Jan 31, 2025

JAX_DPC: Protocol for generating PTC+1 knockout alleles in iPSCs (KOLF2.2J)

DOI
https://dx.doi.org/10.17504/protocols.io.5qpvo92dxv4o/v1

Enrica Pellegrino[1], Justin McDonough[1], William C. Skarnes[1], Justin McDonough[1]

[1]The Jackson Laboratory

MorPhiC Consortium

Enrica Pellegrino
The Jackson Laboratory

OPEN ACCESS

DOI: https://dx.doi.org/10.17504/protocols.io.5qpvo92dxv4o/v1

Protocol Citation: Enrica Pellegrino, Justin McDonough, William C. Skarnes, Justin McDonough 2025. JAX_DPC: Protocol for generating PTC+1 knockout alleles in iPSCs (KOLF2.2J). **protocols.io**
https://dx.doi.org/10.17504/protocols.io.5qpvo92dxv4o/v1

Protocol status: Working
We use this protocol and it's working

Created: January 24, 2025

Last Modified: January 31, 2025

Protocol Integer ID: 119040

Keywords: targeted gene knockout, gene knockout, precise genome engineering, copy of the target gene, target gene, recombinant cas9 protein, synthetic oligonucleotide, single guide rna, gene function, specific gene, gene, rna, induced pluripotent stem cell, pluripotent stem cell, degenerate base insertion, specific gene of interest, transcript isoform, premature termination codon, protocol for the generation

Funders Acknowledgements:
JAX MorPhiC DPC
Grant ID: 1 UM1 HG012651-01

Abstract

The ‘PTC+1’ knockout strategy offers a robust and efficient approach to eliminate gene function in human induced pluripotent stem cells (iPSCs). The method utilizes precise genome engineering to introduce a premature termination codon (PTC) and a degenerate base insertion (+1) within an early coding exon that is shared across all transcript isoforms. Here, we describe the protocol for the generation of ‘PTC+1’ alleles in KOLF2.2J iPSCs to achieve a targeted gene knockout. Using nucleofection, we co-deliver a synthetic oligonucleotide harboring the PTC+1 mutation along with recombinant Cas9 protein and a single guide RNA (sgRNA) designed to target the specific gene of interest. This protocol ensures efficient editing and generation of loss-of-function alleles in both copies of the gene. The degenerate base allows us to rule out on-target effects such as loss of one copy of the target gene and to identify independent, non-sibling clones.

Troubleshooting

Reagent list

1

	A	B	C
		Vendor	**Catalog #**
	DPBS, no calcium, no magnesium (Gibco)	ThermoFisher Scientific	14190-094
	Vitronectin (VTN-N) Recombinant Human Protein	ThermoFisher Scientific	A14700
	StemFlex Medium (Gibco)	ThermoFisher Scientific	A33494-01
	CloneR2	STEMCELL Technologies	100-0691
	Accutase	STEMCELL Technologies	07920
	P3 Primary Cell 4D-Nucleofector X Kit S	Lonza	V4XP-3032
	Synthetic, chemically modified sgRNA (in 15 ul TE)	Synthego	N/A
	Alt-R S.p. Cas9 Nuclease V3	Integrated DNA Technologies	1081058
	Alt-R HDR Enhancer V2	Integrated DNA Technologies	10007921
	Alt-R HDR Donor Oligo, 2 nmol (in 10ul TE)	Integrated DNA Technologies	N/A
	Cell Lifter (Corning)	Fisher Scientific	C3008
	5 ml capped polypropylene tubes	Globe Scientific	6101S

	A	B	C
	TipOne filter P200 tips (P200)	USA Scientific	1120-8810
	Synthemax II_SC substrate	Corning	3535
	ReLeSR	Stem Cell Technologies	05872

Pre-nucleofection preparation

2 **1.** Resuspend the synthetic, modified sgRNAs and epegRNAs overnight at 4^{o}C in TE buffer at a concentration of 4 mg/ul. Check the concentration by Nanodrop of a 1:5 dilution.

2. Resuspend the Alt-R HDR Donor oligo (modified ssODN) at room temperature in TE buffer at a concentration of 200 pmol/ul.

Note: Guides, oligos, should be kept at -80 ^{o}C for long- term storage.

Before nucleofection, the iPSC cells should be passaged with ReLeSR to Synthemax-coated wells of a 6W plate at least once after thawing.

N.B.Preparation of 40X Synthemax stock: Dissolve 10 mg Synthemax in 10 ml $H_2$0. Store at 4^{o}C for up to 2 months.

Recomendation: We recommend a 1:10 dilution for the first passage after thawing and a 1:15 dilution thereafter. Change media at least 2 hours prior to nucleofection. One well of a confluent 6W plate contains approximately 2-3 million cells.

Note: Cells grown on Synthemax substrate do not detach from surface following Accutase treatment!

3. For each 20ul **16W-strip nucleofection**, prepare 1 ml StemFlex media + CloneR2 + HDRe v2 (IDT, 1 uM final and coat one well of a 24W plate with 0.5 ml vitronectin (1X in DPBS) for 2 hours at 37^{o}C.

Preparation of recovery media

5ml	4.5ml StemFlex	0.5ml CloneR2	7.2ul HDRe v2 (stock 690 uM)	

4. Aspirate vitronectin and add 100 ul of StemFlex media containing CloneR2+ HDRe to each well
and return to the incubator. Pre-warm Accutase in a 37^{O}C waterbath.

2.1 **P3 Mix preparation**
For a well in a 16W strip (160,000 cells, 20ul volume)
- Cold shock + HDRe + bridging oligo (20ul, 16W strip)
18 ul P3 buffer
4ul Supplement
0.4 ul WT Cas9 (4ug)
0.8 ul sgRNA (3.2ug)
-resuspend 160,000 cells in 20ul P3 mix
-culture @ 32^{O}C in SF+cR2+HDRe media (see section "Nucleofection of cells", Step 3)

Nucleofection

3 **16W strip (200,000 cells, 20ul volume)**

1. Switch on the nucleofector unit and select 'X unit' and single cuvette. Use the 'Primary Cell P3' program and set the pulse code to 'CA-137'.

2. Wash the cells once with room temperature D-PBS.

3. Add 1.5ml of pre-warmed Accutase to each well of a 6 well plate and incubate in a 37^{O}C/5% CO_2 incubator for 7 minutes.

Note: At 80% confluence, each well of a 6W plate contains 2 - 3 × 10^6 cells, enough for 2-3 single cuvette or 8-10 16W strip nucleofections.

4. Aspirate the Accutase solution (cells plated on Synthemax will not detach) and add 3ml StemFlex + CloneR2 media.

5. Using a cell lifter, scrape the cells from the surface of the plate. Triturate twice using a 5 ml pipette and count the cell suspension in a haemocytometer.

6. Pellet 2×10^5 cells at 300 x g for 5 minutes in well of a V-bottom plate. With a 200ul pipetteman, remove the media, taking care not to disturb the cell pellet.

7. While the cells are spinning, remove the 24W plate containing StemFlex + CloneR2 + HDRe media from the incubator and place in hood.

8. Using a p20 pipetman, gently resuspend the cell pellet in 20ml P3 mix by trituration 2-3 times and transfer the cell suspension to one well of the 16W strip. Tap the cuvette on a flat surface to eliminate bubbles and nucleofect immediately using the 'Primary Cell P3' program and 'CA-137' pulse code.

9. With a p200 pipetteman, add 100ul of media from the 24W plate and transfer the cells from the nucleocuvette into one well of a vitronectin-coated 24W plate containing 1 ml StemFlex + CloneR2 + HDRe media. Wash the cuvette once with 100 ul media to collect the remaining cells.

10. Culture the cells in a 32^{O}C/5% CO_2 incubator (cold shock for two days).

12. Replace the media the next day with StemFlex + CloneR2 *without* HDR enhancer. Change the media to StemFlex on Day 3, returning the plate to the 37^{O}C/5% CO2 incubator and changing the media every two days thereafter. When 60-80% confluent, cells are passed with Accutase for single cell cloning and for freezing the cell pool.

Single cell cloning

4 Prepare a vitronectin-coated 10 cm dish with 6 ml of 1X vitronectin (in PBS) and incubate for 2hr at 37^{O}C. Pre-warm 2 ml of Accutase in 37^{O}C bead bath. Add 70 µl Revitacell (100X stock solution) to 7 ml StemFlex media and warm to room temperature. Transfer 3.9 ml of StemFlex + Revitacell to a 5ml tube labeled '1:40 dilution'. Prepare 9 ml StemFlex + 1ml CloneR2 and transfer to the vitronectin-coated 10cm dish.

1. Wash the cells once with 3 ml of D-PBS then add 2 ml of pre-warmed Accutase.

2. Incubate the 6W plate in a 37^{O}C/5% CO2 incubator for 20 minutes.

3. Aspirate the Accutase solution (the cells should remain attached) and add 3 ml StemFlex + Revitacell medium.

4. Using a cell lifter, scrape the cells from the surface of the plate. Triturate twice using a 5 ml pipette
and immediately transfer 0.1 ml of the cell suspension to the tube containing 3.9 ml StemFlex +
Revitacell (1:40 dilution).

5. Count the undiluted cell suspension in a haemocytometer.

6. Mix the 1:40 diluted sample well by pipetting and transfer 500 single cells to the vitronectin-coated 10cm dish containing 10 ml SF + CloneR2.

7. Place cells in a 37^{o}C/5% CO2 incubator.

8. After 2 days, replace the media with StemFlex without CloneR2. Change the media every 2 days
thereafter until colonies are approximately 1 mM in diameter, usually about 9-10 days after plating
single cells.

Picking single cell-derived colonies

5 With the aid of a dissecting microscope and using a p200 pipetman, individual colonies are picked and transferred into replicate vitronectin-coated 96W plates, one for freezing single cells and one for DNA lysis and genotyping. Vitronectin is used in place of Synthemax to permit the freezing of single cells from 96W plates without having to scrape each well. To avoid recovering clones that could be formed by more than one cell, do not pick the largest colonies on the dish. Select the perfectly round colonies of intermediate size.

1. For each set of 96 colonies to be picked, prepare 12 ml of D-PBS + 120 µl vitronectin (100X). Transfer to a reservoir and with a multichannel pipettor add 50 µl per well of two 96W flat-bottom plates. Place in a 37^{o}C/5% CO2 incubator for 2 hours.

Note: Vitronectin-treated plates can be prepared one day in advance, wrapped in plastic film and stored at 4^{o}C. Do not let the well dry.

2. For each set of 96 colonies, prepare 15 ml of StemFlex medium containing 1X Revitacell supplement (150 µl of 100X stock) and warm to room temperature in the hood.

3. Use the p200 tip to break apart and dislodge the colony from the plate and collect the cell fragments
in a volume of 50 µl of media. Transfer each colony to a well of a U-bottom 96W plate.

4. Once 96 colonies are picked, add 15 ml of StemFlex + Revitacell media to a reservoir and transfer 150µl to each well of the U-bottom plate with a multichannel pipettor.

5. With a multichannel pipettor set to 100 µl, triturate the cells extensively (10-20 times) in the U-bottom plate. Transfer 100 µl of the cell suspension to corresponding rows of the duplicate vitronectin-coated 96W flat bottom plates. Repeat the process row by row

(or column by column) changing tips between each row (column) until the plate is filled, each well containing 100 µl of the cell suspension. Check under the microscope to confirm that the cells have been broken into small clumps and are evenly divided between the two replicate wells.

6. After 18-24 hours, add 100 µl of StemFlex media (without Revitacell supplement).

7. Change the StemFlex media every two days until the wells reach confluence.

Freezing clones in 96W format

6 Accutase is used to dissociate the cells in the vitronectin-coated 96W replicate plate. After addition of freezing media, the cells are transferred to a 96W Matrix plate containing individual 0.5 ml cryovials, frozen overnight at -80^{o}C, then transferred to liquid nitrogen for long-term storage.

1. Pre-warm Accutase in a 37^{o}C bead bath for 15 minutes. Prepare 20 ml of cold freezing media (KnockOut Serum Replacement + 11.5% DMSO) by adding 2.6 ml DMSO (Sigma-Aldrich, D2650) to 20 ml of KnockOut Serum Replacement (ThermoFisher, 10828028), mixing well, sterilize with a 0.2 micron syringe filter and store at 4^{o}C.

2. Aspirate the media from the wells of the vitronectin-coated 96W plate and wash the cells once with 150µl room temperature D-PBS. Remove D-PBS with a multichannel pipettor set at 175 ul and change tips between each row.

3. With a multichannel pipettor, add 25 µl of Accutase to each well and incubate in the 37^{o}C/5% CO2
incubator for 7-10 minutes. Periodically, tap the plate gently and check under the microscope that all of
the cells have detached.

4. Once the cells have detached, add 175 µl of freezing media to each well containing 25 µl Accutase. Triturate one time and then transfer the 200 µl single-cell suspension to a 96W Matrix plate.

5. Overlay each vial with 100 ml filter-sterilized mineral oil.

6. Place the Matrix plate in a -80^{o}C freezer overnight then transfer the plate to liquid nitrogen for long-term storage.

Thawing and expansion of edited clones

7 After selecting the edited clones by genotyping, thaw and expand the edited clones. One Matrix cryovial contains sufficient numbers of cells for plating onto one well of a vitronectin-treated well of a 24-W plate. For optimal thawing, residual Accutase and DMSO must be removed prior to plating. Cells are pelleted in V-bottom 96W plates and washed once with media.

1. For every clone to be thawed, treat one well of a 24W plate with 0.5 ml vitronectin (1X) in a 37^{O}C/5%CO2 incubator for at least 2 hours. Pre-warm 1 ml of StemFlex media containing CloneR2 to room temperature and add it to one well of the vitronectin-coated 24W plate.

2. Remove the 96W Matrix 0.5 ml cryovial from the liquid nitrogen tank and place into a row of an empty Matrix box.

3. Allow cells to partially thaw in a 37^{O}C/5%CO2 incubator, continue thawing in the hood until complete.

4. Once thawed, using a multichannel pipettor, remove 0.16 ml of the cell suspension under the oil layer and transfer to one well of a V-bottom 96W plate. Pellet the cells by low speed centrifugation (300x g for 5 minutes) and carefully remove the freezing medium.

5. Resuspend cell pellets in 0.16 ml of StemFlex media and pellet the cells again by low speed centrifugation (300 x g for 5 minutes) and carefully remove the medium.

6. With a p200 pipettor, remove 150 µl of StemFlex media + CloneR2 from the 24W plate. Gently resuspend the cell pellet in the 96W V-bottom plate and then return the cell suspension to the well of the 24W plate. Mix the cells by swirling the plate and check for even plating under a microscope.

7. On day 2, change the media to StemFlex without CloneR2 and every 2 days after until cells reach 80%
confluence.

Freezing cells

8 Once cells are confluent:

1. Wash the cells once with room temperature D-PBS.

2. Add 1.5ml of pre-warmed Accutase to each well of a 6 well plate and incubate in a 37^{O}C/5% CO2 incubator for 7 minutes.

Note: At 80% confluence, each well of a 6W plate contains 2 - 3 × 10^6 cells.

3. Aspirate the Accutase solution (cells plated on Synthemax will not detach) and add 3ml StemFlex + CloneR2 media.

4. Using a cell lifter, scrape the cells from the surface of the plate. Triturate twice using a 5 ml pipette.

5. Pellet cells at 300 x g for 5 minutes and aspirate the media, taking care not to disturb the cell pellet.

6. Remove media and add 2.5 ml filter-sterilized 10%DMSO/KOSR freezing media.

7. Transfer 0.5 ml to 5 cryotubes and place in -80^{O}C freezer.

Thawing cells

9 Prepare two vitronectin-coated wells of a 6W plate. Add 120 ul Revitacell (100X) to 12 ml of StemFlex media and warm to room temperature.

1. Remove cryotubes from -80^{O}C freezer and thaw quickly in your hand.

2. Transfer 0.5 ml of cells to a 5 ml tube containing 2.5 ml StemFlex + Revitacell

3. Spin cells at 300xg for 3 minutes. While spinning, add 3 ml of StemFlex + Revitacell to two vitronectin-coated wells of a 6W plate.

4. Aspirate media, taking care not to disturb cell pellet.

5. With a p1000, resuspend cell pellet in 0.5 ml media from the destination well and return the cells to
the well. Mix cells in well by swirling back and forth, then side-to-side for even plating.