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AAVS1 Knock-in

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ABSTRACT

This protocol describes the standard procedure to knock-in constructs to the AAVS1 safe harbor locus in hPSCs.

General Notes:

1. The AAVS1 knock-in construct, AAVS1-SA-neo-CAGGS-PE2-2A-GFP, can be found at AddGene (Catalog: 180014, RRID:Addgene_180014)
2. Throughout this protocol, the term hPSC is used to collectively refer to both hiPSCs and hESCs. All described procedures have been tested and work equally well for hiPSCs and hESCs.

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PROTOCOL CITATION

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KEYWORDS

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MATERIALS TEXT

Item	Vendor	Catalog #
G418	Life Technologies	11811031
PrimeStar GXL DNA polymerase	Takara	R050B
DMEM/F12	Thermo Fisher	11320082
Fetal Bovine Serum (FBS)	Corning	35-011-CV
Knockout Serum Replacement	Thermo Fisher	10828-028
L-Glutamine	Sigma	G8540
Penicillin & Streptomycin (100X)	Thermo Fisher	15140163
MEM Non-Essential Amino Acids (100X)	Thermo Fisher	11140050
Heat Stable Recombinant Human FGF2	Thermo Fisher	PHG0360
2-Mercaptoethanol	Sigma	M3148
Y-27632	Chemdea	CD0141
BSA	Sigma	A4503
DMSO	Fisher Scientific	BP231-100

Note: This protocol makes reference to protocols in other collections. Please check for any materials found in those protocols, which might not be listed here

1 One day before nucleofection, prepare two DR4 MEFs 6-well plates.

2 Nucleofection of Cas9/sgRNA RNP (protospacer sequence, ACCCCACAGTGGGGCCACTA) and AAVS1 knock-in targeting vector is performed using the nucleofection of ribonucleoprotein

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(RNP) into human pluripotent stem cells (hPSCs) protocol as described in the collection "Nucleofection (Amaxa) and electroporation (Biorad) of hPSCs;" dx.doi.org/10.17504/protocols.io.b4qnqvve

After nucleofection, seed all cells onto two 6-well plates containing hPSCs medium + Rock Inhibitor.

2.1 hPSCs Medium

A	B
DMEM/F12	385 ml
Fetal Bovine Serum (FBS)	75 ml
Knockout Serum Replacement	25 ml
L-Glutamine (100X)	5 ml
Penicillin & Streptomycin (100X)	5 ml
MEM Non-Essential Amino Acids (100X)	5 ml
2-Mercaptoethanol (10,000X)	50 µl
Heat Stable Recombinant Human FGF2 (25µg/ml)*	80 µl

*While we prefer Heat Stable Recombinant Human FGF2, we also have used regular FGF2.
Final volume: 500ml

L-Glutamine (100X)

L-Glutamine, powder	14.6 g
MilliQ H2O	500 ml

2-Mercaptoethanol (10,000X)

2-Mercaptoethanol	0.78 ml
MilliQ H2O	9.22 ml

Heat Stable Recombinant Human FGF2 (25µg/ml)

A	B
Heat Stable Recombinant Human FGF2	500 µg
0.1% BSA	20 ml

Final volume: 20ml

Y-27632 (1,000X)

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Y-27632	5 mg
DMSO	1.56 ml

hPSCs Medium + Rock Inhibitor

A	B
hPSCs medium	500 ml
Y-27632 (1,000X)	500 µl

Final volume: 500ml

3 From day 3, change medium daily for 10 days with hPSCs medium with 70 µg/ml G418. Most of the hPSCs will die during the G418 selection.

4 When large hPSC colonies emerge, manually pick and re-plate them individually in 12-well ICR MEFs plates, as described in the collection "Standard operating procedure for the isolation of genetically engineered hPSCs clones in a high-throughput way;" dx.doi.org/10.17504/protocols.io.b4mmqu46

5 When these expanded clones grow to 50%, passage 1/4 to a new well of a 6-well plate for further expanding.

For a detailed protocol on passaging hPSCs, refer to the collection "Thawing, Passaging and Freezing of hPSCs on MEFs;" dx.doi.org/10.17504/protocols.io.b4msqu6e

6 Prepare crude cell lysate from the rest of the cells for genotyping as described in the collection “Genotyping by next generation sequencing;" dx.doi.org/10.17504/protocols.io.b4n3qvgn

7 Freeze the expanded cells when they grow up.

For a detailed protocol on freezing hPSCs, refer to the collection "Thawing, Passaging and Freezing of hPSCs on MEFs;" dx.doi.org/10.17504/protocols.io.b4msqu6e

8 Genotype crude cell lysate from step 6 using the primers flanking each homologous arm with GXL DNA polymerase. Use unedited cells as a negative control.

8.1 Primer Sequences & Product Size

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Primers	Sequence	Product Size
SP-AAVS1-HR-L	CCCGCTTCAGTGACAACGTC	1313bp
ASP-AAVS1-HR-L	GAACTCTGCCCTCTAACGCT	
SP-AAVS1-HR-R	TGCATCGCATTGTCTGAGTAG	1184bp
ASP-AAVS1-HR-R	TACCCCGAAGAGTGAGTTTGC	

9 PCR with GXL DNA polymerase

9.1 PCR with GXL DNA polymerase - Setup

A	B
Ultrapure H2O	11 µl
5x GXL buffer	4 µl
2.5 mM dNTP	1.6 µl
10 µM primer Forward	0.5 µl
10 µM primer Reverse	0.5 µl
PrimeStar GXL DNA polymerase	0.4 µl
Crude cell lysis	2 µl

10 Touch-down PCR program

10.1 Touch-down PCR program

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A	B
98°C	3 min
98°C	30 s
70°C (touch down, 1C/cycle)	30 s
72°C	1 min
Go to 2	12 cycles in total
98°C	30 s
58°C	1 min
72°C	30 s
Go to 6	23 cycles in total
72°C	7 min
4°C or 12°C	forever

11 Run PCR products in agarose gels.

12 Gel purify the bands of positively targeted clones and perform sanger sequencing to confirm.

13 Thaw and expand the correctly targeted clones

14 Test clones for mycoplasma, stain for pluripotent markers, and karyotype

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