Protocol for the Generation of Human Pluripotent Reporter Cell Lines Using CRISPR/Cas9

Reporter cell lines based on human pluripotent stem cells (hPSCs) are highly desirable for studying differentiation, lineage tracing, and target cell selection. However, several technical bottlenecks, such as DNA transduction, low homology recombination rate (HDR), and single-cell cloning, have made this effort an arduous process in hPSCs. Here, we provide a step-by-step protocol and practical guide for generating reporter lines in hPSCs via CRISPR/Cas9-mediated HDR. We also elaborate on the process of generating a TBXT-GFP reporter line as an example.
Protocol for the Generation of Human Pluripotent Reporter Cell Lines Using CRISPR/Cas9

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SUMMARY
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BEFORE YOU BEGIN

© Timing: 1 day

1. All primers in this protocol are designed using open web resource Primer 3 http://bioinfo.ut.ee/primer3/. Primers can be ordered from IDT or other companies.

   Note: We use the Gibson cloning method to construct the donor plasmid. The 5× Isothermal Buffer used in the Gibson Assembly Master Mix, and the Gibson Assembly Master Mix are prepared by ourselves in lab following the published method (Gibson et al., 2009).

2. Prepare 5× Isothermal Buffer

| Component     | Amount   | Final Concentration |
|---------------|----------|---------------------|
| PEG-8000      | 0.75 g   | 25%                 |
| 1 M Tris-HCl pH 7.5 | 1.5 mL   | 500 mM              |
| 2 M MgCl2     | 75 μL    | 50 mM               |
| 1 M DTT       | 150 μL   | 50 mM               |
| 100 mM dATP   | 30 μL    | 1 mM                |
| 100 mM dTTP   | 30 μL    | 1 mM                |
| 100 mM dCTP   | 30 μL    | 1 mM                |

(Continued on next page)
a. Combine Tris-HCl, dNTPs, MgCl₂, DTT, and NAD. Slowly add the PEG-8000 to the mixture to ensure it dissolves completely. Add ddH₂O to a final volume of 3 mL. Divide the solution into 80 µL aliquots and store at −20°C for up to 6 months.

3. Prepare Gibson Assembly Master Mix

a. Mix thoroughly and divide the solution into 15 µL aliquots. Store it at −20°C for up to 6 months.

 Alternatives: Gibson Assembly Master Mix can be purchased from NEB (Catalog# E2611S).

4. High quality hPSCs (hESC lines/hiPSC lines) in the absence of spontaneous differentiation should be used to perform the knockin

△ CRITICAL: It is important to perform quality controls on the hPSCs such as karyotyping, mycoplasma testing, and STR profiling.

5. Matrigel preparation

a. Thaw Corning Matrigel hESC-Qualified Matrix on ice for 20–24 h at 4°C. Store in 250 µL aliquots in −80°C for up to 6 months.

b. Thaw an aliquot of the Matrigel for at least 2 h, or up to 24 h at 4°C on ice. Dilute the Matrigel 1:100 with cold DMEM/F12 medium and store at 4°C for up to a week.

c. Coat 1 mL/well for a 6-well plate, 0.5 mL/well for a 12-well plate, or 0.25 mL/well for a 24-well plate. Place the coated plates in a 37°C incubator and wait at least an hour before using.

Note: Matrigel will solidify and become a gel when allowed to warm to 20–22°C. Keep the Matrigel aliquots on ice prior to dilution in DMEM/F12. When diluted, coat the plates immediately after removing from 4°C storage, and place back in 4°C storage after coating.

Note: Growth factors that influence cell growth and spontaneous differentiation are present in Matrigel, albeit at very low levels, but lot-to-lot variability should be monitored as this could introduce variability in cultures. Internal quality control of lots is recommended.

6. hPSC culture medium preparation
STAR Protocols

Protocol

a. Stemflex Medium: 450 mL Stemflex Basal Medium + 50 mL Stemflex Supplement
b. Stemflex Medium + 1 × CloneR
c. Stemflex Medium + 1 × CloneR + 0.5 µg/mL Puromycin

**Alternatives:** Other widely used hPSC medium, such as mTESR1 (Stemcell Technologies) and E8 (Thermo Fisher Scientific) can be also used for hPSC culture. We use StemFlex medium since it is better to support hPSC single-cell splitting and single-cell clone survival.

7. 0.5 mM EDTA: Used to passage hPSCs. Dilute 0.5 M EDTA into DPBS at a 1:1,000 ratio to make a final concentration of 0.5 mM EDTA. Make freshly each time.

**Note:** By using the protocol we have successfully generated multiple reporter lines based on H1 hESCs, H9 hESCs or other iPSC lines for our own and for other labs at MSK.

8. Prepare chemicals for mesoendoderm differentiation (specifically for TBXT-GFP reporter line characterization).

9. CHIR99021 (15 mM Stock): Dissolve 50 mg CHIR99021 in 7.16 mL DMSO to a final concentration of 15 mM. Divide the solution into 200 µL aliquots and store at −80°C for up to 6 months.

10. IWP2 (2.5 mM Stock): Dissolve 10 mg IWP2 in 8.57 mL DMSO to a final concentration of 2.5 mM. Divide the solution into 200 µL aliquots and store at −80°C for up to 6 months.

**Note:** The mesoendoderm differentiation reagents used here is for characterization of TBXT-GFP reporter line which we used as an example. For characterization of other tissue-specific gene reporter line, the specific differentiation protocol is needed.

**KEY RESOURCES TABLE**

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Antibodies          |        |            |
| Human/Mouse Brachyury APC-conjugated Antibody | R&D | IC2085A |
| **Bacterial and Virus Strains** | | |
| MultiShot™ StripWell TOP10 Chemically Competent E. coli | Thermo Fisher Scientific | C409601 |
| **Chemicals, Peptides, and Recombinant Proteins** | | |
| Matrigel hESC-Qualified Matrix | Corning | 354277 |
| EDTA | Fisher Scientific | MT-46034CI |
| CHIR99021 | TOCRIS | 4423 |
| IWP2 | TOCRIS | 3533 |
| B-27 Supplement, minus insulin | Fisher Scientific | A1895601 |
| CloneR | Stemcell Technologies | 05888 |
| PEG-8000 | Fisher Scientific | 65-101-KG |
| 1 M Tris-HCl pH 7.5 | Fisher Scientific | 15567027 |
| 2 M MgCl₂ | Sigma | M1028 |
| 1 M DTT | Sigma | 1019777701 |
| 100 mM dATP, dTTP, dCTP, dGTP | Fisher Scientific | 50-183-024 |
| 100 mM NAD | Sigma | 10127965001 |

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### REAGENT or RESOURCE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Carbenicillin       | Thermo Fisher Scientific | 10177012 |
| Puromycin           | Fisher Scientific         | A1113803 |

### Critical Commercial Assays

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| KOD Xtreme Hot Start DNA Polymerase | EMD Millipore | 71975-3 |
| BbsI-HF             | NEB    | R3539S     |
| Smal                | NEB    | R0141S     |
| QS High Fidelity 2X Master Mix | NEB | M0492L |
| OneTaq Hot Start Quick-Load 2X Master Mix with GC Buffer | NEB | M0489L |
| Cell Culture PBS (1x) | Fisher Scientific | MT21040CV |
| Stemflex Medium     | Thermo Fisher Scientific | A3349401 |
| DMEM/F12            | Fisher Scientific         | MT10092CV |
| Accutase            | Innovative Cell Technologies | AT104 |
| RPMI 1640 Medium with L-Glutamine | Fisher Scientific | MT10041CV |
| Lysis Solution for Blood | Sigma | L3289 |
| Neutralization Solution for Blood | Sigma | N9784 |
| Gibson Assembly Master Mix | NEB | E2611S |
| Taq DNA ligase      | NEB    | M0208L     |
| T5 Exonuclease      | NEB    | M0363L     |
| Phusion High Fidelity DNA Polymerase | NEB | M0530L |
| BD Cytofix Fixation Buffer | BD Bioscience | 554655 |
| LB Broth Base       | Thermo Fisher Scientific | 12780052 |
| P3 Primary Cell 4D-Nucleofector X Kit L | Lonza | V4XP-3024 |
| QIAquick Gel Extraction Kit | Qiagen | 28706 |
| QIAprep Spin Miniprep Kit | Qiagen | 27106 |
| QIAprep Plasmid Midi Kit | Qiagen | 12145 |
| QIAquick Gel Extraction Kit | Qiagen | 28706 |

### Experimental Models: Cell Lines

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs) | N/A | N/A |
| H1 hESCs (to generate TBXT-GFP reporter line) | WiCell | WA01 |

### Oligonucleotides

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Primer: M13 Forward 5’ GTTTTCCCCAGTCA CGAC 3’ | This Paper | N/A |
| Primer: STCLM0118R 5’ CTGAACCTTGCCGT TTACG 3’ | This Paper | N/A |
| Primer: STCLM0072F 5’ CGCAGCAACAGATGGAAGG 3’ | This Paper | N/A |

(Continued on next page)
| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Primer: M13 reverse 5'-CGGATAACAATTTCACACAG 3' | This Paper | N/A |
| Primer: STCLM0119F 5'-CCGACAAACCACCTAAGC 3' | This Paper | N/A |
| Primer: STCLM0051R 5'-AATGTGTGGGCGCCAGAG 3' | This Paper | N/A |
| Primer: hPGK-F 5'-CATTCTGACACGCTTTCAAAAGC 3' | This Paper | N/A |
| Primer: STCLM0153R 5'-CTAAAACCTGCTGTCCTCAACTATG 3' | This Paper | N/A |
| Primer: TBXT-PCR-F1 5'-CTACACACCTCCCTACCCCATC 3' | This Paper | N/A |
| Primer: TBXT-PCR-R1 5'-TAAACCTGAGACTGCACAGG 3' | This Paper | N/A |
| Primer: U6-Fwd 5'-GACTATCATATGGCTTACCGT 3' | This Paper | N/A |
| H1-TBXT-GFP sgRNA target: 5'-ACCTTCCATGTGAGC 3' | This Paper | N/A |
| Primer: STCLM0014F 5'-CTCGAGGATATCGGCAGCGGGGCGCCACCAACT 3' | This Paper | N/A |
| Primer: STCLM0015R 5'-GTCGACATAACTTCTGAGAGGGCCGACAGCAGCAGCAGCAGGCTGGGGGCCCAGTTCTTCCGGGGCTCCCAGCGCACTACACACCCCTCACCCA TCCGGTCTCGGCGCCCTCTTCCTCGGGATCCCCACTGTACGAAGGGGCGGC CGCGGCCACAGACATCGTGGACACCCAGTACGACGCCGCAGCCCAAG GCCGCCTCATAGCCTCATGGACACC TGTGTCGCCACCTTCCATGCTCGAGG 3' | This Paper | N/A |

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RESOURCE AVAILABILITY

Lead Contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Ting Zhou (zhout@mskcc.org).

Materials Availability
The H1 TBXT-GFP reporter line generated in this study will be made available on request, but we may require a payment and/or a completed Materials Transfer Agreement if there is potential for commercial application.

Data and Code Availability
This study did not generate or analyze any datasets or code.

STEP-BY-STEP METHOD DETAILS

sgRNA Cloning and Donor Plasmids Construction

Continued

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| H1-TBXT-GFP Donor HAR: | This Paper | N/A |
| 5’gaagttatgtgacAGCAGCAA GCCCCAGTCCCCGAAAGATGCAG TGACTTTTTTGGTCGGCAGCCAG TGTTGACTGGATTGACCTAATAGG TACCCAGTGGCACTCTCAAGG TTAAGAAGAAATGCGACGCTCAGT AACTTCCTTTTCAAAGCAG TGAGGGAGCAGCCGGCACC TTTCGCCAGAGCCCGACATCC CTGCTCAACACCTGGAGTACGCG TGCTGTCACCCAGTTGTACAGA TGAAACCAACTGTTGAGATATG TCAAGGGCAACCACTTGACTGACG GTGAAAAATGTTTGCCAGGG TCCAGAAACTTTTTTGTATTATT TCTCATACAGTGATTTGCAAC TTTGGCCACACCAAGATTGTAAC TCCACGTCTCCTATTATTGAG ATAGATAGAAAGCACA ggatcgagtata3' |
| Recombinant DNA PX330 | Addgene | 42230 |
| PX335 | Addgene | 42335 |
| PUC57Bsal | Addgene | 128859 |
| PUC19 | Addgene | 50005 |
| PAX6 donor plasmid | Addgene | 105239 |
| Software and Algorithms SnapGene | SnapGene | https://www.snapgene.com/ |
| Other 4D-Nucleofector X Unit | Lonza | AAF-1002X |
| 4D-Nucleofector Core Unit | Lonza | AAF-1002B |
| Cellometer K2 Fluorescent Viability Cell Counter | Nexcelom Bioscience | N/A |
sgRNA Design

We design sgRNA targets using the Benchling CRISPR Design Tool (https://www.benchling.com/#). The choice of N vs. C terminal tagging should be based on a literature search and the functional properties of the protein of interest. For example, if an important domain is localized to one end, that end should be avoided. The TBXT gene, the example we used in writing the protocol, is an embryonic transcription factor which binds to a specific DNA element through its N terminus (Kispert and Herrmann, 1993). Therefore, we chose its C terminus for tagging.

For TBXT-GFP knockin, the sgRNA target sequence: 5’ ACCTTCCATGTGAAGCAGCA 3’ which the CRISPR is expected to cut right after the STOP codon of the TBXT gene.

sgRNA Cloning

1. Order Forward and Reverse sgRNA oligos:
   - Forward: 5’ CACC+ “G+ 20 bp sgRNA sequence” 3’
   - Reverse: 5’ AAAC+ reverse complement of the “G+ 20 bp sgRNA sequence” 3’
   As an example of TBXT-GFP knockin, we order the:
   - Forward: 5’ CACC+ “G+ ACCTTCCATGTGAAGCAGCA” 3’
   - Reverse: 5’ AAAC+ “TGCTGCTTCACATGGAAGGT+C” 3’

2. Oligos annealing. Prepare the following reaction in an Eppendorf tube, put the reaction tube in boiled water for 10 min, and then let the water temperature gradually cool down to 20°C–22°C.

| Component                  | Volume (µL) |
|----------------------------|-------------|
| NEB buffer 2               | 10          |
| sgRNA top (100 µM)         | 1           |
| sgRNA bottom (100 µM)      | 1           |
| ddH₂O                      | 88          |
| Total                      | 100         |

3. Golden gate cloning to ligate the annealed oligos to PX330 vector. Prepare the following reaction in 0.2 mL PCR tube.

| Components        | Volume (µL) |
|-------------------|-------------|
| PX330(100 ng/µL)  | 0.25        |
| Annealed oligos   | 0.5         |
| Tango buffer, 10× | 0.5         |
| DTT, 10 mM        | 0.5         |
| ATP, 10 mM        | 0.5         |
| FastDigest BbsI   | 0.25        |
| T7 ligase         | 0.125       |
| ddH₂O             | 2.375       |
| Total             | 5           |

4. Run the following program in the thermocycler.
5. Transformation (day 1). Transform the product in Step 2.4 into a competent E. coli strain (Thermo Fisher Cat# C409601) according to the protocol supplied with the competent cells.

6. Day 2, pick colonies. Use a sterile pipette tip to inoculate a single colony into a 3 mL culture of LB medium with 100 μg/mL Carbenicillin. Incubate and shake the cultures at 37°C for 16–18 h.

7. Day 3, isolate the plasmid DNA from the cultures by using a QIAprep spin miniprep kit according to the manufacturer’s instructions.

8. Sequence validation of CRISPR plasmid. Verify the sequence of each colony by sanger sequencing using the U6-Fwd primer: 5’GACTATCATA TGCTTACCGT3’.

Note: The sgRNA cloning efficiency is generally high. Normally, there are tens to hundreds of colonies on the plate. Pick two or three colonies should be enough to get the sequence correct sgRNA plasmid.

9. Make endotoxin free midi-prep plasmid by using ZymoPURE II Plasmid Midiprep Kit according to the manufacturer’s instructions.

Note: A good quality and endotoxin free plasmid is important for successful electroporation in hPSCs.

Donor Plasmid Design

⊙ Timing: ~1–2 weeks

Gibson assembly tool in Snapgene software is a very helpful tool to make the donor plasmid construction strategy. Generally, the donor plasmid contains four parts: (1) the vector backbone; (2) the left homology arm (HAL); (3) the insertion cassette (P2A-H2B-GFP-loxp-PGK-puro-loxp); and (4) the right homology arm (HAR). The structure of the TBXT donor plasmid is shown in Scheme 1.

10. Vector backbone. A simple vector backbone, such as PUC57Bsal (Addgene #128859), PUC19 (Addgene #50005), or others can be served for the donor plasmids construction. To generate the TBXT-GFP donor plasmid, we used restriction enzyme SmaI (NEB Cat# R0141S) to linearize the PUC57Bsal vector to process the Gibson cloning.

11. Homology Left Arm (HAL) and Right Arm (HAR). 400–1,000 bp of HAL and HAR works efficiently for knockin in our hands. The dsDNA of HAL and HAR can be ordered from IDT or other similar companies. To generate the TBXT-GFP donor plasmids, we chose a 400-bp nucleotide sequence before the sgRNA cutting site as HAL, and a 400-bp nucleotide sequence after the sgRNA cutting site as HAR. With this information, we used the Gibson assembly tool in Snapgene software to generate full size of the dsDNA sequence of HAL and HAR with 15 bp identical to the vector backbone and the insert cassette at both sides for the Gibson cloning. The dsDNA sequences of HAL and HAR are listed in Key Resources Table.

12. The Insertion Cassette (P2A-H2B-GFP-loxp-PGK-puro-loxp). A PCR from an existed donor plasmid is the simplest way to obtain the reporter insertion cassette. To obtain the insertion cassette (P2A-H2B-GFP-loxp-PGK-puro-loxp) for TBXT-GFP reporter, we performed a PCR from a previous published
PAX6 donor plasmid (Addgene #105239) using a designed pair of primer Forward (STCLM0014F) and reverse primer (STCLM0015R) (listed in Key Resources Table).

**Note:** H2B fragment is a nuclear localization signal tag, which allows the H2B fusion protein to localize into the nucleolus. The H2B-GFP fragment is only used for tagging nuclear protein, such as the transcription factors. For tagging a protein that not located in the nucleus, the H2B fragment needs to be removed to allow GFP reflect the subcellular localization of the endogenous protein.

**Note:** We recommend that whenever possible, choose a sgRNA target site as close as to the KI location. This could not only increase KI efficiency, but the insertion could also disrupt the original sgRNA recognition site. However, if your CRISPR cutting site is far away from the KI location and the insertion does not disrupt the sgRNA target sequence after KI, a silent mutation can be designed on the repair template (e.g. on HAL or HAR) to prevent the CRISPR from re-cutting the recombined sequence. To do that, we usually mutate the PAM (NGG) by changing one of the G to an A, T or C (avoid changing NGG to NAG), without changing the resultant amino acid.

### Donor Plasmid Cloning

**Timing:** ~1–2 weeks

After each part of the donor plasmid are designed and obtained, we used the Gibson cloning method to ligate each part to construct the full donor plasmid.

12. Gibson assembly reaction. Prepare the following reaction in 0.2 mL PCR tube, and run the 50°C for 30 min, and then hold at 4°C in the thermocycler.

| Components               | Amount  |
|--------------------------|---------|
| Gibson master mix        | 15 µL   |
| pUC57 vector backbone    | 50 ng   |
| Fragment1 (HAL)          | 50 ng   |
| Fragment2 (HAR)          | 50 ng   |
| Fragment3 (GFP)          | 50 ng   |
| ddH2O                    | to 20 µL|
| **Total**                | 20 µL   |

13. Transform the product in Step 12 into a competent *E. coli* strain (Thermo Fisher Cat# C409601), according to the protocol supplied with the cells.
14. Use PCR to screen the positive colonies. Design the PCR primer pairs that one targets the vector backbone, and the other one targets the insert fragment. For TBXT-GFP donor plasmids, we used M13 forward/STCLM0118R and STCLM0072F/M13 reverse to screen the positive colonies. The sequences of the primers are listed in Key Resources Table.

15. Pick at least 2 PCR positive colonies using a sterile pipette tip and then inoculate each single colony into a 3 mL culture of LB medium with 100 µg/mL carbenicillin. Incubate and shake the culture at 37°C for 16–18 h.

16. Isolate the plasmid DNA from cultures by using a QIAprep spin miniprep kit according to the manufacturer’s instructions.

17. Sequence validation of the donor plasmid by using M13 forward, M13 reverse primers and the internal primers. For TBXT-GFP donor plasmid, primers (STCLM0118R, STCLM0072F, STCLM0119F, STCLM0051R and PGK-F) are used for sequencing to validate the whole inserts. The sequencing primers are listed in Key Resources Table.

18. Make endotoxin free midi-prep plasmid by using ZymoPURE II Plasmid Midiprep Kit according to the manufacturer’s instructions.

Note: A good quality and endotoxin free plasmid is important for successful electroporation in hPSCs.

Plasmids Electroporation and Single-Cell Clone Generation

Total Timing: 20–30 days

When the sgRNA plasmid and donor plasmid are ready, hPSCs can be prepared for the knockin experiment which involves plasmids electroporation, puromycin selection and single-cell clone generation.

Electroporation

Timing: ~1 h

19. hPSCs preparation. hPSCs are maintained in StemFlex medium. We routinely passage hPSCs at a ratio of 1:6 every 3–4 days using 0.5 mM EDTA. High quality hPSCs with standard growth rate in the absence of random differentiation are important for achieving highly efficient hPSC electroporation.

20. hPSCs digestion for electroporation. When the hPSCs reach ~70%–80% confluency, aspirate the medium, wash the cells once with DPBS, and then add 1 mL Accutase to the cells and incubate at 37°C for 10 min. Add 3 mL of medium and detach cells by slowly pipetting up and down. Collect the cells to a 15 mL conical tube and spin at 120 x g for 3 min. Aspirate the supernatant, add 1 mL StemFlex medium and count the number of cells using the Nexcelom Bioscience Cellometer K2.

21. Prepare cells and reagents for one electroporation reaction:
   a. 2 x 10⁶ cells
   b. Mix the reagents: 82 µL P3 Primary Cell Nucleofector Solution and 18 µL Supplement 1
   c. Plasmids: 4 µg sgRNA plasmids + 5 µg Donor Plasmids. Eg. We electroporated 4 µg of TBXT-GFP sgRNA and 5 µg of TBXT-GFP donor plasmid for TBXT-GFP reporter line generation.

Note: A single GFP expression plasmid provided in the supplied reagents should be electroporated separately as a positive control for the electroporation.

Note: Scale up accordingly if doing multiple electroporation reactions. In our experience, 70%–80% confluent of one 6-well of hPSCs is ~4 x 10⁶ cells, which can yield two electroporation reactions.
22. Transfer the cell mixture to the Nucleocuvette Vessel using a P200 pipet.  

*Note:* Avoid creating bubbles when transferring; if there are bubbles, tap the cuvette gently or manually remove them as bubbles may impact the efficiency of the electroporation.

23. Transfer the cuvette to the 4D-Nucleofector and electroporate the cells. Select Solution “Primary Cell P3”, Pulse Code “CB-150”, and press “Start”. 

*Note:* Pulse Code for your hPSC lines can be optimized according to the protocol supplied with the reagents. In our hands, Pulse Code “CB-150” with the Solution “Primary Cell P3” is the best condition for H1 and H9 hESCs nucleofection, and the cell survival rate of post-nucleofection in this condition is routinely around 60% calculated by Cellometer K2 Fluorescent Viability Cell Counter. 

*Alternatives:* Lonza 2B-Nucleofector or other Nucleofector equipment that you may have in lab can be also tested for hPSCs electroporation. Lonza 4D-Nucleofector works best for hPSCs electroporation in our hands, considering the electroporation efficiency and cell survival rate.

24. Using the provided single-use-pipet, transfer the cell mixture from the cuvette to a 15 mL conical tube containing 12 mL of StemFlex + CloneR medium. Rinse the cuvette once with a small amount of StemFlex + CloneR medium using the pipet and transfer to the 15 mL conical tube. 

25. Transfer the 12 mL cells equally to a fresh Matrigel-coated 6-well plate (2 mL cells in each well, and totally six wells). Place the plate in a 37°C, 5% CO2 incubator for 24 h. 

*Note:* Gently shake the plate back and forth and front to back to evenly distribute the cells. Avoid circular motions to prevent concentrating cells in the middle or around the edge of the well.

26. 24 h post electroporation, change to 3 mL fresh StemFlex medium for each well, and check the efficiency of the GFP control plasmid electroporation. We routinely get ~50% efficiency based on fluoresces microscopic images and flow cytometry results (Figure 1A and 1B).

**Puromycin Selection and Collecting the Surviving Clones**

© Timing: 4–6 days

27. Continue culturing the cells for 2 or 3 days until the cells reach to 70%–80% confluency (Figure 2A), change to 3 mL StemFlex + puromycin (0.5 mg/mL) + CloneR medium for each of the well.

28. Puromycin selection will be continued for the following 4–6 days. A dramatic cell death will happen on the first day of puromycin selection and survived small colonies will appear in each well after 4–6 days selection (Figure 2B).

*Note:* In rare cases, there are some differentiated colonies after puromycin selection. It is optimal to remove them before collecting and passaging.

29. After puromycin selection complete, add 1 mL of 0.5 mM EDTA to each well, and leave the plate at 20°C–22°C for 10 min, fully detach the cells in all six wells and collect the cells together in a 15 mL conical tube.

30. Spin at 120 x g for 3 min. Resuspend the cell pellet in 1 mL StemFlex medium and transfer the cells to one well of a Matrigel-coated 24-well plate.
31. At the following 2–3 days, culture the cells in StemFlex medium until cells reach ~80% confluence.

Pause Point: At this point, the cells are in a mixed population which contains corrected knockin cells or other cells. The mixed cells can be collected and frozen. PCR with the mixed population can be performed to confirm the presence of any targeted KI event before splitting for single-cell clone generation.

Single-Cell Clone Generation

زادن: 2–3 weeks

32. Detach the mixed cell population with Accutase and incubate in 37°C for 10 min. Resuspend the cell pellet in 1 mL of StemFlex media and count the cell number.

33. We perform single-cell clone generation in Matrigel-coated 96-well plates. For knockin in H1 hESCs, we seeded the cells to 2 × 96-well plates as the number 20 cells/per well. Place the plates in a 37°C, 5% CO2 incubator. We consider this splitting day to be day 0.

Note: The number of splitting cells to each well is the key condition to generate single-cell clones. hPSC single-cell clone survival rate is extremely low when just splitting one cell/per well. However, if you split many cells per well, the clones will merge together so that the resulted “clones” are not actually a single-cell clone. To balance well, we suggest testing the

Figure 1. Electroporation Efficiency of hPSCs
(A) Fluoresces Microscopic analysis of H1 hESCs at 24 h post-electroporation with a GFP expression plasmid included in the P3 Primary Cell 4D-Nucleofector X Kit L. Scale bars, 250 μm.
(B) Flow cytometry analysis of GFP+ hESCs at 24 h post-electroporation.

Figure 2. Representative Images of the Cells after Puromycin Selection
(A) Bright field image of H1 hESCs at 3 days post electroporation with TBXT-GFP sgRNA and donor plasmids. It shows the cells reach to ~70%–80% density which is the start point for puromycin selection.
(B) Bright field image shows the small survived clones after 6 days of puromycin selection. Scale bars, 250 μm.
cell number for splitting for your hPSC line before the knockin experiments. 10–20 cells/per well is a good start for testing.

**Note:** The best condition we have tested for the H1 hESCs cell based single-cell clone generation. As a result, we routinely can get ~30 clones/per 96-well plate, ~60 clones for two plates.

**Note:** StemFlex medium along (without the CloneR or Rock inhibitor) supports for single-cell clone survival and growth in 96-well plate. We try to avoid adding CloneR or Rock inhibitor when it is not necessary since whether CloneR or Rock inhibitor affects pluripotency or cell differentiation of hPSCs it is not fully clear yet.

34. Day 0 to day 3, do not move the plate.
35. At day 4, change each well of 96-well plates to 100 μL fresh StemFlex. Tiny colonies will show up.
36. Day 5–day 7, no need to change medium.
37. At day 8, change the medium in each well of 96-well plates to 100 μL fresh Stemflex. Around day 7 to day 8, the clones should be discernable easily, where one (the most cases), two or merged clones can be found in each well (Figures 3A–3E). The potential single-cell clones have round edge and without boundary inside a clone (Figure 3A and 3B), while clones of possible two or multiple-cell origin or clones being very close to each other should be excluded (Figures 3C–3E).

38. Day 9, no need to change medium.
39. At day 10, the colonies are usually big enough to be picked (Figure 3B), and split at a ratio of 1:2 (split one clone to two wells of a 96-well plate). There are no digesting enzymes needed. Directly scratch the bottom of the well using a P200 pipet for the splitting. We usually split 20–50 single-cell clones for the follow up assays.
   a) Mark the location of the single-cell clone on the bottom of the well under the microscope. This way you will easily see the location of the clone in culture hood.
   b) Using a P200 pipet, gently scratch the marked location on the bottom of the well in a horizontal, vertical, and a circular motion to detach the clone. Pipet up and down gently once, then transfer and split evenly between two wells of a Matrigel-coated 96-well plate.
   c) Mark the number for the splitted clones, such as “1”, “2”, “3” … for each clone. You have now two copies for each clone.
   d) Place the plates in a 37°C, 5% CO2 incubator.

**Note:** Clear boundaries can be noticed by eyes if two or more colonies merge together. Do not pick non-single-cell clone in gene editing experiments.

e) Change to StemFlex medium for the following 2–3 days, and the cell density will be then ~30%–50% of the well. At this point, 1 copy of each clone is ready for following lysis, PCR and sequencing, and the other copy of each clone is keeping maintained in culture.

**Single-Cell Clone Screening by PCR and Sequencing**

**Total Timing:** 3–7 days

**Lysis to Collect Genomic DNA**

**Timing:** 2–4 h

40. Remove medium from each one copy of the clone and wash once by adding ~100 μL of 1× DPBS.
41. Remove 1× DPBS wash and add 20 μL of Sigma Lysis Solution for Blood to each well.
42. Using a P200 pipet, scratch the bottom of the well in a vertical, horizontal, and circular fashion. Slowly transfer cell suspension into the tip and expel into a PCR tube or PCR plate well, labeled respective to the particular clone. (eg. Clone #1 goes into well 1, etc.). Seal tubes/plate.

Note: Change tips for each well when scratching colonies for lysis.

43. Quickly spin down the PCR tubes or plate and immediately move to a thermal cycler.

44. Incubate at 75°C for 15 min followed by a 4°C hold.

45. Carefully remove the seal and add 180 μL of Sigma Neutralization Buffer to each well. Re-seal and store at 4°C until ready for use as PCR template.

Note: The prepared lysis is stable for PCR for at least 6 months in our testing.

**PCR to Verify the Positive Single-Cell Clones**

© Timing: 1–3 days

PCR primers design: there are several ways to design PCR primers to amplify the inserts:

a) Using a pair of the primers that targets to the nucleotide outside of the both homologous arm (Scheme 2). This will be the idea case since one PCR reaction can detect knockin clones and can also differentiate heterozygous or homozygous knockin clones. However, this PCR amplify product is long (>2 kb) and for some cases the PCR reaction does not work well.
b) Using one primer target to the inserts and the other primer target to the nucleotide outside of homologous arm (Scheme 3). In this way, two PCR reactions (5'-end and 3'-end junction PCR) are needed to verify the inserts, and additional a PCR reaction to detect the WT sequence is also needed for detecting heterozygous or homozygous knockin clones. The benefits of this design is the PCR product is usually <1 kb and it is easier to make the PCR works, but more PCR reactions are need for the screening.

c) In our lab, we use one primer targets to one homologous arm and one other primer targets to the nucleotides outside another homologous arm (Scheme 4). The design is similar as a), but with shorter PCR length.

d) As the example of PCR screening for H1 TBXT-GFP reporter knockin single-cell clones (Scheme 5): We use primer TBXT-PCR-F1 targets to the HAL, and STCLM0153R targets to the nucleotide outside of the Right homologous arm (HAR) for screening the single-cell clones.

**Scheme 2. One pair of the Primers to Amplify the Whole Inserts**

**Scheme 3. 5'-End and 3'-End Junction PCR Primers Design**

**Scheme 4. Modified PCR Primers Design for Screening Positive Knockin Clones**

**Scheme 5. PCR Primers Design for Screening of Positive TBXT-GFP Clones**

For the PCR reaction:

50. Prepare the PCR mix

| Components               | Amount (μl) |
|--------------------------|-------------|
| 2× Xtreme™ Buffer        | 12.5        |
| dNTPs (2 mM each)        | 5           |
| KOD Taq                  | 0.5         |
| Cell lysate              | 2           |
| Primer                   | 0.75        |
| ddH2O                    | 4.25        |
| Total                    | 25          |
Note: We recommend using KOD Xtreme Hot Start DNA Polymerase from EMD Millipore for the lysis PCR reaction to screen positive single-cell clones.

51. Set the PCR program

| PCR Cycling Conditions | Temperature | Time  | Cycles |
|------------------------|-------------|-------|--------|
| Initial Denaturation   | 94°C        | 4 min | 1      |
| Denaturation           | 98°C        | 10 s  | 35 cycles |
| Annealing              | 64°C        | 30 s  |        |
| Extension              | 68°C        | 3 min 20 s |        |
| Final Extension        | 68°C        | 5 min | 1      |
| Hold                   | 4°C         | Forever |        |

52. Gel electrophoresis for H1 TBXT-GFP single-cell clones.

a) Clone 1, 9, 10, 13, 16, 19, 20 which only have the larger band (3,227 bp) are homologous knockin clone candidates.

b) Clone 2, 3, 5, 7, 14, 17, 18, 22 which have the larger band (3,227 bp) and the smaller band (653 bp) are heterozygous knockin clone candidates.

c) Clone 4, 6, 8, 11, 12, 15, 21 which only have the smaller band (653 bp) are wildtype clone candidates.

Sanger Sequencing Verification for PCR Product

© Timing: 2–5 days
53. For which only have one clear band of the PCR products, we do enzymatic cleanup using ExoSAP before sending for sequencing. Prepare the following reagent, and 37°C for 30 min and then 4°C hold.

| Components     | Amount (µL) |
|----------------|-------------|
| PCR product    | 9           |
| ExoSAP         | 1           |
| Total          | 10          |

54. For which have two bands of the PCR products, we do gel extraction to purify them before sending for sequencing.

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**Figure 4. An Example of H1-TBXT-GFP Single-Cell Clone Sequencing Results**

(A) Sanger sequencing results with primer TBXT-PCR-F1 confirms the 5’ end junction of the insert in the genome of the single-cell clone 1.

(B) Sanger sequencing results with primer STCLM0072F confirms the 3’ end junction of the insert in the genome of the single-cell clone 1.
55. Send the purified PCR product for sequencing to confirm the junctions between the original genomic DNA sequence and the insert sequence and the sequence of the wildtype allele.

56. An example of H1-TBXT-GFP single-cell clone sequencing and the alignment results are shown in Figures 4A and 4B.

**Note:** To make sure the KI clones are bona fide one-cell origin, a subsequent round of sub-cloning is required to verify the selected clones are indeed a homogeneous population by Sanger sequencing.

**Functional Characterization of the Corrected Knockin Reporter Lines**

**Total Timing:** ~1 week

For generating tissue-specific reporter line in hPSCs, it is important to perform the functional characterization of the line through tissue-specific differentiation. In this protocol, we generated TBXT-GFP reporter lines based on H1 hESCs. TBXT gene encodes Brachyury, an embryonic nuclear transcription factor involves in the transcriptional regulation of genes required for mesoderm formation and differentiation, and TBXT gene is widely used as a marker gene in hPSC mesoendoderm differentiation (Lian et al., 2013). Here, we performed a one-day mesoendoderm differentiation using an established protocol (Lian et al., 2013) to monitor the GFP signal by imaging and flow cytometry analysis.

**Mesoendoderm Differentiation**

57. We performed mesoendoderm differentiation for one homozygous knockin clone (clone #1) and one heterozygous knockin clone (clone #2). When cells are 70%–80% confluency, detach the cells with Accutase and count the cell number.

**Note:** Freeze all the sequencing-confirmed knockin clones for backing up in case aberrant karyotyping and differentiation happened in the selected clones.

58. For each clone, split ~250,000 cells / well to three wells of a Matrigel-coated 24-well plate. The rest of the cells can be expanded or frozen down.

59. On the next day, add 1 mL of RPMI + B27(-insulin) + 10 μM CHIR99021 medium to each well (Day 0).

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**Figure 5. Functional Characterization of the TBXT Reporter Lines**

(A–H) Fluorescence microscopic analysis of a H1 TBXT-GFP homozygous reporter line clone #1 (A–D), and a heterozygous reporter line clone #2 (E–H) before and after mesoendoderm differentiation. Day 0 indicated the cells before differentiation and day 1 indicate the cells after differentiation. Representative bright field and GFP images for Clone #1 at day 0 (A and B), Clone #1 at day 1 (C and D), Clone #2 at day 0 (E and F), and Clone #2 at day 1 (G and H). Scale bars, 250 μm.
60. Day 1, GFP+ cells can be detected in the differentiated cells under fluorescence microscope, but not in the cells at the undifferentiated stage (Figures 5A–5H).

61. The undifferentiated cells (day 0), and the differentiated cells (day 1) was also fixed with BD Cytofix Fixation Buffer at 20°C–22°C for 10 min, and then stained by Brachyury APC-conjugated Antibody for flow cytometry analysis. The results showed >90% of the differentiated cells became Brachyury and GFP double positive, while Brachyury and GFP expression were both negative at day 0, suggesting a faithful GFP expression of the TBXT-GFP reporter line (Figure 6).

EXPECTED OUTCOMES
We routinely obtain an electroporation efficiency between 33% to 50% using the GFP control plasmid included in the P3 Primary Cell 4D-Nucleofector X Kit L.

We seed 20 cells/well for the H1 hESCs based single-cell clone generation, and routinely obtain around 30 single-cell clones per 96-well plate.

PCR screening results suggest the efficiency of homozygous knockin clones is up to 32%, and heterozygous knockin clones is up to 90%. Variation exists and depends on the different genes and different sgRNA target sites. However, single-cell clones generated in 2 × 96-well plates is usually enough for us to screen the correct knockin clones.

LIMITATIONS
The CRISPR vector PX330 (addgene #42230) used in the protocol contains sgRNA expression cassette, as well as a wildtype CAS9 endonuclease (Ran et al., 2013). While it is the most efficient CRISPR tool vector, it creates double-strand break (DSB) in the genome which may potentially cause more off-targets and DNA damage response (Ihry et al., 2018)(Haapaniemi et al., 2018). PX335 (addgene #42335), the SpCas9 nickase (D10A) based the CRISPR vector may be better for your knockin experiments to decrease the DSB side effect. Alternatively, ribonucleoprotein (RNP) which contain Cas9 protein and gRNA generated in vitro may be used (Chen et al., 2016). Since RNP only stay in cells shortly it may also decrease the off-target effects. Using deep sequencing such as whole genome sequencing (Veres et al., 2014) to verify potential random integration of the plasmids and any off-target effect is needed for clinical or transnational use of the edited cells.

Inserting a reporter tag into a gene locus can potentially affect the expression or the function of the gene. If possible, we prefer the 3’ end for knockin and recommend Cre-mediated excision of the

Figure 6. Flow Cytometry Analysis of the H1 TBXT-GFP Homozygous Reporter Line Clone #1 and the Heterozygous Reporter Line Clone #2 before and after Mesoendoderm Differentiation.
The day 1 differentiated cells showed >90% of Brachyury+GFP+, while both Brachyury and GFP expression were negative at day 0.
drug selection cassette in the final product. We also favor the generation of heterozygous clones. Since CRISPR/Cas9 is very efficient to generate DSB in both alleles in this protocol, expect roughly half of the heterozygous clone candidates to contain indels in the non-KI allele. Therefore, please do not rely on PCR product size but use Sanger sequencing to confirm the sequence of each allele at the edited locus.

Aberrant karyotyping and differentiation ability could exist in some clones. Appropriate characterization is highly recommended.

TROUBLESHOOTING

Problem
In Electroporation:

Rampant cell death after electroporation.

Potential Solution
Double check that the electroporation program is optimized for your cell line and media choice. Also, ensure that your plate has been covered with Matrigel for at least an hour.

Problem
In Single-Cell Clone generation and Mesoendoderm Differentiation:

My medium choice is different from Stemflex.

Potential Solution
Using other medium (ie. mTeSR or E8) is fine. However, the Pulse Code must be optimized for each hPSC cell line and culture medium beforehand when using the 4D-Nucleofector for electroporation reaction. When splitting to single-cell clones, dilute Rock inhibitor in your medium to a concentration of 10 µM before seeding to the 96-well plate. Change to fresh media without Rock inhibitor after 2 days, and every 4 days after.

Problem
In Puromycin Selection and Collecting the Surviving Clones:

Cells do not die after antibiotic selection

Potential Solution
In our case, dramatic cell death will be apparent in the first 4 days of puromycin selection. If not, either an increase in antibiotic concentration or an increase in days of exposure to the antibiotic could help with this selection.

Problem
In Puromycin Selection and Collecting the Surviving Clones:

Cells become ball-like after antibiotic selection

Potential Solution
If cells become ball-like, passage once with EDTA. Cells will grow normally after passing once before splitting to single-cell.

Problem
In PCR to verify the positive single-cell clones:

PCR amplify the insert yield no band or non-specific band
Potential Solution
Optimize the PCR system or PCR program. If the result does not improve, design and synthesize more pairs of primers and try again.

Problem
In Sanger sequencing verification for PCR product:

PCR product sequencing fail.

Potential Solution
Amplify more PCR product and using gel extraction to get more clean sequencing sample.

SUPPLEMENTAL INFORMATION
Supplemental Information can be found online at https://doi.org/10.1016/j.xpro.2020.100052.

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AUTHOR CONTRIBUTIONS
A.Z. and M.L. performed the experiments and analyzed the data. T.Z., A.Z., and M.L. wrote the manuscript.

DECLARATION OF INTERESTS
The authors declare no competing interests.

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