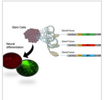

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Single-step generation of genetic reporter lines using CRISPR/Cas9 and transient antibiotic selection

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1 Works for me This protocol is published without a DOI.

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ABSTRACT

The following protocol describes the generation of genetic reporters in human iPSC using in vitro synthesized Cas9 mRNA and guideRNA, a reporter plasmid for homologous recombination, and a homology-directed repair plasmid. The major advantage of this procedure compared to other gene editing schemes is the introduction of a system for the selection of homology-directed repair competent cells in *trans* (not physically linked to the genomic gene edition of interest). While this system was initially described in Flemr M. et al. (2015) Cell Reports in murine ESC, we introduced here some modifications that enable its efficient application in human PSC and with longer *knock-ins*.

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KEYWORDS

CRISPR/Cas9, Gene editing, Reporter lines

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MATERIALS TEXT

pIVTR(T7A)-Cas9-T2A-mClover3 (provided by Verstreken lab and soon through Addgene)
Q5®High-Fidelity DNA Polymerase. NEB. Cat no. M0491L
HighYield T7 Cap 1 AG (3'-OMe) mRNA Synthesis Kit. Jena Biosciences. Cat no. RNT-122-L
HighYield T7 RNA Synthesis Kit. Jena Biosciences. Cat no. RNT-101
RNeasy Mini Kit (250). Qiagen. Cat no. 74106
RiboLock RNase Inhibitor (40 U/µL). ThermoFisher Scientific. Cat no. EO0382
pMB1610_pRR-Puro plasmid. Addgene. Plasmid no. #65853
SacI. NEB. Cat no. R0156S
AatII. NEB. Cat no. R0117S
mTESR Plus. StemCell Technologies. Cat no. #100-0276
UltraPure™ 0.5M EDTA, pH 8.0. ThermoFisher Scientific. Cat no. AM9260G
StemPro™ Accutase™ Cell Dissociation Reagent. ThermFisher Scientific. Cat no. A1110501
StemMACS Y27632. Miltenyi Biotech. Cat no. 130-106-538
Lipofectamine MessengerMAX. ThermoFisher Scientific. Cat no. LMRNA001
Apex 2X Taq Red PCR master mix. Genesee Scientific. Cat no. 42-138

ABSTRACT

The following protocol describes the generation of genetic reporters in human iPSC using in vitro synthesized Cas9 mRNA and guideRNA, a reporter plasmid for homologous recombination, and a homology-directed repair plasmid. The major advantage of this procedure compared to other gene editing schemes is the introduction of a system for the selection of homology-directed repair competent cells in *trans* (not physically linked to the genomic gene edition of interest). While this system was initially described in Flemr M. et al. (2015) Cell Reports in murine ESC, we introduced here some modifications that enable its efficient application in human PSC and with longer *knock-ins*.

Preparing Cas9 mRNA and the gene-specific guide RNA through in vitro transcription (IVTR) 2d

1 Amplify Cas9-T2A-mClover3 IVTR template from pIVTR(T7A)-Cas9-T2A-mClover3 using the following primers 1d

Primer	Sequence (5'-3')
Pre_T7_Fwd	ACATTTCCCCGAAAAGTGCC
HBB_3UTR_150T_Rv	TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTGCAA

Use an annealing temperature of 66ºC when using Q5 Polymerase from NEB. Extend for 3' (30 sec/kb)

This PCR generates an IVTR template encoding for the Cas9-T2A-mClover3 HBB gene 5' and 3' UTRs, the

Gel-purify the PCR product and resuspend in RNAse-free water. Concentration should be >100 ng/µL for the IVTR. Generally, that yield is attained with 3-4x 50 µL PCR reactions, but yield may vary depending on the polymerase and the thermocycler used.

Perform IVTR following the vendor's instructions. It is important to produce capped mRNA. We use HighYield T7 Cap 1 AG (3'-OMe) mRNA Synthesis Kit from Jena Biosciences (see Materials section).

- We typically perform a 2-hour 20 µL IVTR with 1 µg purified IVTR template.
- Purify in vitro transcribed mRNA with the preferred RNA purification method. We use column-based purification.
- With the indicated IVTR kit, we generally obtain 70-120 µg of mRNA per 20 µL reaction.
- After measuring the mRNA concentration, add a broad spectrum RNAse inhibitor to the purified mRNA (see Materials section).
- Aliquot the mRNA and keep it @ -80°C until needed.

2 Generate the gene-specific **guideRNA IVTR** template using nested oligos as indicated in this protocol with minor modifications: 2d

- We use Q5 polymerase for amplifying the IVTR template, hence we adapt the denaturing step temperature to 98°C and the timings to the ones indicated by NEB.

- We have found that adding two Gs instead of one before the protospacer sequence in the T7FwdVar oligo significantly increases the yield of the IVTR RNA.
- With the indicated IVTR kit and protocol modifications, we generally obtain 70-120 µg of mRNA per 20 µL reaction.
- After measuring the mRNA concentration, add a broad spectrum RNAse inhibitor to the purified mRNA (see Materials section).
- Aliquot the mRNA and keep it @ -80ºC until needed.

* GuideRNAs are not capped since they are not translated within the cells. Hence we use a regular IVTR kit such as HighYield T7 RNA Synthesis Kit (see Materials).

Generate the gene-specific homology-directed repair (HDR) template and recombination reporter plasmids

1w

3 **pHDR plasmid**

For reporter gene knock-in experiments, we typically use ~800 bp-long homology arms, which we amplify from the genomic DNA of the human PSC line that is going to be gene-edited. Left homology arm spans ~800 bp before the gene of interest (GOI) stop codon (last GOI's exon and adjacent intron). Right homology arm spans ~800 bp after the stop codon (typically the GOI's 3'UTR and the adjacent intergenic sequence).

- Make sure you remove the stop codon and fuse the T2A-Fluorescent_Protein cassette to the last coding codon of the GOI. It is recommended to include a Gly-Ser-Gly linker right before the T2A. The fluorescent protein should contain its own stop codon.
- There is no special requirement for choosing the backbone plasmid, though it is advisable to only maintain the indispensable elements (AmpR and Ori) in order to minimize its size.

Scheme of a typical HDR plasmid. The size of the different elements of the GOI is illustrative.

pRR-Puro plasmid

- pRR-Puro plasmid with the intact multiple cloning site was deposited by the Bühler lab and can be obtained from Addgene is.
- Cut the plasmid with SacI and AatII and gel-purify the digested vector.
- Order oligos for oligoannealing. Oligos should contain the following overhangs so that they will anneal to the SacI/AatII-cleaved pRR-Puro:

Oligo design (5'-3')
5bp+Spacer_sequence+PAM+5bp+ACGT
5bp+PAM+Spacer_sequence+5bp+AGCT

* Annealed oligos should contain the gRNA-specific spacer sequence, the PAM sequence, and some extra base pairs on each side. We always include 5bp on each side of the Spacer_sequence+PAM.

- Anneal oligos and ligate them into the pRR-Puro vector as indicated in this guide by Addgene.

Endofree preparation of plasmids is generally advised. Given that the plasmids are to be mixed with RNAs for the transfection it is important to have very clean preps.

Transfecting hPSC with the gene editing cocktail and subsequent Puromycin selection

1w 3d

2d

4 **Seeding hPSC for transfection:**

We maintain hPSCs with mTESR Plus (mTESR+) (StemCell Technologies), but the protocol works indistinctly with other media formulations. We also use 0.5 mM EDTA (Life Technologies; AM9260G) (diluted in Ca2+/Mg2+-free PBS) for regular passaging and Accutase (Life Technologies; A11105-01) for seeding cells to be transfected.

- hPSCs are ready to be seeded for transfection when they are When hPSC reach 80% confluency. Change the medium of hPSCs with fresh medium supplemented with 10 µM ROCK inhibitor (RI) (Miltenyi Biotech; 130-106-538) at least 2 hours before starting the dissociation.
- Remove the medium containing RI from the cells (this medium can be kept for later). Add 1 mL of pre-warmed Accutase and keep the cells in the incubator for 1-2 minutes. Remove the accutase once the cells start to round and the separation between them becomes evident. Detach the cells by flushing medium on top of the colonies. Break up the colonies to small clumps of 2-3 cells by gently pipetting up and down the suspension of cells 2-3 times.
- Count the cells and seed $1 \cdot 10^6$ cells per well of a 6-well plate in the presence of RI (see **Note 1** below).

Note 1: We have observed that lipid-mediated transfection is more efficient when hPSCs are seeded in a 1:1 mix of mTESR+/KSR (Knockout Serum Replacement medium). It helps to keep cells less tightly packed more evenly distributed over the plate.

30m

5 **Transfecting DNA/RNA gene editing mix:**

We found Lipofectamine MessengerMAX (Life Technologies; LMRNA001) to be the best reagent to simultaneously transfect RNA and DNA.

- **Tube 1:** 125 µL of OptiMEM (Life Technologies; 31985070) + 7.5 µL Lipofectamine MessengerMAX. Wait for 10 minutes (Please see **Note 1**).
- **Tube 2:** 125 µL of OptiMEM + 1 µg pHDR + 500 ng pRR. After 9 minutes of having prepared **Tube 1** mix add + 750 ng Cas9-T2A-mClover3 mRNA + 250 ng guideRNA (Please see **Note 2**).

- After the initial 10' incubation, add 125 µL from **Tube 1** to **Tube 2** and wait for 5 more minutes.
- Remove the medium from the cells, add 1 mL to the eppendorf tube containing the 250 µL transfection mix and add the resulting 1.25 mL rapidly to the cells.
- Incubate the hPSCs with the transfection mix for 2-4 hours and add a total of 2 mL of fresh mTESR+ medium after that.

Note 1: we generally prepare a mix for all the transfections to be carried out. E.g. 250 µL OptiMEM + 15 µL Lipofectamine MessengerMAX for 2 transfections.

Note 2: we recommend to include a negative control transfection mix with non-matching pRR and gRNA. It is a

good indicator of spontaneous (and unwanted) pRR plasmid recombination.

6 **Puromycin selection:** 3d

- The morning after, replace the medium plus the transfection mix with fresh medium supplemented with RI and 0.5 µg/mL Puromycin (Invivogen; ant-pr-1) selection (Please see **Note 1**).
- 2 days after transfection, replace the medium with fresh medium supplemented with RI and 0.5 µg/mL Puromycin.
- 3 days after transfection, replace the medium with fresh medium supplemented with RI.
- RI can be removed from the medium once colonies start to have >4/5 cells.
- Approximately 8-12 days post-transfection, the colonies are big enough to be manually isolated into 96-well plates. Please see **Notes 2** and **3**. We find the stripper pippettes (Cooper Surgical) are the best suited for accurately isolating monoclonal colonies.

Note 1: whereas this concentration has worked fine with most of the lines, you may have to perform kill curves for your own line.

Note 2: we generally try to stay away from very big colonies as they may be the result of two independent gene editing events. However, it is often obvious when big colonies originated from two merging medium-sized colonies. In that case, it is safe to pick the most distant parts of each colony.

Note 3: we try not to isolate entire colonies. If part of the colony is left on the original plate, it is possible to prepare a pellet from the leftovers, which can be used to optimize the screening PCR reaction. It must be taken into account that it very convenient to be sure that the screening PCR conditions are optimized before screening the 96-well plates

Screening clones in 96-well plates 1d

7 **Sampling the wells:** 3h

- With the use of a multi-channel pipette and a reagent reservoir, remove the medium from the cells, wash once with 0.5 mM EDTA and treat with 0.5 mM EDTA for 5 minutes in the incubator.
- After the 5-minute incubation time, discard the EDTA and vigorously flush 100 µL of medium in the first column (in general this detaches most of the cells in the well, otherwise pipette up and down). Take approximately 70 µL to a 96-well PCR plate and leave the remaining 30 µL in the original plate. Proceed sequentially with the 11 remaining rows. Once the whole plate is sampled, add 70 µL of fresh medium to each well.
- Centrifuge the PCR plate at 3000g for 5 minutes and discard the supernatant.
- Add 35 µL of the following lysis solution to the cell pellets:

Reagent	Vol (µL) per sample
PBS	35
10% SDS	0,16884
Proteinase K (600 amu)	0,08736

* This recipe was adapted from this protocol from the Soriano Lab

- Incubate at 55ºC for 1-2 hours and heat-inactivate the Proteinase K at 85ºC for 15 minutes.
- At this point the cell lysate can be used directly as a PCR template.

8 **Setting-up the PCR reaction and sequencing:** 2d

- We typically use a PCR mix (Apex 2X Taq Red PCR master mix; 42-138), which includes all the necessary reagents (including loading buffer) but primers and template (see **Note 1** for details on primer design):

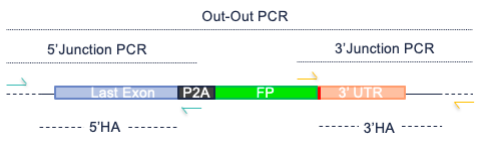
Reagent	Volume (µL)
Template*	1
Primer (F+R) (10 µM each)	1
PCR mix	8
Water	10

* It is easier to pipette the lysate when it is still warm from the Proteinase K inactivation. Otherwise, it gets too slimy.

Note 1: primer design is crucial to determine whether the reporter construct has been knocked-in in the target locus. We perform the following PCR:

- 5' Junction (5'J) PCR: forward primer lies in the region immediately adjacent to the start of the 5'HA and reverse primer binds to the 2A sequence.
- 3' Junction (3'J) PCR: forward primer binds to the Ct part of the knocked-in cassette and the reverse primer binds to the region immediately downstream to the region covered by th 3'HA.
- Out-Out PCR: combines the forward primer from the 5'J and the reverse primer from the 3'J PCRs.

Out-Out PCR is important to discard weird gene editing outcomes (except large deletions or inversions).



- Select clones that are positive for the 5'J and/or the 3'J PCRs. Ideally both, however, some of these PCR reactions might be challenging.
- Perform Out-Out PCR in those clones that were positive for the Junction PCRs. This PCR is also important for identifying bi-allelic *versus* mono-allelic modifications. Mono-allelic clones show a *wild type* allele in the gel (see **Note 2** for additional considerations).

Note 2: It is common that actual bi-allelic clones show the band corresponding to the *wild type* allele in the first PCR (with the DNA harvested from the 96-well plate). In some instances, this allele disappears in subsequent samplings. We attribute it to free-floating genomic DNA from cells killed during the Puromycin selection step, which slowly gets washed away. In case it does not disappear, it could be convenient to consider subcloning the cells from the positive well.

Characterization of correctly-targeted clones

9 Once the positive clones have been verified by Sanger sequencing, it is convenient to perform the following analyses:

- Karyotype analysis through G-banding or ideally CGH array.
- Sequencing predicted off-target sites
- Southern blotting to verify the integrity of the genomic region and to discard non-specific integrations.