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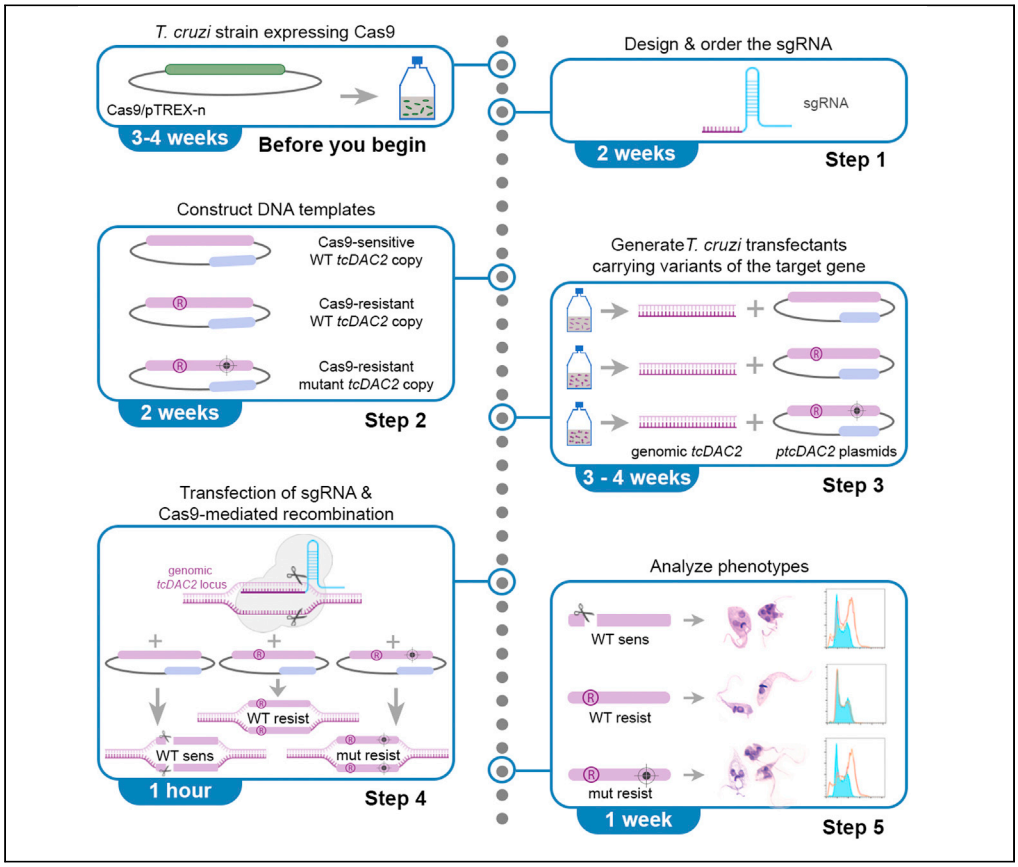
Efficient CRISPR-Cas9-mediated genome editing for characterization of essential genes in *Trypanosoma cruzi*



This protocol outlines a new genetic complementation strategy to investigate gene function in *Trypanosoma cruzi*, the parasite causing Chagas disease. We combine CRISPR-Cas9 technology with recombination of variants of the target gene containing the desired mutations that are resistant to Cas9-cleavage, which enables detailed investigation of protein function. This experimental strategy overcomes some of the limitations associated with gene knockouts in *T. cruzi*.

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Highlights

Genetic complementation strategy to investigate gene function in *Trypanosoma cruzi*

Analysis of specific mutations on protein function *in vivo*

CRISPR-Cas9-based gene knockout and gene replacement

Rapid analysis of *Trypanosoma cruzi* essential genes

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Efficient CRISPR-Cas9-mediated genome editing for characterization of essential genes in *Trypanosoma cruzi*

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SUMMARY

This protocol outlines a new genetic complementation strategy to investigate gene function in *Trypanosoma cruzi*, the parasite causing Chagas disease. We combine CRISPR-Cas9 technology with recombination of variants of the target gene containing the desired mutations that are resistant to Cas9-cleavage, which enables detailed investigation of protein function. This experimental strategy overcomes some of the limitations associated with gene knockouts in *T. cruzi*. For complete details on the use and execution of this protocol, please refer to Marek et al. (2021).

BEFORE YOU BEGIN

Trypanosoma cruzi is the etiological agent of Chagas disease, a neglected tropical disease for which no vaccine neither safe nor efficient treatment is available. *T. cruzi* belongs to the Kinetoplastida group of parasitic protozoans which has evolved peculiar biological features, including polycistronic organization of protein coding genes, absence of canonical transcription factors and transcription regulation and, control of gene expression taking place mostly at the posttranscriptional level. These genetic features, along with absence of RNA interference machinery has drastically hindered the development of efficient genetic tools for molecular studies and characterization of genes with unknown function in this pathogen. New experimental strategies that enable the study of gene function, especially for the essential genes, will contribute to gene target validation and positively influence the initial stages of drug discovery to develop new therapeutic strategies for Chagas disease.

Strategies based on gene silencing by using RNA interference (RNAi) have been in general successful for functional characterization of genes and validation of targets for development of inhibitors in many organisms. However, such a strategy cannot be used in *T. cruzi* since this organism lacks the enzymes of the RNA interference machinery (Darocha et al., 2004). Strategies based on gene knockout are not suitable for the characterization of essential single-copy genes, since the cells are not viable after deletion of the two alleles. In addition, there is currently no regulated system available for conditional depletion of essential genes that allows for characterization of the cellular

and molecular phenotypes. Several studies have proposed a combination of homologous recombination of a small DNA template with the CRISPR/Cas9 methodology to knockout *T. cruzi* genes (Burle-Caldas et al., 2018; Lander et al., 2015; Pavani et al., 2016; Peng et al., 2015; Romagnoli et al., 2018; Souza et al., 2010). Although this is possible for non-essential genes when the knockout cells can grow to a number that allows for subsequent analysis, it is basically useless for essential genes, since null mutant *T. cruzi* cells die within a few days preventing any characterization of the phenotypes caused by the gene knockout.

Thus, a tool that allows for gene replacement is extremely useful to investigate the phenotypes caused by knockout of essential genes even in a transient manner. The protocol herein proposes a strategy for the knockout of the target gene by CRISPR/Cas9 combined with its replacement for a mutated copy that could carry any kind of desired alterations. The method involves providing suitable DNA repair templates to induce homolog-driven repair following cleavage of Cas9 from the target gene. In addition, the simultaneous gene knockout and replacement strategy described in this protocol allows for testing different variants of the same gene to obtain relevant functional information such as the role of specific amino acid residues, motifs and protein domains.

The protocol below outlines the steps used in the analysis of the *Trypanosoma cruzi* deacetylase 2 gene. However, it can be applied for all targets of interest, especially for essential genes.

Obtaining a *T. cruzi* strain stably expressing a Cas9 endonuclease

Timing: ~4 weeks

A *T. cruzi* strain stably expressing a Cas9 endonuclease is a starting point as it will be necessary for gene knockout in this CRISPR/Cas9-based strategy. The proposed protocol uses the *Streptococcus pyogenes* Cas9 (SpCas9) endonuclease fused to a green fluorescent protein. The GFP fusion facilitates selection and monitoring of the Cas9 expressing cells.

1. *T. cruzi* Dm28c strain epimastigotes are maintained at 28°C in liver infusion tryptose (LIT) medium supplemented with 10% heat-inactivated fetal bovine serum (FSB).
2. Collect early-log phase *T. cruzi* epimastigotes by centrifugation at 20°C–25°C for 5 min at 3,000 × *g*.
   a. Use 5 × 10^6 cells for each transfection.
3. Wash in sterile PBS (pH 7.4). Spin down for 5 min at 3,000 × *g*.
4. Recover cells in transfection buffer.
   a. Use 100 μL of buffer for 5 × 10^6 cells.

   Note: Transfection buffer should be at 20°C–25°C at the moment of use.

5. Mix 100 μL of cells and 20 μg of Cas9/pTREX-n plasmid in the test tube.

   Note: It is important that the DNA volume added does not exceed 20% of the initial volume.

6. For a negative control of transfectant selection, mix 100 μL of cells and 15 μL of TE buffer in the negative control tube.
7. Transfer each mixture to an individual electroporation cuvette and electroporate cells using the X-014 program in an Amaxa Nucleofector Device.
8. After one electric pulse, transfer the cells to a sterile 15 mL conical tube containing 3 mL of LIT medium supplemented with 10% FBS. Incubate at 28°C.
9. At 24 h post-transfection, add 15 μL of G418 (final concentration 250 μg/mL) to the cultures.
10. At 72 h post-transfection, dilute cultures in a sterile 15 mL conical tube using 500 μL of the previous culture in 2.5 mL of LIT medium supplemented with 10% FBS containing 250 μg/mL of G418.

11. Repeat culture dilution every 3 days until transfectant selection is completed.

Alternatives: When culture density reaches 1 × 10^7 cells/mL, transfer 1 × 10^6 cells/mL to a new sterile 15 mL conical tube containing LIT medium supplemented with 10% FBS and 250 μg/mL of G418.

12. Sort GFP-positive cells 15 days post-transfection using a BD FACSARIA II.

Note: Cells can be selected based on green fluorescence found in the nuclei of Cas9-GFP expressing parasites.

Alternatives: Any suitable cell sorter can be used. If a cytometer is not available, it is possible to clone by limiting dilution and check for the presence of GFP by fluorescence microscopy or even by western blotting.

13. *T. cruzi* cell line stably expressing Cas9-GFP is maintained in LIT medium supplemented with 10% FBS and 250 μg/mL of G418 at 28°C.

⚠ CRITICAL: It is important that the selected cells are expressing the endonuclease Cas9 for the next steps. This can be easily verified by flow cytometry, fluorescence microscopy due to its fusion with GFP or by western blotting using a commercial GFP antibody.

Obtaining a sgRNA scaffold

Timing: ~2.5 weeks

A scaffold sequence must be present in the sgRNA since it is required for Cas9-binding. It can be amplified using specific forward and common reverse primers to generate the template molecule that will be used for *in vitro* transcription to produce the sgRNA.

14. Obtain the scaffold sequence for the sgRNA and clone it in the pUC19 vector between the restriction sites *Eco*RI and *Hind*III (indicated below as underlined letters).

Note: In this work, the sgRNA scaffold sequence was acquired cloned into pUC19 from GenScript, but the sgRNA scaffold can be cloned in any other vector.

a. Scaffold sequence: GAATTCCATGGGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGC TAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTTTTAAGCTT.

Alternatives: It is also possible to use the scaffold plasmids pUC_sgRNA (Lander et al., 2015) or pX330 (Peng et al., 2015).

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
DH5α *E. coli*	Invitrogen	Cat# 18265017
Chemicals, peptides, and recombinant proteins		
Ampicillin	Sigma-Aldrich	Cat# A9393
Blasticidin S HCl (10 mg/mL)	Gibco	Cat# A1113903
Fetal bovine serum (FBS)	Gibco	Cat# 12657029

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
G418	Gibco	Cat# 10131027
LB broth medium	Sigma-Aldrich	Cat# L3022
Liver Infusion Tryptose (LIT) medium	Prepared in house	(Camargo, 1964)
NP40 (Nonidet P-40)	Sigma-Aldrich	Cat# 74385
PBS pH 7.4	Gibco	Cat# 70011044
Permount Mounting Medium	Fisher Scientific	Cat# SP15-100
Propidium iodide (1 mg/mL solution in water)	Invitrogen	Cat# P3566
Restriction enzyme: *Apa*I	New England Biolabs	Cat# R0157S
Restriction enzyme: *Sac*II	New England Biolabs	Cat# R0114S
RNase A	Sigma-Aldrich	Cat# R5503
Transfection buffer	Prepared in house	n/a
Critical commercial assays		
MEGAshortscript T7 Transcription kit	Invitrogen	Cat# AM1354
Nucleospin Gel and PCR clean up kit	MACHEREY-NAGEL	Cat# 740609.250
Nucleospin Plasmid EasyPure kit	MACHEREY-NAGEL	Cat# 740727.250
Panoptic Staining Solutions	Laborclin	Cat# 620529
pGEM-T easy Vector Systems	Promega	Cat# A1360
Platinum SuperFi DNA polymerase	Invitrogen	Cat# 12351010
T4 DNA ligase (1 U/μL)	Invitrogen	Cat# 15224-017
Taq DNA polymerase	Invitrogen	Cat# 10342053
Experimental models: Organisms/strains		
Trypanosoma cruzi Cas9-GFP expressing strain	Previous work from G. Picchi-Constante team	(Romagnoli et al., 2018)
Trypanosoma cruzi Dm28c strain	Stock from Fiocruz Paraná	(Contreras et al., 1988)
Oligonucleotides		
bsdR 5′-AAAActcgagGCCCTCCCACACATAACCAGA-3′	Sigma-Aldrich	n/a
DAC2_F1 5′- agcGAGCTCCTGGAAAGACGTCACTGCCA-3′	Sigma-Aldrich	(Marek et al., 2021)
DAC2_R1 5′- agcGAGCTCAAGAGCGGTGATGCCATGAA-3′	Sigma-Aldrich	(Marek et al., 2021)
DAC2_F3 5′- TCATGCgAAaAAgAGcACgGCtGGGGGTTTTTGC TTTGCAAATGATGTGGT-3′	Sigma-Aldrich	(Marek et al., 2021)
DAC2_R3 5′- ctaCTTATCGTCATCGTCCTTGTAATCAATGTCG TGGTCCTTGTAATCGCCGTCGTGATCCTTGTAGTC tgctgcggctgcTGGTTCTTCCTCCTCCTCTTC-3′	Sigma-Aldrich	(Marek et al., 2021)
sgRNAF_205 5′-ggaggccggagaattgtaatacgactcactataggga ga**gctgcgattcattggtttgg**gttttagagctagaaatag caag-3′	Sigma-Aldrich	(Marek et al., 2021)
sgRNAF_238 5′-ggaggccggagaattgtaatacgactcactataggg aga**gctaagaaaagtactgcggg**gttttagagctagaa atagcaag-3′	Sigma-Aldrich	(Marek et al., 2021)
sgRNAF514 5′-ggaggccggagaattgtaatacgactcactataggg aga**gctaagaaaagtactgcggg**gttttagagctagaa atagcaag-3′	Sigma-Aldrich	(Marek et al., 2021)
sgRNA_ScaffoldR 5′-aaaaaagcaccgactcggtgccactt-3′	Sigma-Aldrich	n/a
SP6 5′-ATTTAGGTGACACTATAG-3′	Sigma-Aldrich	n/a
T7 5′-TAATACGACTCACTATAGGG-3′	Sigma-Aldrich	n/a
Recombinant DNA		
Cas9/pTREX-n plasmid	(Lander et al., 2015)	Addgene Cat# 68708
pnEA/3CH-tcDAC2 plasmids	Previous work from C. Romier team	(Marek et al., 2021)

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
pScaffold	GenScript	n/a
pTc2KO-bsd	Provided by Fragoso lab, from Fiocruz Paraná	(Pavani et al., 2016)
Software and algorithms		
Eukaryotic Pathogen CRISPR guide RNA/DNA Design Tool	(Peng and Tarleton, 2015)	http://grna.ctegd.uga.edu/
FlowJo v10.1 r7	BD Bioscience,	Version 10.1 r7 http://www.flowjo.com/
Other		
Amaxa Nucleofector Device	Lonza	n/a
BD FACSARIA II cytometer	BD	n/a

MATERIALS AND EQUIPMENT

Transfection Buffer	
Reagent	Final concentration
KCl (1 M)	5 mM
$CaCl_2$ (100 mM)	0.15 mM
Na_2HPO_4 (200 mM)	90 mM
HEPES pH 7.3 (1 M)	50 mM
ddH_2O	to 50 mL
Store at 4°C. Stable for several months. Use at 20°C–25°C.	

TE Buffer	
Reagent	Final concentration
Tris-HCl pH 8 (1 M)	10 mM
EDTA	1 mM
ddH_2O	to 10 mL
Store at 4°C. Stable for several months. Use at 20°C–25°C.	

P1 resuspension solution	
Reagent	Final concentration
Tris-HCl (1 M)	50 mM
EDTA pH 8 (500 mM)	10 mM
ddH_2O	to 100 mL
Store at 4°C. Stable for several months.	

P2 lysis solution	
Reagent	Final concentration
NaOH	0.15 M
SDS (10%)	1%
ddH_2O	to 100 mL
Store at 18°C–25°C. Stable for several months. If precipitates, warm up before use.	

P3 neutralization solution – pH 4.8	
Reagent	Final concentration / Amount
Potassium acetate	3 M / 60 mL
Acetic acid glacial	11.5 mL
ddH_2O	to 100 mL
Store at 18°C–25°C. Stable for several months.	

Wash buffer	
Reagent	Final concentration
Tris-HCl pH 7.5 (1 M)	10 mM
96% ethanol	80%
ddH_2O	to 50 mL
Store at 18°C–25°C. Stable for several months.	

Elution buffer	
Reagent	Final concentration
Tris-HCl pH 8.5	10 mM
ddH_2O	to 50 mL
Store at 18°C–25°C. Stable for several months.	

Propidium iodide solution	
Reagent	Final concentration
Tris HCl pH 7.4 (1 M)	3.4 mM
NP40	0.1%
RNase A (10 mg/mL)	10 µg/mL
NaCl (5 M)	10 mM
Propidium Iodide	30 µg/mL
ddH_2O	to 10 mL
Store at 4°C. Stable for several months.	

Alternatives: This protocol uses an Amaxa Nucleofector Device for cell transfections, but any other suitable electroporation equipment can be used.

Alternatives: This protocol uses a BD FACSARIA II for cell sorting, enrichment and cell cycle analyses but any other suitable flow cytometer instrument can be used.

STEP-BY-STEP METHOD DETAILS

Knocking out the target gene—Defining the best protospacer sequence

Timing: 3 weeks

In this step, the objective is to design, test and select a specific protospacer that will be used to generate specific sgRNA to knock out the target gene.

1. Design the protospacer sequences for the target genes using the Eukaryotic Pathogen CRISPR guide RNA/DNA Design Tool (EuPaGDT, http://grna.ctegd.uga.edu/).
   a. There is no need to change parameters other than mandatory options:
      i. Choose a "Job Name".
      ii. On "RNA guided nuclease selection", choose "SpCas9: 20 nt gRNA, NGG PAM on 3′ end".
      iii. On "genome", choose the appropriate one. In this work, genomes "*T. cruzi* Dm28c TritrypDB-26" or "*T. cruzi* Dm28c TritrypDB-28" were selected in the Trypanosomatid option.
      iv. On "sequence", paste a single nucleotide sequence in FASTA format of the target gene.
   b. Click on "Get guide RNA".
   c. On Results page, click on "gRNA sequence and score" and choose 3 different gRNA sequences, based preferentially on:
      i. Score: analyze total and efficiency scores. The higher the value, the better.

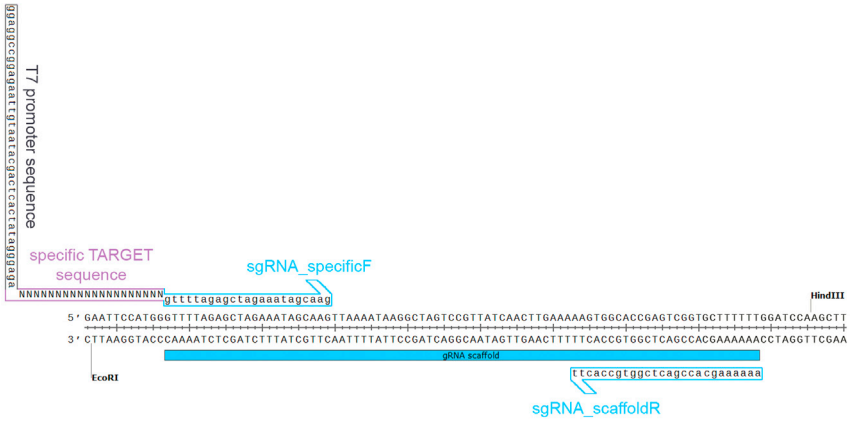

Figure 1. Representative scheme to design specific sgRNA molecules

ii. On and off-target hits. The best choice is a perfect match on a single specific target and no match on non-specific targets.

2. Design a specific forward primer for each sgRNA containing the T7 promoter, the specific target sequence and the scaffold complementary sequence:
   a. Insert the sequence GN(19–24) defined above into the primer backbone: 5′-GGAGGCCGGA GAATTGTAATACGACTCACTATAGGGAGA-**GN(19–24)**-GTTTTAGAGCTAGAAATAGCAAG-3′.

   Note: GN(19–24) corresponds to the sequence defined in step 1 (N19-24) preceded by a G nucleotide.

3. Order the designed specific forward primers and common reverse primer (Figure 1).
   a. sgRNA_ScaffoldR: 5′-CAGTGGATCCAAAAAAGCACCGACTCGGTG-3′.
   b. Specific primers used in this work are listed in key resources table as sgRNAF_205, sgRNAF_238 and sgRNAF_514.

   Alternatives: It is possible to purchase the entire DNA fragments from DNA synthesis companies.

4. Amplify the complete DNA templates for each sgRNA from pScaffold by PCR (Figures 2A and 2B).

PCR reaction master mix	
Reagent	Amount
Buffer Taq DNA polymerase	1×
dNTPs mix	0.2 mM each
Specific Primer F*	0.2 μM
sgRNA_ScaffoldR	0.2 μM
pScaffold	1 ng
Taq DNA Polymerase	2.5 U
ddH_2O	to 50 μL

*Note: In this work, 3 independent reactions were performed using sgRNAF_205, sgRNAF_238 and sgRNAF_514 separately as forward primer.

PCR cycling conditions			
Steps	Temperature	Time	Cycles
Initial Denaturation	94°C	5 min	1
Denaturation	94°C	45 s	35 cycles
Annealing	56°C	45 s	
Extension	72°C	45 s	
Final extension	72°C	5 min	1
Hold	4°C	forever	

5. Verify PCR product amplification using 2% agarose gel electrophoresis. Troubleshooting 1.
6. Purify the products using a DNA purification method.
   a. In this work, the PCR cleanup protocol of Nucleospin Gel and PCR Clean-up Kit is used with the following modification:
      i. Elution is done twice with 15 µL of the elution buffer provided in the kit (NE buffer) pre-heated at 70°C.
7. Verify product purification yield using 2% agarose gel electrophoresis.
8. Generate the sgRNA by *in vitro* transcription using the MEGAshortscript T7 Transcription Kit (Figure 2C).

Note: Thaw the T7 10× buffer, ribonucleotide solutions and water at 18°C–25°C. Briefly vortex and spin down all components before using. Keep the T7 enzyme mix on ice.

Reagent	Amount
T7 buffer 10×	2 µL
ATP solution (75 mM)	2 µL
CTP solution (75 mM)	2 µL
GTP solution (75 mM)	2 µL
UTP solution (75 mM)	2 µL
Purified PCR product	8 µL
T7 Enzyme mix	2 µL
Total	**20 µL**

   a. Set up the reaction in a nuclease-free tube at 18°C–25°C. Mix by gently flicking the tube, spin down and incubate at 37°C for 16 h.
   b. Add 1 µL of Turbo DNase, incubate at 37°C for 15 min and transfer to ice.
   c. Add 115 µL of nuclease-free water and 15 µL of ammonium acetate stop solution.

Alternatives: This protocol uses the MEGAshortscript T7 Transcription Kit, but any other transcription kit can be tested for this purpose.

   d. Purify the sgRNA using a phenol/chloroform extraction.

⚠ CRITICAL: The phenol/chloroform solution should not be used if it is oxidized. Oxidation of the phenol can be detected by pink/brown products and can cause DNA nicks and RNA degradation.

      i. Add 100 µL of nuclease-free saturated phenol:chloroform:isoamyl alcohol (25:24:1) in each tube. Mix by inversion 5 times. Centrifuge at 13,000 × *g*, for 5 min at 20°C–25°C.
      ii. Recover the upper aqueous phase and transfer to a new nuclease-free 1.7 mL microcentrifuge tube. Add 100 µL of nuclease-free saturated chloroform:isoamyl alcohol (24:1) in each tube. Mix by inversion 5 times. Centrifuge at 13,000 × *g*, for 5 min at 20°C–25°C.

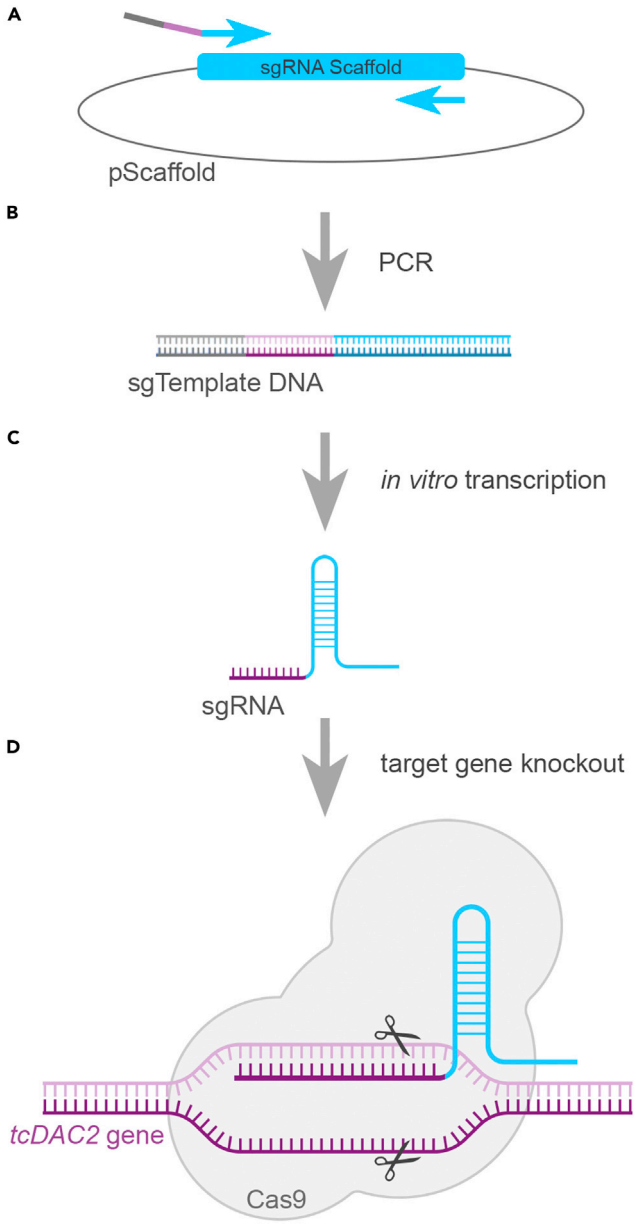

Figure 2. Representative steps to knock out the target gene by CRISPR-Cas9
(A) Amplify the sgRNA scaffold.
(B) Obtain sgTemplate DNA.
(C) Generate the sgRNA by *in vitro* transcription.
(D) Knock out target gene using the obtained sgRNA.

iii. Recover the aqueous phase and transfer to a new nuclease-free 1.7 mL microcentrifuge tube.

e. Precipitate the RNA by adding 300 μL of ethanol and mixing well. Chill the mixture for 2 h at −20°C.

Alternatives: It is possible to reduce the incubation time to a minimum of 15 min at −20°C.

Pause point: It is also possible to leave 16 h or longer at −20°C.

f. Centrifuge at 4°C for 15 min at maximum speed (at least 13,000 × *g*). Carefully remove the supernatant.
g. Wash RNA pellet with 1 mL of cold nuclease-free 75% ethanol. Centrifuge at 4°C for 15 min at maximum speed (at least 13,000 × *g*). Carefully remove the supernatant.
h. Let the RNA pellet to dry at 20°C–25°C for 5–10 min.

⚠ CRITICAL: Do not let it dry completely as this will decrease its solubility. However, if ethanol is left, it will also prevent the RNA from dissolving.

i. Resuspend the RNA pellet in 40 μL of nuclease-free water and keep the tube on ice.
j. Transfer 3 μL to another nuclease-free tube and store the remaining sgRNA at −80°C until use.

Alternatives: It is possible to store at −20°C if −80°C is unavailable.

Pause point: Purified sgRNAs can be stored at −80°C for several weeks.

k. From the aliquoted sgRNA, use 1 μL of each sample to quantify on a Nanodrop spectrophotometer (or another similar). It should be around 2 μg/μL.
l. Analyze 0,5 μL of each sample by electrophoresis on a 2% agarose gel.

Note: If problems arise in the sgRNA transcription step, follow the instructions of the troubleshooting section in the MEGAshortscript kit manual.

9. Knock out target gene by transfecting *T. cruzi* stably expressing Cas9-GFP cells with sgRNA (Figure 2D).
   a. Collect the early-log phase *T. cruzi* epimastigotes by centrifugation at 20°C–25°C for 5 min at 3,000 × *g*.
      i. Use 5 × 10^6 cells for each transfection.
   b. Wash in sterile PBS (pH 7.4). Spin down for 5 min at 3,000 × *g*.
   c. Recover cells in transfection buffer.
      i. Use 100 μL of buffer for 5 × 10^6 cells.
   d. Denature the sgRNA by heating at 70°C for 5 min. Transfer immediately to ice.
   e. Mix 100 μL of cells and 20 μg of sgRNA in the test tube.

Note: It is important that the sgRNA volume added does not exceed 20% of the initial volume.

   f. For the negative control, mix 100 μL of cells and 15 μL of TE buffer in the negative control tube.
   g. Transfer each mixture to an individual electroporation cuvette and electroporate cells using the U-033 program in an Amaxa Nucleofector Device.
   h. After one electric pulse, transfer the cells to a sterile 15 mL conical tube containing 5 mL of LIT medium supplemented with 10% FBS. Maintain at 28°C.
10. If the phenotypes of *T. cruzi* epimastigotes after target gene knockout are already known, use the appropriate tests to verify the efficiency of the chosen sgRNAs. However, if the phenotypes are still unknown, analyze daily for four days following the transfections:
   a. Cell proliferation by cell counting.
      i. Use 100 μL of the 5 mL transfected culture to monitor cell proliferation by cell counting in a Z series Coulter counter.
   b. Cell cycle by DNA content analyses.
      i. Transfer 100 μL of the 5 mL transfected culture to 400 μL of PBS in a tube. Centrifuge at 3,000 × *g*, for 5 min at 20°C–25°C. Suspend cells in 100 μL of PBS and mix with 100 μL of propidium iodide staining solution.

ii. Perform cell cycle analysis in a Flow Cytometer instrument. Propidium iodide is excited by a 488 nm laser and emitted light is recorded using a 616/23 bandpass filter (PE-Texas Red channel).

Note: This protocol uses a BD FACSAria II, but any other suitable flow cytometer instrument can be used.

iii. Collect data from at least 10,000 single cell events and gate them based on pulse area versus pulse width of PE-Texas Red channel, excluding aggregates and debris.
iv. Analyze DNA content of gated cell population using FlowJo software (or similar).

Alternatives: It is also possible to analyze cell death/apoptosis using flow cytometry.

c. Cell morphology by panoptic staining in clarified cells.
   i. Transfer 100 μL of the 5 mL transfected culture to a 1.5 mL microcentrifuge tube containing 500 μL of PBS.
   ii. Centrifuge at 3,000 × *g*, for 5 min at 20°C–25°C. Suspend cells in 20 μL of PBS.
   iii. Deposit 15 μL of cells on a glass slide, allow to air dry and fix the samples by dipping the slide in cold methanol for 5 min. Let dry.

Pause point: If necessary, it is possible to pause at this point. Store slides at 20°C–25°C taking care of the surface containing the samples.

iv. Clarify *T. cruzi* cells by dipping the glass slide in 5 M HCl for 3 min.
v. Wash thoroughly by dipping the glass slide 5 times in a beaker containing 2 l of water. Let air dry.

⚠ CRITICAL: Panoptic solutions should be at 20°C–25°C before use.

vi. Stain cells using panoptic solutions by dipping the glass slide for 5 min in solution 1, then 20 min in solution 2 and 15 min in solution 3.
vii. Prewash the glass slides by dipping it 5 times in a beaker containing 1 l of water. Then, wash thoroughly by dipping the glass slide 5 times in a beaker containing 5 l of water. Let air dry.
viii. Finalize by mounting slides using a small drop of Permount Mounting Medium and a coverslip.
ix. Analyze the cells by light microscopy on a Nikon Eclipse E600, a Leica DMi8 microscope or similar.

⚠ CRITICAL: To ensure that the observed phenotypes are due to the absence of the gene and not the transfection itself, it is important to always compare the transfected knocked out cells with the transfected wild-type control cells. Troubleshooting 2.

Alternatives: If a specific antibody is available, it is also possible to test the protein presence by western blotting and immunofluorescence microscopy.

d. Editing confirmation at DNA level.
   i. It is recommended that deletion or substitution of the target gene is confirmed at DNA level using DNA sequencing, PCR or any other suitable method.

11. Based on the analyses performed, check which of the sgRNA sequences was able to knock out the target gene more efficiently and choose those that presented the best result considering the observed phenotypes.

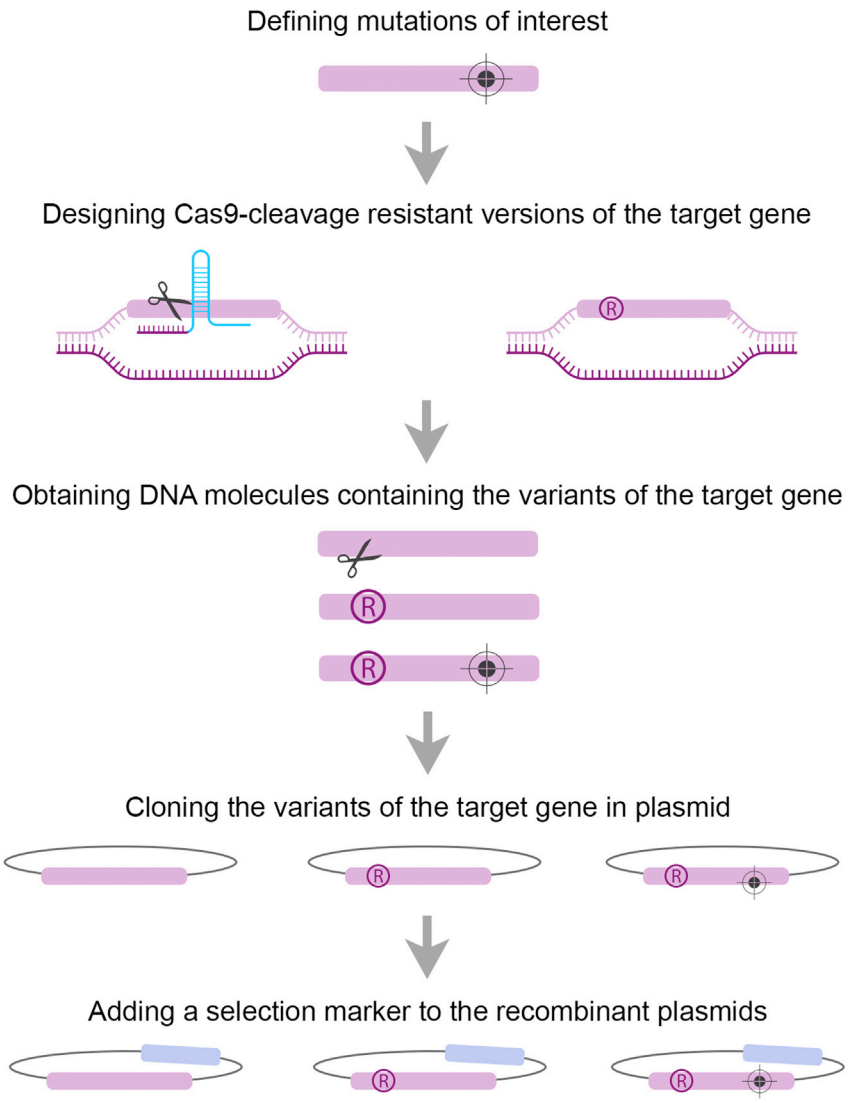

Figure 3. Representative scheme of the construction of plasmids containing the DNA repair templates of the target gene

Construction of plasmids carrying the DNA repair templates of the gene of interest for the complementation assays

Timing: 3 weeks

In this step, the objective is to obtain Cas9-cleavage resistant variants of the target gene that will be used as DNA templates in the complementation assays (Figure 3). A wild-type Cas9-resistant variant should be used as a control to determine the efficiency of knockdown complementation in parallel with test variants such as single point and deletion mutants.

12. Based on the information about the target protein such as the presence of catalytic sites, protein binding regions or other relevant feature, define the points of interest to be mutated in the gene.
    a. In this work, the tcDAC2-coding sequence containing mutation of the catalytic tyrosine 371 to phenylalanine (Y371F) was tested. This mutation was obtained previously by a fusion PCR strategy and cloned into an *E. coli* expression vector (Marek et al., 2021).

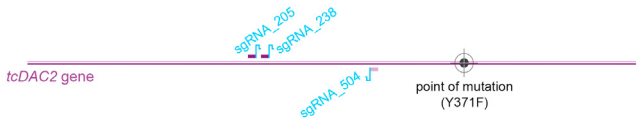

Figure 4. Location of tested sgRNAs and mutation of interest

13. Compare the previously selected sgRNA sequence (step 11) and choose the best one considering its position so that it does not overlap with any of the desired changes in the target sequence (Figure 4).
    a. In this work, the sgRNA_238 was chosen.
14. Design molecules resistant to Cas9 cleavage by inserting silent mutations into the sgRNA recognition site so that it is no longer recognized and cleaved by the Cas9 endonuclease but keeping the original amino acid sequence unchanged (Figure 5).

Note: In this work, 6 mismatches were inserted to prevent sgRNA recognition. Although good to be conservative, the silent mutations can result in rare codons that would hinder protein production. Alternatively, it is possible to introduce a silent mutation in the PAM sequence.

15. Design primers to amplify all the DNA templates including:
    a. all mutations of interest previously defined to investigate gene function that, in addition, are resistant to Cas9 cleavage (such as tcDAC2_Y371F of this work);
    b. a Cas9-cleavage resistant copy that serves as positive control since it encodes a functional protein with no changes in the original amino acid sequence, and replaces the knocked-out gene (such as tcDAC2_resist of this work);

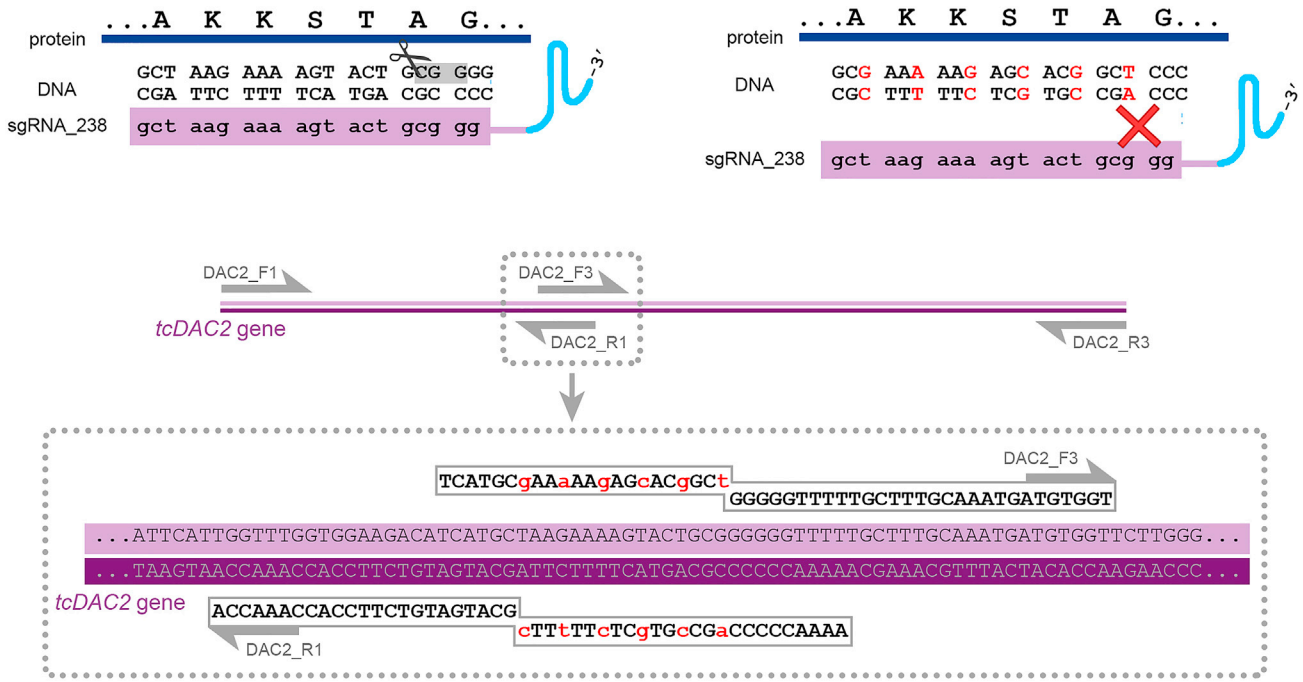

Figure 5. Design of Cas9-cleavage resistant molecules

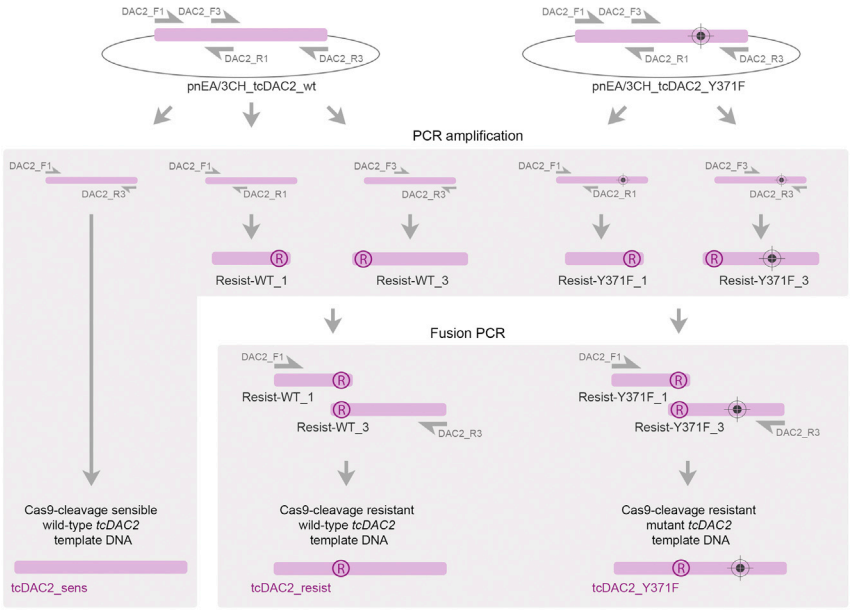

Figure 6. Obtention of DNA repair templates of the variants of the gene of interest by PCR amplification

c. a Cas9-cleavage sensitive copy that serves as knockout control since it has no alteration in the original gene sequence being cleaved by Cas9 at the same time that genomic *TcDAC2* copy (such as tcDAC2_sens of this work).

16. Obtain the designed primers.
    a. Specific primers used in this work are listed in key resources table as DAC2_F1, DAC2_R1, DAC2_F3 and DAC2_R3.

Alternatives: It is possible to purchase the entire DNA fragments from DNA synthesis companies.

17. Amplify DNA fragments by PCR following the instructions below (Figure 6 – PCR amplification).

PCR primers and DNA template			
Fragment ID	Forward primer	Reverse primer	Input DNA
Resist-WT_1	DAC2_F1	DAC2_R1	pnEA/3CH_tcDAC2_wt
Resist-WT_3	DAC2_F3	DAC2_R3	pnEA/3CH_tcDAC2_wt
Resist-Y371F_1	DAC2_F1	DAC2_R1	pnEA/3CH_tcDAC2_Y371F
Resist-Y371F_3	DAC2_F3	DAC2_R3	pnEA/3CH_tcDAC2_Y371F
tcDAC2_sens	DAC2_F1	DAC2_R3	pnEA/3CH_tcDAC2_wt

PCR reaction master mix	
Reagent	Amount
SuperFi Buffer	1×
SuperFi GC Enhancer	1×
dNTP mix	0.2 mM each
Forward primer*	0.5 µM
Reverse primer*	0.5 µM
Input DNA*	1 ng
Platinum SuperFi DNA Polymerase	0.5 U
ddH_2O	to 25 µL

*Note: Use specific primers and plasmid input DNA for each construction as defined above.

PCR cycling conditions			
Steps	Temperature	Time	Cycles
Initial Denaturation	98°C	30 s	1
Denaturation	98°C	10 s	35 cycles
Annealing	56°C	20 s	
Extension	72°C	30 s/Kb	
Final extension	72°C	5 min	1
Hold	4°C	forever	

18. Verify PCR product amplification using agarose gel electrophoresis.
19. Purify PCR products using a PCR Purification Kit.
    a. The Nucleospin Gel and PCR Clean-up Kit was used in this work with the following modification:
        i. Elution is done twice with 15 µL of NE buffer preheated at 70°C.

Optional: Fragments can be purified from agarose gel using the same Nucleospin Gel and PCR Clean-up kit. Exposure to UV light should be minimized to avoid formation of pyrimidine dimers.

20. Verify PCR product purification using agarose gel electrophoresis.
21. Combine fragments to reconstitute the complete genes containing the Cas9 resistant site by fusion PCR (Figure 5 – Fusion PCR).

Input DNA		
Fragment ID	Fragment 1	Fragment 2
tcDAC2_resist	Resist-WT_1	Resist-WT_3
tcDAC2_Y371F	Resist-Y371F_1	Resist_Y371F_3

PCR reaction master mix	
Reagent	Amount
SuperFi Buffer	1×
SuperFi GC Enhancer	1×
dNTP mix	0.2 mM each
Forward primer - DAC2_K7_F1	0.5 µM
Reverse primer - DAC2_K7_R3	0.5 µM
Input DNA*	1 ng
Platinum SuperFi DNA Polymerase	0.5 U
ddH_2O	to 25 µL

*Note: Input DNA in the fusion PCR is the combination of fragments previously generated in first reactions. Combinations are defined above in the "input DNA" table.

PCR cycling conditions			
Steps	Temperature	Time	Cycles
Initial Denaturation	98°C	30 s	1
Denaturation	98°C	20 s	35 cycles
Annealing	56°C	45 s	
Extension	72°C	5 min	
Final extension	72°C	10 min	1
Hold	4°C	forever	

Note: PCR performed in a Proflex PCR System with the advanced option "simulating Eppendorf Mastercycler Gradient" to maintain previously used conditions of ramp rates.

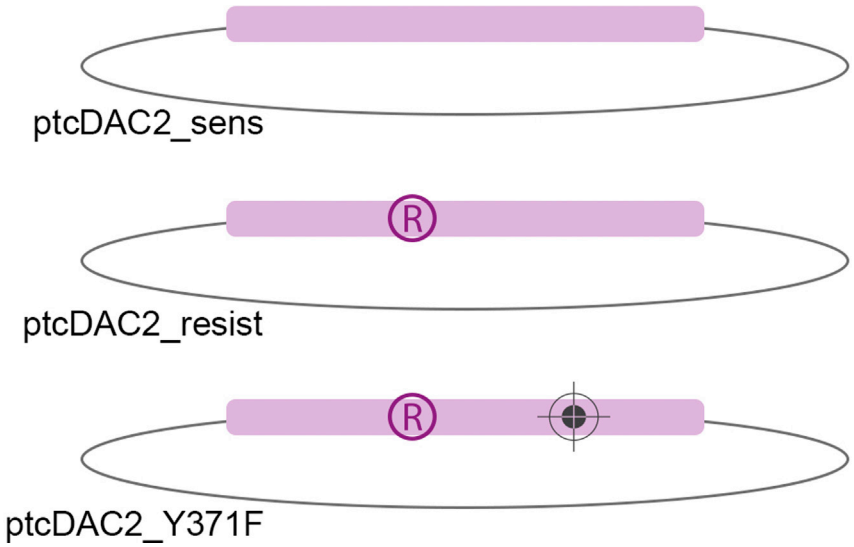

Figure 7. Cloning of the reconstitute gene variants to be used as templates in DNA recombination after Cas9 cleavage

22. Verify PCR product amplification using agarose gel electrophoresis. Troubleshooting 3.
23. Purify PCR products as indicated above (items 19 and 20).

24. Clone the reconstituted DNA templates of the gene of interest in a cloning vector (Figure 7).

Note: In this work, the DNA fragments of tcDAC2 variants were cloned into the pGEM-T easy vector.

a. If a high-fidelity DNA polymerase, like Platinum SuperFi, is used, it is necessary to add a 3′ A-tail in the PCR products before cloning in T-vector systems.

3′ A-tailing	
Reagent	Amount
Taq DNA Buffer	1×
dATP	0.2 mM
Taq DNA polymerase	0.25 U
PCR product	to 5 µL

Alternatives: If dATP alone is not available, it is possible to use dNTP mix in order to have a final concentration of 0.2 mM of each nucleotide.

i. Mix by pipetting.
ii. Incubate for 10 min at 72°C.

b. Ligate into the pGEM-T vector immediately.

pGEM-T ligation reaction	
Reagent	Amount
2× rapid ligation Buffer	1×
pGEM-T vector	25 ng
T4 DNA ligase	1 µL
PCR product	to 10 µL

i. Mix by pipetting.
ii. Incubate 12–16 h at 4°C.

Alternatives: It is possible to incubate ligation reactions for just 1 h at 20°C–25°C.

c. Transform DH5α *E. coli* competent cells with the ligation reaction.

Note: Use any suitable *E. coli* cloning strain with an efficiency of 5×10^6 UFC/μg DNA at least.
i. Mix the ligation reaction and competent cells and incubate 30 min on ice.
ii. Heat shock at 42°C for 1 min and 30 s and transfer to ice immediately for 2 min;

Alternatives: It is possible to heat shock cells for shorter time (20 sec).

iii. Add 1 mL of LB medium and incubate with agitation at 37°C for 1 h.
iv. Plate 100 and 200 μL of each transformation on LB plates containing 100 μg/mL ampicillin for selection.

d. Confirm the correct cloning directly from bacterial colonies by PCR using T7 and SP6 primers.
i. Pick a part of a bacterial colony with a toothpick;
ii. Rub it in the bottom of an 0.2 mL PCR tube;
iii. Prepare a PCR mix and distribute 15 μL in each tube.

Colony PCR

Reagent	Amount
Taq DNA Buffer	1×
dNTP mix	0.2 mM each
T7 primer	0.2 μM
SP6 primer	0.2 μM
Taq DNA polymerase	0.15 U
ddH_2O	to 15 μL

PCR cycling conditions

Steps	Temperature	Time	Cycles
Initial Denaturation	94°C	5 min	1
Denaturation	94°C	45 s	
Annealing	56°C	45 s	35 cycles
Extension	72°C	1 min/Kb	
Final extension	72°C	5 min	1
Hold	4°C	Forever	

iv. Visualize PCR products by agarose gel electrophoresis and choose 3 different clones of each construction.

e. Isolate plasmid DNA from the three positive clones using a plasmid preparation protocol.

Note: The Nucleospin Plasmid EasyPure kit without modifications was used in this work, but any plasmid purification protocol can be used.

f. Verify the nucleotide sequence by DNA sequencing.
g. Choose one clone of each construction (ptcDAC2_sens, ptcDAC2_resist and ptcDAC2_Y371F) to proceed to next steps.

25. Add a blasticidin-resistance cassette (bsd) in the selected plasmids containing the DNA repair templates of the variants of the gene of interest (Figure 8).

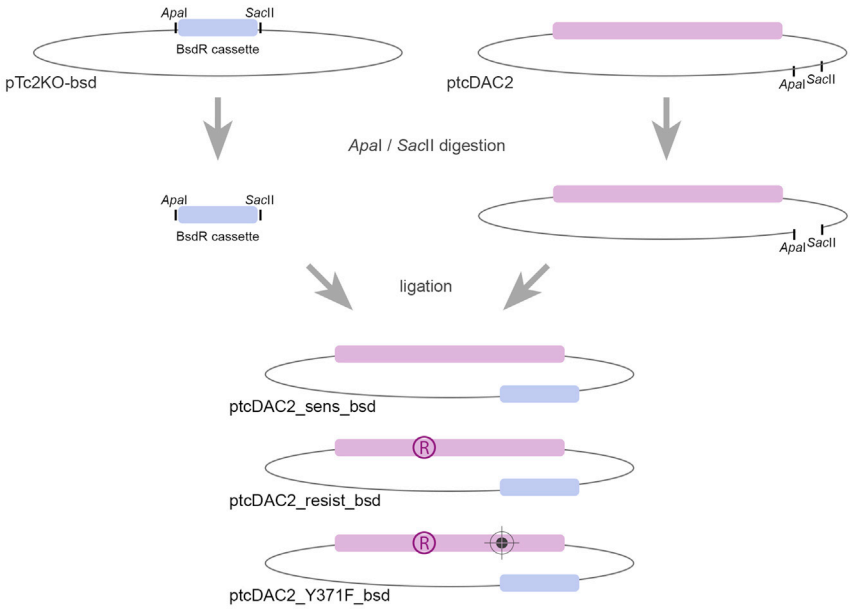

Figure 8. Insertion of blasticidin-resistance cassette in ptcDAC2 plasmids containing the DNA repair templates of the variants of the gene of interest

Note: ptcDAC2 plasmids are those obtained by cloning DAC2 gene variants (ptcDAC2_sens, ptcDAC2_resist and ptcDAC2_Y371F).

Note: The bsd-cassette should contain the blasticidin S deaminase gene flanked by *T. cruzi* intergenic sequences for proper expression.

Alternatives: In this work, the bsd-cassette is obtained from pTc2KO-bsd since it provides the bsd gene with flanking sequences, but any other suitable plasmid can be used.

a. Digest pTc2KO-bsd and the previously obtained ptcDAC2 plasmids (ptcDAC2_sens, ptcDAC2_resist and ptcDAC2_Y371F) with *Apa*I and *Sac*II restriction enzymes.

*Apa*I + *Sac*II restriction digestion

Reagent	Amount
CutSmart Buffer	1×
Plasmideal DNA	10 µg
NEB *Apa*I (50 U/µL)	1 µL
ddH_2O	to 30 µL

b. Incubate for 2 h at 25°C.
c. Add 20 U of NEB *Sac*II (20 U/µL) restriction enzyme, transfer to 37°C and incubate for 2 h.
d. Purify bsd-resistance cassette and ptcDAC2 plasmid fragments from agarose gel. The Nucleospin Gel and PCR Clean-up Kit was used in this work with the following modification:
   i. Elution is done twice with 15 µL of NE buffer preheated at 70°C.
e. Verify PCR product purification by agarose gel electrophoresis.
f. Measure DNA concentration.
g. Ligate the ptcDAC2 plasmids with the bsd-resistance cassette.

ptcDAC2 and bsd-resistance cassette ligation

Reagent	Amount
5× ligation Buffer	1×
bsd-resistance cassette	500 ng
Receptor plasmid	50 ng
T4 DNA ligase (1 U/μL)	1 μL
ddH_2O	to 10 μL

i. Mix by pipetting.
ii. Incubate 12–16 h at 16°C.

h. Transform *E. coli* competent cells (as indicated in step 24c).

i. Confirm correct cloning directly from bacterial colonies by PCR using T7 vector primer and a bsd-cassette specific primer.
   i. Pick a part of a bacterial colony with a toothpick;
   ii. Rub in the bottom of a 0.2 mL PCR tube;
   iii. Prepare a PCR mix and distribute 15 μL in each tube.

Colony PCR

Steps	Temperature	Time	Cycles
Initial Denaturation	94°C	5 min	1
Denaturation	94°C	45 s	35 cycles
Annealing	56°C	45 s	
Extension	72°C	1 min/Kb	
Final extension	72°C	5 min	1
Hold	4°C	forever	

PCR cycling conditions

Reagent	Amount
Taq DNA Buffer	1×
dNTP mix	0.2 mM each
T7 primer	0.2 μM
bsdR primer	0.2 μM
Taq DNA polymerase	0.15 U
ddH_2O	to 15 μL

iv. Visualize PCR products (~ 0.9 kb) by electrophoresis in a 1% agarose gel. Troubleshooting 4.

j. Aiming to recover a larger amount of high concentration plasmid DNA, isolate positive clones using an improved plasmid purification protocol (Pronobis et al., 2016) with modifications:
   i. Inoculate a single colony in 20 mL of appropriate selective media and incubate under agitation at 37°C for 12–16 h.
   ii. Transfer the culture to a centrifuge tube and spin at 4,000 × *g* for 10 min. Discard the supernatant.
   iii. Suspend the cell pellet in 2 mL of P1 resuspension solution freshly supplemented with 500 μg/mL of RNase A.
   iv. Add 2 mL of P2 lysis solution. Mix gently 4 times. Incubate 3 min at 20°C–25°C.
   v. Add 2 mL of P3 neutralization solution. Mix gently.
   vi. Centrifuge at 13,200 × *g* for 10 min at 20°C–25°C.
   vii. Transfer the supernatant to a clean 15 mL tube, taking care not to carry the precipitate. Add 1 volume of 96% ethanol and mix well by inversion.

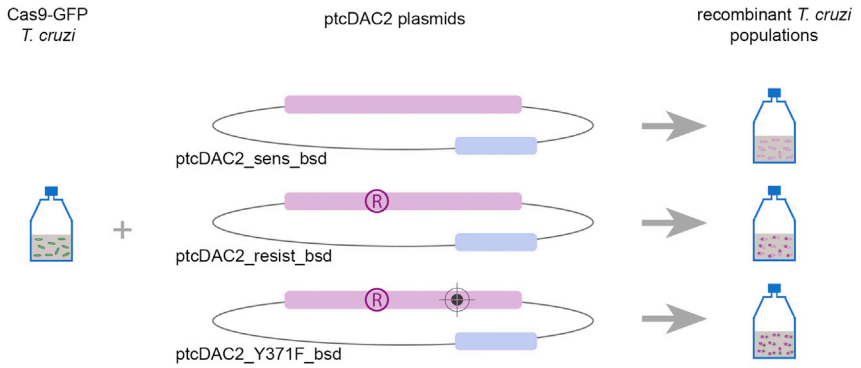

Figure 9. Generation of *T. cruzi* clones bearing the tcDAC2 DNA repair template plasmids for subsequent recombination after Cas9 cleavage

viii. Load 700 μL aliquots on a spin-column and centrifuge 30 s at 13,200 × *g*. Discard flow-through. Repeat loading on the same spin-column and centrifugation until all sample is loaded.
ix. Wash using 700 μL of washing buffer and centrifuge at 13,200 × *g* for 30 s at 20°C–25°C. Discard the flow-through.
x. Wash again using 500 μL of washing buffer and centrifuge at 13,200 × *g* for 1 min at 20°C–25°C. Discard the flow-through.
xi. Centrifuge again at 13,200 × *g* for 1 min to remove all the remaining ethanol. Discard the flow-through.
xii. Transfer the spin-column to a new 1.7 mL microcentrifuge tube.
xiii. Add 70 μL of elution buffer preheated at 70°C and incubate for 2 min.
xiv. Centrifuge at 13,200 × *g* for 1 min at 20°C–25°C.
xv. Repeat steps xiii and xiv 3 more times.

Optional: Add 10 μg/mL of RNase A to the DNA solution.

xvi. Measure DNA concentration.

Note: In this work, the Nucleospin Plasmid EasyPure columns was used, but any plasmid purification columns can be used.

Alternatives: It is possible to invert the cloning strategy, making it simpler by cloning first bsd-resistance cassette and then the variants of the target gene. It is also possible to acquire commercially all DNA sequences already inserted in a plasmid.

Obtaining *T. cruzi* clones bearing the tcDAC2 DNA repair template plasmids

Timing: ~4 weeks

In this step, the objective is to obtain *T. cruzi* clones bearing the DNA repair template of each tcDAC2 variants (Figure 9).

26. Collect early-log phase *T. cruzi* epimastigotes stably expressing Cas9-GFP by centrifugation at 20°C–25°C for 5 min at 3,000 × *g*.
    a. Use 5 × 10^6 cells for each transfection.
27. Wash in sterile PBS (pH 7.4). Spin down for 5 min at 3,000 × *g*.
28. Recover cells in transfection buffer.
    a. Use 100 μL of buffer for 5 × 10^6 cells.

29. Mix 100 μL of cells and 25 μg of each tcDAC2 plasmid in the test tube.
    a. In this work, the following plasmids were used:
        i. ptcDAC2_Y371F_bsd: a plasmid containing a tcDAC2 variant resistant to Cas9 cleavage and a bsd-resistance cassette.
        ii. ptcDAC2_resist_bsd: a plasmid containing a tcDAC2 copy resistant to Cas9 cleavage and a bsd-resistance cassette.
        iii. ptcDAC2_sens_bsd: a plasmid containing a tcDAC2 copy sensitive to Cas9 cleavage and a bsd-resistance cassette.

Note: It is important that the DNA volume added does not exceed 20% of the initial volume.

30. For a negative control of the transfection, mix 100 μL of cells and 15 μL of TE buffer in the negative control tube.
31. Transfer each mixture to an individual electroporation cuvette and perform electroporation using the X-014 program in an Amaxa Nucleofector Device.
32. After one electric pulse, transfer the cells to a sterile 15 mL conical tube containing 3 mL of LIT medium supplemented with 10% FBS. Incubate at 28°C.
33. At 24 h post-transfection, add G418 (final concentration of 250 μg/mL) and blasticidin (final concentration of 12.5 μg/mL) to the cultures.
34. At 72 h post-transfection, dilute the cultures in sterile 15 mL conical tubes using 500 μL of the transfection culture in 2.5 mL of LIT medium supplemented with 10% FBS containing 250 μg/mL of G418 and 12.5 μg/mL of blasticidin.
35. Repeat dilution every 3 days to maintain the cultures under selection until the transfectant selection is completed.
    a. Alternatively, when culture density reaches 1×10^7 cells, transfer 1×10^6/mL cells to a new sterile 15 mL conical tubes containing LIT medium supplemented with 10% FBS and 250 μg/mL of G418 and 12.5 μg/mL of blasticidin.

Performing complementation assays

Timing: 5 days

In this step, the objective is to verify if a variant version of the target gene is able to recover the wild-type phenotype lost after the knockout of the target gene. This is especially useful to define regions that are important to the target protein function since mutations that affect protein function will not recover the wild-type phenotype.

36. Transfect each of the *T. cruzi* cultures bearing the variants of the gene of interest with the specific sgRNA (Figure 10).
    a. Collect the early-log phase *T. cruzi* epimastigotes by centrifugation at 20°C–25°C for 5 min at 3,000 × *g*. Use 5×10^6 cells for each transfection:
        i. Cas9-GFP expressing *T. cruzi* clone bearing ptcDAC2_Y371F_bsd plasmid as DNA template for the catalytic site mutation replacement.
        ii. Cas9-GFP expressing *T. cruzi* clone bearing ptcDAC2_resist_bsd plasmid as DNA template for functional tcDAC2 replacement.
        iii. Cas9-GFP expressing *T. cruzi* clone bearing ptcDAC2_sens_bsd plasmid as DNA template for tcDAC2 knockout maintenance.
        iv. Parental *T. cruzi* cells expressing Cas9-GFP.
    b. Wash in sterile PBS (pH 7.4). Spin down for 5 min at 3,000 × *g*.
    c. Recover cells in transfection buffer.
        i. Use 100 μL of buffer for 5×10^6 cells.
    d. Prepare the sgRNA defined in step 13a as indicated in steps 4–8.
    e. Denature sgRNA by heating at 70°C for 5 min. Then, transfer immediately to ice.

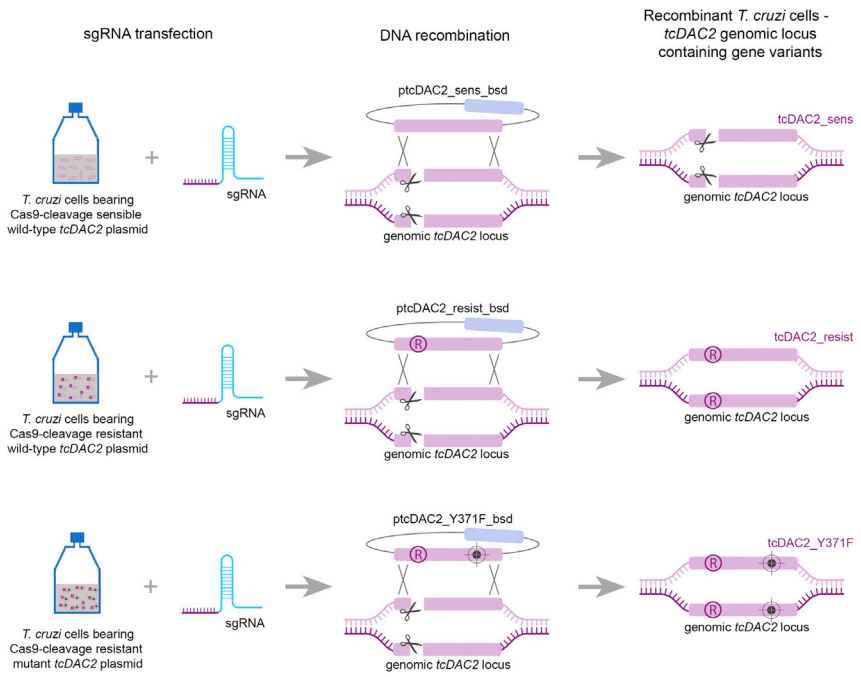

Figure 10. Complementation assay

f. Mix 100 μL of cells and 20 μg of sgRNA in each test tube.

Note: It is important that the sgRNA volume added does not exceed 20% of the initial volume.

g. For a negative control, mix 100 μL of the parental *T. cruzi* strain and 15 μL of TE buffer in the negative control tube.

h. Transfer each mixture to an individual electroporation cuvette and electroporate cells using the U-033 program in an Amaxa Nucleofector Device.

i. After one electric pulse, transfer the *T. cruzi* cells to a sterile 15 mL conical tubes containing 5 mL of LIT medium supplemented with 10% FBS. Maintain at 28°C.

37. Analyze the phenotypes daily for four days following the transfections as indicated in step 10 (Figure 11). Troubleshooting 5.

EXPECTED OUTCOMES

It is expected that knockout *T. cruzi* cells complemented with functional versions of the target protein present a phenotype identical to the wild type cells. Those supplemented with non-functional versions of the target protein, on the other hand, present the same characteristics as cells deficient for the target protein.

LIMITATIONS

The complementation assay is indicated to restore wild type phenotype after knockout of target genes or to define functional domains of the protein function. Therefore, it is essential that knockout cells show a detectable phenotype to be analyzed. Besides that, off-target effects could also generate similar phenotypes making analysis more complicated.

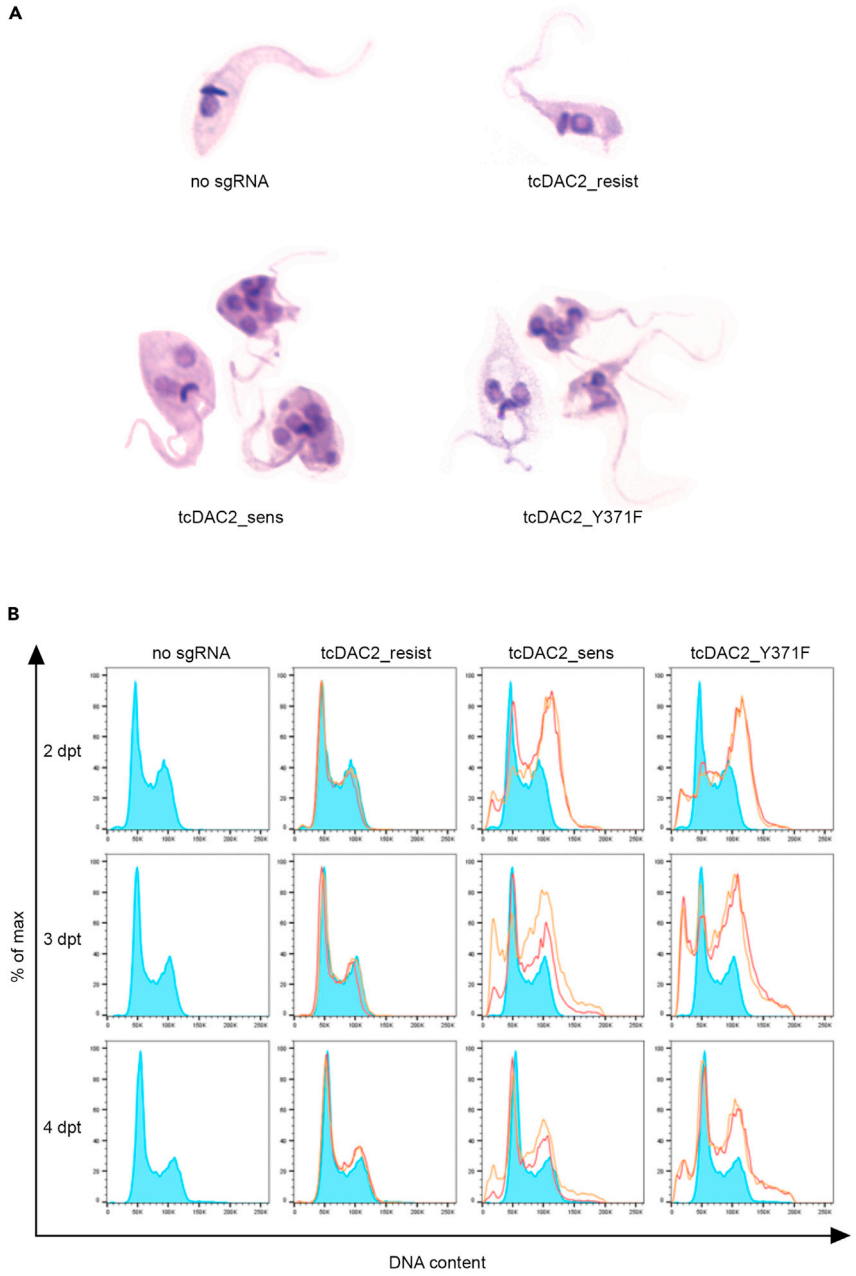

Figure 11. Phenotypes observed in wild-type and tcDAC2-deficient *T. cruzi* cells

(A and B) Deficient tcDAC2 cells present an abnormal phenotype with only one kinetoplast and multiple nuclei and flagella (A) suggesting proliferation defects and genome instability corroborated by the abnormal profile observed by cell cycle analysis (B). Cyan curves show wild-type cell profile and red and yellow line curves show mutant cell profiles. dpt – days post-transfection.

Another limitation is the time window for carrying out the analyzes when studying essential genes since knocked out *T. cruzi* cells end up dying while non-transfected cells remain viable and continue to divide and can overtake in number the transfected cells in a short period of time.

In addition, spontaneous undesired mutations of the target sequence can also abolish Cas9 targeting, leading to a high survival rate of wild-type population after targeting of essential genes.

In addition, loss of the complementation plasmid while maintaining resistance to the selection marker even though it is undesirable, could happen in *T. cruzi*, compromising the experiments.

Another important limitation is that the proposed methodology is suitable only for target genes that are essential for *T. cruzi* epimastigotes or with a visible mutant phenotype.

This is a method that allows a rapid analysis of essential genes by gene editing (knockout and gene complementation), because the mutant populations are analyzed just after transfection with sgRNA (4 days). However, stable or clonal populations are not obtained.

TROUBLESHOOTING

Problem 1

No PCR product obtained (Major step 5).

Potential solution

Check DNA template integrity.

Problem 2

No detectable effect seen when knocking out the target gene (Major step 10).

Potential solution

Check *T. cruzi* cell viability after transfection. If less than 80% of the cells remain alive, repeat transfection process using new cuvettes and solution (step 9).

Check sgRNA quality in steps 8i and j. If the sgRNA is degraded or contaminated, repeat from step 4.

If the sgRNA quality is ok, choose new sequences to use as sgRNA (return to step 1).

Problem 3

No PCR product obtained in fusion PCR (Major step 22).

Potential solution

Check DNA templates integrity.

Check if overlapping regions are present to allow annealing and extension of fusion PCR.

Problem 4

No PCR product obtained in colony PCR (Major step 25i).

Potential solution

Ensure that a small part of the colony has been rubbed into the bottom of the 0.2 mL PCR tube.

Problem 5

Expected outcomes are not observed in complementation assays (Major step 37).

Potential solution

Check if *T. cruzi* transfected cells are still bearing DNA template plasmids. If not, repeat from step 26.

Check the sgRNA sequence to knockout the target gene.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Nilson Ivo Tonin Zanchin (nilson.zanchin@fiocruz.br).

Materials availability

All unique/stable materials generated in this study will be made available upon request but may require a complete Materials Transfer Agreement if there is potential for commercial applications.

Data and code availability

This study did not generate dataset or original code. Any additional information required is available from the lead contact upon request.

ACKNOWLEDGMENTS

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AUTHOR CONTRIBUTIONS

Conceptualization and methodology, G.F.A.P.-C., M.M., C.R., and N.I.T.Z.; formal analysis, G.F.A.P.-C. and P.M.H.; investigation, G.F.A.P.-C., P.M.H., M.M., V.Z.R., and E.P.G.-S.; writing – original draft, G.F.A.P.-C.; writing – review & editing, G.F.A.P.-C. and N.I.T.Z.; funding acquisition, G.F.A.P.-C., C.R., and N.I.T.Z.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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