Stem Cell Research

Generation of an INSULIN-H2B-Cherry reporter human iPSC line

--Manuscript Draft--

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Abstract:	Differentiating human induced pluripotent stem cells (hiPSCs) into insulin (INS)-producing β-like cells has potential for diabetes research and therapy. Here, we generated a heterozygous fluorescent hiPSC reporter, labeling INS-producing β-like cells. We used CRISPR/Cas9 technology to knock-in a T2A-H2B-Cherry cassette to replace the translational INS stop codon, enabling co-transcription and T2A-peptide mediated co-translational cleavage of INS-T2A and H2B-Cherry. The hiPSC-INS-T2A-H2B-Cherry reporter cells were pluripotent and showed multi-lineage differentiation potential. Cells expressing the β-cell specific hormone INS are identified by nuclear localized H2B-Cherry reporter upon pancreatic endocrine differentiation. Thus, the generated reporter hiPSCs enable live identification of INS hormone-producing β-like cells.

02nd March, 2020

To the Editors of Stem Cell Research

Please find enclosed our manuscript with the title "**Generation of an INSULIN-H2B-Cherry reporter human iPSC line**" by Anna Karolina Blöchinger, Johanna Siehler, Katharina Wißmiller, Alireza Shahryari, Ingo Burtscher and Heiko Lickert, which we submit to *Stem Cell Research* as a Lab Resource for review.

Autoimmune destruction of β-cells results in insulin deficiency in patients with type 1 diabetes. Generation and transplantation of insulin producing β-like cells from pluripotent stem cells *in vitro* is a promising approach to compensate for the loss of endogenous β-cells in type 1 diabetes. Here, we generated a human INS-T2A-H2B-Cherry induced pluripotent stem cell (iPSC) reporter line for identification of β-like cells during endocrine pancreatic differentiation *in vitro*.

We would be thankful for a review in *Stem Cell Research* and we would like to thank you in advance for your time and consideration.

Sincerely,

Heiko Lickert

Generation of an INSULIN-H2B-Cherry reporter human iPSC line

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Abstract

Differentiating human induced pluripotent stem cells (hiPSCs) into insulin (INS)-producing β-like cells has potential for diabetes research and therapy. Here, we generated a heterozygous fluorescent hiPSC reporter, labeling INS-producing β-like cells. We used CRISPR/Cas9 technology to knock-in a T2A-H2B-Cherry cassette to replace the translational INS stop codon, enabling co-transcription and T2A-peptide mediated co-translational cleavage of INS-T2A and H2B-Cherry. The hiPSC-INS-T2A-H2B-Cherry reporter cells were pluripotent and showed multi-lineage differentiation potential. Cells expressing the β-cell specific hormone INS are identified by nuclear localized H2B-Cherry reporter upon pancreatic endocrine differentiation. Thus, the generated reporter hiPSCs enable live identification of INS hormone-producing β-like cells.

Resource table

Unique stem cell line identifier	HMGUi001-A-1
Alternative name(s) of stem cell line	hiPSC-INS-T2A-H2B-Cherry (+/-), AB001
Institution	Institute of Diabetes and Regeneration Research, Helmholtz Zentrum München, 85764 Neuherberg, Germany
Contact information of distributor	Heiko Lickert, heiko.lickert@helmholtz-muenchen.de
Type of cell line	iPSCs
Origin	Human, HMGUi001-A hiPSCs described in Wang et al., 2018
Additional origin info	Age: N/A Sex: Female Ethnicity: Caucasian
Cell Source	Fibroblasts
Clonality	Clonal
Method of reprogramming	Nucleofection

Genetic Modification	YES
Type of Modification	Heterozygous insertion of a fluorescent reporter
Associated disease	N/A
Gene/locus	Insulin gene (INS)/11p15.5
Method of modification	CRISPR/Cas9
Name of transgene or resistance	Thosea asigna virus 2A (T2A)-histone 2B (H2B)-Cherry
Inducible/constitutive system	N/A
Date archived/stock date	February 2018
Cell line repository/bank	N/A
Ethical approval	The choice of appropriate human donors, the procedures for skin biopsy, isolation of dermal fibroblasts, generation of iPSCs and their use in further scientific investigations were performed under the positive vote of the Ethics Committee of the Medical Faculty of the Eberhard Karls University, Tübingen (file numbers 629/2012BO2 and 130/2018BO2). The study design followed the principles of the Declaration of Helsinki. All study participants gave informed consent prior to entry into the study.

Resource utility

The generated heterozygous hiPSC-INS-T2A-H2B-Cherry reporter line enables live identification of INS producing β-like cells during pancreatic differentiation. Expression of nuclear H2B-Cherry co-localizes with the β-cell-specific hormone INS and its cleavage peptide (C-peptide; C-PEP).

Resource Details

Insulin (INS) is a hormone secreted by pancreatic β-cells in the islets of Langerhans. By releasing INS, β-cells promote glucose uptake in peripheral organs and consequently regulate constant blood glucose levels. Autoimmune destruction of β-cells results in INS deficiency in patients with type 1 diabetes (T1D). To compensate for the loss of endogenous β-cells in T1D, generating INS producing β-like cells from hiPSCs *in vitro* is a promising approach for cell-replacement therapy. Monitoring the expression of INS throughout the differentiation is a powerful tool to improve differentiation of functionally relevant β-like cells. Here, we generated a hiPSC-INS-T2A-H2B-Cherry reporter to monitor live β-like cell formation during pancreatic endocrine differentiation *in vitro*. We targeted exon 3 of the *INS* gene of the previously described hiPSCs HMGUi001-A (Wang et al., 2018) to generate a C-terminal fusion of INS with T2A-H2B-Cherry, using CRISPR/Cas9 genome editing (Fig. 1A). The T2A self-cleaving sequence was used to generate a bi-cistronic reporter cassette enabling equimolar expression of H2B-Cherry and INS-T2A and thus avoiding loss of one copy of the endogenous *INS* gene. The heterozygous insertion of the targeting vector in clone A was confirmed by PCR analysis (Fig. 1B). The integrity of the non-targeted allele in clone A was confirmed by Sanger sequencing of the single guide RNA (sgRNA) target site in the non-targeted allele (Fig. S1A). Correct

integration of the targeting vector was validated by sequencing the 5' and 3' recombination borders of the knock-in (KI) allele (Fig. S1B and S1C).

The generated reporter cells had a normal karyotype (Fig. 1C) and formed colonies with normal hiPSC morphology (Fig. 1D). Short tandem repeat (STR) analysis confirmed derivation from the parental HMGUi001-A hiPSC line and the generated hiPSC line was not contaminated with mycoplasma (Fig. S1D).

We performed sgRNA off-target analysis on coding regions with the highest off-target scores. The analysis revealed no off-target effects (Fig. S1E). The differentiation ability of the newly generated reporter cell line was assessed by directed *in vitro* differentiations. INS-H2B-Cherry hiPSCs expressed markers for endoderm (FOXA2/SOX17), mesoderm (smooth muscle 22 alpha (SM22-α)) and ectoderm (NESTIN), and thus markers of all three germ layers (Fig. 1G).

Stage-wise pancreatic endocrine differentiation according to Rezania et al., 2014 was performed to test β–like cell differentiation ability. INS-H2B-Cherry hiPSCs showed expression of the pancreatic progenitor transcription factors PDX1 and NKX6.1 after 10 days of differentiation (Fig. 1H). INS-H2B-Cherry hiPSCs showed a bright nuclear H2B-Cherry signal in live imaging at day 15 (Fig. 1J) and at day 20 (Fig. 1K) during differentiation. Further characterization by immunocytochemistry showed co-localization of nuclear H2B-Cherry signal (stained with anti-RFP antibody (AB)) with cytoplasmic INS (Fig. 1I & Fig.1I') and cytoplasmic C-PEP staining (Fig. S1F & S1F') after 20 days of differentiation.

Materials and Methods

CRISPR/Cas9 genome editing

The INS-T2A-H2B-Cherry targeting vector was cloned by traditional cloning. The targeting vector contained a 1338bp 5' homology region (HR), T2A-H2B-Cherry coding sequences cloned from a pCAG-T2A-H2B-Cherry plasmid and a 754bp 3' HR. HRs were amplified by PCR using genomic DNA extracted from HMGUi001-A hiPSCs. For CRISPR/Cas9 mediated targeting we designed a sgRNA binding upstream of the INS Stop codon sequence (Fig. 1A) using CRISPOR website (http://crispor.tefor.net). The specific sgRNA was cloned into the BbsI site of the pU6-(BbsI)-sgRNA-CAG-Cas9-Venus-bpA plasmid (Addgene plasmid #86986). Approximately $2x10^5$ HMGUi001-A hiPSCs were transfected with Lipofectamine™ Stem Transfection Reagent (Fisher Scientific, Cat# STEM00003) to deliver 1.25 µg sgRNA/Cas9-Venus expressing plasmid and 1.25 µg targeting vector. Cells expressing sgRNA/Cas9-Venus were selected by sorting highly GFP expressing cells using flow cytometry. Cells were seeded at low density to obtain colonies derived from single cells. Single colonies were picked and expanded. Detailed transfection, sorting and expansion conditions are described in Yumlu et al., 2017. Correct insertion of the targeting construct was validated by PCR and Sanger sequencing.

Cell culture

Cells were cultured on Geltrex (Life Technologies, Cat# A1413302) coated plates using StemMACS™ iPS-Brew XF medium (Miltenyi Biotec, Cat# 130-104-368). The culture medium was replaced daily. Cells were passaged every three to four days using 5 mM EDTA (AppliChem, Cat#A4892) in PBS. 10 µM ROCK Inhibitor (Y-27632, Santa Cruz Biotechnology, Cat# sc-281642A) was added for 24 h after splitting. Incubation was performed at 37 °C, 5 % CO2 and 21 % O2.

Three germ layer differentiation

Directed three germ layer differentiation was performed using StemMACS™ Trilineage Differentiation Kit (Miltenyi Biotec, Cat# 130-115-660) according to manufacturer's instructions. Cells were stained for expression of endoderm, mesoderm and ectoderm markers.

Pancreatic differentiation

Differentiation towards pancreatic β-like cells was performed according to Rezania et al., 2014. 1.25 µM IWP-2 (Tocris-Bioscience, Cat# 3533/10) was added during stage 2 (S2) of differentiation. After 10 days of differentiation cells were collected and re-seeded to form 3D aggregates (~ 600 cells/aggregate) by using AggreWell™ 400 plates (Stem Cell Technologies, Cat# 34415). Cells were fixed after three days (S1), ten days (S4) and 20 days (S6) of differentiation. Cells were stained for endoderm markers (FOXA2/SOX17), pancreatic progenitor markers (PDX1/NKX6.1) and hormones (INS/C-PEP). For labelling H2B-Cherry expressing cells, RFP AB was used (Tab. 2).

Immunocytochemistry

Fixation and staining of adherent cells were performed as described in Wang et al., 2018. Embedding, sectioning and staining of 3D cell aggregates were performed according to Bastidas-Ponce et al., 2017. Detailed information about 1° ABs and 2° ABs is listed in Tab. 2.

Flow cytometry

Flow cytometry was used to quantify cellular expression of pluripotency markers. Cells were washed twice with PBS and detached with TrypLE™ Select Enzyme (Thermo Fisher Scientific, Cat# 12563011) for 3–4 min. Approximately $1x10^6$ cells were stained with conjugated surface ABs SSEA-4-FITC and TRA-1-60-PE according to manufacturer's instructions. Unstained samples and isotype controls were included. Detailed information about surface ABs and isotype controls is listed in Tab. 2.

STR Analysis and Karyotyping

STR analysis was performed using the AmpFℓSTR™Identifiler™ PCR Amplification Kit (appliedbiosystems, Cat# 4322288) according to manufacturer's instructions. Karyotyping was performed as described before (Wang et al., 2018). Karyotyping and STR analysis were performed by the Institute of Human Genetics, Technische Universität München and Helmholtz Zentrum München, Munich, Germany.

Acknowledgements

We thank G. Lederer, G. Eckstein and T. Meitinger for the karyotyping and for the STR analysis of our cells. We thank X. Wang for generating the parental hiPSC line. We are grateful to Ralf Kühn for discussion and advice. The project was funded by the German Federal Ministry of Education and Research (project number 01EK1607A)**.**

References

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Wang, X., Sterr, M., Burtscher, I., Chen, S., Hieronimus, A., Machicao, F., Staiger, H., Häring, H.-U., Lederer, G., Meitinger, T., Cernilogar, F. M., Schotta, G., Irmler, M., Beckers, J., Hrabě de Angelis, M., Ray, M., Wright, C. V. E., Bakthi, M., Lickert, H., 2018. Genome-wide analysis of PDX1 target genes in human pancreatic progenitors, Molecular Metabolism, March; 9: 57–68.

125 **Table 1: Characterization and validation**

Classification	Test	Result	Data
Morphology	Photography	Normal morphology	Fig. 1 panel D Scale bar: 100µm
Phenotype	Immunocytochemistry	Staining for OCT3/4 and SOX2	Fig. 1 panel E Scale bars: 50µm
	Flow Cytometry	SSEA-4 (98.4 %) TRA-1-60 (98.0 %)	Fig. 1 panel F
Genotype	Karyotype (G-banding) and resolution	46, XX Resolution 450-525 bands	Fig. 1 panel C
Identity	Microsatellite PCR (mPCR) OR	Not performed	
	STR analysis	AmpFℓSTR™Identifiler™ PCR Amplification Kit, 16 sites tested, all matched	submitted in archive with journal
Mutation analysis (IF APPLICABLE)	Sequencing	Heterozygous insertion of reporter cassette determined by PCR and confirmed by Sanger sequencing, three sgRNA putative off-target sites analyzed by Sanger sequencing	Fig. 1 panel B, Supplementary Fig. S1 panel A, B, C Supplementary Fig. S1 panel E
	Southern Blot OR WGS	Not performed	
Microbiology and virology	Mycoplasma	Biochemical luminescence MycoAlert™ Plus Mycoplasma Detection Kit, Lonza, Negative	Supplementary Fig. S1 panel D
Differentiation potential	Directed differentiation	Three germ layer formation: FOXA2/SOX17: endoderm SM22-α: mesoderm Nestin: ectoderm	Fig. 1 panel G Scale bars: 50µm
Donor screening (OPTIONAL)	HIV 1 + 2 Hepatitis B, Hepatitis C	N/A	
Genotype additional info (OPTIONAL)	Blood group genotyping	N/A	
	HLA tissue typing	N/A	

126 **Table 2: Reagents details**

Antibodies used for immunocytochemistry/flow-citometry			
	Antibody	**Dilution**	**Company Cat # and RRID**
Pluripotency Markers	Goat anti-OCT3/4	1:500	Santa Cruz Biotechnology Cat# sc-8628, RRID:AB_653551
	Rabbit anti-SOX2	1:400	Cell signaling Technology Cat# 3579S, RRID:AB_2195767
	Human anti-SSEA4-FITC	1:11, used for FACS	Miltenyi Biotec Cat# 130-098-371, RRID:AB_2653517
	Human anti-TRA-1-60-PE	1:11, used for FACS	Miltenyi Biotec Cat# 130-100-347, RRID:AB_2654227
Isotype controls	REA Control (S)-PE-Vio615	1:11, used for FACS	Miltenyi Biotec Cat# 130-107-146, RRID:AB_2661694
	REA Control (S)-FITC	1:11, used for FACS	Miltenyi Biotec Cat# 130-104-610, RRID:AB_2661688
Germ layers	Rabbit anti-FOXA2	1:1000	Cell signaling Technology Cat# 8186S, RRID:AB_10891055
	Goat anti-SOX17	1:1000	Neuromics Cat# GT15094, RRID:AB_2195648
	Rabbit anti-SM22-α	1:100	Abcam Cat# ab14106, RRID:AB_443021
	Mouse anti-Nestin	1:300	Abcam Cat# ab22035, RRID:AB_446723
Pancreatic progenitor Markers	Goat anti-PDX1	1:500	R&D Systems Cat# AF2419, RRID:AB_355257
	Rabbit anti-NKX6.1	1:300	Arcis Cat# NBP1-82553, RRID:AB_11023606
Hormone Markers	Rabbit anti-INS	1:400	Cell signaling Technology, Cat# 3014, RRID:AB_2126503
	Guinea pig anti-C-PEP	1:100	Abcam Cat# ab30477, RRID:AB_726924
Cherry Marker	Rat anti-RFP	1:1000	Chromotek Cat# 5F8, RRID:AB_2336064
Secondary antibodies	Donkey anti-rabbit Alexa Fluor 555 IgG	1:800	Invitrogen Cat# A31572, RRID:AB_162543
	Donkey anti-goat Alexa Fluor 488 IgG	1:800	Invitrogen Cat# A11055, RRID: AB_2534102
	Donkey anti-rabbit Alexa Fluor 488 IgG	1:800	Invitrogen Cat# A21206, RRID:AB_2535792
	Donkey anti-mouse Alexa Fluor 555 IgG	1:800	Invitrogen Cat# A31570, RRID:AB_2536180
	Donkey anti-guinea pig DyLight 649	1:800	Dianova Cat# 706-605-148, RRID:AB_2340476
		1:800	

	Donkey anti-rat Cy3 IgG (H+L)		Dianova Cat# 712-165-153, RRID:AB_2340667
Primers			
	Target	**Forward/Reverse primer (5'-3')**	
Cloning	T2A-H2B-Cherry/1238bp 5' homology arm/1338bp 3' homology arm/754bp	ACTAGTTTACTTGTACAGCTCGTCCATGCCG/CCTGGGCAACGTGCTGGTTATTG GCGGCCGCAAGAGGCCATCAAGCAGGTCTGTTC/ACGCGTCGTTGCAGTAGTTCTCCAGCTGGTAGAG GTCGACGCTGGAGAACTACTGCAACTAGAC/GGTACC AGCTCATGGTGCCATCTGAC	
Oligo for cloning sgRNA	Guide 4A	CACCGGGCTGGTAGAGGGAGCAGATGC/AAACGCATCTGCTCCCTCTACCAGCCC	
Genotyping	INS 5' knock-in (KI) allele/1590bp INS 5' wild-type (WT) allele/2266bp INS 3' knock-in (KI) allele/1059bp INS 3' wild-type (WT) allele/1851bp	GTGCTGACGACCAAGGAGATCTTC/CTCAACGTCGCCGCATGTTAG GTGCTGACGACCAAGGAGATCTTC/CAGCTCATGGTGCCATCTGAC AACAGTACGAACGCGCCGAG/AGCCAAGCAGCCCTGCTTAC GTGAGCCAACTGCCCATTGC/AGCCAAGCAGCCCTGCTTAC	
Sequencing	Amplification of the non-targeted allele Seq sgRNA target site in the non-targeted allele INS 5' KI allele/1590bp Seq 5' recombination border INS 3' KI allele/1073bp Seq 3' recombination border	GTGCTGACGACCAAGGAGATCTTC/TCACAACAGTGCCGGGAAGTGGG TCACAACAGTGCCGGGAAGTGGG GTGCTGACGACCAAGGAGATCTTC/CTCAACGTCGCCGCATGTTAG GTGCTGACGACCAAGGAGATCTTC AACAGTACGAACGCGCCGAG/TAGCAAAGGAAGCCAGCCAAG AGCCAAGCAGCCCTGCTTAC	
Off-target analysis	CAAP1/587bp COA1/622bp RADIL/584bp	CAGGTTGGCACTGCTATTG/AGGTCATGCCACTGCACAC GCTTTGACCACAGCACAAAC/ACCAGATCAGACCCTCAGTAAC GAGGAGTTCTCCCTGAAG/GTCTCATAGCACCAGGAC	

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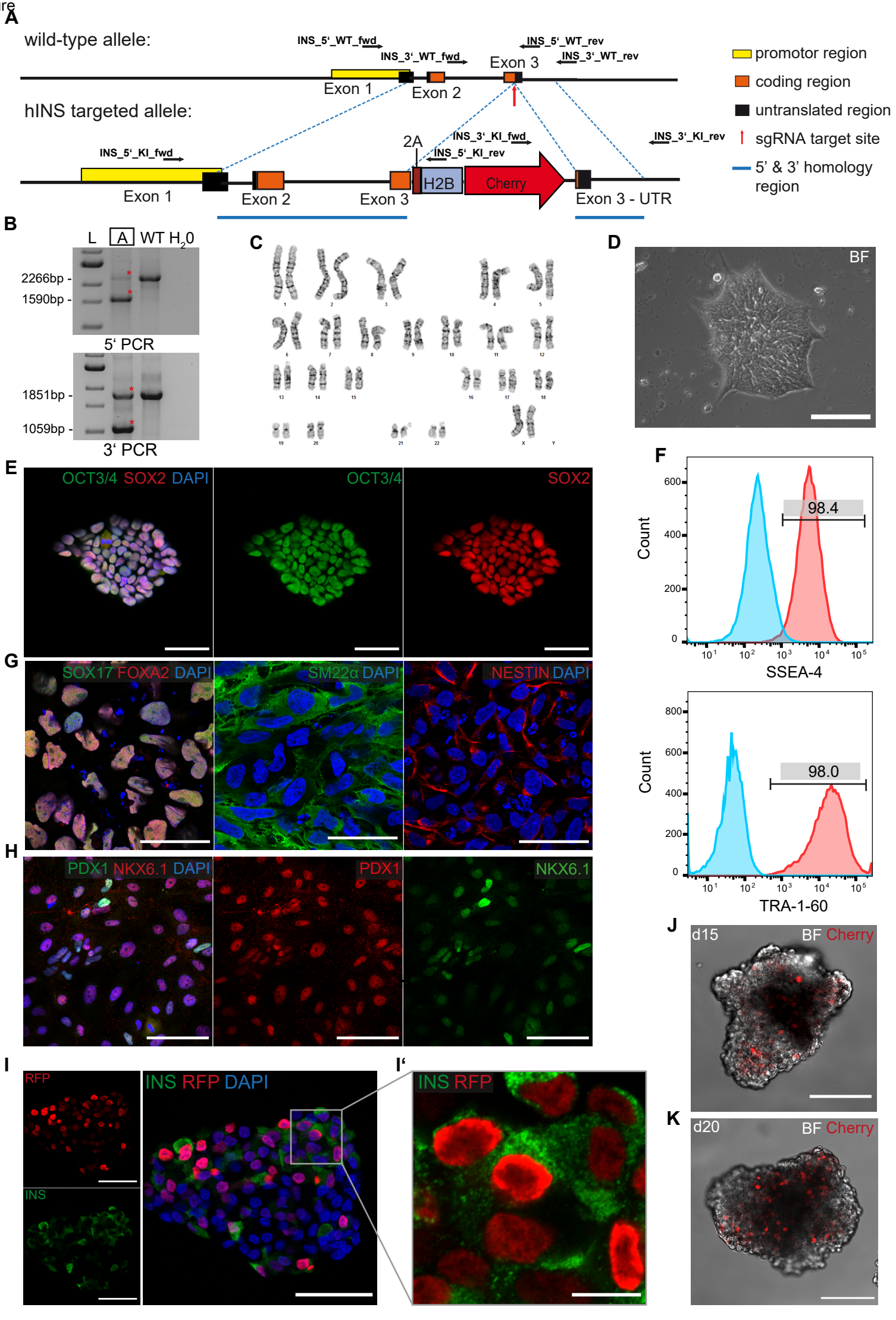
Figure


A
wild-type allele:
INS_5'_WT_fwd
INS_3'_WT_fwd
INS_5'_WT_rev
INS_3'_WT_rev
Exon 1
Exon 2
Exon 3
hINS targeted allele:
INS_5'_KI_fwd
2A
INS_3'_KI_fwd
INS_5'_KI_rev
INS_3'_KI_rev
H2B
Cherry
Exon 1
Exon 2
Exon 3
Exon 3 - UTR
promotor region
coding region
untranslated region
sgRNA target site
5' & 3' homology region
B
L
A
WT
H20
2266bp -
1590bp -
5' PCR
1851bp -
1059bp -
3' PCR
C
D
BF
E
OCT3/4 SOX2 DAPI
OCT3/4
SOX2
F
Count
98.4
SSEA-4
98.0
TRA-1-60
G
SOX17 FOXA2 DAPI
SM22α DAPI
NESTIN DAPI
H
PDX1 NKX6.1 DAPI
PDX1
NKX6.1
I
RFP
INS
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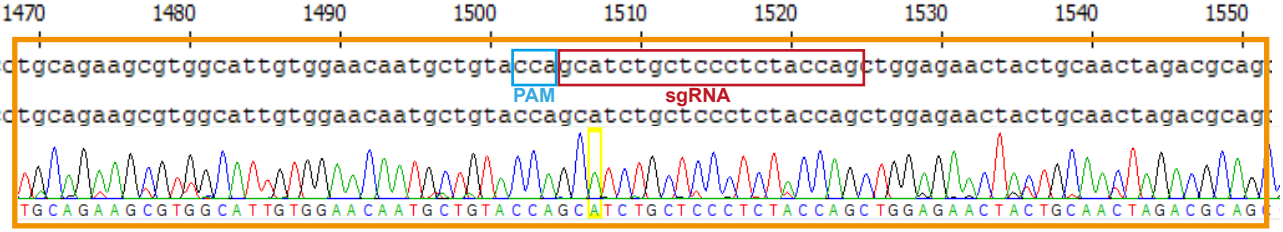
I'
INS RFP
J
d15
BF Cherry
K
d20
BF Cherry

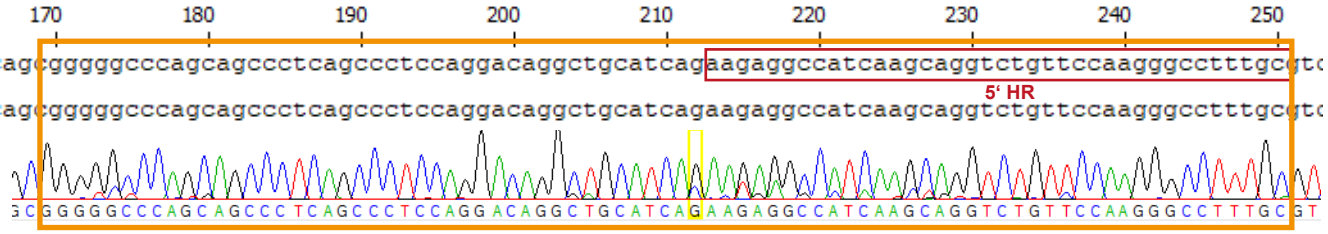
S1A



sgRNA target site: 1505 - 1524bp

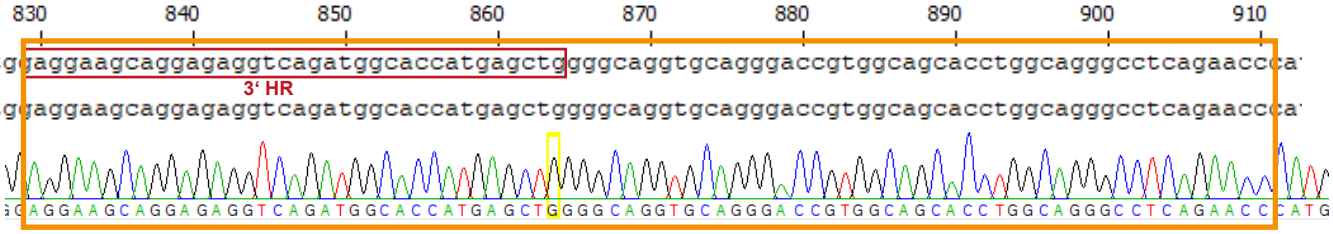
Cas9 cutting site: 1507/1508 bp

S1B



5‘ recombination border: 212bp

S1C



S1D

3‘ recombination border: 864bp

Mycoplasma results

Cell line: HMGUi001-A-1 **Passage number:** P30

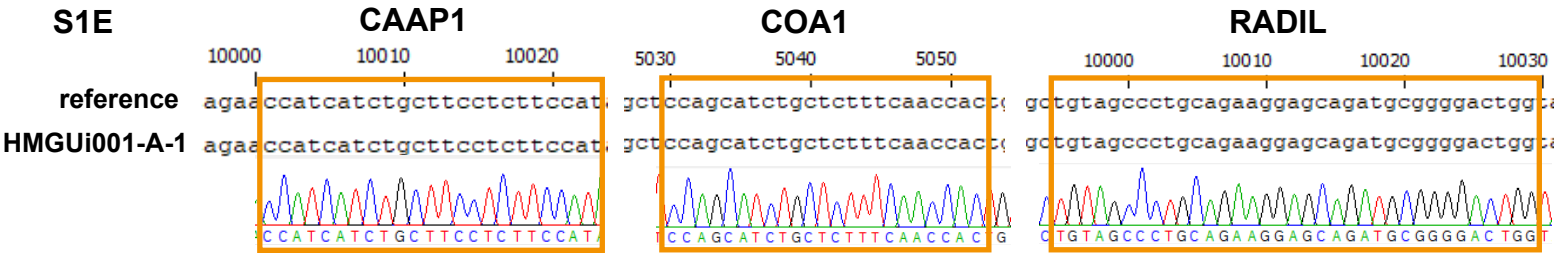
Test method: Biochemical luminescence MycoAlert™ Plus Mycoplasma Detection Kit, Lonza

Reading A: 5446 **Reading B:** 4013 **Ratio (B/A):** 0.74

Comments: Ratios <0.9 are negative for Mycoplasma
Ratios 0.9-1.2 are boderline
Ratios >1.2 are positive for Mycoplasma

Result: Mycoplasma negative

S1E

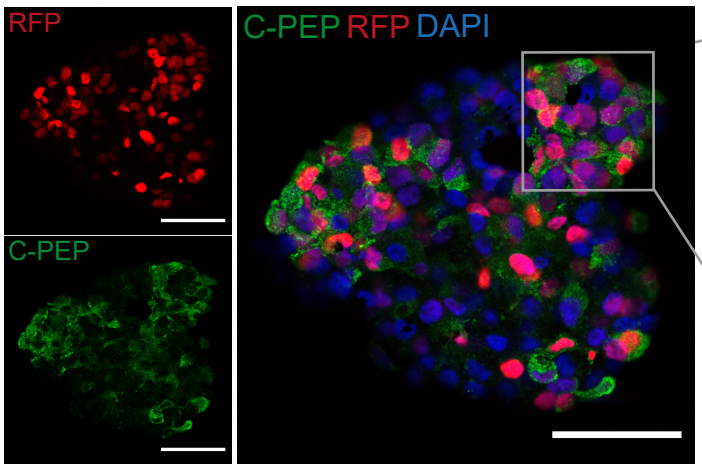


sgRNA recognition site: 10001 - 10023bp

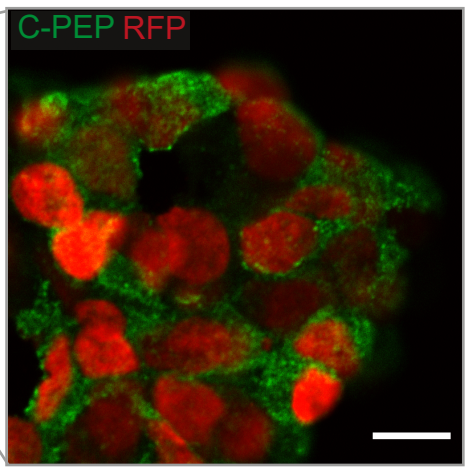
5030 - 5052bp

9995 - 10029bp

S1F



S1F‘



Conflicts of Interest

All authors declare no conflicts of interest.