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Efficient and precise targeting of the AAVS1 safe harbour locus in hPSCs. V.2

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We use this protocol and it's working

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Disclaimer

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Abstract

Stably genetically-modified human pluripotent stem cells (hPSCs) are increasingly being used for studies relying on the consistent expression of the transgene of interest in human stem cells and their derivatives. Most often, the robustness of the transgene expression is achieved by its introduction into one of the members of an ever-expanding set of so-called "safe harbour"loci in the human genome. Here we describe a process of an efficient generation of high-quality hPSC clones with precise homology-directed targeting of the AAVS1(PPP1R2C) locus assisted by local DNA cutting using TALEN user-customisable nucleases.

Protocol materials

ACCUTASE™ 100 mL **STEMCELL Technologies Inc. Catalog #**7920	Step 2
Corning® Matrigel® **Corning Catalog #**354277	Step 2
Puromycin Dihydrochloride **Gibco - Thermo Fisher Catalog #**A1113803	Step 3

Preparation of reagents for successful transfection

1 Preparation of targeting and nuclease-encoding vectors for a successful transfection

1.1 Targeting of the *AAVS1/PPP1R12C* locus could be aided, for instance, by the use of the 2 TALEN plasmids available from theAddgene repository (addgene.org),
AAVS1 TALEN-L **addgene Catalog #**59025
AAVS1-TALEN-R **addgene Catalog #**59026 These vectors are compatible with a wide range of AAVS1-targeting plasmids, e.g. those based on
AAVS1 targeting vector with puromycin selection **addgene Catalog #**73503 Sufficient amount of the plasmid should be prepared using a midi/maxiprep commercial plasmid prep kit, preferably with an endotoxin-free purification option.

1.2 High-quality bulk or manually-passaged hPSC culture is used as a cell source for transfection. Typically, depending on survival due to passaging and electroporation which is highly hPSC line-specific, 1-2 wells of a 6-well plate with 30-60% confluent stem cell culture provides a sufficient cell number for plating into 3/6 wells after transfection.

Transfection for gene targeting

2 1. Prepare a desired number of wells in a 6-well plate to accommodate the hPSC cell suspension after electroporation, and become "master" wells for establishing targeted clones after antibiotic selection. The wells are coated with
Corning® Matrigel® **Corning Catalog #**354277 or similar ECM with 2x higher concentration relative to the manufacturer's instructions.
2. Prepare a suspension of hPSCs for transfection using a Neon electroporator kit (or similar device)

Equipment

new equipment

NAME

Thermo Fisher Scientific Neon™ Transfection System

BRAND

MPK5000S

SKU

This is achieved by the generation of a single-cell suspension from the existing cultures using accutase ACCUTASE™ 100 mL **STEMCELL Technologies Inc. Catalog #**7920 digestion (5-7 minutes at RT), followed by a rinse/spin with culture medium.
3. According to the manufacturer's instructions, use 100µL tip to transfect 3-4x10^6 hPSCs with 3µg of AAVS1 targetting vector, and 2µg of each of the TALEN vectors (see 1.1). Use a customised protocol or this set of parameters: 1250V / 15 ms / 2 pulses. Let cells recover after the pulses in the tip for 2 mins.
4. Plate cells in 3 wells of a 6-well plate (2.1) (density could be adjusted depending on the specific cell line used), in the antibiotic-free medium (1.5mL/well) supplemented with CloneR2 **STEMCELL Technologies Inc. Catalog ##** 100-0691 or a similar hPSC survival-promoting agent.

Selection of the correctly-targeted clones

3 After 5 days in culture or ~95% confluency (whichever comes first), selection of the single transfected cell-derived clones could be started. For instance, for AAVS1-Px vectors (see 1.1), Puromycin Dihydrochloride **Gibco - Thermo Fisher Catalog #**A1113803 selection at 2µg/mL for 1-2 days. Single cell-derived colonies should be allowed to grow for 5-10 days after transfection (depending on the cell line/colony density/selection regiment used) before being manually passaged into individual plate wells to establish clonal lines for downstream use and characterization (genotyping).

Correctly-targeted clones could be identified by genotyping PCR from genomic DNA using a primer set, with one anchored in the AAVS1 locus outside the homology arms of the AAVS1 vector, while the reverse primer is targetted to the puromycin resistance coding region.
Primers used for genotyping of the AAVS1 targetting SA-Puro vectors:
AAVS1_PC_F
CTG CCG TCT CTC TCC TGA GT
AAVS1-SA-Puro_R3
TCG TCC GCG ACC CAC ACC TT

gDNA-based PCR: use 100-200ng of high-purity gDNA prep for a >=20uL reaction using standard PCR reaction setup (using a conventional Taq or similar polymerase)

PCR conditions used for the amplification on a conventional PCR machine:

35 cycles of:
98°C for 15 secs
68°C (decreasing 0.2 deg every cycle) for 20sec (touchdown PCR)
72°C for 30 sec,
Followed by a final extension

72°C for 5 min
Keep at 4°C

The bands can then be separated on a TAE agarose gel (0.8-1%) with a ~1.1kb band indicative of the presence of at least one targetted allele. Make sure (especially in the first runs) to include negative and positive controls for this genotyping PCR. For new AAVS1-targeting vectors Sanger sequencing is recommended.