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SOP002 Human pluripotent stem cell HDR-mediated gene editing using CRISPR/Cas9 and PiggyBac technology

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ABSTRACT

The piggyBac transposon system can be combined with CRISPR/Cas9 to efficiently perform genetic manipulations in animal models or cells lines, including stem cells, without leaving behind any accessory DNA sequences like for example it happens in the Cre system. Simply put, the piggyBac transposon (e.g. our in-house pMV vector or Transposagen commercially available vectors) containing Puro positive and Thymidine kinase negative selection markers is included in a standard homology directed repair template to facilitate the selection of cells containing your desired edit. The selection cassette is then seamlessly removed using a piggyBac excision only transposase (e.g. pBX). To achieve this type of gene editing a CRISPR/Cas9 + specific gRNA plasmid is transfected into your cells of interest along the piggyBac transposon containing your repair template (e.g. mutation you want to insert or correct) and selection cassette. After the Cas9 nuclease + gRNA generates a site-specific DNA cleavage, the piggyBac donor is used by the cell's host machinery for DNA repair by homologous recombination, which results in the incorporation of your specific edit into the host genome together with the selection cassette (Figure 1). You can now select your gene edited cells with puromycin so you don't have to screen hundreds of clones (like it occurs with other methods). Once you isolate gene edited stem cell clones that carry out your edit of interest you can remove the selection cassette by transfecting your cells with an excision-only piggyBac transposase (e.g. PBx). The removal of the cassette by pBX is not 100% efficient. It is likely some of your cells will still carry the cassette. To remove all traces of cells containing the cassette you can take advantage of the thymidine kinase negative selection marker. Add Ganciclovir to your cells post pBX transfection and recovery to kill any remaining cells that still carry the cassette. The complete and detailed protocol below leads to Footprint-Free precise genome editing of PSCs.

ATTACHMENTS

[dh4ebiq7.pdf](#)

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PROTOCOL CITATION

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KEYWORDS

SOP002, Human pluripotent stem cells, HDR-mediated gene editing, CRISPR/Cas9 technology, PiggyBac technology

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GUIDELINES

Construct Design:

PiggyBac Vector:

The PiggyBac multivector I suggest using is commercially available from Transposagen (see materials). This vector contains a PGK promoter driving the expression of Puromycin and Thymidine Kinase (i.e. +/- selection markers) followed by a SV40 polyA. The piggyBac vector absolutely requires "TTAA" sequences flanking the inverted terminal repeats (ITRs) to work (these are already present in this vector, see Figure 2). To knock in the +/- selection cassette of this construct (along with your edit), in your loci of interest you must clone left and right homology arms right next to the TTAA sites that flank each PiggyBac ITRs. You can use your left or right homology arm to carry your edit of interest. This vector contains unique PacI and BsiW1 sites to clone your left homology arm in and NsiI, HpaI and AscI sites to clone your right homology arm (you can use any of these). For small edits (e.g. 1bp to 100bp changes) I would recommend homology arms of 500bp, these have been shown to work best. Since the homology arms are short, I would suggest synthesizing them and add homology regions that would allow cloning right next to the TTAA sites using Gibson assembly (NEB) or In-Fusion (Clontech). Once the PiggyBac is knocked-in, you can remove it from the genome using an excision only transposase (e.g. PbX vector) which will recombine the ITRs+TTAA sites and excise the whole PGKPuro-2a-TK-SV40polyA cassette, leaving behind the edit you inserted in your homology arm.

Cas9 Vector:

Use a Cas9 vector that carries a fluorophore reporter, pX458 from Feng Zhang works well and a detailed protocol to synthesize and clone your gRNAs into this vector can be found at the link below. In terms of designing the gRNAs I find the Wellcome Trust Sanger Institute site has the most comprehensive tools for gRNA design. The link to access their website is also below.

HDR-mediated gene editing of pluripotent stem cells:

For this method you will need 6 million PSCs per desired gene editing, this is about three 80% confluent PSC wells (6-well plate). Nucleofections are carried out per 2 million PSCs, I split the 6 million PSCs into 3 samples of 2 million each, one is the negative control and the other two are duplicate of the same gene editing (this duplicate effort ensures enough gene edited colonies are generated).

MATERIALS TEXT

Materials:

[StemFlex Medium](#) **Thermo Fisher**

Scientific Catalog #A3349401

[BIOLAMININ 521 LN](#)

(LN521) **Biolamina Catalog #LN521**

[Lonza Nucleofector](#)

4d Lonza Catalog #AAF-1002X

[P3 Primary Cell 4D-NucleofectorTM X Kit](#)

L Lonza Catalog #V4XP-3024

[Y-27632 \(Dihydrochloride\) 1 mg](#) **Stemcell**

Technologies Catalog #72302

[CloneR™](#) **Stem Cell**

Technologies Catalog #05888

[GenCrispr NLS-Cas9-EGFP](#)

Nuclease Genscript Catalog #Z03393

[pSpCas9\(BB\)-2A-GFP](#)

(PX458) **addgene Catalog #48138**

[Excision only piggyBac Transposase expression vector](#) **System**

Biosciences Catalog #PB220PA-1

[Accutase cell dissociation reagent](#) **Gibco - Thermo**

Fisher Catalog #A1110501

[STEM-CELLBANKER - GMP](#)

Grade amsbio Catalog #11890

A	B	C
Description	Manufacturer	Catalogue No.
StemFlex	Thermofisher	A3349401
Laminin-521	Biolamina	LN521-25
Amaya4D	Lonza	AAF-1002X
Amaya P3 Primary Cell4D-Nucleofector x Kit L	Lonza	V4XP-3024
Rock Inhibitor Y-27632	Stem Cell Technologies	72302
CloneR	Stem Cell Technologies	05888
Cas9:GFP protein	Genscript	Z03393
pX458 Cas9GFPplasmid	Addgene	48138
pMV PiggyBac plasmid	In-house	-20C Plasmid Box
Gene editing PiggyBac	Transposagen	Catalog #: SGK-005
pBX excision onlyTransposase	System Biosciences	PB220PA-1
Synthetic sgRNA	Synthego	Special request
Acutase	Thermofisher	A1110501
pMV PiggyBac	In-house plasmid	NA
StemCellBanker	Clontech	11890

Plate preparation

1 

Day -1

Coat each well of a 6-well plate with **75 µl** of Laminin-521 (Biolamina **100 µg/ml** stock) diluted in **1 mL** of DPBS+Ca+Mg to make a final coating concentration of **7.5 µg/ml**. Incubate at **4 °C** **Overnight**. Prepare three wells of a 6 well plate per desired gene editing per PSC cell line.

Note: It is important to use the best possible matrix for the HDR mediated CRISPR Cas9 gene editing of PSCs. Use Laminin-521 for this purpose as directed above and, preferably coat plates **Overnight** at **4 °C** to give an optimal coating.

Note: Make sure the PSCs have been cultured on Lam-521 matrix for at least 2 passages. They need to adjust to this matrix. Splitting PSCs from a Matrigel matrix into a Laminin-521 together with the nucleofection and gene editing tools will stress the cells much more than necessary, so avoid it.

Nucleofection Cas9/PiggyBac gene editing toolkit (Cas9 Plasmid DNA/Cas9+gRNA RNP)

30m

2

PSCs are split for gene editing using Acutase (Invitrogen) when they are about 80% confluent and look pluripotent and healthy. Do not use PSCs that are overgrown or with very packed cells in the middle of the colony, this will reduce nucleofection efficiency.

Replace the medium of the PSCs with fresh **1.5 mL** StemFlex + **1 Micromolar (µM)** rock inhibitor at least 1 hour before splitting, this ensures a good recovery of the PSCs following nucleofection.

3 Prepare **350 µl** P3 solution following the Lonza Nucleofector kit instructions (Cat No. V4XP-3024). Split P3 solution into three **100 µl** vials. Take pX458 Cas9 GFP and PiggyBac plasmid (e.g. pMV) out of the freezer and allow to reach **Room temperature**.

RNP Alternative: Order synthetic gRNA from Synthego using their online gRNA design tool (e.g. for SNCA). Order the Cas9:GFP fusion protein from Genscript. Follow Synthego instructions to resuspend gRNA. Gently mix 20 µg of Cas9 protein with 15 µg gRNA per 2 million PSC nucleofection, leave mi8x at room temperature for 20 minutes to form the Cas9 gene editing RNP complex.

Note: Using this new Cas9:GFP fusion protein allows you to see whether the RNP complex has been transfected efficiently into your PSCs. Also, since this is an RNP complex with a half-life of only a few hours, this allows for fast gene editing and reduced off targeting.

4 

Add **1.5 µg** px458 Cas9 GFP plasmid and **1.5 µg** PiggyBac plasmid (e.g. pMV) per **100 µl** P3 reaction. Do this for two out of the three tubes and leave the last tube without plasmid or pMV plasmid only (random plasmid integration control) or px458 GFP with no gRNA (gene editing control). Mix thoroughly and leave at **Room temperature** in the hood.

Note: I usually transfect $1\ \mu\text{g}$ of plasmid DNA/million iPS cells so adjust plasmid DNA or number of cells according to this ratio. The minimum I would electroporate is 1 million iPS cells, the less iPS cells you nucleofect the less gene edited clones you will get.

RNP Alternative: Mix the RNP complex now formed with $100\ \mu\text{l}$ of the P3 reaction per sample (i.e. 2 million PSCs), add $1.5\ \mu\text{g}$ PiggyBac plasmid (e.g. pMV) in the same way as done for the DNA plasmid gene editing protocol. From here on the RNP protocol and DNA protocol are exactly the same.



10m

Remove medium from wells. Wash once with 1X DPBS and then add $1\ \text{mL}$ Acutase per well. Incubate at $37\ ^\circ\text{C}$ for $00:10:00$ if cells have been grown on Laminin-521.

Note: PSCs grown on laminin-521 require longer incubation with Acutase to detach.



While cells are incubating on acutase wash the Lam-521 coated plate that you prepared on step 1 with PBS-Ca-Mg to remove all traces of the PBS+Ca+Mg. Add $2\ \text{mL}$ StemFlex media containing $1\ \mu\text{M}$ Rock inhibitor and put back in $37\ ^\circ\text{C}$ incubator.



Flush cells from the plates surface (See step 5) and then transfer to a $15\ \text{mL}$ falcon (pool all three same cell line wells into the same falcon tube). Wash wells with $2\ \text{mL}$ HES medium each and add to falcon. Pass cells through a $100\ \mu\text{M}$ cell strainer to remove large debris clumps. Count live cells using the Contessa II and add 2 million cells per $15\ \text{mL}$ falcon tube. Do this for three falcons. Spin cells down at $900\ \text{rpm}$. Remove medium and gently resuspend pellet in the $100\ \mu\text{l}$ P3 solution + Cas9 + PiggyBac plasmid (don't forget the negative control too).

8 Transfer each of the three samples into three $100\ \mu\text{l}$ Lonza Nucleofector cuvettes (make sure you don't insert any bubbles). Take samples to the Amaxa 4D nucleofector and nucleofect using program CL113.





Note: I optimized nucleofection for our PSCs and found CL113 to be the most efficient program for plasmid delivery.

RNP Alternative: If you observe little to no GFP expression 24 post RNP delivery and you get no surviving PSC clones following puro selection, you may use program CA137 which I found to be better for RNP complex delivery

(remember you are also delivering the pMV plasmid repair template so there has to be some compromise).

9 

10m


Transfer the nucleofected  **100 µl** samples to the previously prepared Lam-521 coated 6-well plates containing 2 ml StemFlex + Rock inhibitor per well,  **100 µl** of nucleofected sample per well. Wash the cuvette from any remaining cells using StemFlex and add also to the well. Place back in the  **37 °C** incubator and incubate  **Overnight** .

10 24 hours post nucleofection remove rock inhibitor and replace media with fresh StemFlex.


Puromycin selection and picking up clones 30m

11 48 hours post nucleofection select cells with StemFlex + puromycin at  **0.5 µg/ml** + rock inhibitor.

Note: When carrying out HDR-mediated gene corrections there will be a lot of cell death in the plate. Adding rock inhibitor ensures that you will not lose any gene edited cells due low cell density, so it is important to have it during the first 24 hours of Puro selection.

12 72 hours post nucleofection (24 hours post Puro selection) remove rock inhibitor and continue puro selection at  **0.5 µg/ml** .

Note: You may need to keep rock inhibitor for a little longer depending on the survival of your particular cell line.

13 96 hours post nucleofection (48 hours post Puro selection), check cells and keep at  **0.5 µg/ml** puro. At this point some clear clones will be forming in the plate, see Figure 4I. Use the objective marker to mark clonal colonies (Figure 4).

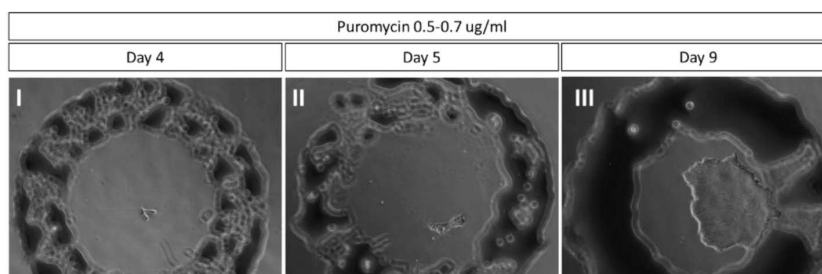


Figure 4: Gene edited and puro selected PSC colonies at different days of selection/proliferation.

- 14 7 days post nucleofection and 5 days post puro selection at **0.5 µg/ml** (See Figure 3), your negative control sample (Cas9 with no gRNA or PiggyBac only controls) should be all dead. At this point you can continue at 0.5 or go ahead and increase the concentration of puromycin to **0.7 µg/ml**. At day 9 puro resistant PSC colonies should look like Figure 4III.

Note: If your negative control is all dead after selection at **0.5 µg/ml** puro you may keep the cells at this puro concentration, increasing puro selection to **0.7 µg/ml** just ensures that the selected cells carry the PiggyBac (i.e. pMV). Do not increase puromycin selection above 0.7 µg/ml, especially for gene corrections of a single allele. Only use 1 µg/ml puro for gene corrections that need both alleles gene edited. If you use too much puromycin you will kill the PSCs that carry the PiggyBac in one allele only.

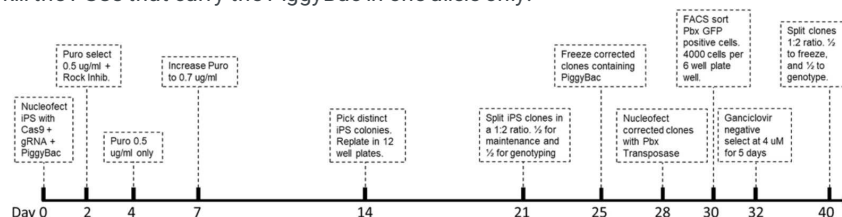


Figure 3: Flow diagram overview of the gene editing stages.

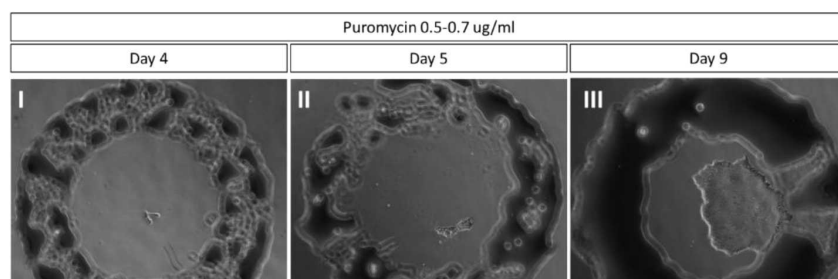


Figure 4: Gene edited and puro selected PSC colonies at different days of selection/proliferation.

15

At around day 14 your puro resistant colonies should be about the same diameter as your objective marker mark. Now it is a good time to pick them and transfer them to a Lam-521 coated 12 well plate. 1 hour before picking colonies, add **1.5 mL** fresh StemFlex media with Rock inhibitor (this reduces cell death upon colony picking). Also add **1 mL** of StemFlex+rock inhibitor to each well of the Lam-521 coated plate.


Note: Do not pick colonies that are smaller than the objective marker mark.

- 16 Use the dissection microscope under the tissue culture hood for the picking. Using a **10 µl** tip scratch the PSC

colony in a squared pattern, making small PSC clumps of cells out of the whole PSC colony. Then suck all the clumps and transfer them to the 12 well plate well. If clumps are too large you may pipette up and down a couple of times but do so very gently as the cells can get stressed and die at this point.

17 

10m

Allow the picked clones to recover  **Overnight** under StemFlex + Rock inhibitor. 24 hours later replace media with fresh StemFlex only.

Genomic DNA extraction and PCRs

30m

18 

48 hours post colony picking and, once the PSC colonies have recovered, add **0.5 µg/ml** - **0.7 µg/ml** puromycin and keep under selection.

19 At around day 21 (see Figure 3), the 12 well plate wells should be around 30% confluent. Split them at 1:2 ratio into a new lam-521 coated 12-well plate. Keep ½ of the cells for genomic extraction using Quickextract reagent (see manufacturer instructions).

Note: You do not need a lot of cells for Quickextract genomic DNA extraction. Pellet the cells at **5000 rpm** for **00:05:00** and resuspend in **50 µl** of Quickextract reagent. This volume tends to give me a gDNA concentration of about **50 Mass Percent** (in my hands).

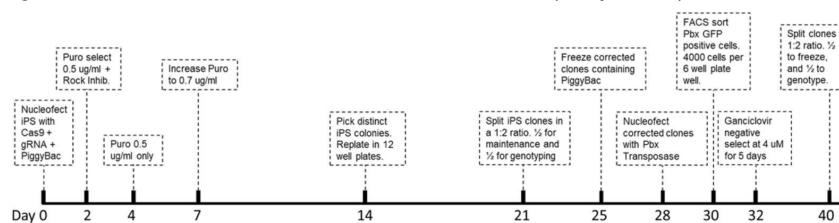


Figure 3: Flow diagram overview of the gene editing stages.

20 Once you have extracted your genomic DNA (gDNA) carry out PCRs on the gDNA to amplify your gene editing region of interest and for the presence of the left and right homology arms of your PiggyBac plasmid (Figure 5A). For example, for SNCA E46K repair, use the NGS primers to PCR the region of interest and primers 11 and 22 for the left homology arm and primers 12 and 23 for the right homology arm. Use Q5 polymerase (NEB) for the NGS PCR and 2xTaq polymerase (NEB) for the homology arm PCRs. **Figure 5Ai** shows a diagram of where the homology arm primers are binding. **Figure 5Bi** shows the presence of left and right homology arm PCR products, indicating the CRISPR/Cas9 HDR mediated knock-in of the PiggyBac cassette containing your repair DNA sequence was successful.

Note: I find it is a good idea to check for the correction/mutation at this point before moving forward. Carrying out the pBX transfection and isolating clones following Ganciclovir treatment can take two to three weeks. Doing this to later find there are no gene edited clones wastes a lot of time. Find if there is correction at this step and then move forward to remove the PiggyBac with only the positively corrected clones.

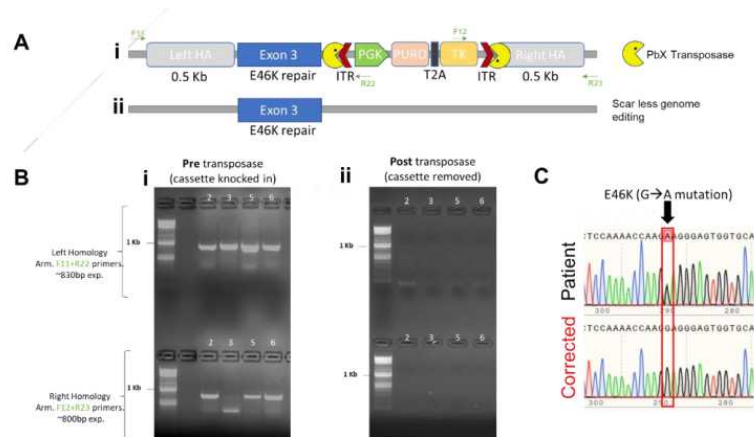


Figure 5. Screening for successfully targeted iPS clones by Junction PCR and targeted sequencing. A) Junction PCR strategy to amplify left and right homology arms (HA) using F1/R1 and F2/R2 primer pairs, these are locus specific and PiggyBac donor plasmid specific. i) SNCA gene edited loci containing the repaired SNCA Exon 3 and the selection cassette. Yellow cartoon denotes the Pbx transposase, an excision only transposase, which is responsible for binding to the PiggyBac inverted terminal repeats (ITR), recombining these ends and excising the cassette from the genomic DNA. ii) Following Pbx plasmid transfection the transposase excises the selection cassette leaving scarless precise genome editing behind. B) Junction PCR products for left and right homology arms of four clones of a E46K iPS cell line. i) Junction PCR for the left homology arm and right homology arm shows PCR product at approximately 830 bp and 800bp respectively, which are the expected molecular weights if the PiggyBac donor plasmid containing the SNCA Exon 3 repair template was knocked in at the correct locus. ii) Junction PCR for left and right homology arms of the same E46K iPS clones post Pbx transposase transfection showing no PCR products, meaning the selection cassette had been excised from the genomic DNA. C) Targeted sequencing using primers flanking the SNCA Exon 3 point mutation (400bp at each side of the mutation). Sequencing shows the parent unmodified patient iPS cell line harboring the G→A heterozygous G/A disease-causing mutation. Following PiggyBac based gene editing of the same patient iPS cell line, the disease-causing mutation is repaired back to a healthy G/G genotype.

- 21 Send the PCR products of the NGS PCR products for sanger sequencing using primer F27. If the sequencing shows correction and left and right homology are present for a particular clone this indicates that there is a good chance that that particular clone is corrected. Discard the clones that show no homology arms or correction.

Nucleofection of excision only transposase (e.g. pBX) to remove PiggyBac selection cassette and leaving behind gene edit correction

30m

- 22 At around day 25 split the gene edited puro resistant clones (Once they are about 80% confluent) and also freeze 1 vial of each. Use **500 µl** StemCell Banker reagent for the freezing, this reagent allows you to store the cells at **-80 °C** for a short time and is more stable than other options. Culture positive clones under Lam-521 and StemFlex conditions and puromycin [**0.5 µg/ml** - [**0.7 µg/ml** until they are 80% confluent.
- 23 At around day 28, nucleofect positive clones with an excision only transposase (e.g. pBX plasmid) to remove the PiggyBac selection cassette and leave only the gene edit of interest behind in a scar-less manner. (Figure 5AII). To do so nucleofect 3 million cells of your iPS clone with 3uf of pBX plasmid using the same directions as steps 2 to 9, in terms of plating, media and process.
- 24 48 hours post pBX transfection of each positive clone, day 30, check for GFP expression under the epifluorescence microscope and sort all GFP sorted cells into a Lam-521 coated plate containing StemFlex and CloneR (sort anything between 500 (ideally) to 4000 live GFP+ve cells per 6-well plate well).

Do not sort more than that or you will not be able to isolate clones from that same plate. Alternatively, you can do single cell sorting.

25 At day 32, if PSC cells have recovered and started forming colonies, you can start Ganciclovir negative selection at **4 Micromolar (μM)** for **120:00:00** . 5d

Note: It is very important that you use a fresh aliquot of GCV and that you replace the StemFlex+GCV media every day, otherwise it will not work. Do not select with GCV for longer than 5 days as longer culture with GCV may cause unnecessary toxicity to the gene edited cells.

26 At day 37 remove GCV from the media and allow cells to recover for 3 days. Pick up to 4 clones per each pBX transfected positive clone and transfer to a Lam-521 coated plate, follow steps 15 to 20. Carry the PCR of the gene edited region of interest using the NGS primers but also primers 14 and 15, this primer set flanks the PiggyBac cassette meaning that if the PiggyBac cassette remains present in that particular clone you will not be able to amplify it because the cassette is still integrated. If the cassette is not there any longer you should get a product that you can Sanger sequence. For Sanger sequencing of the primers 12 and 15 PCR product use F27 and R29 primers.

Note: This second clone isolation step from that initial positive gene edited clonal PSC allows for selecting a clone from the clone pool that does no longer carry the PiggyBac cassette but also ensures that the final isolated footprint free gene edit clone is truly clonal (as it has gone through two rounds of colony picking).

27 The resulting genotyped positive gene edited clones following pBX transfection and GCV selection should show no homology arm PCR products as shown in Figure 5Bii. The final Sanger sequencing result should be very clean and give the same result when sequencing the NGS of primers 14+15 PCR product, chromatogram should look something like Figure 5C.

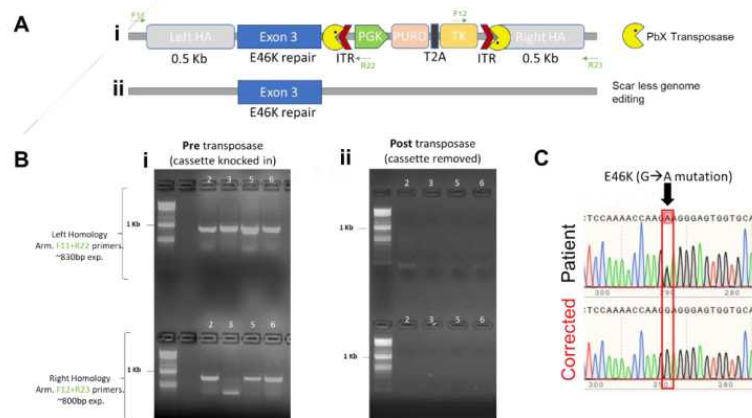


Figure 5. Screening for successfully targeted iPS clones by Junction PCR and targeted sequencing. A) Junction PCR strategy to amplify left and right homology arms (HA) using F1/R1 and F2/R2 primer pairs, these are locus specific and PiggyBac donor plasmid specific. i) SNCA gene edited loci containing the repaired SNCA Exon 3 and the selection cassette. Yellow cartoon denotes the Pbx transposase, an excision only transposase, which is responsible for binding to the PiggyBac inverted terminal repeats (ITR), recombining these ends and excising the cassette from the genomic DNA. ii) Following Pbx plasmid transfection the transposase excises the selection cassette leaving scarless precise genome editing behind. B) Junction PCR products for left and right homology arms of four clones of a E46K iPS cell line. i) Junction PCR for the left homology arm and right homology arm shows PCR product at approximately 830 bp and 800bp respectively, which are the expected molecular weights if the PiggyBac donor plasmid containing the SNCA Exon 3 repair template was knocked in at the correct locus. ii) Junction PCR for left and right homology arms of the same E46K iPS clones post Pbx transposase transfection showing no PCR products, meaning the selection cassette had been excised from the genomic DNA. C) Targeted sequencing using primers flanking the SNCA Exon 3 point mutation (400bp at each side of the mutation). Sequencing shows the parent unmodified patient iPS cell line harboring the G□A heterozygous G/A disease-causing mutation. Following PiggyBac based gene editing of the same patient iPS cell line, the disease-causing mutation is repaired back to a healthy G/G genotype.

