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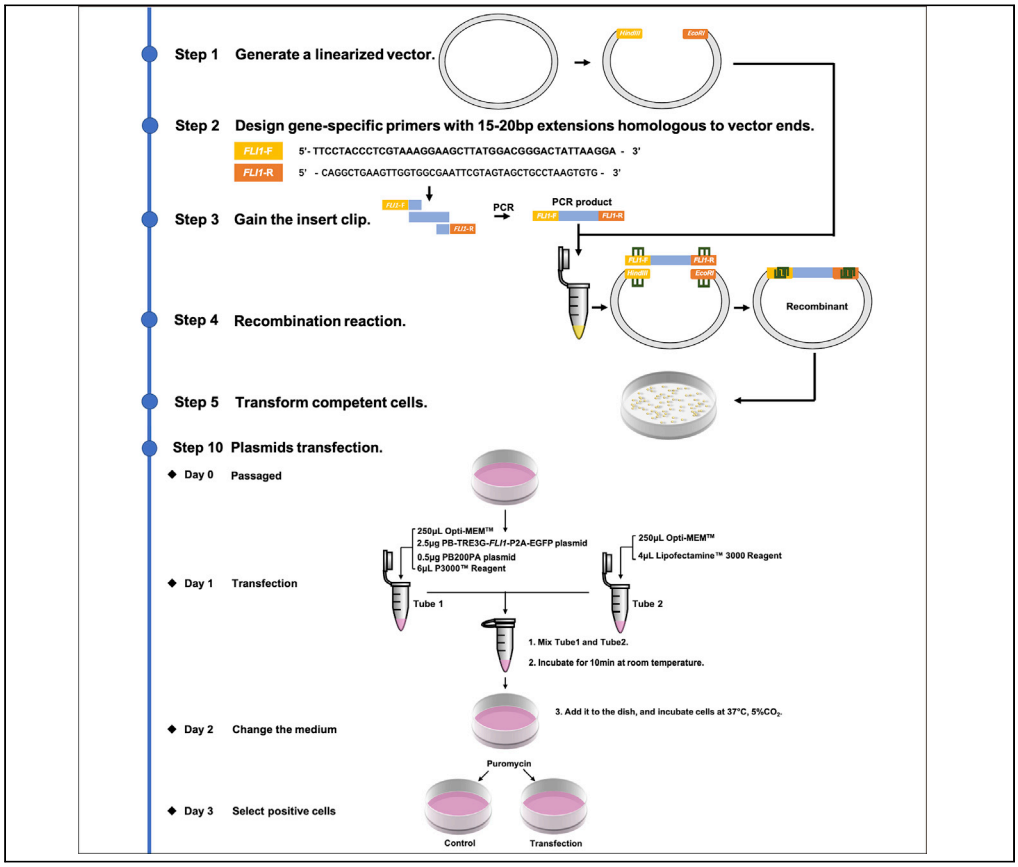
Protocol for inducible piggyBac transposon system for efficient gene overexpression in human pluripotent stem cells



In human pluripotent stem cells (hPSCs), traditional approaches for gene overexpression have low efficiency and are often laborious. Here, we provide a relatively simple protocol for gene overexpression with the Dox-inducible PiggyBac transposon system. We detail the steps for overexpression of *FLI1* and/or *YAP* in H1 embryonic stem cells (H1 ESCs) as an example. Our protocol can be applied to any gene of interest in a variety of hPSCs.

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Highlights

Gibson Assembly allows for quick construction of inducible piggyBac transposon plasmids

Doxycycline-inducible gene overexpression in stable H1 ESCs

Details for FLI1 overexpression or FLI1/YAP5SA double overexpression

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Protocol for inducible piggyBac transposon system for efficient gene overexpression in human pluripotent stem cells

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SUMMARY

In human pluripotent stem cells (hPSCs), traditional approaches for gene overexpression have low efficiency and are often laborious. Here, we provide a relatively simple protocol for gene overexpression with the Dox-inducible PiggyBac transposon system. We detail the steps for overexpression of *FLI1* and/or *YAP* in H1 embryonic stem cells (H1 ESCs) as an example. Our protocol can be applied to any gene of interest in a variety of hPSCs.

For complete details on the use and execution of this protocol, please refer to Quan et al. (2021).

BEFORE YOU BEGIN

PCR primer design

Timing:20 min

1. Introduce Gibson Assembly experimental method to construct recombinant plasmid (Gibson et al., 2009).Therefore, it is necessary to design specific primers, which include homologous region of the vector ends and gene-specific amplification sequences. Here, we take the construction of an inducible PB-TRE3G-*FLI1*-P2A-EGFP recombinant plasmid as an example. The primers can be designed with SnapGene software.
   a. Open SnapGene, input and save the CDS sequence (without stop codon) of *FLI1* from NCBI, and name it "*FLI1*-CDS".
   b. Use SnapGene to open sequence file of the PB-TRE3G-P2A-EGFP vector, and select the restriction enzyme sites *HindIII* and *EcoRI* on the map.
   c. Select the toolbar "Actions", click "Gibson Assembly®" → "Insert Fragment…".
   d. Show (Vector-Fragment-Product) options on the upper bar, click "Fragment".
   e. On the right sidebar "Source of Fragment", drop down and select the "*FLI1*-CDS".
   f. Click *FLI1* CDS sequence to select all, then click "Product" in the upper bar.
   g. Click "Choose Overlapping PCR Primers…" on the right sidebar, and a new interface will pop up.
   h. Enter the appropriate T_m value, overlapping is 15–20 bp, choose whether to "Regenerate the enzyme restriction site", etc.
   i. After choosing according to your needs, click "Choose Primers".
   j. Click "Assemble" on the right sidebar to generate primers for PCR, and a map of the recombinant plasmid.

k. Order primers. We recommend using PAGE purification to increase the successful cloning rate.

Note: We suggest the homologous region of vector ends is 15–20 bp, gene-specific sequence is 18–25 bp, and a GC-content of primers is between 40%-60%.

Note: The T_m should be calculated based upon the gene-specific sequence of the primer, and not the entire primer. If the calculated T_m is too low, increase the length of the gene-specific portion of the primer until you reach a T_m of between 58°C–65°C.

2. If the Snapgene Software is not available, we recommend to use the free NEB online tool, NEBuilder Assembly Tool, for primer design. Here, we describe how to use the NEBuilder Assembly Tool to generate overlap sequences for the assembly of one fragment into a vector.
   a. Open the website of NEBuilder Assembly Tool.
   b. click "Settings", the "Current Settings" interface appears to start editing.
   c. Select "NEBuilder HiFi DNA Assembly Cloning Kit" in the "Product/Kit" column.
   d. Input overlapping sequence size 15–25 nt in the "Minimum Overlap (nt)" column.
   e. Click to tick in the "Circularize" column.
   f. Select "Phusion High-Fidelity DNA Polymerase (HF Buffer)" in the "PCR Polymerase/Kit" column. (This Polymerase is commonly used in our laboratory)
   g. Keep the default value or input value according to the actual situation in the "PCR Primer Conc. (nM) " and "Min. Primer Length (nt)" column.
   h. Click the "Done" button, the "Get started designing primers" interface appears to start editing.
   i. Click the "+NEW FRAGMENT" button, enter the "Add a new fragment" interface.
   j. In step "1. Input source sequence", click "Paste Sequence", copy and paste the vector sequence (PB-TRE3G-P2A-EGFP).
   k. Click "Process text", and click to tick "Vector" and "Circular" in the "Parsed Sequence (5′→3′)" column.
   l. Name this vector in step "2. Name/rename fragment [optional]."
   m. In step "3. Select method for production of linearized fragment." select the linearization method "Restriction Digest".
   n. Enter the 5′ restriction enzyme HindIII-HF and the 3′ restriction enzyme EcoRI-HF, click the "Add" button.
   o. Click the "+NEW FRAGMENT" button on the new interface, copy and paste the CDS sequence of *FLI1*, and name it.
   p. Select the "PCR" amplification method and click the "Add" button.
   q. Primer sequences appear in the new interface "Required Oligonucleotides".
   r. Click the "Done" button to export the primer sequences.

Note: This primer design method does not contain restriction enzyme sites, if necessary, we can manually add.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
CD144 (VE-Cadherin) MicroBeads, human	Miltenyi Biotec	Cat#130-097-857
Bacterial and virus strains		
DH5α Competent cell	Vazyme	Cat#C502-02

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
LB Broth Agar	Sangon Biotech	Cat#A507003-0250
LB Broth	Sangon Biotech	Cat#A507002-0250
Ampicillin, Sodium Salt	Solarbio	Cat#A8180
Agarose	Sigma-Aldrich	Cat#V900510-100G
Tris	Solarbio	Cat#T8086
$Na_2 \cdot EDTA \cdot 2H_2O$	Sigma-Aldrich	Cat#E5134
Trizma® base	Sigma-Aldrich	Cat#V900483
Sodium Hydroxide (Granulated)	Adamas	Cat#01485557
SDS	Sigma-Aldrich	Cat#V900859
Potassium acetate	Sigma-Aldrich	Cat#V900213
RNase A	Sigma-Aldrich	Cat#V900498
Acetic acid	Aladdin	Cat#A116170
DPBS basic (1×)	Gibco	Cat#C14190500BT
ncEpic hPSC Medium	Nuwacell	Cat#RP01001-01
ncEpic 125× Supplement	Nuwacell	Cat#RP01001-02
Versene (1×)	Gibco	Cat#15040066
DMEM/F-12 (with L-glutamine, HEPES)	Gibco	Cat#C11330500BT
Matrigel hESC-qualified Matrix	Corning	Cat#354277
Blebbistatin	Nuwacell	Cat#RP01008
DMSO	Solarbio	Cat#D8371
Super GelRed™	US EVERBRIGHT	Cat#S2001
Opti-MEM® I Reduced Serum Media	Gibco	Cat#31985070
GeneRuler 1 kb DNA Ladder	Thermo Scientific	Cat#SM0311
Doxycycline	MCE	Cat#HY-N0565B
Puromycin	MCE	Cat#HY-B1743A
Hind III-HF®	New England BioLabs	Cat#R3104S
EcoR I-HF®	New England BioLabs	Cat#R3101S
2-Propanol	Adamas	Cat#01226749
Ethanol	Adamas	Cat#01226776
Neurobasal™ Medium	Gibco	Cat#21103049
B-27® Supplement (50×) without vitamin A	Gibco	Cat#12587-010
N-2 Supplement (100×)	Gibco	Cat#17502-048
CP21R7	MCE	Cat#HY-100207
β-Mercaptoethanol	Sigma-Aldrich	Cat#M6250-10ML
rhBMP-4	R&D Systems	Cat#1401543
Recombinant Human VEGF165	PeproTech	Cat#100-20
Human FGF-basic	PeproTech	Cat#AF-100-18C
EBM®-2	Lonza	Cat#CC-3156
EBM®-2 Supplement	Lonza	Cat#CC-4176
Hydrochloric acid	ZH chemical	N/A
BSA Fraction V (7.5%)	Gibco	Cat#15260-037
Critical commercial assays		
Endofree Mini Plasmid Kit II	TIANGEN	Cat#DP118-02
QIAquick® Gel Extraction Kit (50)	QIAGEN	Cat#28704
Lipofectamine™ 3000 Transfection Reagent	Invitrogen	Cat#L3000001
Phusion High-Fidelity DNA Polymerase with dNTPs	Thermo Scientific	Cat#F530S
TaKaRa Taq™	Takara Bio	Cat#R001A
Gibson Assembly® Master Mix	New England BioLabs	Cat#E2611S
Experimental models: Cell lines		
H1 ESCs	WiCell	WA01
Oligonucleotides		
Primer: *FLI1*-F: 5′-TTCCTACCCTCGTAAAGGA AGCTTATGGACGGGACTATTAAGGA-3′	This paper	N/A

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Primer: *FLI1*-R: 5′-CAGGCTGAAGTTGGTGGC GAATTCGTAGTAGCTGCCTAAGTGTG-3′	This paper	N/A
Primer: *YAP5SA*-F: 5′-TTCCTACCCTCGTAAA GGAAGCTTATGGATCCCGGGCAGCAGCCGCC-3′	This paper	N/A
Primer: *YAP5SA*-R: 5′-CAGGCTGAAGTTGG TGGCGAATTCTAACCATGTAAGAAAGCTTTCT-3′	This paper	N/A
Recombinant DNA		
PB-TRE3G-P2A-EGFP plasmid	This paper	N/A
PB200PA plasmid	From Zhou Lab	N/A
Software and algorithms		
SnapGene software	N/A	https://www.snapgene.com/
NEBuilder Assembly Tool	New England BioLabs	https://nebuilder.neb.com/#!/
Other		
NanoDrop	Thermo Scientific	N/A
37°C incubator	Thermo Scientific	N/A
37°C, 5% CO_2 incubator	Eppendorf	N/A
Shaking incubator	Thermo Scientific	N/A
Centrifuge	Eppendorf	N/A
Gradient PCR Instrument	SimpliAmp	N/A
Water bath	LICHEN	N/A
Autoclave	Panasonic	N/A
Ultrapure Water Production Unit	Milli-Q	N/A
0.22 mm Vacuum driven filter	Corning	Cat#431097
15 mL centrifuge tube	JET BIOFIL	Cat#CFT711150
50 mL centrifuge tube	JET BIOFIL	Cat#CFT511500
0.2 mL PCR tube	AXYGEN	Cat#PCR02C
1.5 mL eppendorf tube	JET BIOFIL	Cat#CFT001015
35 mm dish	Thermo Scientific	Cat#150460
Bacteria petri dish	JET BIOFIL	Cat#MCD-000090

MATERIALS AND EQUIPMENT

H1 ESCs growth medium		
Reagent	Final concentration	Amount
Nuwacell™-ncEpic hPSC Medium	1×	500 mL
Nuwacell™-ncEpic 125× Supplement	1×	4 mL
Total	1×	504 mL
H1 ESCs growth medium can be stored at 4°C for 2 weeks.		

Ampicillin		
Reagent	Final concentration	Amount
Ampicillin, Sodium Salt	100 mg/mL	1.0 g
Sterilized ddH_2O	N/A	10 mL
Total	100 mg/mL	10 mL
Ampicillin can be stored at −20°C for 1 month. Sterile through a 0.22 μm filter.		

LB Broth Agar plate		
Reagent	Final concentration	Amount
LB Broth Agar	40.0 g/L	8.0 g
ddH_2O	N/A	200 mL
Ampicillin	100 μg/mL	200 μL
Total	N/A	200.2 mL
LB Broth Agar plate can be stored at 4°C for 1–2 weeks.		

LB Broth medium		
Reagent	Final concentration	Amount
LB Broth	25.0 g/L	2.5 g
ddH_2O	N/A	100 mL
Total	25.0 g/L	100 mL
LB Broth medium can be stored at 4°C for 1–3 months.		

50× TAE buffer		
Reagent	Final concentration	Amount
Tris	2 mol/L	242 g
$Na_2 \cdot EDTA \cdot 2H_2O$	100 mmol/L	37.2 g
Acetic acid	1 mol/L	57.1 mL
ddH_2O	N/A	Add to 1 L
Total	50×	1 L
50× TAE buffer can be stored at 18°C–25°C for 3 months.		

1× TAE buffer		
Reagent	Final concentration	Amount
50× TAE buffer	1×	10 mL
ddH_2O	N/A	490 mL
Total	1×	500 mL
1× TAE buffer can be stored at 18°C–25°C for 1 week.		

Buffer P1		
Reagent	Final concentration	Amount
Trizma® base	50 mM	3.03 g
$Na_2 \cdot EDTA \cdot 2H_2O$	16 mM	1.86 g
Hydrochloric acid	N/A	Adjust the pH to 8.0
ddH_2O	N/A	Add to 500 mL
Total	N/A	500 mL
Buffer P1 can be stored at 4°C for 3 months. Add RNase A (final concentration 100 μg/mL) before use.		

Buffer P2		
Reagent	Final concentration	Amount
Sodium Hydroxide (Granulated)	200 mM	0.8 g
SDS	35 mM	1.0 g
ddH_2O	N/A	Add to 100 mL
Total	N/A	100 mL
Buffer P2 can be stored at 18°C–25°C for 3 months.		

Buffer P3		
Reagent	Final concentration	Amount
Potassium acetate	3 M	29.45 g
Acetic acid	N/A	Adjust the pH to 5.5
ddH_2O	N/A	Add to 100 mL
Total	N/A	100 mL
Buffer P3 can be stored at 18°C–25°C for 3 months.		

RNase A		
Reagent	Final concentration	Amount
RNase A	100 mg/mL	100 mg
ddH_2O	N/A	1 mL
Total	100 mg/mL	1 mL
RNase A can be stored at −20°C for 1 month.		

1.0% Agarose		
Reagent	Final concentration	Amount
Agarose	1%	0.2 g
1× TAE buffer	1×	20 mL
Super GelRed™	1×	2 μL
Total	N/A	20 mL
1.0% Agarose can be stored at 4°C for 3 days.		

Blebbistatin		
Reagent	Final concentration	Amount
Blebbistatin (10 mM)	2.5 mM	10 μL
DMSO	N/A	30 μL
Total	2.5 mM	40 μL
Blebbistatin can be stored at −20°C for 1 month.		

Puromycin		
Reagent	Final concentration	Amount
Puromycin dihydrochloride	1 mg/mL	5 mg
ddH_2O	N/A	5 mL
Total	1 mg/mL	5 mL
Puromycin can be stored at −20°C for 1 month.		

Doxycycline		
Reagent	Final concentration	Amount
Doxycycline	2 mg/mL	100 mg
ddH_2O	N/A	50 mL
Total	2 mg/mL	50 mL
Doxycycline can be stored at −20°C for 1 month.		

5 mM CP21R7		
Reagent	Final concentration	Amount
CP21R7	5 mM	1.0 mg
DMSO	N/A	630 μL
Total	5 mM	630 μL
5 mM CP21R7 can be stored at −80°C for 6 months.		

1 mM CP21R7		
Reagent	Final concentration	Amount
5 mM CP21	1 mM	10 μL
DMSO	N/A	40 μL
Total	1 mM	50 μL
1 mM CP21R7 can be stored at −20°C for 1 month.		

β-Mercaptoethanol		
Reagent	Final concentration	Amount
β-Mercaptoethanol	50 mM	3.5 μL
DMEM/F12	N/A	1 mL
Total	50 mM	1.0 mL
β-Mercaptoethanol can be stored at −20°C for 1 month.		

VEGF		
Reagent	Final concentration	Amount
Recombinant Human VEGF165	50 μg/mL	50 μg
ddH_2O	N/A	1 mL
Total	50 μg/mL	1 mL
VEGF can be stored at −20°C for 1 month.		

bFGF		
Reagent	Final concentration	Amount
Human FGF-basic (146 a.a.)	25 μg/mL	10 μg
ddH_2O	N/A	400 μL
Total	25 μg/mL	400 μL
bFGF can be stored at −20°C for 1 month.		

1 M HCl		
Reagent	Final concentration	Amount
Hydrochloric acid	1 M	820 μL
ddH_2O	N/A	10 mL
Total	1 M	10.82 mL
1 M HCl can be stored at −20°C for 1 month.		

4 mM HCl-0.1% BSA		
Reagent	Final concentration	Amount
1 M HCl	1 M	4 μL
BSA Fraction V (7.5%)	0.1%	13 μL
ddH_2O	N/A	1 mL
Total	4 mM	1,017 μL
4 mM HCl-0.1% BSA can be stored at −20°C for 1 month.		

rhBMP4		
Reagent	Final concentration	Amount
rhBMP-4	25 μg/mL	5 μg
4 mM HCl-0.1% BSA	N/A	200 μL
Total	25 μg/mL	200 μL
rhBMP4 can be stored at −20°C for 1 month.		

Mesoderm differentiation medium		
Reagent	Final concentration	Amount
DMEM/F12	48.45%	24.225 mL
Neurobasal™ Medium	48.45%	24.225 mL

(*Continued on next page*)

Continued		
Reagent	Final concentration	Amount
B27 (50×)	1×	1 mL
N-2 (100×)	1×	0.5 mL
β-Mercaptoethanol (50 mM)	50 μM	50 μL
CP21 (1 mM)	1 μM	50 μL
hBMP4 (25 μg/mL)	25 ng/mL	50 μL
Total	N/A	50 mL
Complete Mesoderm differentiation medium can be stored at 4°C for 1 week.		

Endothelial cells differentiation medium			
Reagent		Final concentration	Amount
EBM®-2		1×	48.38 mL
EBM®-2 Supplement	CC-4101A FBS	N/A	1 mL
	CC-4113A hFGF-B	N/A	200 μL
	CC-4112A Hydrocortisone	N/A	20 μL
	CC-4114A VEGF	N/A	50 μL
	CC-4115A R3-IGF-1	N/A	50 μL
	CC-4116A Ascorbic Acid	N/A	50 μL
	CC-4117A hEGF	N/A	50 μL
	CC-4381A GA-1000	N/A	50 μL
	CC-4396A Heparin	N/A	50 μL
VEGF (50 mg/mL)		50 μg/mL	50 μL
bFGF (25 mg/mL)		25 μg/mL	50 μL
Total		N/A	50 mL
Complete Endothelial cells differentiation medium can be stored at 4°C for 1 week.			

STEP-BY-STEP METHOD DETAILS

Construction of inducible PB-TRE3G-*FLI1*-P2A-EGFP recombinant plasmid (Figure 1A)

Timing: 7 days

1. Preparation of linearized vector.
   a. Select the *Hind III* -HF and *EcoR I*-HF restriction enzyme to digest PB-TRE3G-P2A-EGFP vector. The size of this vector is 7,253 bp, and TRE3G is the promoter for specific gene expression.

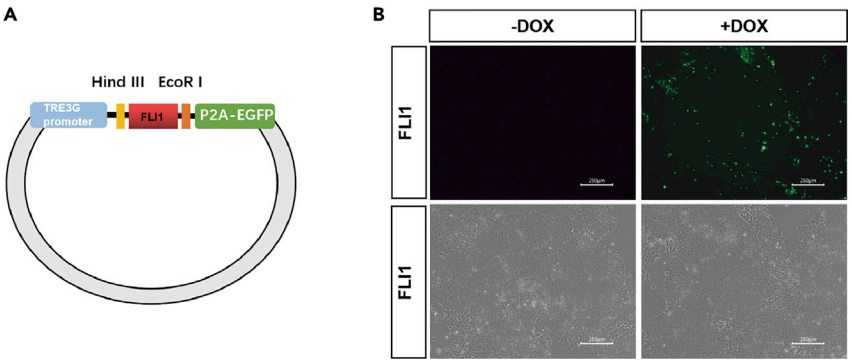

Figure 1. The inducible FLI1 overexpression stable H1 ESCs

(A) Schematic view of an inducible piggyBac transposon plasmid with the FLI1 gene.

(B) The fluorescence and phase images for the stable H1 ESCs with inducible FLI1 overexpression without or with DOX treatment.

Scale=250μm.

The vector contains an anti-puromycin gene as selectable marker and the EGFP as a tag, the map of the vector is available in Supplemental information (Figure S1). Prepare the following Enzyme digestion reaction system in 0.2 mL PCR tube.

Component	Amount per reaction (μL)
ddH_2O	33
rCutSmart Buffer	5
PB-TRE3G-P2A-EGFP Vector (100 ng/μL)	10
HindIII-HF	1
EcoRI-HF	1

b. Digest vector at 37°C for 15–30 min.
c. Take the reaction product for 1.0% Agarose electrophoresis.
d. Use the Qiaquick Gel Extraction Kit to purify the linearized vector according to the manufacturer's protocol (https://www.qiagen.com/us/).
e. Detect the concentration of DNA using NanoDrop.

Note: We recommend using double enzymes for digestion, which obtain complete linearized vector and fewer false positive clones.

2. Design of primers for insert gene *FLI1*.
   a. As mentioned at the beginning, primers include homologous region of the linearized vector ends and gene-specific amplification sequences.
   b. The F primer has a *HindIII*-HF restriction enzyme site, and the R primer has an *EcoRI*-HF restriction enzyme site, the sequences are:

FLI1-F: TTCCTACCCTCGTAAAGGAAGCTTATGGACGGGACTATTAAGGA

FLI1-R: CAGGCTGAAGTTGGTGGCGAATTCGTAGTAGCTGCCTAAGTGTG

3. PCR amplification of insert (*FLI1* cDNA).
   a. Amplify the insert by PCR according to the following PCR reaction.

Component	Amount per reaction (μL)
ddH_2O	to 50
5× Phusion™ HF Buffer	10
10 mM dNTPs	1
Forward primer (10 μM)	1
Reverse primer (10 μM)	1
Template DNA	0.1–0.5 ng
(DMSO, optional)	(1.5)
Phusion™ High–Fidelity DNA Polymerase	0.5

Note: A plasmid with *FLI1* cDNA or endothelial cell cDNA can be used as a PCR template. Phusion™ High–Fidelity DNA Polymerase is capable of amplifying long amplicons such as the 7.5 kb genomic and 20 kb λ DNA.

Note: Addition of DMSO is for GC-rich amplicons. We do not recommend using DMSO for very low GC % amplicons or amplicons > 20 kb.

b. Run the PCR reaction according to the following program.

Steps	Temperature	Time	Cycles
Initial denaturation	98°C	30 s	1
Denaturation	98°C	5–10 s	30
Annealing	X°C	10–30 s	
Extension	72°C	15–30 s/kb	
Final extension	72°C	10 min	1
Hold	4°C	forever	

c. Take the reaction product for 1.0% Agarose electrophoresis.
d. Use the Qiaquick Gel Extraction Kit to purify the PCR products, and measure the concentration of DNA.

Note: Use the T_m calculator and instructions on the website to determine the T_m values of primers and optimal annealing temperature (http://www.thermofisher.com/tmcalculator).

Note: If the PCR product is directly used for the recombination reaction, the volume of PCR product should not exceed 1/5 of the total volume of the recombination reaction.

4. Recombination reaction.
   a. Calculate the amount of the vector and insert. The optimal molar ratio of insert to vector is ≥2:1.

   pmols = (weight in ng) × 1,000 / (base pairs × 650 daltons).
   b. Set up the following reaction on ice.

Component	Experimental group (μL)	Positive control (μL)
ddH_2O	to 20	0
Gibson Assembly Master Mix (2×)	10	10
Vector	50–100 ng	10
Insert	20–200 ng	

   c. Incubate samples at 50°C for 15–60 min (appropriately extend the time to improve assembly efficiency).
   d. Following incubation, store samples on ice or at –20°C for subsequent transformation.

   Note: Optimized cloning efficiency is 50–100 ng of vector with 20–200 ng of insert.

   Note: Use the PCR machine to accurately control the reaction temperature. Insufficient or long reaction time will reduce cloning efficiency.

5. Transformation of reaction products.
   a. Prepare LB Broth medium and LB Broth Agar plate. Autoclave 120°C, 15 min after mixing LB Broth or LB Broth Agar with ddH_2O.
   b. When the temperature of LB Broth Agar buffer drops to 60°C, add Amp to the LB Broth Agar to final concentration 100 μg/mL.
   c. Mix well and pour 20 mL of agar into the bacteria petri dish to cool and solidify.
   d. Thaw chemically competent cells DH5α (50 μL) on ice.
   e. Add 5 μL of the chilled assembly product to the competent cells. Mix by gently flicking the tube 4–5 times.
   f. Place the mixture on ice for 30 min.
   g. Heat shock at 42°C for 30–60 s.
   h. Put the tubes back on ice for 2 min.
   i. Add 900 μL of room-temperature LB medium to the tube. Incubate the tubes by shaking (250 r/min) for 1 h at 37°C. (This step can be skipped when using Amp for antibiotics).
   j. Warm LB Broth Agar plates 30 min in a 37°C incubator.

k. Centrifuge the transformation reaction at 3,000 × *g* for 5 min. Discard the supernatant and resuspend each pellet in 100 μL fresh LB medium.
l. Spread each sample on a separate LB agar plate containing 100 μg/mL Amp.
m. Incubate the plates upside-down 12–18 h at 37°C.

6. Positive clone identification with PCR.
   a. Randomly pick 4–8 single clones into tubes containing 100 μg/mL Amp with 3 mL LB broth medium, and shake at 37°C,300 r/min for 4 h.
   b. Take 100–300 μL of bacteria, centrifuge at 13,000 × *g* for 5 min, discard the supernatant and resuspend the bacterial pellet with 30–50 μL of ddH2O.
   c. Boil in a boiling water for 5 min, centrifuge at 13,000 × *g* for 5 min.
   d. Take 1 μL of supernatant as a template, and set up the following reaction on ice with *FLI1*-specific primers.

Component	Amount per reaction (μL)
10×Taq Buffer (Mg^{2+} plus)	5
Supernatant	1 (< 500 ng)
F primer (10 μM)	1
R primer (10 μM)	1
dNTP Mixture (2.5 mM)	4
Takara Taq™ (5 U/μL)	0.25
ddH_2O	To 50

   e. Run the PCR according to the following program.

Steps	Temperature	Time	Cycles
Initial denaturation	94°C	5 min	1
Denaturation	94°C	30 s	30
Annealing	55°C	30 s	
Extension	72°C	1 min/kb	
Final extension	72°C	3 min	1
Hold	4°C	forever	

   f. Take 10 μL of the reaction product for 1.0% Agarose electrophoresis to identify the positive clones.

7. Plasmid extraction with isopropanol precipitation.
   a. Culture the remaining bacterial with positive plasmid for 12–18 h.
   b. Take 0.5–2 mL bacteria in a 1.5 mL eppendorf (EP) tube, centrifuge at 12,000–13,000 × *g* for 1 min.
   c. Discard the supernatant, add 250 μL Buffer P1 (resuspension buffer), vortex.
   d. Add 250 μL Buffer P2 (lysis buffer) and mix gently to lyse the bacteria and stand it at 18°C–25°C for 2–3 min to fully digest the RNA. Do not exceed 5 min to avoid damage to the plasmid.
   e. Add 350 μL Buffer P3 (neutralization buffer) and mix gently to make a flocculent precipitate. Then centrifuge, 12,000–13,000 × *g*, 10 min.
   f. Pipette the supernatant into a new 1.5 mL EP tube, as far as possible not to aspirate the precipitate. Add 0.7 volume of isopropanol, mix gently, and stand for 3–5 min.
   g. Centrifuge, 12,000–13,000 × *g*, 10 min. After centrifugation, white precipitate at the bottom.
   h. Discard the supernatant, add 1 mL of 70% ethanol to wash the pellet, centrifuge, 12,000–13,000 × *g*, 2 min.
   i. Discard the supernatant, open the lid to dry. When the plasmid precipitation is observed to turn from white to transparent, add 50–100 μL of ddH_2O to resuspend it.

j. Take 100 ng plasmid for 1.0% Agarose electrophoresis identification.

Alternatives: Use plasmid miniprep kit from commercial for plasmid isolation according to the manufacturer's instructions.

8. Restriction enzyme identification.
   a. Set up the following reaction on ice.

Component	Amount per reaction (μL)
ddH_2O	To 50
rCutSmart Buffer	5
Vector	1 μg
HindIII-HF®	1
EcoRI-HF®	1

   b. Digest plasmid at 37°C for 2 h.
   c. Take 25 μL of reaction product for 1.0% Agarose electrophoresis identification.

9. Sequence validation of the recombination plasmid. Verify the sequence of plasmid by sanger sequencing using the pCAGGS-F primer:

5′-ACGTGGTTGGTTAATTGTGCTGTC-3′.

10. Long term storage of constructs Frozen bacteria.
    a. Add 400 μL of 60% glycerol to each 2 mL cryotube.
    b. Add 1.2 mL of bacteria containing validated plasmid, mix well and store at −80°C (the final concentration of glycerol is 15%).
11. Plasmid Purification.
    a. Use EndoFree Mini Plasmid Kit II to isolate and purificate the recombined transposon PB-TRE3G-*FLI1*-P2A-EGFP plasmid and transposase PB200PA plasmid.
    b. Prepare H1 ESCs for transfection.

Note: The plasmid for transfecting cells must be endotoxin-free, with the concentration >500 ng/μL and the OD value about 1.8–1.9.

Generation of *FLI1* inducible overexpression H1 ESCs

Timing: 14 days

When the PB-TRE3G-*FLI1*-P2A-EGFP plasmid and transposase PB200PA plasmid are ready, hPSC can be prepared for transfection.

12. Human PSC preparation.
    a. Here, we use H1 ESCs as a sample. We diluted Matrigel with cold DMEM/F12. Coat each 35 mm dish for 1 mL diluted Matrigel and store at 4°C for up to 1 week.
    b. Place the coated dishes in a 37°C, 5% CO_2 incubator for more than 1 h before using. We routinely maintain H1 ESCs in 35 mm dish using Nuwacell ncEpic hPSC Medium, with about 1:10 ratio for passage every 4 days using Versene (1×).

Note: The Matrigel should be thawed on ice and aliquoted according to the Dilution Factor. The Dilution Factor is available from website (http://catalog2.corning.com/lifesciences/en-CN/certificates/retrievecertificate.aspx) by the catalog number and lot number of Corning

Matrigel. Aliquoted Matrigel may be stored at −70°C for up to six months. For example, if Dilution Factor is 291 μL, you can aliquot 291 μL Matrigel in each tube. A vial of Matrigel could be diluted in 25 mL cold DMEM/F12 and then coat the dishes or plates.

13. Plasmids transfection.
    a. When H1 ESCs grow to 90% confluency in a 35 mm dish, passage cells for transfection.
    b. Coat 35 mm dishes with 1 mL diluted Matrigel for 1 h at 37°C in 5% CO_2 incubator.
    c. Aspirate growth medium from dishes and wash cells once with 2 mL of DPBS.
    d. Add 1 mL pre-warmed Versene and incubate 3–5 min at 37°C.
    e. Check cell's detachment under a microscope. When the cells become bright and intercellular spaces become larger, remove the Versene.
    f. Add 1 mL of hPSC Medium and pipette H1 ESCs into single cell suspension, and transfer into a clean 15 mL centrifuge tube, mix well and count the cell number by hemocytometer.
    g. Seed the cells at 3 × 10^5 cells/35 mm dish, and add hPSC Medium to 1.5 mL, add Blebbistatin to final conc. 2.5 μM.
    h. Incubate the cells at 37°C in 5% CO_2 incubator 24 h.
    i. Change the hPSC Medium, prepare for transfection with Lipofectamine™ 3000 Reagent transfection system, as the following in two separated tubes.

    Tube 1.

Component	Content	Ratio
Opti-MEM™	250 μL	
P3000™ Reagent	6 μL	
PB-TRE3G-*FLI1*-P2A-EGFP plasmid	2.5 μg	5:1
PB200PA plasmid	0.5 μg	

    Tube 2.

Component	Content
Opti-MEM™	250 μL
Lipofectamine™ 3000 Reagent	4 μL

    j. Mix tube 1 and tube 2, and incubate for 10 min at 25°C.
    k. Drop the mixed solution into the dish, and incubate cells at 37°C in 5% CO_2 incubator. Prepare a dish of H1 ESCs without transfection as the control for puromycin selection in the next step.
    l. Next day, change the fresh hPSC Medium, 2 mL/dish.
    m. When the cell density reach to 60%–70% confluency (about 48 h after transfection), add puromycin (final conc. 1 μg/mL) to select positive cells, H1 ESCs without transfection as a control. When control H1 ESCs all died (usually 2–3 days), there should be some survived colonies in the dishes with transfected H1 ESCs.
    n. Continuously culture the survived H1 ESCs after puromycin selection, passage the cells 3–4 generations (reduce the concentration of puromycin to final 0.5 μg/mL) to obtain the stable cell line with inducible overexpression of *FLI1*.

Note: At this point, the most of cells should be positive for inducible overexpression of *FLI1*, which could be used for most of *FLI1* function assay in hPSC. We can cryopreserve these cells. If need pure stable cell line, you can perform single-cell clone generation as other protocol (Zhong et al., 2020).

Verify the *FLI1* inducible overexpression stable H1 ESCs

Timing: 2–3 days

14. Induced *FLI1* overexpression in stable cells.
    a. When the stable cells are 30% confluence, DOX was added into the medium to final concentration 2 μg/mL to induce *FLI1* overexpression.
    b. Check the cells under the fluorescence microscope after transfection for 24–48 h, it could be seen EGFP expression in the cells (Figure 1B).
    c. RT-qPCR or WB can be used to detect whether the *FLI1* gene is significantly up-regulated after DOX treatment.

Note: The analysis of mRNA or protein expression level of *FLI1* by RT-qPCR or WB could refer to Quan et al. (2021) .

Differentiation of *FLI1*-inducible H1 ESCs into endothelial cells (ECs)

Timing: 6–7 days

15. *FLI1*-inducible H1 ESCs preparation.
    a. The stable H1 ESCs were routinely maintained in 35 mm dishes using Nuwacell ncEpic hPSC Medium, with about 1:10 ratio for passage every 4 days using Versene.
    b. Two 35 mm dishes of H1 ESCs with 80% confluent are required for EC differentiation.
16. Day 0: Plating *FLI1*-inducible H1 ESCs.
    a. Coat 35 mm dishes with 1 mL diluted Matrigel for 1 h at 37°C in 5%CO_2 incubator.
    b. Remove the medium and wash once with 2 mL of DPBS.
    c. Digest the hPSC with 1 mL pre-warmed Versene for 3–5 min at 37°C.
    d. Remove Versene and add 1 mL of hPSC Medium and pipette H1 ESCs into single cell, transfer cell suspension into a clean 15 mL centrifuge tube, mix well and count the cell number by hemocytometer.
    e. Seed the cells at 3 × 10^5 cells/35 mm dish, and add hPSC Medium to 1.5 mL, add Blebbistatin to final conc. 2.5 μM.
    f. Incubate the cells at 37°C in 5% CO2 incubator 24 h.

Note: The initiated cell density influences the differentiation efficiency and should be optimized for each cell line.

17. Day 1–3: Lateral mesoderm induction.
    a. Wash H1 ESCs once with 2 mL of DPBS. Then change the Mesoderm differentiation medium 2 mL/dish for 3 days without medium change.
    b. On day3, add 2 μL of 2 mg/mL Dox (final conc. 2 μg/mL) to induce *FLI1* expression.
18. Day 4–5: Endothelial cells induction. Wash H1 ESCsonce with 2 mL of DPBS. Then change the Endothelial cell differentiation medium 2 mL/dish every day.
19. Day 6–7: Endothelial cell purification. On day 6–7, Endothelial cells were purified by MACS with CD144 magnetic beads and culture in EBM-2 medium.

Note: For more details of EC purification, please refer to Gao et al. (2018).

Construction of PB-TRE3G-*YAP5SA*-P2A-EGFP plasmid (Figure 2A)

Timing: 7 days

Using inducible piggyBac transposon system, we could not only inducible overexpression one gene, but also two or multiple genes at the same time. The process of multiple gene expression is similar to that of single gene expression, and we apply inducible overexpression of *FLI1* and *YAP5SA* (An active mutant version of *YAP*) at the same time as an example.

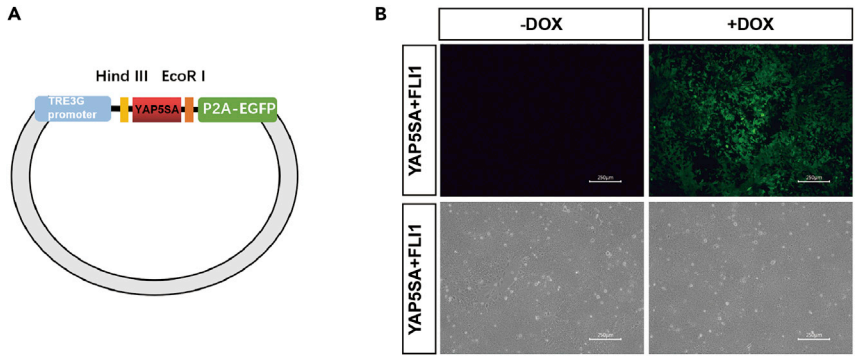

Figure 2. The YAP5SA-FLI1 inducible overexpression stable H1 ESCs

(A) Schematic view of an inducible piggyBac transposon plasmid with the YAP5SA gene.

(B) The fluorescence and phase images for the stable H1 ESCs with inducible YAP5SA-FLI1 overexpression without or with DOX treatment.

Scale=250μm.

20. Construction PB-TRE3G-*YAP5SA*-P2A-EGFP plasmid.
    a. Construction method refer to "steps 1–11".
    b. The sequences of *YAP5SA* primers are:

YAP5SA-F: TTCCTACCCTCGTAAAGGAAGCTTATGGATCCCGGGCAGCAGCCGCC

YAP5SA-R: CAGGCTGAAGTTGGTGGCGAATTCTAACCATGTAAGAAAGCTTTCT

Generation of *YAP5SA-FLI1* double-inducible over-expressing H1 ESCs

Timing:14 days

21. Plasmid preparation. When the PB-TRE3G-*YAP5SA*-P2A-EGFP, PB-TRE3G-*FLI1*-P2A-EGFP and transposase PB200PA plasmids are ready, H1 ESCs can be prepared for transfection.
22. Human PSCs preparation. Refer to "step 12".

23. Two plasmids transfection.
    a. Refer to "steps 13 a–h".
    b. Change the hPSC Medium 24 h later, prepare for transfection with Lipofectamine™ 3000 Reagent transfection system, as the following in two separated tubes.

Tube 1.

Component	Content	Ratio
Opti-MEM™	250 mL	
P3000™ Reagent	6 μL	
PB-TRE3G-*YAP5SA*-P2A-EGFP plasmid	2.5 μg	2.5: 2.5: 1
PB-TRE3G-*FLI1*-P2A-EGFP plasmid	2.5 μg	
PB200PA plasmid	1.0 μg	

Tube 2.

Component	Content
Opti-MEM™	250 mL
Lipofectamine™ 3000 Reagent	4 μL

    c. Mix tube 1 and tube 2, and incubate for 10 min at 25°C.

d. Refer to "steps 13 k–h".
e. Continuously culture the survived H1 ESC after puromycin selection, passage the cell 3–4 generations (change the final concentration of puromycin to 0.5 μg/mL) to obtain the inducible overexpression of *YAP5SA-FLI1* cell line.

Verify the *YAP5SA-FLI1* inducible overexpression stable H1 ESCs

Timing: 2–3 days

24. Induced *YAP5SA-FLI1* overexpression in stable cells.
    a. When the stable cells are 30% confluence, DOX was added into the medium to final concentration 2 μg/mL to induce *YAP5SA-FLI1* overexpression.
    b. Check the cells under the fluorescence microscope 24–48 h later, it should be seen EGFP expression in the cells (Figure 2B).
    c. RT-qPCR or WB can be used to detect whether the *YAP5SA* and *FLI1* genes are significantly up-regulated after DOX treatment.

Note: The analysis of mRNA or protein expression level of *YAP5SA-FLI1* by RT-qPCR or WB analysis please refer to Quan et al. (2021). Because the double-inducible gene overexpression could not discriminate EGFP in the cell between single-gene and double-gene overexpression, we recommend performing single cell subcloning experiment to obtain pure double-inducible overexpression cell line (Park et al., 2018).

EXPECTED OUTCOMES

The transfection efficiency of liposomes was around 50%. By performing puromycin selection, the stable cell line is possible to obtain GFP-positive lines for almost all colonies (Quan et al., 2021).

LIMITATIONS

The aim of this protocol is to construction inducible gene(s) overexpression to study the function of gene in hPSCs, such as differentiation regulation of hPSCs. The piggyBac transposon system provide us a technically easy approach to overexpression of specific genes in hPSC. Compared to the other methods to overexpress of specific genes, such as plasmid DNA transfection, retroviral- or lentiviral-based infection, electroporation, the PiggyBac transposon system is technically simpler and higher efficiency, it has a large cargo capacity (up to 10 KB) with multiple genes expression at the same time, and the donor cassette can be excised using excision-only PiggyBac transposase in scarless fashion if we do not need them later. PiggyBac transposon system is broadly used tool that allows DNA cargos inserted into genome in "AATT" sequence with high efficiency, which has been applied in gene overexpression, knockdown, genome editing in various mammalian cells, including stem cell (Schertzer et al., 2019; Sun et al., 2021). However, we did not perform the gene overexpression in other stem cells, such as cancer stem cells. At the same time, we did not do multiple genes (more than two) overexpression at the same time, or the donor cassette removal which could refer to others (Wang et al., 2017).

TROUBLESHOOTING

Problem 1

Low recombination efficiency (step 4).

Potential solution

Increasing the molar ratio of insert to vector to 3–15: 1 for different size fragment of insert. Sometimes, for longer insert, it would be help to extended the length of overlap in PCR primers of insert, as well as increase PCR extension time. Obtaining higher DNA concentration and adding more DNA fragments in recombination reaction will increase the efficiency.

Problem 2
There is no colony growth after the recombinant product spread onto the selection plate (step 5).

Potential solution
Increasing the amount of recombined product, but it should not exceed 1/10 of the competent cell volume. Make sure the competent cells work well.

Problem 3
Low transfection efficiency (steps 13 and 23).

Potential solution
The ratio of transposon to transposase is appropriate. The transfection time can be extended, and the culture medium can be replaced after 24 h of culture.

Problem 4
Obtain less GFP-positive lines (steps 13 and 23).

Potential solution
Can be screened continuously to the 4th generation, it was possible to obtain GFP-positive lines for almost all colonies.

Problem 5
The initiating cell density influences the yield of the transfection (steps 13 and 23).

Potential solution
Control the number of H1ESC around 3×10^5/35 mm dish, or need to optimize the cell density for different cell lines.

RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Yongyu Wang (yywangut@163.com).

Materials availability
Plasmids and cell lines described in this study will be available upon request. Plasmids are also available in Bio-Research Innovation Center Suzhou in China (www.brics.ac.cn): BRICS#SP-3032, SP-3033, SP-3034, SP-3035.

Data and code availability
We did not generate a data set or code.

SUPPLEMENTAL INFORMATION
Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2022.101296.

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AUTHOR CONTRIBUTIONS

J.Y. and M.H. performed the experiments and analyzed the data. Y.W., J.Y., and M.H. wrote the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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