Pac (Puro$^R$) |
| Inducible/constitutive system | N/A |
| Date archived/stock date | June 2015 |
| Cell line repository/bank | https://hpscreg.eu/cell-line/GIBHi002-A-2 |
| Ethical approval | Ethics committee: Institutional Review Board of the University of Hong Kong/Hospital Authority Hong Kong West Cluster (Approval No. UW 13-342) |

## 1. Resource utility

We knocked-in a cassette containing eGFP and Pac into the NKX2-5 locus of normal human iPSCs to generate GIBHi002-A-2 by TALENs-mediate homologous recombination. GIBHi002-A-2 enables us to monitor and optimize cardiac differentiation procedures from human iPSCs, as well as to isolate CPCs and CMs by drug selection (Table 1).

## 2. Resource details

Patient-specific CMs can be differentiated from human iPSCs and are widely used in disease modeling and drug toxicity testing (Burridge

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**Table 1**
Characterization and validation.

| Classification | Test | Result | Data |
|---|---|---|---|
| Morphology | Photography | Normal | Fig. 1B |
| Phenotype | Qualitative analysis | Expression of OCT4, NANOG, Tra-1-60, SSEA4 | Fig. 1E |
| | Quantitative analysis | Relative expression to H9 hESC (set as 1): OCT4, 2.05; SOX2, 1.20; NANOG, 1.81; REX1, 1.15. | Fig. 1D |
| Genotype | Karyotype (G-banding) and resolution | 46, XX. Resolution 320–400 | Fig. 1C |
| Identity | Microsatellite PCR (mPCR) OR | Not performed | N/A |
| | STR analysis | 20 sites tested for engineered iPSCs and 16 sites tested for donor iPSC. All tested sites matched | Submitted in archive with journal |
| Mutation analysis (IF APPLICABLE) | Sequencing | N/A | N/A |
| | Southern Blot OR WGS | N/A | N/A |
| Microbiology and virology | Mycoplasma | Mycoplasma testing by luminescence: Negative | Supplementary Fig. 1F |
| Differentiation potential | Teratoma formation | Proof of three germ layers formation | Fig. 1F |
| 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 |

et al., 2016). However, the heterogeneity of the differentiated cells from hPSCs limits their applications. To facilitate the identification and isolation of the human hPSCs-derived CMs, an eGFP coding sequence was knocked into the NKX2-5 locus of a hESC line (Elliott et al., 2011). This line enables purification or monitoring the differentiation process of hESCs-derived CMs using eGFP. However, as dissociation and sorting may introduce extra stress to CMs, we aimed to generate a human iPSC line with a dual-reporter, eGFP and Pac, driven by a NKX2-5 promotor.

We designed a cassette containing eGFP and Pac, which were linked by a 2A sequence, and this cassette was then inserted into a donor plasmid containing left and right homologous arms of 700-1000 base pairs that flank the genomic site of NKX2-5 locus. A loxP-site-flanked PGK-Neo$^R$ selection cassette enables enrichment of targeted clones (Fig. 1A). Additionally, we designed a pair of TALENs to target the exon 1 of NKX2-5 to initiate a double-strand break (Supplementary Fig. 1A), which has been proved to be able to improve the homologous recombination efficiently (Hockemeyer et al., 2011). Junction PCR (F1/R1 and F2/R2) and the CEL-I PCR (F3/R3) analysis confirmed that several single cell-derived clones have targeted monoallelic integration of donor constructs (Supplementary Fig. 1B-1C). We further used Sanger sequencing to exclude clones that have non-homologous end joining event in the cutting site of the second allele (Supplementary Fig. 1D). We selected clone 18 for further experiment. To remove the drug selection cassette, we overexpressed CRE recombinase in clone C18 and identified a subclone C14 (GIBHi002-A-2) that was free of drug selection cassette (Supplementary Fig. 1E). GIBHi002-A-2 has typical hESC morphology (Fig. 1B, scale bar represents 200 μm), a normal karyotype (Fig. 1C), and express pluripotency markers (Fig. 1D and 1E; scale bars represent 50 μm). In addition, GIBHi002-A-2 can form teratomas containing tissues of three germ layers after subcutaneously injected into SCID mice (Fig. 1F; scale bars represent 50 μm). Moreover, GIBHi002-A-2 was free of mycoplasma contamination (Supplementary Fig. 1F), and has the same STR profile with its parental cells (Submitted and archived with journal).

We further differentiated GIBHi002-A-2 into the cardiac lineage (Supplementary Fig. 1G). As expected, GFP can be observed as early as day-8 of differentiation, and the expression level was stabilized at around day-11 of differentiation (Supplementary Fig. 1H, scale bars represent 200 μm). Quantitative real-time PCR (RT-qPCR) showed that the differentiated cells expressed lineage specific markers of CPCs (ISL-1 and NKX2-5) and CMs (TNNT2; Supplementary Fig. 1I) at the indicated stages. As expected, immunofluorescent staining showed that the GFP$^+$ cells co-expressed NKX2-5, TNNT2 and ACTN2 (Fig. 1G; scale bars represent 200 μm). After treating the day-8 differentiated CMs with puromycin for 8 days, we observed almost all cells are GFP$^+$ (Fig. 1H; scale bars represent 100 μm), indicating GIBHi002-A-2-derived CMs can be enriched by puromycin.

## 3. Materials and methods

### 3.1. Cell culture

Human iPSCs were cultured on Matrigel-coated plates and fed with mTeSR1 medium (STEMCELL Technologies) at 37 °C in a 5% $CO_2$ humidified incubator. When grew confluence, cells were dissociated with Accutase and seeded to a new Matrigel-coated plate with a split ratio of 1:6 in mTeSR1. Five μM Y27632 (Sigma-Aldrich) was used in the culture medium on the first day of passaging to promote cell survival.

### 3.2. Genetic modification

The donor plasmids and the designed TALENs were co-delivered to human iPSCs by nucleofection using Amaxa Nucleofector II Electroporation Machine (Lonza). The transfected cells were plated on Matrigel-coated plate and cultured in mTeSR1 supplemented with 10 μM Y27632. To enrich the targeted cells, cells were treated with 0.5 mg/mL G418 in mTeSR1 supplemented with 10 μM Y27632 for 24 h, then medium was changed to mTeSR1 for recovery. For single cell cloning, the enriched human iPSCs were dissociated into a single cell suspension with Accutase, and seeded to feeder- or Matrigel-coated flasks at a density of 50–200 cells/cm$^2$. Seven to 10 days later, single cell-derived colonies were picked for expansion and characterization. Genomic DNA of cells was obtained using QuickExtract™ DNA Extraction Solution 1.0 (Epicentre), and Junction PCR, CEL-I PCR and CRE-out PCR were employed to identify the targeted clones using primers listed in Table 2.

### 3.3. Cardiac differentiation

Human iPSCs were differentiated into CMs using a PSC Cardiomyocyte Differentiation Kit (Gibco). In brief, when iPSCs grow to an optimal confluence in E8 medium (day-0), change medium with Medium A. 48 h later, change medium with Medium B and cultured for another 2 days. On day-4 and thereafter, change the medium to CMs Maintenance Medium and replace the medium every two days. To enrich CMs, day-8 differentiated cells were treated with 1.5 ug/mL puromycin (Yeasen Biotech) for 8 days.

### 3.4. Immunofluorescence staining

Cells were fixed with 4% paraformaldehyde solution at 4 ℃ overnight or room temperature for 10 min, then washed with PBST solution (PBS supplemented with 0.05% triton-X) for three times. Cells were stained with primary antibody overnight, followed by the corresponding secondary antibody for 1 h at room temperature. Finally, cell nucleus were stained with DAPI (Sigma-Aldrich). Antibodies used in this study are listed in Table 2.

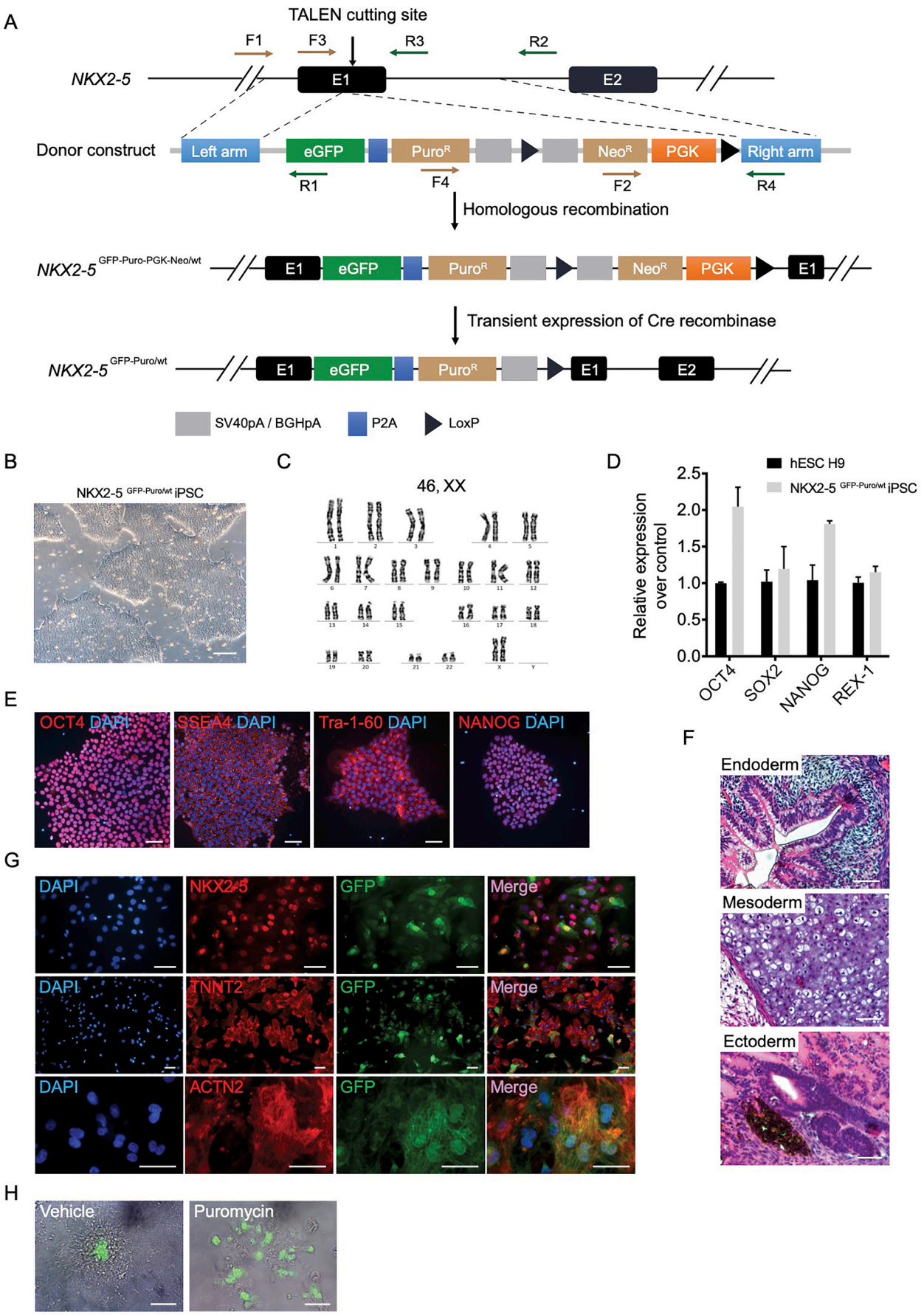


**Fig. 1.** Characterization and validation of GIBHi002-A-2.

**Table 2**
Reagents details.

| Antibodies used for immunocytochemistry/flow-cytometry | | | |
|---|---|---|---|
| | Antibody | Dilution | Company Cat # and RRID |
| Pluripotency Markers | Goat anti-NANOG | 1:20 | R&D Systems Cat# AF1997, RRID: AB_355097 |
| | Rabbit anti-OCT4 | 1:200 | Invitrogen Cat# PA5-27438 RRID: AB_2544914 |
| | Mouse anti-Tra-1–60 | 1:50 | Millipore Cat# MAB4360 RRID: AB_2119183 |
| | Mouse anti-SSEA4 | 1:60 | Abcam Cat# ab16287 RRID: AB_778073 |
| Cardiac Differentiation Markers | Rabbit anti-NKX2-5 | 1:50 | Santa Cruz Cat# sc-14033 |
| | goat anti-TNNT2 | 1:250 | Abcam Cat# ab64623, RRID: AB_1139590 |
| | mouse anti-ACTN2 | 1:250 | Sigma-Aldrich Cat#A7811 RRID: AB_476766 |
| Secondary antibodies | NL557 Donkey Anti-Goat IgG | 1:200 | R&D Systems Cat# NL001 RRID: AB_663766 |
| | Alexa Fluor 555 Goat anti-Rabbit IgG | 1:1000 | Invitrogen Cat# A21428 RRID:AB_141784 |
| | Alexa Fluor 555 Goat anti-Mouse IgG (H + L) | 1:1000 | Invitrogen Cat# A21424 RRID: AB_141780 |
| | Alexa Fluor 594 Goat anti-Rabbit IgG (H + L) | 1:1000 | Invitrogen Cat# A11072 |
| | Alexa Fluor 594 Rabbit anti-mouse IgG (H + L) | 1:1000 | Invitrogen Cat# A11062 |
| | Alexa Fluor 594 Donkey anti-goat IgG (H + L) | 1:1000 | Invitrogen Cat# A11058 |
| **Primers** | | | |
| | **Target** | **Forward/Reverse primer (5′-3′)** | |
| Pluripotency Markers (qPCR) | *OCT4* | AGTTTGTGCCAGGGTTTTTG | ACTTCACCTTCCCTCCAACC |
| | *NANOG* | TTTGGAAGCTGCTGGGGAAG | GATGGGAGGAGGGGAGAGGA |
| | *SOX2* | AGTCTCCAAGCGACGAAAAA | TTTCACGTTTGCAACTGTCC |
| | *REX1* | AGGCCAGTCCAGAATACCAG | TAGGTATCCGTCAGGGAAGC |
| | *GAPDH* | GTGGACCTGACCTGCCGTCT | GGAGGAGTGGGTGTCGCTGT |
| Cardiac Markers (qPCR) | *ISL-1* | GCAGAGTGACATAGATCAGCCTG | GCCTCAATAGGACTGGCTACCA |
| | *NKX2-5* | CATTTTACCCGGGAGCCTAC | CTTTGTCCAGCTCCACTGC |
| | *TNNT2* | CATGTCTTCAGCCACTGACC | TGGCCTTGTCCTGGGTTAT |
| | *GAPDH* | CTAACATCAAATGGGGTGAGG | CGGAGATGATGACCCTTTTG |
| Junction PCR | Left arm (F1/R1) | TCCAAACAGGGGAAACAAGGTG | CGCTGAACTTGTGGCCGTTTAC |
| | Right arm (F2/R2) | GATACTTTCTCGGCAGGAGCA | ACCTATGAATTCTCTCCCGGC |
| CRE-out PCR | F4/R4 | GAGCAAAGACCCCAACGAGA | GGCGCAAAGAAAGAAAGGCA |
| CEL-I PCR | TALEN cutting region (F3/R3) | CCGTGGGCAGCGCCGCTTTCTG | GGGGGCCTGTGTTTCCTCCTCACC |

### 3.5. RT-qPCR

Total RNA was extracted from cell lysates in TRI Reagent® (Sigma-Aldrich) using RNAprep pure kit (TIANGEN) according to the manufacturer's protocol. A PrimeScript™ RT reagent kit with gDNA Eraser (TaKaRa) was used for reverse transcription of 1 μg RNA to cDNA. RT-qPCR was carried out using a Real-Time PCR machine and SYBR Green Premix EX Taq™ (Takara) or a QuantiFast SYBR Green PCR Kit (Qiagen). *GAPDH* was used for normalization, and samples were measured in triplicate. The primers used are listed in Table 2.

### 3.6. Karyotype

At day-2 post passaging, human iPSCs were treated with 100 μg/mL colchicine for 10 min. Then, at least 20 metaphases were randomly selected and analyzed by 320–400 G-banding chromosome analysis.

### 3.7. Teratoma formation

Human iPSCs cultured on Matrigel were dissociated with Accutase, and cells were injected subcutaneously into the flanks, or intramuscularly into the hind legs of SCID mice. Teratomas were dissected and sectioned, and were stained with hematoxylin & eosin. The animal experiment was approved by local Institutional Review Board (CULATR 3404-14).

### 3.8. STR analysis

Genomic DNA was extracted from GIBHi002-A-2 and the donor iPSCs, and were sent to third party diagnostic labs, Genetic Testing Biotechnology or Guangzhou KingMed Centre for Clinical Laboratory, for STR analysis respectively. Fifteen or nineteen STR loci plus Amelogenin were tested.

## 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.2020.102120.

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