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Lab Resource: Genetically-Modified Multiple Cell Lines



## Generation of AAVS1 and CLYBL STRAIGHT-IN v2 acceptor human iPSC lines for integrating DNA payloads

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### ABSTRACT

STRAIGHT-IN is a platform to precisely integrate DNA payloads into the genome of cells, including hiPSCs. Here, we generated two hiPSC acceptor lines each with one copy of an upgraded landing pad (LP). This improved design allows more efficient (~100 %) and rapid (~2–3 weeks) generation of genetically modified hiPSC lines containing the desired payloads. This new LP version was inserted into either the *AAVS1* (LUMCi004-A-1) or *CLYBL* (LUMCi004-A-2) safe harbour loci in the hiPSC line, LUMC0099iCTRL04. The resulting lines can be used for the targeted integration of a wide range of transgenes, thereby making them suitable for numerous research applications.

### Resource Table.

Unique stem cell lines identifier	LUMCi004-A-1 LUMCi004-A-2
Alternative name(s) of stem cell lines	LU99_AAVS1-bxb-v2 LU99_CLYBL-bxb-v2
Institution	Leiden University Medical Center (LUMC)
Contact information of the reported cell line distributor	Dr. Richard P. Davis <a href="mailto:r.p.davis@lumc.nl">r.p.davis@lumc.nl</a>
Type of cell lines	iPSC
Origin	Human
Additional origin info (applicable for human ESC or iPSC)	Age: 30–34 Sex: Female Ethnicity: Caucasian No disease known
Cell Source	Fibroblasts
Method of reprogramming	RNA (ReproRNA, Stem Cell Technologies)
Clonality	Clonal, EBFP + single cell isolation by fluorescence-activated cell sorting (FACS)
Evidence of the reprogramming transgene loss (including genomic copy if applicable)	N/A
Cell culture system used	Feeder-free culture. hiPSCs were maintained in StemFlex™ Medium (ThermoFisher) on laminin-521 (BioLamina)-coated plates.

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### (continued)

Type of Genetic Modification	Targeted heterozygous insertion of STRAIGHT-IN v2 landing pad (containing pGK-EBFP2; <i>BleoR</i> lacking ATG initiation codon; attP, loxP and lox251 sites)
Associated disease	N/A
Gene/locus	Adeno-associated virus integration site 1 ( <i>AAVS1</i> / <i>PPP1R12C</i> , chromosome 19q); or Citrate lyase beta-like ( <i>CLYBL</i> , chromosome 13q)
Method of modification/site-specific nuclease used	CRISPR/Cas ( <i>AAVS1</i> ); TALENs ( <i>CLYBL</i> )
Site-specific nuclease (SSN) delivery method	RNP (CRISPR/Cas); plasmid transfection (TALENs)
All genetic material introduced into the cells	<i>AAVS1</i> -Bxb1-LP-v2-TC vector; <i>CLYBL</i> -Bxb1-LP-v2-TC vector, <i>CLYBL</i> TALEN plasmids
Analysis of the nuclease-targeted allele status	PCR amplification and Sanger sequencing of the targeted loci, droplet digital PCR (ddPCR)
Method of the off-target nuclease activity surveillance	PCR amplification and Sanger sequencing of the untargeted allele and 6 of the top 8 predicted off-target sites
Name of transgene	<i>EBFP2</i> and Bleomycin resistance gene ( <i>BleoR</i> ) lacking ATG initiation codon

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**Table 1**  
Characterization and validation.

Classification	Test	Result	Data
<b>Morphology</b>	Brightfield imaging	Typical hiPSC morphology	Fig. 1D
<b>Pluripotency status evidence for the described cell line</b>	Quantitative analysis at passage 10 (flow cytometry)	Positive for pluripotency markers (OCT3/4 and NANOG)	Fig. 1G
	Qualitative analysis at passage 10 (immunofluorescence)	Positive for pluripotency markers (OCT3/4, NANOG and SSEA4)	Fig. 1F
<b>Karyotype</b>	G-banding at passage 8	46, XX (both lines)Normal	Fig. 1E
<b>Genotyping for the desired genomic alteration/allelic status of the gene of interest</b>	CNV analysis iCS-Digital kit	(both lines)	Supplementary Fig. 1C
	Genomic DNA PCR specific for homology directed insertion (3' and 5' junctions)	Specific integration in one <i>AAVS1</i> allele or one <i>CLYBL</i> allele verified by PCR and Sanger sequence	Fig. 1B and Supplementary Fig. 1A and 1B
<b>Verification of the absence of random plasmid integration events</b>	ddPCR	CNV analysis confirming 1 copy of the landing pad (both lines)	Fig. 1C
<b>Parental and modified cell line genetic identity evidence</b>	STR analysis	Both lines: STR profile matches LUMCi004-A (LUMC0099iCTRL04)	Results available on hPSCreg.eu and submitted in archive with journal
<b>Mutagenesis / genetic modification outcome analysis</b>	Sanger sequencing (genomic DNA PCR)	Sequencing chromatograms verifying integration of LP in one of the <i>AAVS1</i> or <i>CLYBL</i> alleles, and absence of genetic modification in the non-targeted allele	Supplementary Fig. 1A and B
	PCR-based analyses	PCR verifying the presence of integration in single edited allele and absence of integration in wild-type allele (both lines)	Fig. 1B
<b>Off-target nuclease analysis-</b>	PCR across top 5/10 predicted top likely off-target sites, whole genome/exome sequencing	Sanger sequencing of 6 of the top 8 genomic off-target sites indicates no mutations (both lines)	Supplementary Fig. 1F
<b>Specific pathogen-free status</b>	Mycoplasma	Mycoplasma testing at passage 8 using Lonza's mycoplasma detection kit: negative (both lines)	Supplementary Fig. 1G
<b>Multilineage differentiation potential</b>	Directed Trilineage differentiation analysed with immunofluorescence.	Positive IF for three-germ layer specific markers: endoderm (FOXA2 and GATA4), ectoderm (Nestin and PAX6) and mesoderm (Brachyury/T and Vimentin) – both lines	Fig. 1H
<b>Donor screening (OPTIONAL)</b>	HIV 1 + 2 Hepatitis B, Hepatitis C	N/A	–
<b>Genotype - additional</b>	Blood group genotyping	N/A	–
<b>histocompatibility info (OPTIONAL)</b>	HLA tissue typing	N/A	–

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Eukaryotic selective agent resistance (including inducible/gene expressing cell-specific)	Positive (Zeocin)
Inducible/constitutive system details	<i>EBFP2</i> driven by pGK constitutive promoter; <i>BleoR</i> expressed following targeted integration of donor DNA vectors
Date archived/stock date	N/A
Cell line repository/bank	<a href="https://hpscereg.eu/cell-line/LUMCi004-A-1">https://hpscereg.eu/cell-line/LUMCi004-A-1</a> <a href="https://hpscereg.eu/cell-line/LUMCi004-A-2">https://hpscereg.eu/cell-line/LUMCi004-A-2</a> <a href="https://divvly.com/lumc-hipsc-hotel">https://divvly.com/lumc-hipsc-hotel</a>
Ethical/GMO work approvals	Ethical approval: see parental line LUMC0099iCTRL04 at <a href="https://hpscereg.eu/cell-line/LUMCi004-A">https://hpscereg.eu/cell-line/LUMCi004-A</a> LUMC GMO permit: 15-104_I
Addgene/public access repository recombinant DNA sources' disclaimers (if applicable)	<i>AAVS1</i> -Bxb1-LP-v2-TC (Addgene, #194326) <i>CLYBL</i> -Bxb1-LP-v2-TC (Addgene, #194327)

## 1. Resource utility

The STRAIGHT-IN v2 hiPSC lines facilitate 100% enrichment of integrated transgenes, thereby enabling a pooled selection of polyclonal hiPSCs to be used downstream which may reduce clonal variation often observed between hiPSC clones. The LP-v2 was separately targeted to *AAVS1* and *CLYBL*, providing options of loci for sustained transgene expression. Table 1.

## 2. Resource details

The previously described STRAIGHT-IN acceptor hiPSC lines could integrate large DNA constructs into the *AAVS1* locus (Blanch-Asensio et al., 2022). Although *AAVS1*-Bxb1 hiPSCs demonstrated higher integration efficiencies compared to the counterpart *AAVS1*- $\phi$ C31 hiPSCs, enrichment of the integrated payloads was lower with the *BsdR* selection cassette present in *AAVS1*-Bxb1 than with *BleoR* in the *AAVS1*- $\phi$ C31 hiPSCs. Therefore, having hiPSC acceptor lines containing Bxb1-attP and the ATG-less *BleoR* selection cassette could be beneficial in generating polyclonal transgene lines. Additionally, the *EGFP* transgene present in the LP in the original STRAIGHT-IN hiPSC lines was disadvantageous when integrating payloads containing GFP reporters. Consequently, in the new LP (LP-v2) we replaced it with *EBFP2* as BFP reporters are less frequently used.

*CLYBL* supports the expression of transgenes targeted to this locus (Cerbini et al., 2015), providing an additional site to *AAVS1* for targeted gene transfer/knock-in. We therefore separately targeted the LP-v2 to the *PPP1R12C/AAVS1* and *CLYBL* loci using respectively either CRISPR/Cas9 or TALENs (Fig. 1A). PCR screening indicated monoallelic targeting of the LP-v2 into the corresponding loci (Fig. 1B), with ddPCR analysis confirming a single copy was present in the genome (Fig. 1C). Sanger sequencing verified that homologous recombination occurred at both loci, the targeted LP-v2 was intact, and the non-targeted allele did not contain indels (Fig. S1A, B). No mutations were detected at 6 of the genomic sites with the highest off-target editing scores for the sgRNA used for generating *AAVS1*-bxb-v2 (Fig. S1F).

Both *AAVS1*-bxb-v2 and *CLYBL*-bxb-v2 lines displayed typical hiPSC colony morphology (Fig. 1D), were karyotypically normal by G-banding,

Figure 1

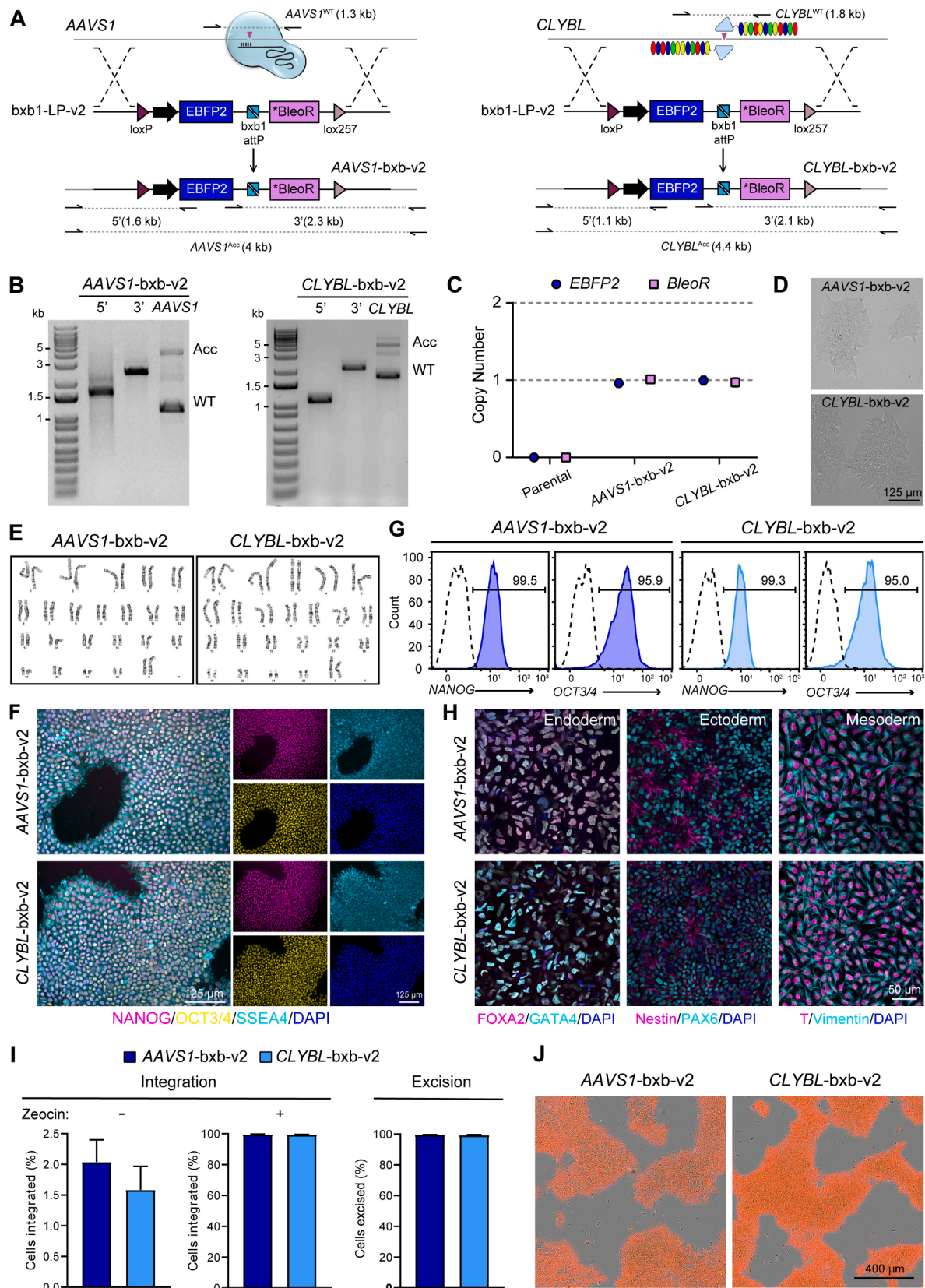


Fig. 1. Generation, characterization and functional validation of LUMCi004-A-1 (LU99\_AAVS1-bxb-v2) and LUMCi004-A-2 (LU99\_CLYBL-bxb-v2).

**Table 2**  
Reagents details.

Antibodies and stains used for immunocytochemistry/flow-cytometry			
	Antibody	Dilution	Company Cat # and RRID
Pluripotency Markers (Flow cytometry)	Anti-Oct3/4 BV421	1:25	BD Biosciences Cat#565644. RRID:AB_2739320 BD Biosciences Cat#560483. RRID:AB_1645522
Pluripotency Markers (IF staining)	Anti-NANOG PE Anti-OCT3/4 (Mouse IgG2b)Anti-Nanog (Rabbit) Anti-SSEA4 (Mouse IgG3)	1:5 1:100 1:200 1:400	Santa Cruz Cat#sc-5279. RRID:AB_628051 Cell Signaling Technology Cat#4903. RRID:AB_10559205 Biolegend Cat#330402. RRID:AB_1089208
Differentiation Markers (IF staining)	FOXA2-Alexa555 GATA4-Alexa647 Nestin-Alexa488 PAX6-Alexa647 Brachyury-Alexa488 Vimentin-Alexa647	1:500 1:200 1:200 1:200 1:200 1:400	Cell Signaling Technology Cat#8186. RRID:AB_10891055 Cell Signaling Technology Cat#36966. RRID:AB_2799108 Cell Signaling Technology Cat#33475. RRID:AB_2799037 Cell Signaling Technology Cat#60433. RRID:AB_2797599 Cell signalling Technology Cat#81694. RRID:AB_2799983 Cell signalling Technology Cat#5741. RRID:AB_10695459
Secondary antibodies	Donkey-anti-Rabbit Alexa555 Goat-anti-Mouse IgG2b Alexa647 Goat anti-Mouse IgG3 Alexa488	1:250 1:250 1:250	Invitrogen Cat#A-31572. RRID:AB_162543 Invitrogen Cat#A-21242. RRID:AB_2535811 Invitrogen Cat#A-21151. RRID:AB_2535784
Nuclear stain Site-specific nuclease Nuclease information	DAPI SpCas9 (AAVS1) and TALENs (CLYBL)	5 µg/mL	ThermoFisher Cat#D3571. RRID: AB_2307445 (D'Astolfo et al., 2015); Addgene #62196 and #62197
Delivery method Selection/enrichment strategy	Electroporation EBFP2		Neon Transfection System (ThermoFisher) Enrichment of EBFP2 + cells by FACS

**Primers and Oligonucleotides used in this study**

	Target	Forward/Reverse primer (5'-3')
Genotyping (desired allele/transgene presence detection)	AAVS1 targeted allele AAVS1 non-targeted allele	5' junction: CCGGAACCTCTGCCCTCTAA/CTCCTGCGCCCTTGCTCACCAT 3' junction: GATCTCATGTGGAGTTCTTCG/GTGAGTTTGCCAAGCAGTCA TTCGGGTACCTCTCACTCC/GTGAGTTTGCCAAGCAGTCA
Genotyping (desired allele/transgene presence detection)	CLYBL targeted allele CLYBL non-targeted allele	5' junction: AGATCATCCAGCCCTAGTCAAAG/GCTAAAGCGCATGCTCCAGACTG 3' junction: GATCTCATGTGGAGTTCTTCG/TGGAGCAGTGGATGACAACCT AGATCATCCAGCCCTAGTCAAAG/TGGAGCAGTGGATGACAACCT
Targeted mutation analysis/sequencing	AAVS1	Left homology arm (LHA): GGAGTGAGAGGTGACCCGAA loxP: GCTAAAGCGCATGCTCCAGACTG attP: CGGACCACACCGGCGAAGTC lox257: GGCTCCATCGTAAGCAAACC Right homology arm (RHA): GGTTTGCTTACGATGGAGCC WT Allele: TTCGGGTACCTCTCACTCC
Targeted mutation analysis/sequencing	CLYBL	LHA: GGCACAGTGTGTTAGTCAGAGC loxP: GCTCTGACTAAACACTGTGCC attP: CGGACCACACCGGCGAAGTC lox257: GCTACTTGGTAGGCTGAGACA RHA: TGTCTCAGCCTACCAAGTAGC WT Allele: GCTCTGACTAAACACTGTGCC
Copy number variation (ddPCR)	RPP30 and BleoR	(Blanch-Asensio et al., 2022)
Copy number variation (ddPCR)	EBFP2	Forward: TCCTTGAAGAAGATGGTGCG Reverse: TGCCCGTGCCCTGCCCCACCTT Probe: TGACCTGAAGTTCATCTGC (FAM-ZEN-IBFQ)
Copy number variation (ddPCR)	Bxb1-attP	Forward: GCATTCTAGTTGTGGTTTGTC Reverse: AAGTCGTCCTCCACGAAAG Probe: CGTGGTTTGTCTGGTCAACCA (HEX-ZEN-IBFQ)
Copy number variation (ddPCR)	Bxb1-attR	Forward: GCATTCTAGTTGTGGTTTGTC Reverse: CCTGCTTGCCGAATATCATG Probe: TCTCCGTCGTCAGGATCATCC. (HEX-ZEN-IBFQ)
gRNA oligonucleotide sequence (AAVS1) Off-target screening	sgRNA CPNE5 (intron) SNORA25/AL603632.1 (intergenic) DAPK2/RP11-111E14.1 (intergenic) RNU6-715P/RNA5SP106 (intergenic)	GGGGCCACTAGGGACAGGAT Forward: GATGTGTATAAGAGACAGgggtgctccctgaagacatc Reverse: CGTGTGCTCTCCGATCTcagggtctctcacatag Forward: CTAACATGAAGCCTTGAGTAAC Reverse: GAGCAGTATAATGAATGCAC Forward: CACAGAGACATGTCTCTTTGTG Reverse: GACAATGGCACATCTGATCCAG Forward: GAGATGAAGACTCCAATTTAG Reverse: CACTAATGATGTTCACTCAACAG

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Table 2 (continued)

Antibodies and stains used for immunocytochemistry/flow-cytometry			
	Antibody	Dilution	Company Cat # and RRID
	MIR7151/CTNNA3 (intergenic)	Forward: CACTCACACACCTCACCAAGAC Reverse: GTTCTTGAGAGCCATTTATTC	
	LINC00905/CTD-2231E14.4 (intergenic)	Forward: TCAACTACCTTATTCTCTGAG Reverse: ACTAGCTGCATGACCCCATCAG	
TALENs	Plasmid	pZT-C13-L1 (Addgene, #62196) pZT-C13-R1 (Addgene, #62197)	
Plasmids used as templates for HDR-mediated site-directed mutagenesis.	Plasmid	AAVS1-Bxb1-LP-v2-TC (Addgene, #194326) CLYBL-Bxb1-LP-v2-TC (Addgene, #194327)	

and mycoplasma negative (Fig. 1E and S1G). The genome integrity of the lines was also corroborated using a ddPCR assay detecting ~ 90 % of recurrent genomic abnormalities in hiPSCs (Fig. S1C). The hiPSC lines were positive for pluripotency markers (Fig. 1F), with > 95 % of the cells expressing NANOG and OCT3/4 as analysed by flow cytometry (Fig. 1G). Both lines differentiated into cell types from the three germ layers, based on immunofluorescence staining for the markers FOXA2 and GATA4 (endoderm), Nestin and PAX6 (ectoderm) and Brachyury/T and Vimentin (mesoderm, Fig. 1H).

We confirmed that DNA payloads could integrate into both AAVS1-bxb-v2 and CLYBL-bxb-v2 hiPSCs (Fig. S1D). As expected, colonies only grew following zeocin selection when the Bxb1-donor plasmid was transfected (Fig. S1E). Prior to selection, on average 1.6 and 2.0 % of the AAVS1-bxb-v2 and CLYBL-bxb-v2 hiPSCs respectively had integrated the Bxb1-donor plasmid, which increased to > 99.4 % after selection (Fig. 1I). Subsequent transfection of a plasmid co-expressing cre recombinase and the puromycin-resistance gene (pEFBOS\_CreIRES-puro), combined with puromycin selection, resulted in a population in which > 99.3 % of the hiPSCs contained only the payload flanked with heterotypic lox sequences (Fig. 1I).

With near 100 % integration and excision efficiencies of the hiPSC acceptor lines containing this updated version of the STRAIGHT-IN platform, multiple assay-ready transgenic cell lines can be generated in parallel within 3 weeks. For example, we integrated a constitutively-expressing tdTomato transgene with nearly all the hiPSCs expressing the reporter following cre-mediated excision of the auxiliary sequences (Fig. 1J).

### 3. Materials and methods

#### 3.1. hiPSCs culture

hiPSCs (LUMC0099iCTRL04, RRID:CVCL\_UK77) were maintained in StemFlex™ Medium (ThermoFisher) on laminin-521 (LN521; BioLamina)-coated (1.5 µg/cm<sup>2</sup>) plates at 37°C and 5% CO<sub>2</sub> as previously described (Blanch-Asensio et al., 2022). Cells were passaged twice a week (at a ratio of 1:8–1:12) by dissociating with 1x TrypLE Select (ThermoFisher).

#### 3.2. Generation of acceptor hiPSC lines

The procedure to target the AAVS1-Bxb1-LP-v2-TC cassette (Addgene #194326) to the AAVS1 locus was performed as previously described (Blanch-Asensio et al., 2022). To target the CLYBL-Bxb1-LP-v2-TC cassette (Addgene #194327) to the CLYBL locus, this plasmid along with the TALEN expression vectors pZT-C13-L1 and pZT-C13-R1 (Addgene #62196 and #62197) (Cerbini et al., 2015) were electroporated (1100 V, 30 ms, 1 pulse) into LUMC0099iCTRL04 hiPSCs. The resulting EBFP2<sup>+</sup> cells were clonally isolated using the single-cell deposition function of a BD FACSAria III (BD Bioscience), expanded and genotyped by PCR using conditions previously described (Blanch-Asensio et al., 2022) followed by Sanger sequencing (primers listed in Table 2).

#### 3.3. Donor vector integration and enrichment

1.2 µg of the donor vectors along with 0.8 µg of pCAG-NLS-HA-Bxb1 (Addgene #51271) were transfected into both lines using Lipofectamine™ Stem Transfection Reagent (ThermoFisher). The rest of the procedure was performed as previously described (Blanch-Asensio et al., 2022).

#### 3.4. Droplet digital PCR

Copy number, as well as integration and excision efficiencies were determined by ddPCR as previously described (Blanch-Asensio et al., 2022). Primers and probes are listed in Table 2.

#### 3.5. Karyotype analysis

Karyotypes were determined by G-banding (Laboratory for Diagnostic Genome Analysis, LUMC) with 20 metaphase spreads examined per line, and using the iCS-digital™ PSC 24-probes kit (Stemgenomics) following manufacturer's instructions.

#### 3.6. STR analysis

Cells were evaluated using the Human 16-Marker STR Profile, Interspecies Contamination Test (Idexx BioAnalytics).

#### 3.7. Immunofluorescence analysis

Undifferentiated and differentiated hiPSCs were fixed with 2% PFA at room temperature (RT) for 30 min, permeabilized with 0.05% Triton-X100/1x PBS and incubated with primary antibodies (Table 2) in PBS/0.05% Triton-4% NSS at 4°C overnight. For unconjugated primary antibodies, the cells were washed three times with 1x PBS/0.05% Tween and then incubated with secondary antibodies (Table 2) for 1 h at RT. After three washes with 1x PBS/0.05% Tween, cells were incubated at RT for 5 min with 1xPBS/DAPI (1 µg/ml) to stain the nuclei, washed with de-ionized water and mounted using Mowiol (Merck Millipore). Images were captured on a SP8 confocal microscope (Leica) or EVOS™ M7000 Cell Imaging System (ThermoFisher).

#### 3.8. Expression of pluripotency markers

hiPSCs for immunofluorescence analysis were plated on LN521-coated coverslips, fixed after 4 days and processed as described above. For flow cytometric analysis, hiPSCs were dissociated with 1x TrypLE Select and filtered. Cells were fixed and permeabilised using the FIX and PERM™ Cell Permeabilization Kit (ThermoFisher), then incubated with the conjugated antibodies OCT3/4-BV421 and NANOG-PE (Table 2). Data was acquired using a MacsQuant VYB flow cytometer (Miltenyi Biotec) and analysed using FlowJo software (FlowJo).

### 3.9. Trilineage differentiation

Cells were seeded on Matrigel-coated (Corning) coverslips and differentiated into ectoderm, mesoderm and endoderm using the STEMdiff™ Trilineage Differentiation Assay Kit (Stem Cell Technologies).

### Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: C. L.M. is a co-founder of Pluriomics B.V. (now Ncardia B.V.) and has advisory roles in Sartorius AG, Mogrify Limited, Angios GmbH and HeartBeat.bio AG. N.G. is co-founder and scientific advisor of NTrans Technologies and Divvly. C.L.M. and R.P.D. declare research funding from Sartorius AG; however, this is for an unrelated study. All other authors declare no potential conflicts of interest.

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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.2022.102991>.

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