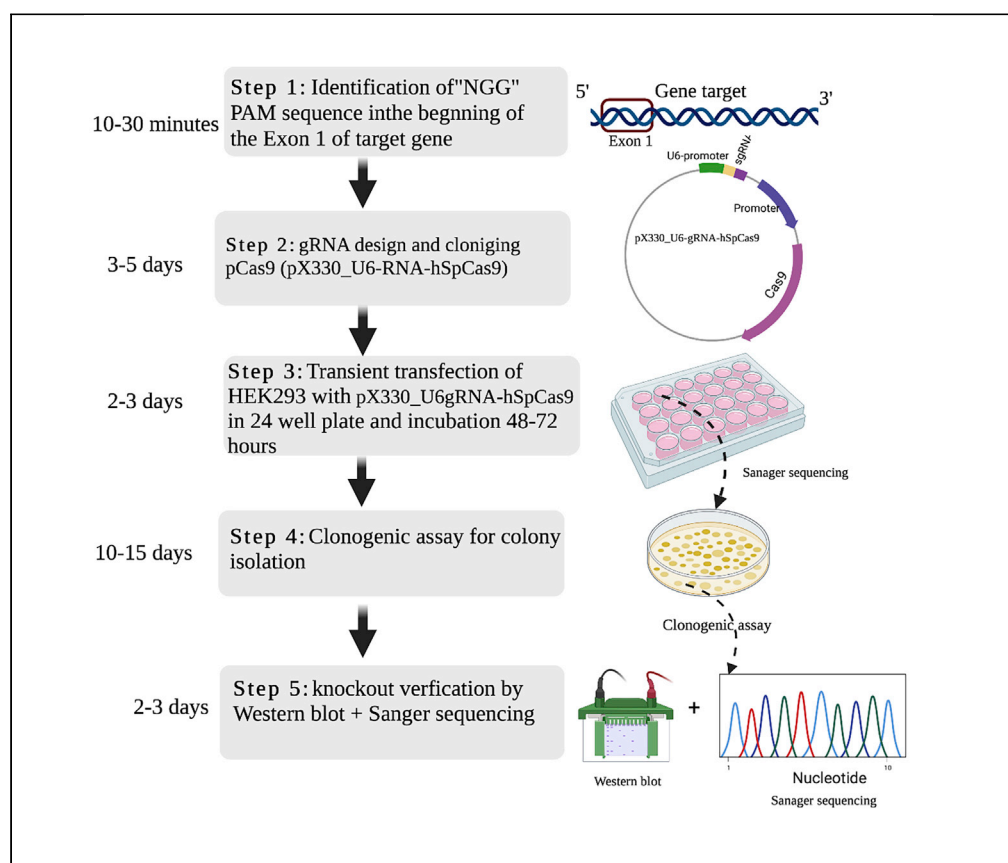


## Protocol

# A simple protocol to isolate a single human cell PRDX1 knockout generated by CRISPR-Cas9 system



Here, we describe a protocol for human PRDX1 gene knockout cells using the CRISPR-Cas9 system. The protocol describes all the steps sequentially: (1) single-guide RNA design, cloning, and transfection; (2) gene editing evaluation by T7EI assay; (3) single-cell isolation; and (4) knockout verification to determine indels in one or both alleles by Sanger sequencing. This strategy is based on the efficiency of DNA editing, avoids antibiotic selection, and bypasses the need for cell sorting.

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### Highlights

Protocol to isolate  
single human cells  
with gene deletion

Step-by-step guide to  
create knockout  
bypassing antibiotic  
selection and cell  
sorting

Gene deletion  
efficiency based on  
T7EI assay

Gene deletion  
confirmation by  
immunoblot assay  
and Sanger  
sequencing

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## Protocol

## A simple protocol to isolate a single human cell PRDX1 knockout generated by CRISPR-Cas9 system

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## SUMMARY

Here, we describe a protocol for human PRDX1 gene knockout cells using the CRISPR-Cas9 system. The protocol describes all the steps sequentially: (1) single-guide RNA design, cloning, and transfection; (2) gene editing evaluation by T7EI assay; (3) single-cell isolation; and (4) knockout verification to determine indels in one or both alleles by Sanger sequencing. This strategy is based on the efficiency of DNA editing, avoids antibiotic selection, and bypasses the need for cell sorting.

## BEFORE YOU BEGIN

The protocol below describes the specific steps for the knockout of PRDX1 gene in HEK293 cells. This gene encodes Peroxiredoxin 1, a protein member of the peroxiredoxin family of antioxidant enzymes, which decomposes hydrogen peroxide and functions as chaperone. However, it can be applied for any gene and any mammalian cells to deliver the CRISPR components and generate a stable knock-out cell line. Obtain the genomic DNA sequence of the gene of interest, for example, we extracted PRDX1 from GeneCards. From the PRDX1 DNA sequence, identify the Protospacer Adjacent Motif (PAM) site (NGG) in exon 1. Design primers in exon 1 to monitor the gene expression by RT-PCR to ensure that the gene is expressed in the HEK293 cells (Figure 1A). If the gene is expressed, design three guide RNAs to target exon 1.

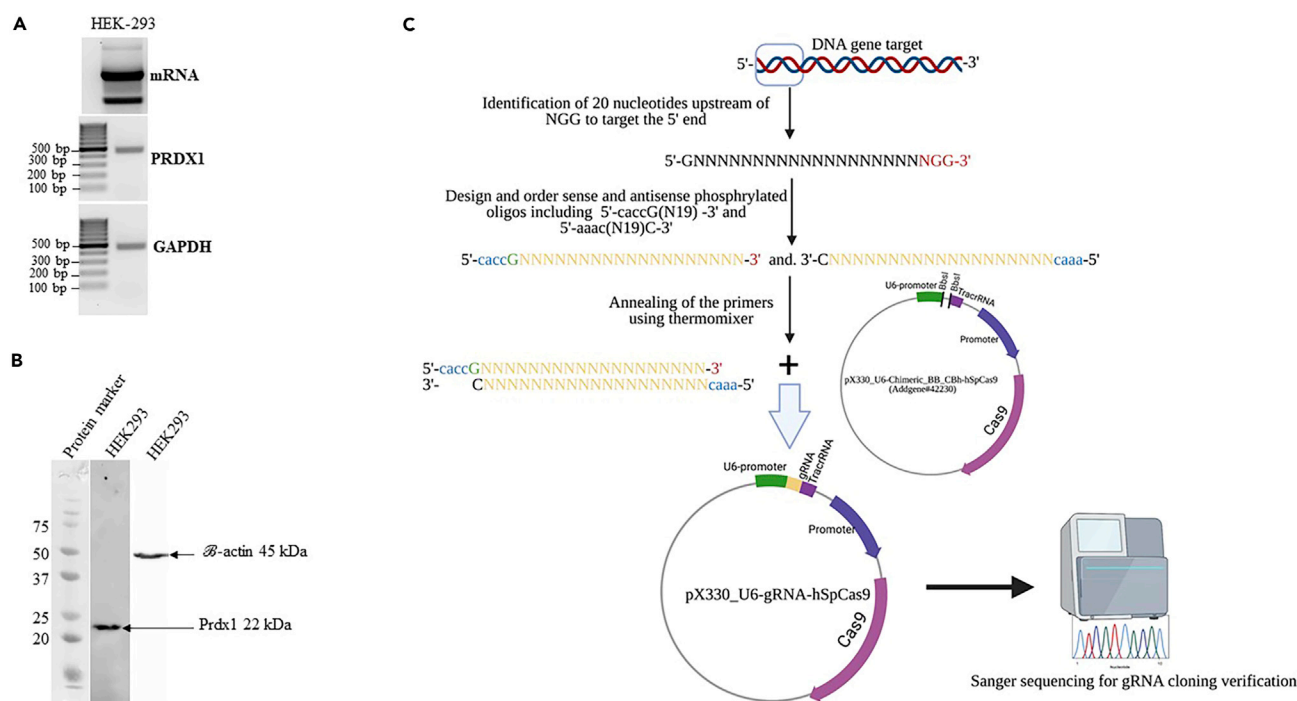
## gRNA, primers design, and antibody

⌚ Timing: 40 min

1. Design of sgRNA (30 mins)
  - a. Use NCBI or GeneCards to extract the gene sequence of interest and import it to SnapGene. In this protocol, we used the PRDX1 gene sequence ([https://www.ncbi.nlm.nih.gov/nucleotide/NC\\_000001.11?from=45511051&to=45522890&report=fasta&strand=true](https://www.ncbi.nlm.nih.gov/nucleotide/NC_000001.11?from=45511051&to=45522890&report=fasta&strand=true)) and for the coding sequence (Figure S1).
  - b. Identify exon 1.

**Note:** It is preferred to choose an early exon (not an intron), i.e., the first translated exon. Introducing the deletion in an early exon by the Non-Homologous End Joining (NHEJ) repair pathway will result in a frame shift mutation leading to a premature stop codon. Thus, no protein will be translated or result in a non-functional truncated protein.





**Figure 1. Workflow of designing and cloning of the gRNA into pX330\_U6-chimeric\_BB\_CbH-hspCas9 (Addgene#42230)**

(A) RT-PCR to check the PRDX1 expression in HEK293.

(B) Western blot to confirm the expression of Prdx1.

(C) workflow to follow to design and clone the gRNA in pCas9 plasmid. The final clone carrying the gRNA and the Cas9 will be sequenced by Sanger sequencing before transfection (Created with [BioRender.com](#)).

- Find the PAM sequence in the sense or antisense strand (at least 15 nucleotides downstream of the start codon in the 5' to 3' direction). It is recommended to find at least three PAM sequences in exon 1.
- Design manually the guide RNA at each PAM site. This is done by copying 5'-G-19N-NGG-3' that are upstream of the PAM site (Figure 1C).
- Each guide RNA is designed and synthesized as a pair of single-strand and reverse oligonucleotides harboring BbsI nucleotides overhang for cloning the fragment (Figure 1C).
- Order primers from any suitable company.
- Resuspend each primer in DNase/RNases free water at 100  $\mu$ M and store at  $-20^{\circ}\text{C}$ .

**△ CRITICAL:** Three guides are needed to finally select the best guide that gives the highest gene editing activity.

## 2. Design RT-PCR primers (10 mins)

Design forward and reverse primers for RT-PCR targeting the coding sequence of exon 1. Ideally, the primers should have  $T_m$  of  $60^{\circ}\text{C}$  or above. The primer length  $\sim 20$  nucleotides, and we used SnapGene for designing the primers. Relative quantification of the target gene expression can be performed by normalization to housekeeping genes such as GAPDH or ACT1.

## 3. Order Antibodies against the product of the target and housekeeping genes if these are commercially available.

### KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
<b>Antibodies</b>		
Anti- $\beta$ -Actin Antibody (1:2000)	Santa Cruz Biotechnology	Cat# sc-69879
Peroxiredoxin 1 Antibody (1:2000)	Novus Biologicals	Cat# NBP1-82558
Donkey anti- Rabbit IgG (H+L) secondary (1:5000)	Jackson ImmunoResearch	Cat# 711-035-152
<b>Bacterial and virus strains</b>		
Top10 competent bacteria	Thermo Fisher Scientific	Cat# C404010
pX330_U6-chimeric_BB_Cb-hSpCasp	Cong et al. (2013)	Addgene# 42230
<b>Chemicals, media</b>		
LB Broth	Invitrogen	Cat# 12780052
LB Agar		Cat# 22700025
Ampicillin	Sigma-Aldrich	Cat# A1593-25G
Kanamycin		Cat# K1637-1G
Bigdye 3.1 Sanger sequencing kit	Thermo Fisher Scientific	Cat# 4337455
Fugene HD	Promega	Cat# E2312
Acrylamide or pre-casting gel	Sigma-Aldrich	Cat# A3574
DMEM Glutamax	Thermo Fisher Scientific	Cat# 10569010
FBS	Thermo Fisher Scientific	Cat# 10082147
OptiMEM	Thermo Fisher Scientific	Cat# 15140122
Pen/strep	Thermo Fisher Scientific	Cat# 15140122
SYBR Safe DNA gel stain	Invitrogen	Cat# S33102
Agarose	Invitrogen	Cat# 16500-500
DNA loading Dye (6 $\times$ )	New England Biolabs	Cat# B70245
100 bp DNA marker	Thermo Fisher Scientific	Cat# SM0241
<b>Critical commercial assays</b>		
Miniprep plasmid extraction kit	QIAGEN	Cat# 27106
Maxiprep endotoxin free kit	QIAGEN	Cat# 12362
QIAEX Gel extraction Kit	QIAGEN	Cat# 20021
PCR purification Kit	QIAGEN	Cat# 28106
RNeasy mini kit	QIAGEN	Cat# 74034
Topo -TA cloning kit pCR4-TOPO vector	Invitrogen	Cat# 45-0030
Quick Extract	Lucigen	Cat# QE09050
Accuprime Polymerase HF	Thermo Fisher Scientific	Cat# 12346094
T7EI	New England Biolabs	Cat# M0302L
<b>Deposited data</b>		
<b>Supplemental information</b>	Mendeley	<a href="https://doi.org/10.17632/nknd6w2kbm.1">https://doi.org/10.17632/nknd6w2kbm.1</a>
<b>Experimental models: Cell lines</b>		
HEK293 T	ATCC	Cat# CRL-3216
<b>Oligonucleotides</b>		
PRDX1-gRNA1 sequence: gTTCAGGAAATGCTAAAATT PRDX1-gRNA1 sequence: gAGCTGTGGCTTTGAAGTTG PRDX1-gRNA1 sequence: GCCACAGCTGTTATGCCAGA	This study	N/A
Primers sequences for gRNA1, 2 and 3 cloning: PRDX1-gRNA1-For: caccGTTTCAGGAAATGCTAAAATT PRDX1-gRNA1-Rev: aaacAATTTTAGCATTTCTGAAG PRDX1-gRNA2-For: caccGAGCTGTGGCTTTGAAGTTG PRDX1-gRNA2-Rev: aaacCAACTTCAAAGCCACAGCTC PRDX1-gRNA3-For: caccGCCACAGCTGTTATGCCAGA PRDX1-gRNA3-Rev: aaacTCTGGCATAACAGCTGTGGC	Integrated DNA Technologies	N/A
Primers for T7EI assay, Sanger sequencing and RT-PCR: PRDX1-T7EI-F: GTGGCATTGTGCATTGGATTCT PRDX1-T7EI-R: GGTTCCTCCAGAAGTGTTTGGTCC M-13-F: GTAAAACGACGGCCAG M-13-R: CAGGAAACAGCTATGAC RT-PCR-PRDX1-F: ATGCTTCAGGAAATGCTAAAATTGGGC RT-PCR-PRDX1-R: ACTAGTCTCAAAGTCTCATCCACAGAGC gRNA-seq-F: CGATACAAGGCTGTAGAGAGATAATT	Integrated DNA Technologies	N/A

(Continued on next page)

**Continued**

REAGENT or RESOURCE	SOURCE	IDENTIFIER
<b>Other</b>		
<b>Consumables:</b>	N/A	N/A
Sterile Cell Culture/Petri Dishes 100 × 15 mm		
Sterile Cell Culture/Petri Dishes 100 × 15 mm		
Sterile Cell Culture/Petri Dishes for bacteria 150 × 25 mm		
Sterile Flask 75 cm <sup>2</sup>		
1, 5, 10 and 25 mL pipettes		
1, 10, 100 and 1,000 µL tips		
1.5 mL microcentrifuge tubes		
15 and 50 mL conical sterile tubes		
0.2 mL PCR strip tubes		
Cell-Culture Treated Multidishes 6 and 24 well		
Counting slide for cell culture		
Cell culture petri dish		
Bacteria petri dish		
2 mL cryotubes for cell storage		
<b>Laboratory Equipment's:</b>	N/A	N/A
Gel Doc system for DNA and protein detection		
Nanodrop		
Sanger sequencing machine		
PCR machine		
Thermo mixer		
Vortex		
Mini centrifuge		
High speed centrifuge		
Biosafety cabinets		
CO2 incubator		
Shaker 37°C		
Bacterial incubator		
Cell counter		
Pump aspiration system		
All set of pipettes (2.5 µL, 10 µL, 20 µL, 100 µL, 200 µL and 1,000 µL)		

## STEP-BY-STEP METHOD DETAILS

### ⌚ Timing: 1 day for step 1

1. Verification of PRDX1 expression in the target cells by RT-PCR and immuno- blot (Figures 1A and 1B, see section 7 for immunoblot).
2. Isolation of total RNA from HEK293 cells
  - a. Grow cells in complete media (DMEM Glutamax (Thermo Fisher) supplemented with 5% Penicillin streptomycin and 10% FBS).
  - b. Harvest a minimum of  $2 \times 10^6$  cells for RNA extraction. Discard the culture medium. Wash the cells with PBS, and trypsinize with 1 mL of Tryp-LE to cover the surface of the cells. Then stop the trypsin enzymatic activity with an equal volume of complete culture medium.
  - c. Pellet cells using a clinical centrifuge at  $201 \times g$  for 5 mins in Eppendorf centrifuge 5810.
  - d. Discard the supernatant and extract total RNA from the cell pellet using RNeasy mini kit (Qia-gen, catg. no.74104)-as per the supplier protocol.
  - e. Quantify the RNA yield using Nanodrop. Typically, expect 300–700 ng/µL of total RNA with an A260/A280 ratio above 1.8.
  - f. Use 2 µg of total RNA to prepare the cDNA using Agilent cDNA synthesis kit.
  - g. Set up RT-PCR reaction to check for target gene expression. Prepare reactions in triplicates for target gene amplification as well as for housekeeping gene amplification (GADPH or ACT1).
  - h. Set up PCR reaction for amplification of the targeted region using 2 µL of this cDNA to check for the amplification of the target gene, see [key resources table](#) for primer sequences. For amplification, Accuprime high fidelity DNA polymerase was used for amplification (Aouida et al., 2015).
  - i. Check PCR amplification using 1% agarose gel pre-stained with SYBR-SAFE dye

**Note:** If antibodies exist for the target gene product, immune-blot analysis can also be performed.

△ **CRITICAL:** If no commercial antibody is available, knockouts can be verified by Sanger sequencing

### gRNA cloning, sequencing and Maxi-prep-endotoxin free (5 days)

⌚ Timing: 2–3 days for step 4

3. Cloning of the guide RNA in plasmid PX330\_U6-Chimeric\_BB\_CBh-hSpCas9 (Addgene # 42230) (Lee et al., 2017)
  - a. Phosphorylate the complementary oligos for each guide (Forward and Reverse, [key resources table](#)).

**Note:** See reaction set up in 25 µL, [Table 1](#) (Lee et al., 2017).

- b. For annealing the oligos, mix the complementary pair of oligos in a final volume of 50 µL in PCR strip tubes at a final concentration of 140 nM of each oligo.
- c. Set up thermomixer at 95°C, insert PCR tubes, incubate for 10 mins, reset the temperature to 25°C to allow annealing of the oligos. This takes ~ 45 mins.
- d. In parallel digest 10 µg of the PX330\_U6-Chimeric\_BB\_CBh-hSpCas9 (Addgene # 42230) plasmid, a gift from Feng Zhang (Addgene plasmid # 42230 ; <http://n2t.net/addgene:42230> ; RRID:Addgene\_42230) (Cong et al., 2013) with BbsI (10 Units) for 2 hrs. There is no need to dephosphorylate the BbsI ends.
- e. Run the digested plasmid on 1% agarose gel and purify the linear plasmid backbone (QIAEX II gel extraction kit) (backbone is 8,484 bp).
- f. Ligate 50 ng of the purified digest plasmid with 1, 2 or 4 µL of the annealed primers in ligation reaction with T4 ligase in 10 µL final volume reaction as indicated in [Table 2](#) and incubate the ligation reaction at 16°C for 16–18 h.
- g. Next day transform 3 µL of the ligation reaction (keep the rest at –20°C) into *E. coli* highly competent TOP10 bacteria (Invitrogen) following the manufacturer protocol ([https://www.thermofisher.com/document-connect/document-connect.html?url=https%3A%2F%2Fassets.thermofisher.com%2FTFS-Assets%2FLSG%2Fmanuals%2Foneshot10\\_chemcomp\\_man.pdf](https://www.thermofisher.com/document-connect/document-connect.html?url=https%3A%2F%2Fassets.thermofisher.com%2FTFS-Assets%2FLSG%2Fmanuals%2Foneshot10_chemcomp_man.pdf)). Alternatively, use chemically prepared laboratory competent bacteria. Plate all the transformation mix (~150 µl) onto LB agar plates containing ampicillin (100 µg/mL) and incubate 16–18 hrs at 37°C. Check the following day for colony formation.
- h. Pick at least 10 colonies and inoculate in 5 mL of LB medium with ampicillin (100 µg/mL) and grow 16–18 hrs.
- i. Next day extract the plasmid using mini prep kit (Qiagen).

**Note:** store 100 µL of the 16–18 hrs culture for maxi prep with Endotoxin free Qiagen kit.

- j. Positive clones are identified by DNA Sanger sequencing (Saifaldeen et al., 2021) using a pair of forward and reverse primers (see [key resources table](#)).
- k. The 100 µL of stored culture carrying the correct plasmid PX330 with the Cas9 and the specific guide RNA can be subcultured into 100 mL of LB with Amp and used for Maxi prep Endotoxin free (Qiagen) to isolate a sufficient amount of the plasmid expressing the gRNA for transfection.

△ **CRITICAL:** It is important to test different gRNAs and use the best gRNA with high efficiency of gene editing determine by the T7EI assay. For the cloning reaction, it is important to troubleshoot with different amount of digested plasmid and annealed primers.

**Table 1. Primers phosphorylation reaction**

Reagent	Amount
10× T4 DNA ligase reaction buffer	2.5 $\mu$ L
5 U T4 Polynucleotide Kinase (10 U/ $\mu$ L)	0.5 $\mu$ L
dATP (10 mM)	2.5 $\mu$ L
Oligonucleotide (10 $\mu$ M)	1 $\mu$ L
ddH <sub>2</sub> O	18.5 $\mu$ L

4. Transfection of HEK293 cells with PRDX1 CRISPR components for gene deletion.
  - a. HEK293 cells are cultured in complete media to 80% confluency.
  - b. The cells were collected by trypsinization using TrypLE, resuspended in DMEM with 10% FBS medium without antibiotics and counted using cell counter.
  - c. Coat 24-well plate with 500  $\mu$ L of 0.1% of sterile filtered gelatin for 10 mins at 37°C and dry the wells by aspirating the gelatin using aspiration pump.

**Note:** the coat with 0.1% gelatin is optional but we found that coating with 0.1% improve the efficiency of the transfection and thus the efficiency of editing.

- d. Seed ~ 80,000 living cells in 500  $\mu$ L of media into the coated 24-well plate. Cells are left to adhere 16–18 hrs.
- e. Next day cells are transfected with the indicated plasmid (e.g., pCas9-gRNA-PRDX-1) using Eugene HD (Promega) reagent in Opti-MEM. The transfection master mix is prepared as follow, 2  $\mu$ L of pCas9-gRNA-PRDX1 (500 ng/ $\mu$ L) + 20  $\mu$ L of Opti-MEM + 3.4  $\mu$ L Eugene HD mix by pipetting and incubate 7 mins at 22°C–25°C.
- f. Add the final master mix to each well and shake the 24 well plate by hands.
- g. Incubate at 37°C for 48–72 hrs (see [Figure 2](#)).
- h. After the 2–3 days post-transfection, collect cells from each well by trypsinization, centrifugation and resuspend in 1 mL of complete DMEM GlutaMax media ([Figure 2](#)).
- i. Save some of the cells (1,000 cells) to isolate single cell colonies with potential PRDX1 knockout (See step 6) and centrifuge the remaining cells to be used for extracting genomic DNA using the Quick Extract DNA extraction kit, which will be used to verify and calculate the gene editing efficiency using T7EI assay (See step 5) ([Figure 2](#))

**△ CRITICAL:** Note we recommend transfection with a GFP or m-RFP expressing plasmid in separate wells for monitoring transfection efficiency of the suggested protocol with the chosen cell line followed by fluorescence microscopy 48–72 hrs post-transfection.

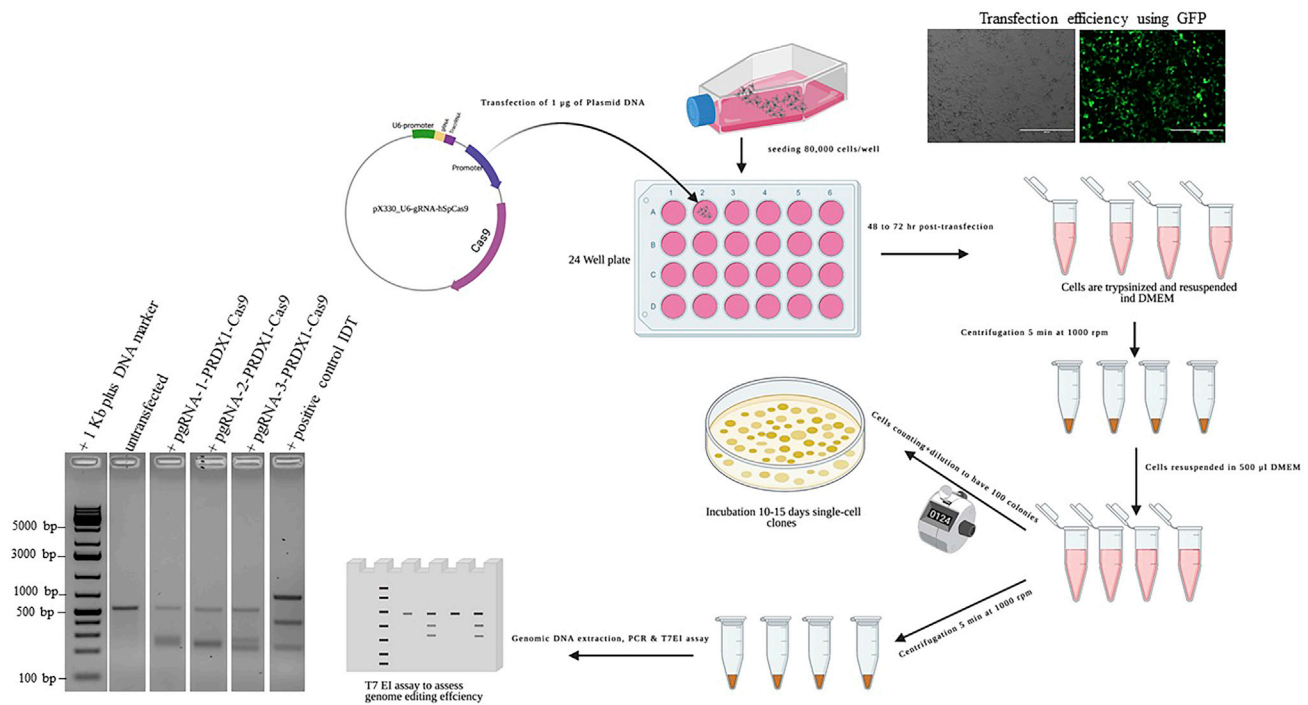
**Note:** pSpCas9(BB)-2A-GFP (PX458: Addgene# 48138) can be used simultaneously to monitor the transfection efficiency as well as to perform the specific editing with the specific gRNA.

5. T7EI assay to assess the gene editing efficiency
  - a. Extract the genomic DNA from the transfected cells (4i) using Quick Extract DNA ([Aouida et al., 2015](#); [Lee et al., 2017](#)).

**Table 2. T4 Ligation reaction**

Reagent	Amount
Digested plasmid (50 ng/ $\mu$ L)	1 $\mu$ L
Annealed primers (140 nM)	1 or, 2 or 4 $\mu$ L
5× T4 Ligase buffer	2 $\mu$ L
T4 Ligase	0.5 $\mu$ L
ddH <sub>2</sub> O	5.5 or 4.5 or 3.5 $\mu$ L





**Figure 2. Workflow to follow to engineer PRDX1 knockout in HEK293 using CRISPR-Cas9 system**

The editing activity is tested by T7EI assay in cells un-transfected or transfected with pgRNA-1-PRDX1-Cas9, or pgRNA-2-PRDX1-Cas9 or pgRNA-3-PRDX1-Cas9 (Created with [BioRender.com](https://www.biorender.com)).

- Use 1 µL of the extracted genomic DNA as a template to amplify the gene of interest; PRDX1 generated a fragment of 703 bp using a pair of primers ([key resources table](#)) and the Accu-prime High fidelity enzyme following the instructions of the manufacturer ([https://www.thermofisher.com/document-connect/document-connect.html?url=https%3A%2F%2Fassets.thermofisher.com%2FTFS-Assets%2FLSG%2Fmanuals%2Faccuprimetaqhifi\\_man.pdf](https://www.thermofisher.com/document-connect/document-connect.html?url=https%3A%2F%2Fassets.thermofisher.com%2FTFS-Assets%2FLSG%2Fmanuals%2Faccuprimetaqhifi_man.pdf)) (Table 3) and the PCR program indicated in Table 4.
- Load 5 µL of the PCR reaction onto a 1% agarose gel pre-stained with SYBER safe to confirm the PCR amplification of the target gene (e.g., PRDX1) and purify the remaining PCR reaction using QIAGEN PCR purification kit. Elute the PCR product using 35 µL of H<sub>2</sub>O that is DNase/RNase free.
- Quantify the PCR amplified DNA using Nanodrop and normalize the concentration to 12 ng/µL.
- Prepare the reaction that is required for the T7EI assay as indicated in Table 5. Denature the total reaction follow by renaturation and digestion with the T7 endonuclease (T7EI) according to the conditions indicated in Table 5 and incubate at 37°C for 1 h (Aouida et al., 2015; Lee et al., 2017).
- Add 6 µL of DNA loading dye (0.5 M EDTA with 6× DNA loading dye) to the reaction, and load the entire content onto a 2 % agarose gel. Run the gel until the bands are well separated, typically 1 hr at 110 V. When imaging the gel, make sure that the exposure time is properly adjusted so that none of the bands is saturated as this will interfere with accurate quantification of the band intensities (Figure 2).
- Estimate the gene editing activity using the free software ImageJ. The percent of alleles that show evidence of NHEJ can be calculated from the band intensities according to the formulas reported by Lee et al. (2017). The gene editing efficiency and specificity can be confirmed by Sanger sequencing using Topo-TA cloning kit and M-13 forward and Reverse primers ([key resources table](#)).



**Table 3. PCR Reaction master mix to amplify PRDX1 fragment**

Reagent	Amount
Genomic DNA	1 $\mu$ L
DNA Polymerase (Accuprime HF)	0.25 $\mu$ L
PRDX1-T7EI-F (10 $\mu$ M)	1 $\mu$ L
PRDX1-T7EI-R (10 $\mu$ M)	1 $\mu$ L
10 $\times$ Accuprime HF-Blue-Buffer	5 $\mu$ L
ddH <sub>2</sub> O	41.75 $\mu$ L

**△ CRITICAL:** To evaluate the gene editing activity by T7EI assay for PRDX1 gene or any other gene targeted with CRISPR-Cas9 system, the PCR amplification of the target should be specific and always do a PCR optimization to determine the T<sub>m</sub> that amplify the specific DNA fragment.

6. Single cell cloning of PRDX1 transfected HEK293 cells
  - a. Count the transfected cells and serially dilute the cells in culture media to achieve a final concentration of 1,000 cells/mL. Dispense 100  $\mu$ L of cell suspension (~100 cells) into 150 mm tissue culture plates (Figure 2).
  - b. Top up the plates with enough volume (25 mL) of complete culture media.
  - c. Incubate the plates for 10–15 days to form visible and separate colonies (Figure 3).
  - d. After enough colony expansion, the visible colonies can be picked up by 10  $\mu$ L pipette with 5  $\mu$ L of TrypLE (Pick up only colonies that are very well separated from others to avoid cross contamination).
  - e. Transfer each of 24 single colonies into separate wells of a 24 well plate for expansion (2–3 days). Expand the single colonies further by transferring the cells to 6-well plates and then finally to Sterile Cell Culture/Petri Dishes 100 $\times$ 15 mm (Figure 3).
  - f. Store some of the expanded single colonies in storage media (50% FBS, 40% DMEM-Gluta-Max, 10% DMSO) for further use that include total protein extract for immunoblot analysis (see Figure 3 for anti-PRDX1). Collect some cells for genomic DNA extraction using the Quick Extract DNA extraction kit for PCR amplification using a primer-pair (Key resources table for PRDX1). Purify the PCR product and proceed with sanger sequencing to identify the type of mutation (see Figure 3 and section 8)

**△ CRITICAL:** For colony isolation, we strongly recommend using the Sterile Petri Dishes for cell culture with a large size such 150 $\times$ 25 mm to have enough separation between the clones and to avoid any cross contamination between the colonies.

7. PRDX1 knockout verification by immunoblot.
  - a. Harvest enough cells from a culture dish or a T75 flask at 70–80% confluency.
  - b. Discard the culture media and wash twice with 1 $\times$  PBS.
  - c. Add 1 mL of TrypLE to trypsinize the cells and incubate at 37°C for 1–2 mins. Do not leave TrypLE for extended periods.
  - d. Add 5–10 mL media to collect the cells and transfer them to a 15 mL tube.
  - e. Centrifuge at 201 $\times$ g for 3 mins in Eppendorf centrifuge 5810. Discard the supernatant. Add 200  $\mu$ L of ice-cold RIPA lysis buffer supplemented with 1% of 100  $\times$  Protease Inhibitors

**Table 4. PCR program conditions for PRDX1 target gene**

Steps	Temperature	Time	Cycles
Initial Denaturation	94°C	2 min	1
Denaturation	94°C	30 s	40 cycles
Annealing	58°C	30 s	
Extension	68°C	30 s	
Hold	4°C	Forever	

**Table 5. Reaction with T7EI assay**

Reagent	Amount	Incubation
PRDX1 PCR product (12 ng/ $\mu$ L)	16.2 $\mu$ L	10 min at 95°C, then decreasing at 0.1°C/s down to 25°C
10 $\times$ NEBuffer #2	1.8 $\mu$ L	
10 $\times$ NEBuffer #2	0.2 $\mu$ L	n/a
T7EI Endonuclease	0.5 $\mu$ L	n/a
ddH <sub>2</sub> O	1.3 $\mu$ L	n/a

and mix gently by pipetting up and down. Incubate on ice for 5 mins. Transfer the lysate to a pre-chilled micro-centrifuge tube.

RIPA lysis buffer

Reagent	Final concentration	Amount
NaCl (1 M)	n/a	7.5 mL
NP-40 (10%)	n/a	0.5 mL
Sodium deoxycholate (0.5%)	n/a	0.25 mL
SDS (10%)	n/a	0.5 mL
Tris (0.05 M)	n/a	25 mL
ddH <sub>2</sub> O	n/a	16.25 mL
<b>Total</b>	<b>n/a</b>	<b>50 mL</b>
Storage 4°C		

- f. Sonicate twice at 40% amplification for 5 seconds. Clean the sonication probe with dH<sub>2</sub>O before and after every use. Note that sonication generates heat which might disturb your proteins, therefore let the samples chill on ice between sets. Make sure the sample is fully submerged but do not let the probe touch the sides or the bottom of the tube.
- g. Centrifuge the tubes at 9,391  $\times g$  for 10 mins at 4°C in an Eppendorf microcentrifuge 5424R. Transfer the supernatant to a new micro-centrifuge tube. Keep the samples on ice.
- h. Quantify your proteins using pierce BCA protein assay kit (Thermo Fisher). Mix 20–30  $\mu$ g of your protein with 4  $\mu$ L 5  $\times$  Laemmli loading buffer and up to 20  $\mu$ L dH<sub>2</sub>O in a microcentrifuge tube.  
5  $\times$  Laemmli loading buffer

Reagent	Final concentration	Amount
SDS (10%)	n/a	2.5 mL
Stacking buffer	n/a	3.13 mL
Glycerol (100%)	n/a	2.5 mL
2-mercaptoethanol	n/a	1.25 mL
Bromophenol blue	n/a	10 mg
ddH <sub>2</sub> O	n/a	0.62 $\mu$ L
<b>Total</b>	<b>n/a</b>	<b>n/a</b>
Storage 22°C–25°C		

- i. Place the tube on a thermal mixer at 95°C for 5 mins to denature the proteins. Load 3  $\mu$ L of the ladder followed by 20  $\mu$ L from each sample in each well of a 12.5% Acrylamide gel.  
12.5% Acrylamide gel

Reagent	Final concentration	Amount
Acrylamide (30%)	n/a	2.1 mL
Separating buffer	n/a	1.25 mL

(Continued on next page)

**Continued**

Reagent	Final concentration	Amount
Ammonium Persulfate (10%)	n/a	2.5 mL
TEMED	n/a	33 $\mu$ L
ddH <sub>2</sub> O	n/a	1.65 mL
<b>Total</b>	<b>n/a</b>	<b>n/a</b>
Freshly prepared		

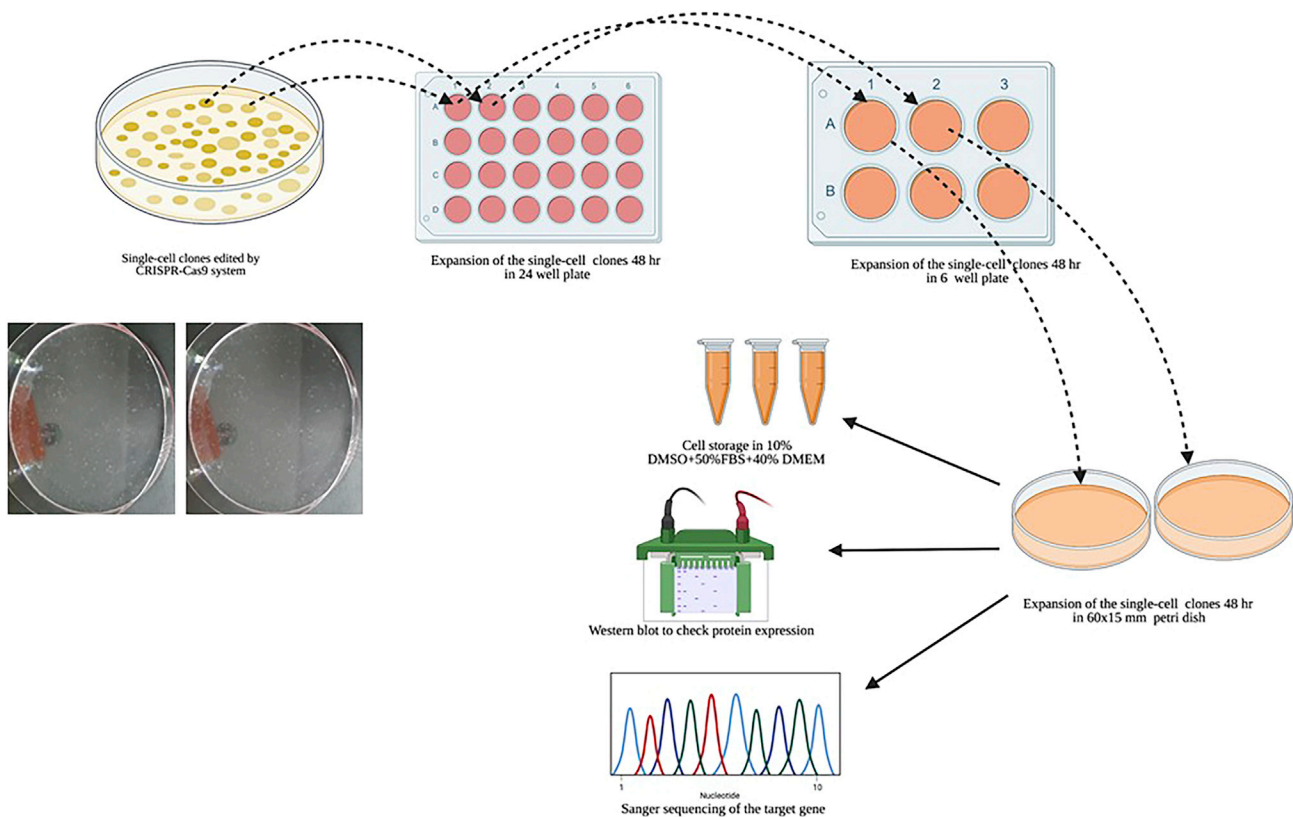
- j. Run the gel at 90 V for 130 mins.  
Transfer the proteins to a nitrocellulose membrane in Transfer Buffer using Trans-Blot Turbo Transfer System (BioRad) at 1.3 A and 25 V for 10 mins  
Transfer buffer

Reagent	Final concentration	Amount
Tris-base	n/a	3 g
Glycine	n/a	14.4 g
Methanol (100%)	n/a	200 mL
ddH <sub>2</sub> O	n/a	800 mL
<b>Total</b>	<b>n/a</b>	<b>1,000 mL</b>
Storage 22°C–25°C		

- k. Wash the membrane with dH<sub>2</sub>O a few times to remove any gel residue. Block the membrane in 5% milk 1  $\times$  TBST for 30 mins.
  - l. Incubate the membrane with rabbit anti-PRDX1 primary antibody (Cell signaling) at 1:2,000 dilution 16–18 hrs at 4°C.
  - m. Wash the membrane three times with 1  $\times$  TBST, 10 mins each.
  - n. Incubate the membrane with anti-rabbit HRP secondary antibody (Jackson Immuno-research) at 1:5,000 dilution for 1 h at 22°C–25°C.
  - o. Wash three times with 1  $\times$  TBST, 10 mins each.
  - p. Visualize using Pierce™ ECL Western Blotting Substrate (Thermo Fisher) (Figure 4).
8. Prdx1 knockout verification by Sanger sequencing.
    - a. Take the PCR product from section 6f and subject it to Topo-TA cloning following the manufacturer instructions ([https://www.thermofisher.com/document-connect/document-connect.html?url=https%3A%2F%2Fassets.thermofisher.com%2FTFS-Assets%2FLSG%2Fmanuals%2Ftopotaseq\\_man.pdf](https://www.thermofisher.com/document-connect/document-connect.html?url=https%3A%2F%2Fassets.thermofisher.com%2FTFS-Assets%2FLSG%2Fmanuals%2Ftopotaseq_man.pdf)).
    - b. Transformations of the Topo-TA cloning product in Top10 competent bacteria and colony selection on solid LB + Kanamycin (50  $\mu$ g/mL) plates or on solid LB + Ampicillin (100  $\mu$ g/mL).
    - c. Select five colonies and inoculate into 5 mL of LB + Kanamycin (50  $\mu$ g/mL) followed by mini-prep plasmid DNA extraction (Qiagen mini prep kit).
    - d. Perform Sanger sequencing using M-13 primers (key resources table) and using the protocol described by Saifaldeen et al. (2021).
    - e. Use SnapGene or any other DNA analysis software in order to determine the mutations (Figure 5).

## EXPECTED OUTCOMES

This genetic engineering method of creating a prdx1 knockout based on the efficiency of DNA editing detected by T7EI assay presents two major advantages (i) it avoids the use of antibiotic selection, and (ii) it bypasses the need for cell sorting, a sophisticated and expensive approach, and the equipment might not be available to some researchers.



**Figure 3. Steps for single colony expansion**

Workflow of PRDX1 knockout for (i) colony expansion (ii) colony storage (iii) Western blot for Prdx1 protein expression and (iv) colony Sanger sequencing for PRDX1 gene to identify the mutations (Created with [BioRender.com](https://www.biorender.com/)).

Testing several gRNAs showed that gRNA targeting the DNA sequence close to the ATG is more efficient in generating the expected PRDX1 knockout (3 times more for gRNA1-PRDX1 vs gRNA2-PRDX1 and gRNA3-PRDX1) ([Figure S1](#) for the gRNA locations).

One of the advantages of using this protocol is that it can be applied to any gene even for the ones encoding proteins that do not have antibodies since the knockout can be confirmed at the genomic level.

## LIMITATIONS

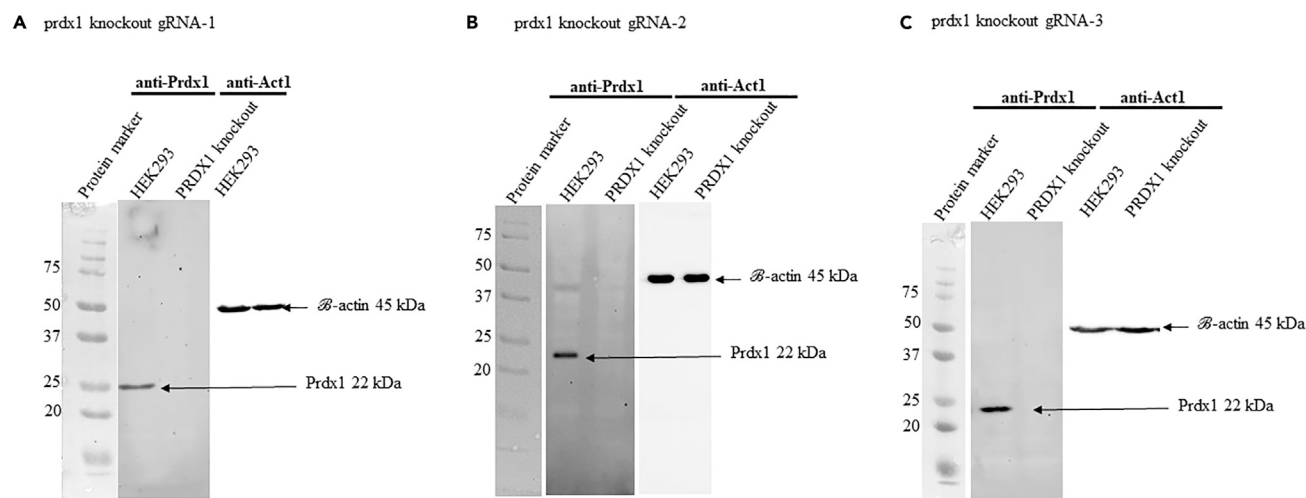
Because the efficiency of this protocol is based on the T7EI assay required to isolate the potential knockout, it has the limitation that it does not work for genes that cannot be specifically amplified with the designed T7EI primers.

Since this gene editing is performed with a group of cells and every single cell will have its specific genotype, some of the potential prdx1 knockouts engineered by CRISPR editing will result in indels with multiples of 3 nucleotides, thus resulting in deletion or insertion of one or multiple amino acids to create a truncated protein. These clones are not a knockout and in immunoblot assay they showed PRDX1 expression.

## TROUBLESHOOTING

### Problem 1

No available PAM sequence close to ATG.



**Figure 4. Characterization of 3 prdx1 knockout derived from gene editing using CRISPR-Cas9 system by western blot analysis**

(A) prdx1 knockout derived from CRISPR-Cas9-gRNA-1.

(B) prdx1 knockout derived from CRISPR-Cas9-gRNA-2.

(C) prdx1 knockout derived from CRISPR-Cas9-gRNA-3.

#### Potential solution

If no NGG sequence is identified in the exon 1 region close to ATG, it is advisable to check 30–60 nucleotides upstream of the ATG. If not, other CRISPR-Cas systems can be used that are available in *Addgene* that use different PAM sequences such as CRISPR-Cas12 (*Cpf1*).

#### Problem 2

No commercial antibody is available.

#### Potential solution

If there is no available antibody for the protein encoded by the targeted gene, skip step 7 and proceed directly with genotyping to sequence the potential knockouts by Sanger sequencing (step 8). Other methodology can be used to identify the potential knockout such as screening for morphological changes or phenotypes.

#### Problem 3

Primers designed for T7EI assay generated more than one fragment

#### Potential solution

Make sure to design good primers for RT-PCR (step 2) and for the T7EI assay (step 5b) that have  $T_m$  above 60°C. Before starting to amplify the target gene, run a PCR reaction using a gradient of  $T_m$  to determine the one giving the specific PCR product. This ensures that the T7EI assay will work efficiently.

#### Problem 4

This protocol is specifically for transfecting HEK293 cells known to be efficiently transfected and it is not likely to be applied to other cell lines.

#### Potential solution

Use an optimized method of transfection with other cells lines, such as lipofectamine, electroporation, lentivirus. Alternatively, it is possible to deliver the CRISPR components as Ribonucleoprotein (RNP) Complex into hard-to-transfect cell lines.

#### PRDX1-gRNA-1

Wild-type: ATGTTCTTCAGGAAATGCTAAATGGGCACCTGCCCACTTCAAAGCCACAGCTGTTATGCCAGATGGTCAGTTTAAAGATATCAGCCTGTCTGACTACAAAGGAAATATGTTGTG

Prdx1-knockout: ATGTTCTTCAGGAAATGCTAA--TTGGGCACCTGCCCACTTCAAAGCCACAGCTGTTATGCCAGATGGTCAGTTTAAAGATATCAGCCTGTCTGACTACAAAGGAAATATGTTGTG (-2)

#### Prdx1 protein sequence:

MSSGNKIGHHPAPNFKATAVMPDGGQKDISLSDYKGYVVFYPLDFTFVCPTEIIAFSDRAEEFKLNCQVIGASVDSHFCHLAWVNTPKQGGGLGPMNIPLVSDPKRTIAQDYGLVKADEGISFRGLFIIDDKGILRQITVND  
LPVGRSVDLRLVQAFQFTDKHGEVCPAGWKPGSDTIKPDVQKSKEYFSKQK\*

#### Prdx1 knockout protein sequence:

MSSGNANWAPCPQLQSHSCYARWSV\*

#### PRDX1-gRNA-2

Wild-type: ATGTTCTTCAGGAAATGCTAAATGGGCACCTGCCCACTTCAAAGCCACAGCTGTTATGCCAGATGGTCAGTTTAAAGATATCAGCCTGTCTGACTACAAAGGAAATATGTTGTG

Prdx1-knockout: ATGTTCTTCAGGAAATGCTAAATGGGCACCTGCCCACTTCAAAGCCACAGCTGTTATGCCAGATGGTCAGTTTAAAGATATCAGCCTGTCTGACTACAAAGGAAATATGTTGTG (-1)

#### Prdx1 protein sequence:

MSSGNKIGHHPAPNFKATAVMPDGGQKDISLSDYKGYVVFYPLDFTFVCPTEIIAFSDRAEEFKLNCQVIGASVDSHFCHLAWVNTPKQGGGLGPMNIPLVSDPKRTIAQDYGLVKADEGISFRGLFIIDDKGILRQITV  
NDLPVGRSVDLRLVQAFQFTDKHGEVCPAGWKPGSDTIKPDVQKSKEYFSKQK\*

#### Prdx1 knockout protein sequence:

MSSGNKIGHHPAPNTSKPQLLCQMVSLKISACLTTKENMLCSSLTLLTSPLCAPRRSLLSVIGQKNLRNSTAK\*

#### PRDX1-gRNA-3:

Wild-type: ATGTTCTTCAGGAAATGCTAAATGGGCACCTGCCCACTTCAAAGCCACAGCTGTTATGCCAGATGGTCAGTTTAAAGATATCAGCCTGTCTGACTACAAAGGAAATATGTTGTG

Prdx1-knockout: ATGTTCTTCAGGAAATGCTAAATGGGCACCTGCCCACTTCAAAGCCACAGCTGTTATGC-AGATGGTCAGTTTAAAGATATCAGCCTGTCTGACTACAAAGGAAATATGTTGTG (-1)

#### Prdx1 protein sequence:

MSSGNKIGHHPAPNFKATAVMPDGGQKDISLSDYKGYVVFYPLDFTFVCPTEIIAFSDRAEEFKLNCQVIGASVDSHFCHLAWVNTPKQGGGLGPMNIPLVSDPKRTIAQDYGLVKADEGISFRGLFIIDDKGILRQITV  
NDLPVGRSVDLRLVQAFQFTDKHGEVCPAGWKPGSDTIKPDVQKSKEYFSKQK\*

#### Prdx1 knockout protein sequence:

MSSGNKIGHHPAPNFKATAVMQMVSLKISACLTTKENMLCSSLTLLTSPLCAPRRSLLSVIGQKNLRNSTAK\*

### Figure 5. Characterization of 3 prdx1 knockouts derived from gene editing using CRISPR-Cas9 system by Sanger sequencing analysis

(A) prdx1 knockout derived from CRISPR-Cas9-gRNA-1.

(B) prdx1 knockout derived from CRISPR-Cas9-gRNA-2.

(C) prdx1 knockout derived from CRISPR-Cas9-gRNA-3.

## Problem 5

Primers for cloning the gRNA into the PX330\_U6-Chimeric\_BB\_Cbh-hSpCas9 did not work

## Potential solution

This could happen (i) if the ratio of the absorbance 260/280 of the digested plasmid pX330 is below 1.8, however, this can be easily resolved by adding more steps of washing with PE buffer and using completely dried DNA or (ii) if the ratio between Vector (V) vs Insert (I) used during the ligation reaction is not optimal. It is strongly recommended to troubleshoot with different ratios of V:I (1:1; 1:3; 1:6; 3:1; 3:3; 3:6) during the ligation reaction.

## RESOURCE AVAILABILITY

### Lead contact

Further information and requests for resources should be directed to and will be fulfilled by the lead contact, Dr. Mustapha Aouida ([maouida@hbku.edu.qa](mailto:maouida@hbku.edu.qa)).

### Materials availability

Reagents generated within this study are available upon request from the lead contact.

### Data and code availability

The Supplementary information's have been deposited to Mendeley data: <https://doi.org/10.17632/nknd6w2kbn.1>.

## SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.xpro.2022.101216>.

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## AUTHOR CONTRIBUTIONS

M.A. and D.R. conceived the idea and designed the experiments. M.A. and D.J. performed the experiments. M.A., D.R., and D.J. analyzed the data. M.A. and D.R. contributed reagents/materials/analysis tools. M.A., D.R., and R.A. wrote the paper. M.A. and D.J. prepared the figures. M.A., D.R., R.A., and D.J. reviewed the manuscript.

## DECLARATION OF INTERESTS

The authors declare no competing interests.

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