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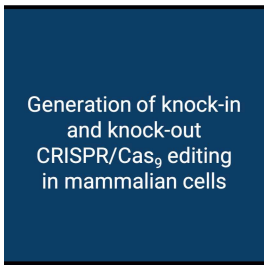
Generation of knock-in and knock-out CRISPR/Cas9 editing in mammalian cells



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Abstract

This protocol is to help with the generation of knock-in and knock-out genomically edited mammalian cells lines using CRISPR/Cas9.

Attachments

PDF

Generation of knock-...

78KB

Materials

Required buffers

Sorting buffer

1x dPBS

0.02% EDTA

0.2% FBS

Safety warnings

Please refer to the Safety Data Sheets (SDS) for health and environmental hazards.

Before start

Knock-out mammalian cell lines

General overview of generating clonal knock-out mammalian cells lines using CRISPR/Cas_9

a. The purpose of this part of the protocol is to explain the methods of generating a clonal cell line that has been edited at the endogenous locus to knock-out a gene of interest. In particular, this part of the protocol focuses on using hTERT-RPE1 cells (https://www.atcc.org/products/crl-4000) as they are chromosomally stable, are generally easy amenable to CRISPR/Cas_9 genomic editing, reliably diploid, and have a flat nature ideal for imaging of organelles. However, this protocol has been applied to other cell types, but subtleties in protocols are not discussed here.

b. This protocol for generating knock-out cells uses a single guide RNA (gRNA) sequence usually directed at an early exon (e.g., exon 2). Importantly, this protocol utilizes the well-established PX458 plasmid (Addgene # 48138) to express the gRNA and Cas_9 protein with a cleavable GFP that serves as a reporter for cloning.

c. All imaging and further cell culture techniques can be found here: DOI of other protocol

Knock-in mammalian cell lines

General overview of generating clonal knock-in mammalian cells lines using CRISPR/Cas_9

a. The purpose of this part of the protocol is to explain the methods of generating a clonal cell line that has been edited at the endogenous locus to knock-in a fluorescent protein tag in gene of interest. In particular, this part of the protocol focuses on using HeLa cells (https://www.atcc.org/products/ccl-2?geo_country=au) as they are easy to transfect with DNA and amenable to genomic editing. However, this protocol has been successfully applied to other cell types, but subtleties in protocols are not discussed here.

b. This protocol for generating knock-in cells uses a single guide RNA (gRNA) sequence usually directed toward the start ATG codon, but also at internal sites. Importantly, this protocol utilizes the now established homology independent targeted integration (HITI) ORANGE system and accompanying plasmid (Addgene # 131471) to express the gRNA, Cas_9 protein, and sequence of the fluorescent protein tag to be inserted at the endogenous locus. A more detailed protocol and explanation about the engineering can be found here: **10.1371/journal.pbio.3000665**

Knock-out mammalian cell lines - Choosing a gRNA for editing endogenous locus of gene of interest

1 All gRNA sequences used in these protocols were identified using the publicly available CRISPR/Cas_9 gRNA design program CHOPCHOP (https://chopchop.cbu.uib.no/). For the generation of $SHIP_{164}$ knock-out cells, a gRNA directed toward exon 2 was selected based on the predicted efficiency score and low off-target sites. For the exact sequences used, please see Table X in our publication.

Knock-out mammalian cell lines - Cloning the gRNA into PX458

2 Linearize PX458 plasmid by using the restriction enzyme BbsI (New England Biolabs) following the manufacturers guidelines. Run the linearized plasmid on an agarose gel (e.g., 1% supplemented with Midori Green or Ethidium Bromide for visualization) and purified using a NucleoSpin Gel and PCR Clean-up kit (Takara)

3 Synthesize a single stranded oligonucleotide (ssOligo) containing the desired 20bp gRNA sequence by IDT. The ssOligo contained 25bp of homology at each end that corresponds with the flanking sequence of the linearized PX458 plasmid.

4 Ligate the ssOligo into the purified linearized PX458 plasmid using NEB HiFi assembly mixture following manufacturers protocol (https://www.neb.com/-/media/nebus/files/applicationnotes/appnote_bridging_dsdna_with_ssdna_oligo_and_nebuilder_hifi_dna_assembly_to_create_sgrna-cas9_expression_vector.pdf?rev=8537d5c90b07406b85b9f2221e2410f6).

Knock-out mammalian cell lines - Transfection of PX458-gRNA into RPE_1 cells

2d 8h

5 Plate 1.0x10^5 wild-type RPE_1 cells in to one well of a 6-well plate.

6 Replace 24:00:00 later, the complete media with media without antibiotics.

1d

7 Transfect cells with 1 µg of PX458-gRNA plasmid using FugeneHD (Promega) following the manufacturers protocol.

8 Incubate cells with the transfection reagent for 08:00:00 - 24:00:00 be sorting. 1d

Knock-out mammalian cell lines - FAC sorting of PX458-gRNA transfected RPE_1 cells

1d 0h 5m

9 Remove RPE1 cells transfected for 24:00:00 with the PX458-gRNA plasmid from the 6-well dish
using standard trypsinization. 1d

10 Spun down the cells at 500 g for 00:05:00 to pellet them, and aspirate the media off. 5m

11 Resuspend the cell pellet in 500 µL of Room temperature sorting buffer.

12 Based on the presence of GFP signal compared with empty-PX458 control cells, a gate was created to
sort individual cells into wells of a 96-well plate.

Knock-out mammalian cell lines - Identification and verification of clonal knock-out RPE_1 cells

13 Once cells in 96-wells have grown into a discernable colony (visualized by brightfield microscope), move colonies to 24-well plates to expand them.

14 When the colonies have grown to near confluency (~80%) of the 24-well, there are enough cells to then
collect for genomic DNA purification and to also maintain on a new 24-well plate.

15 Performe genomic DNA of clonal cells using the DNeasy purification kit (Qiagen 69056).

16 Use purified genomic DNA to amplify a 250-300bp region surrounding the predicted gRNA cut site using Taq polymerase.

17 Run the resulting PCR product on an agarose gel to confirm correct size and then purified as described above.

18 Ligate the purified PCR product into the pTOPO-TA sequencing vector following the manufacturers protocol. (https://www.thermofisher.com/order/catalog/product/450071?ICID=search-450071#/450071?ICID=search-450071)

19 Pick enough colonies of the resulting transformation to sequence ~20 clones for each clonal cell line being characterized. Typically, 5-8 cell clones were characterized at a time.

20 Keep only clonal cell lines where every sequence confirmed an indel that would result in a premature stopcodon.

21 Expand the selected clonal cell lines, and aliquots frozen down for safe keeping.

22 Confirm confirmation of protein expression levels consistent with a knock-out by immunoblotting using a specific antibody against the protein of interest compared against polyclonal empty-PX458 cells.

Knock-in mammalian cell lines - Choosing a gRNA for editing endogenous locus of gene of interest

23 Choose gRNA sequences as described above.

24 One important difference is finding a gRNA based on the ideal location for inserting your fluorescent
protein tag.

Knock-in mammalian cell lines - Cloning the pORANGE plasmid for fluorescent protein tag knock-in

25 Clone the gRNA sequence into pORANGE as described above for the gRNA cloning into PX458.

26 Once the gRNA sequence insertion into pORANGE was confirmed by sequencing, linearize the plasmid using HindIII/XhoI.

27 Purify linearized plasmid as described above.

28 Ligate the fluorescent protein DNA amplified by PCR (typically mNeonGreen or mScarlet-i) with flanking
reverse complement gRNA + PAM sequences, Gly-Ser linkers, and corresponding cut sites into the linearized pORANGE plasmid using standing cloning techniques.

Knock-in mammalian cell lines - Transfection of pORANGE into HeLa cells

2d 8h

29 Plate 5.0x10^4 wild-type HeLa cells in to one well of a 6-well plate.

30 Replace 24:00:00 later, the complete media with media without antibiotics. 1d

31 Transfect cells with 500 ng of the pORANGE plasmid using FugeneHD (Promega) following the manufacturers protocol.

32 Incubate cells with the transfection reagent for 08:00:00 - 24:00:00 . 1d

33 Exchange the media after transfection with complete media and cells were allowed to grow to near confluency (2-3 days) before being split 1:5 onto another well of a 6-well plate.

34 A key component of the knock-in process is to wait at least five days before continuing on as the
genomic editing is a slow process.

Knock-in mammalian cell lines - Identification, cloning, and verification of clonal knock-in HeLa cell lines.

35 Split HeLa cells after five days post-transfection and plate ~1.0x10^4 cells on a 35mm glass bottom dish (Mattek).

36 Cells were allowed to grow to near confluency on the dish (~3-4 days).

37 Image the dish using a confocal microscope to identify cells positive for the fluorescent marker.

38 The reason behind plating the glass bottom dish so low initially is to allow colonies to grow, which

makes finding cells that are positive for the fluorescent marker easier.

39 If there are cells positively identified, then split the cells on the dish and serially dilute for subcloning.

40 Subcloning begins with plating 10 cells per well in a 96-well glass bottom plate.

41 Allow cells in the well to grow to ~50-60% confluency before imaging of wells using a confocal microscope.

42 Split wells with positively identified cells—for the fluorescent marker— to a well of a 24-well plate and allowed to expand.

43 Split the cells once the 24-well is near confluency, serially dilute, and plate at one cell per well of a 96-well glass bottom plate.

44 Allow again, cell colonies to expand until they are easily visualized by a brightfield microscope.

45 Image colony using a confocal microscope to identify the fluorescent protein marker.

46 Split clones positively identified for the fluorescent protein marker to a new 24-well and allow to expand.

47 Split colonies once the well is nearly confluent, and some will be used for purifying the genomic DNA as described above.

48 Use primers directed against the region of the genome surrounding the site of endogenous editing to amplify by PCR this area and run on an agarose gel as described above.

49 Purify the DNA based on the size of the PCR band and ligate into TOPO-TA sequencing vector.

50 Keep only clonal cell lines where every sequence confirmed the correct knock-in sequence.

51 Expand the selected clonal cell lines and aliquots frozen down for safe keeping.

52 Confirm confirmation of protein expression levels consistent with a knock-in by immunoblotting using a specific antibody against the protein of interest and fluorescent protein tag compared against polyclonal empty-pORANGE cells.