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TITLE:

Use of LeishGEdit CRISPR-Cas9 technology for genetic manipulation of protozoan parasite *Leishmania*

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CRISPR-Cas9; *Leishmania*; gene tagging; gene deletion; transfection; mutants.

SUMMARY

The establishment of a robust CRISPR-Cas9 methodology for genetic manipulation of *Leishmania* has accelerated the understanding of key biological processes of this parasite. Here, we describe in detail all the steps to generate knockout or *in situ* fluorescent-tagged parasites of virtually any gene of interest using LeishGEdit methodology.

ABSTRACT

The cell biology of a parasitic protozoan as well as the impact of the infection in host cells can be addressed using modification of their genomes. The development of robust methods eases the burden to obtain gene mutants and contributes to answer specific biological questions. Here we describe the LeishGEdit CRISPR-Cas9 high-throughput method that allows for *Leishmania* in situ gene tagging and deletion in a short span of time (7-10 days). Briefly, a transgenic cell line expressing SpCas9 and T7 RNA polymerase serves as the background for electroporation of DNA fragments generated by PCR: (1) a fragment containing a T7 promoter and the gene specific guide RNA expressed with a Cas9 scaffold; and (2) a homologous recombination (HR) fragment to

introduce a resistance marker and/or a fluorescent tag/epitope to the desired genome location. Our protocol will cover (1) primer design, (2) DNA fragment production and confirmation, (3) transfection, and (4) cell line confirmation methods. We hope the article will allow for easy reproduction of the protocol for genome manipulation by CRISPR-Cas9 and make the method largely available to the parasitology community, enabling advances in the understanding of the biology of *Leishmania* and other protozoan pathogens of medical and veterinary importance.

INTRODUCTION

The leishmaniases are a group of neglected tropical diseases present in nearly 100 countries, caused by more than 20 species of parasites from the genus *Leishmania*. The disease can manifest as a self-healing cutaneous lesion, mucocutaneous lesion, or visceral disease, which if not treated can be fatal. According to the World Health Organization (WHO), around 1 million of new cases of cutaneous leishmaniasis and 50,000-100,000 cases of visceral leishmaniasis are reported annually, resulting in 20,000-30,000 deaths per year[1]. During its life cycle *Leishmania* shifts between an invertebrate and a vertebrate host, forcing the parasite to adapt to different environmental conditions to survive and establish the infection[2]. The mechanisms used by *Leishmania* to adapt to these conditions are still poorly understood and the application of methodologies that allow the genetic manipulation of the parasite can contribute to the understanding of the cellular pathways involved in these mechanisms. Indeed, this might also contribute to the identification of drug targets to the development of new and needed treatments for leishmaniasis.

The postgenomic era had significantly increased the understanding of *Leishmania* biology coupled to the development of genetic manipulation tools. For several years, attempts to genetically manipulate *Leishmania* were restricted to the use of homologous recombination-based gene replacement[3]. This significantly limited the success in obtaining gene deletion mutants, due to the need of at least two rounds of transfection and the compensatory effects in the parasite over time, reflected in the few examples of genes subjected to loss of function studies until recently[4]. Also, attempts to generate *Leishmania* null mutant parasites frequently resulted in the amplification of the gene of interest (GOI), even after several rounds of transfection[5]; chromosome copy number variation is a common compensatory adaptation mechanism that occurs in *Leishmania* in response to environmental changes[6, 7]. Fortunately, with the advent of Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) technology this scenario has rapidly changed and these numbers have increased to more than 500 genes so far investigated[8–13].

Here we describe the CRISPR-Cas9 method recently developed by Eva Gluenz's group, called LeishGEdit, that was applied successfully in Old World and New World *Leishmania* species[14,15]. The method is based on the transfection of a cell line expressing *Streptococcus pyogenes* Cas9 (SpCas9) nuclease and T7 RNA polymerase constitutively. The *L. major* ß-tubulin sequence was used for SpCas9 integration, and is compatible with most species of *Leishmania*, but depending on the conservation between the homologous loci of ß-tubulin this would need to be adapted. Alternatively, pTB007 can be transfected as a stable circular episome, as recently demonstrated in *L. braziliensis*[16]; the selection of transfectants can be performed using hygromycin B. The

suitable antibiotic concentration will largely depend on the *Leishmania* species and specific strains and must be determined through titration curves prior to the experiments. It is important to mention that *L. mexicana* was used as the reference species for all steps described in this protocol. The genetic modification is performed by electroporation of two DNA fragments generated by PCR; one corresponding to the cassette for expression of the guide RNA (sgRNA) from a T7 RNA polymerase promoter that determines the exact region where Cas9 will insert the double strand break on the DNA molecule (specific for the targeted gene); and the repair template, amplified from plasmids containing the marker gene for selection. This approach has been applied to *in situ* gene tagging and deletion of hundreds of genes of *Leishmania mexicana*, *Leishmania braziliensis*, *Leishmania donovani, Leishmania major*, *Leishmania infantum*[7, 9–11, 16] and *Trypanosoma brucei*[14]; from DNA production/transfection, selection and validation, mutants (gene knockout and *in situ* tagging) can be produced in approximately 20 days[14].

One of the many advantages of LeishGEdit toolbox is the availability of a bank of plasmids to be used as templates to generate the transfection DNA fragments by PCR, herein named repair template, for homologous recombination (HR) of tags at the 5'or 3'-end of the GOI or to delete genes or locus of *Leishmania*'s genome. There are different plasmids for several experimental setups (e.g.: fluorescent tags, bioluminescent proteins, biotin ligase for proximity labelling assays, etc) and a number of resistance markers. The system also makes available a primer design script that can be accessed online (http://www.leishgedit.net/Home.html), which design primers compatible with the plasmid series (pPLOT and pT). Previously, it was shown that at least 100 nucleotides (nt) were required to allow for homologous recombination in wildtype *Leishmania*[17]. Using CRISPR-Cas9, repair templates containing target-specific 30 nt homology flanks are enough to promote homologous recombination allowing for addition of those regions into oligonucleotides, followed by common primer binding sites of pPLOT and pT, such that a single set of primers enables generation of gene deletion and fluorescent mutants, for example. In order to facilitate homologous recombination, Cas9 requires single guide RNAs for precise introduction of double strand breaks (DSB) into the genome. In LeishGEdit the system uses *Leishmania* heterologous expression of T7 RNA Polymerase and requires transfection of a PCR product produced using a forward primer containing the T7 promoter, the DSB target sequence without the PAM region, and a complementary SpCas9 scaffold, to be annealed with a universal reverse primer containing the entire SpCas9 recognition site of the final sgRNA (for sequence, please consult the materials section). Transcription from the T7 promoter begins with the GG adjacent to the target sequence, thereby extending the sgRNA by 2nt.

The LeishGEdit primer design tool provides six primer sequences for each given GOI:

(1) A primer with 30 nt for recombination upstream the GOI (**upstream forward primer**);
(2) A primer with 30 nt for recombination immediately downstream the GOI start codon (**upstream reverse primer**);
(3) A primer containing a sgRNA for DSB insertion at the 5' UTR of the GOI (**5' sgRNA primer**);
(4) A primer with 30 nt for recombination downstream the GOI (**downstream forward primer**);
(5) A primer with 30 nt for recombination immediately upstream the GOI stop codon

(**downstream reverse primer**);

(6) A primer containing a sgRNA for DSB insertion at the 3' UTR of the GOI (**3' sgRNA primer**)

Although different CRISPR-Cas9 methods have been used for genetic manipulation of *Leishmania* parasites, varying from constitutive to transient expression of Cas9 and sgRNA; *in vitro* sgRNA transcription; transfection of recombinant Cas9-sgRNA complex (reviewed in[18]), the LeishGEdit methodology introduced here has been proven to be the most effective[8, 13-15]. One great advantage of this method is that there is no need for molecular cloning, PCR purifications or *in vitro* transcription steps prior to transfection, which allows generation of mutant parasites in a short span of time. Indeed, a collection of plasmids bearing different selection marker genes and/or "tags" (fluorescent or not), are available as templates to obtain the specific DNA fragments (repair cassettes) for gene deletion or *in situ* gene tagging. More information about this plasmid collection can be found at LeishGEdit online platform (http://www.leishgedit.net).

Since the establishment of LeishGEdit, two main improvements have been developed: (1) the possibility to introduce a barcode in the locus of the GOI for further phenotypic analyses using a large cohort of mutants, instead of performing individual experiments[8–10, 13]; and (2) the design of an inducible system combining the CRISPR-Cas9 and DiCre recombinase advantages that allow the study of essential genes[18, 1920]. Though we have performed these methodologies in our laboratory, we will not describe them here and for more information, please consult references[18, 21].

Thus, thanks to the LeishGEdit methodology, gene replacement in *Leishmania* has rapidly progressed from being cumbersome and time consuming to relatively straightforward, contributing to the understanding of key biological processes for this parasite. In this article, we provide a step-by-step protocol to facilitate its proper implementation and use for genetic manipulation of the parasite[9, 11–13, 16, 22, 231].

PROTOCOL

1. Primer design for knockout and *in situ* tagging

1.1. To design GOI specific primers, enter the Tritrypdb (https://tritrypdb.org/tritrypdb/app) gene ID at the LeishGEdit website (www.leishgedit.net). First, select the option **Primer Design** and choose the strategy (N-terminal tagging; C-terminal tagging; Knockout; or Tagging and knockout) and the plasmid system (pT and pPLOT plasmids). See below the combination of primers necessary to generate the choice of mutants:

1.1.1. For tagging (whether at the N- or C-terminus), use 3 primers:
A 5'sgRNA primer or 3'sgRNA primer (for generating the DSB in 5' or 3' UTR, respectively);
An upstream forward primer and upstream reverse primer (for generating the protein tag in frame with the corresponding gene in the N terminus);
A downstream forward primer and downstream reverse primer (for generating the protein tag in frame with the corresponding gene in the C terminus).

1.1.2. For gene disruption/deletion, use 4 primers:
A 5'sgRNA primer and 3'sgRNA primer (for generating the DSB in 5' and 3' UTR of the gene/locus, respectively);
An upstream forward primer (with a 30 nt homology arm located upstream to the 5' DSB)
A downstream reverse primer (with a 30 nt homology arm located downstream to the 3' DSB)

2. Transfection DNA preparation

2.1. For transfection, generate the products for both the repair and the single guide RNA (sgRNA) templates by PCR. During PCR preparation, keep all reagents on ice.

2.1.1. For amplification of the 5' and 3' sgRNA templates, prepare the following reaction: 2 µM of Reverse primer; 2 µM of Forward primer; 250 µM of dNTPs Mix; 3.5 mM of $MgCl_2$; 5 U of High-fidelity DNA Polymerase; and H_2O Milli-Q sufficient for 20 µL of reaction.

2.1.1.1. Use the following thermocycler - PCR reaction conditions: 98 °C for 30 s; 35 cycles of 98 °C for 10 s, 60 °C for 30 s, 72 °C for 15 s; then 72 °C for 10 min.

2.1.2. For amplification of the HR fragment use the conditions below: 2 µM of Reverse primer; 2 µM of Forward primer; 125 µM of dNTPs Mix; 2 mM of $MgCl_2$; 30 ng of plasmid (template); 5 U of High-fidelity DNA Polymerase; and H_2O Milli-Q sufficient for 40 µL of reaction.

2.1.2.1. Use the following thermocycler - PCR reaction conditions: 98 °C for 30 s; 35 cycles of 98 °C for 10 s, 60 °C for 30 s, 72 °C for 2 min 15 s; then 72 °C for 10 min.

2.1.3. After PCR reactions, confirm the correct amplification of DNA fragments by running part of the samples (2 µL) on agarose gels; 0.8% for repair templates and 1.5% for sgRNAs cassettes, to check the presence of the expected product. PCR product purification prior transfection is recommended, but not mandatory.

3. Transfection and cell cloning

3.1. Cell culture and transfection

3.1.1. Prepare *Leishmania* Cas9 T7Pol promastigote cell culture to be transfected. Cells must be in mid-logarithmic phase of proliferation (approx. 5.0-8.0 x 10^6 cells/mL), usually a 48-72 h culture diluted 1:50 or 1:100 (depending on the *Leishmania* species) from a healthy and dense culture. Add the appropriate concentration of hygromycin for each parasite species to maintain *Leishmania* Cas9 T7Pol mutants. For *L. mexicana* we recommend 50 µg/mL of hygromycin. Cell culture media appropriate to *Leishmania* must be used, as M199 or HOMEM supplemented with 10% or 20% of heat inactivated fetal calf serum (HI-FCS), depending on the *Leishmania* species.

3.1.2. Count parasites to obtain 2.0 x10^7 cells per electroporation, including enough for a negative control (cells transfected with sterile water).

3.1.3. Centrifuge cells at 1,000 x *g* for 10 min and wash it once with 1 mL of room temperature 1x PBS pH 7.4.

3.1.4. Resuspend cells in filter-sterilized transfection buffer (66.7 mM Na_2HPO_4, 23.3 mM NaH_2PO_4, 5 mM KCl, 50 mM HEPES pH 7.3 supplemented with 150 µM $CaCl_2$)[24]. Calculate the volume required as 150 µL or 200 µL per transfection for *in situ* tagging and gene KO, respectively. The DNA resuspended in sterile water or buffer must be considered in the final volume.

3.1.5. Transfer cells into 0.2 mm gap electroporation cuvettes containing DNA prepared in step 2. Heat-sterilize repair templates and sgRNA cassettes (95 °C for 10 min followed by chilling in ice). Remember to prepare a cuvette with sterile water for the negative control.

3.1.6. Place the cuvette in the electroporation device. There are protocols available for *Leishmania* electroporation using different machines[15, 25].

3.1.7. Immediately transfer 500 µL of pre-warmed culture media to electroporation cuvettes under sterile conditions, and then to 25 cm^2non-vented flasks containing 5 mL of appropriate culture media supplemented with 20% of HI-FCS.

3.1.8. Leave flasks on their sides to recover overnight at 26 °C.

3.2. Antibiotic selection and cloning

3.2.1. For tagging and double marker KO add the appropriate concentration of antibiotic(s) to the electroporated cultures for selection of transfectants. For *L. mexicana*, we recommend 10 µg/mL of blasticidin, 75 µg/mL of puromycin and 50 µg/mL of G418.

3.2.2. For single marker KO dilute the recovered culture 1:5 by adding 20 mL of culture media supplemented with 20% HI-FCS and appropriate concentration of selection antibiotic to the flasks. Reserve 2.5 mL in a 50 mL tube for further dilution.

3.2.3. Dilute the reserved 2.5 mL culture (1:5) by adding 22.5 mL of culture media supplemented with 20% HI-FCS and appropriate concentration of selection antibiotic for a final dilution of 1:50. Reserve 2.5 mL of the 1:50 dilution and repeat step 3.2.3 for a final dilution of 1:500.

3.2.4. Distribute the dilutions (1:5; 1:50; and 1:500) into three 96 well flat clear bottom plates by dispensing the culture in sterile reagent reservoirs and transferring 200 µL/well using a multichannel micropipette.

3.2.5. Seal the plates with parafilm tape to avoid evaporation and pool the remains of the diluted cultures into a flask to be treated as a population to control for cell growth and recovery.

This culture will not be used for further analysis, but can be diluted for 1 cell/well and plated after recovery in case no clones grow in the 1:5, 1:50 and 1:500 dilution plates.

3.2.6. Incubate cultures at 26 °C, with flasks on their sides and plates in a wet-chamber to avoid wells to dry during selection. Populations (*in situ* tagging and double marker KO) must recover within 5-10 days, which may vary depending on the *Leishmania* species.

3.2.7. After recovery (7-10 days), split population (1:10) into fresh culture media and resistance antibiotics before further analysis.

3.2.8. After 10-15 days clones can be detected in positive wells (observed by changes in cell culture media color and opacity) and cell growth can be attested by checking wells under the microscope. Aspirate the 200 µL and add to 5 mL culture media with resistance antibiotics. Always try to select clones from more diluted plates where clones can be seen, and check at least 5 clones for each intended mutant (details on Part 4 of this protocol).

NOTE: For null mutants, absence of recovery on population and cloning plates is an indication of gene essentiality. Transfections must be repeated to eliminate the possibility of technical failure.

4. Cell line confirmation

4.1. Confirmation of knockout cell lines

NOTE: The first step to confirm the knockout cell lines is to obtain total genomic DNA of the clones selected previously. For that, there are several approaches that can be used. We recommend using a commercial genomic DNA extraction kit (please refer to the manufacturer instruction). Alternatively, use a standard DNA extraction protocol, as described below. Remember to prepare the parental cell line genomic DNA as well.

4.1.1. Harvest the cells (~$1x10^8$ total) by centrifugation at 3,500 x *g* for 10 min.

4.1.2. Wash the pellet with 1x PBS.

4.1.3. Add 300 µL of TELT buffer (50 mM Tris-HCl, pH 8.0; 62.5 mM EDTA pH 8.0; 2.5 mM LiCl; 4% Triton X-100). Lyse the cells by pipetting up and down a number of times.

4.1.4. Add 300 µL of phenol/chloroform/isoamyl alcohol (25:24:1) and vortex vigorously for at least 30 s.

4.1.5. Centrifuge the mixture at 13,000 x *g* for 15 min.

4.1.6. Recover the aqueous phase and add 500 µL of 100% ethanol. Mix well and incubate at room temperature for 5 min.

4.1.7. Centrifuge at 13,000 x *g* for 5 min.

4.1.8. Discard the supernatant and wash the pellet with 1 mL of 70% ethanol.

4.1.9. Centrifuge at 13,000 x g for 5 min. Discard the supernatant.

4.1.10. Air dry the DNA at room temperature for 5-10 min.

4.1.11. Dissolve the DNA in TE (10 mM Tris-HCl, pH 8.0; 1 mM EDTA pH 8.0) or water.

4.2. Confirmation of *in situ* tagged cells

NOTE: There are a variety of fluorescent and non-fluorescent tags that can be used for *in situ* tagging and the methods used to confirm these cells will vary depending on the tag. For fluorescent tagged cells, a fast and easy way to confirm mutants is to perform flow cytometry, while the best way to confirm non-fluorescent tag is by Western blotting.

4.2.1. Flow cytometry confirmation

4.2.1.1. Harvest cells (parental and fluorescent tagged) by centrifugation at 3,000 x *g* for 5 min.

4.2.1.2. Wash the pellet with 1x PBS and resuspend at a density of 10^5-10^6 cells/mL with 1x PBS. The cell density is important to keep the narrow bores of the flow cytometer and its tubing from clogging up.

4.2.1.3. Set the equipment to collect 20,000 events inside the gate determined using the parental non-fluorescent cell line based on the forward scatter and side scatter of the population.

4.2.1.4. Run samples using the desired laser, which will vary depending on the fluorescent protein being expressed in tandem with the GOI.

4.2.1.5. Analyze the data comparing parental and fluorescent tagged cells to determine the percentage of positive cells (**Figure 3A**).

4.2.1.6. To increase the positive cells, depending on the population, perform cell sorting for enrichment.

4.2.2. Western blotting confirmation

NOTE: Western blotting assays will be mandatory to *in situ* tagging with non-fluorescent tags for cell line confirmation, and very important to confirm if the expression of protein fused to fluorescent tags generate the expected size. It is important to mention that all LeishGEdit plasmids for *in situ* tagging with fluorescent tags also have a c-myc epitope, easing the

confirmation of these cells by Western blotting with anti-c-myc antibody.

4.2.2.1. Harvest parasites (parental and tagged) by centrifugation at 3,000 x *g* for 5 min. For confirmatory Western blotting assays $1x10^7$ cells are enough to obtain good results.

4.2.2.2. Wash the pellet with 1x PBS and resuspend the cells with Sample Buffer (1x Laemmli buffer), and pipette up and down to lysis the parasites and obtain total protein extracts. For specific preparations, the use of a lysis buffer containing protease inhibitors may be required.

4.2.2.3. Boil samples at 95 °C for 5 min and load in an SDS-PAGE gel. Bis-acrylamide concentration of the resolving gel will be determined based on the size of the proteins to be analyzed.

4.2.2.4. After running the samples in SDS-PAGE gel, transfer the proteins to nitrocellulose or PVDF membranes. Use standard washing and incubation conditions with primary anti-c-myc tag antibody at suggested dilution of 1:2,500 and secondary antibody Goat Anti-Mouse (suggested 1:10,000). Secondary antibodies conjugated to peroxidase (HRP) can also be used. Our laboratory uses TBSt (Tris-buffered saline with 0.05% Tween-20) for washing steps and TBSt with 3% skimmed milk for blocking and dilution of the antibodies.

4.2.2.5. Probe membranes with loading control primary antibodies, usually a highly constitutively expressed protein as tubulin, actin or aldolase. Fluorescent quantitative systems will allow for concomitant incubation of both primary antibodies, as long as they were produced in distinct animals, as mouse and rabbit, for example. Perform detection by incubating membranes with correspondent secondary antibodies conjugated with red and green fluorophores. For HRP-conjugated secondary antibodies, strip membranes and repeat Western blotting for loading control detection.

4.2.2.6. Analyze the results using an available imaging system. It is expected to detect positive signals for samples from tagged cells and negative signals for parental cells (**Figure 3B**).

4.2.3. Fluorescence assays using *in situ* fluorescent tagged cells

NOTE: Another approach to confirm as well as analyze the localization of the *in situ* tagged protein is by fluorescence microscopy. There are two ways to do that: direct fluorescence microscopy or immunofluorescence using the anti-c-myc tag antibody. We prefer the former method in our initial analyses, as detailed below.

4.2.3.1. Collect $1x10^7$ cells (parental and fluorescent tagged) at mid-log phase by centrifugation at 2,000 x *g* for 3 min.

4.2.3.2. Wash the pellet twice with 1x PBS and resuspend in 500 µL of 1x PBS.

4.2.3.3. Fix the cells with paraformaldehyde 4% at room temperature for 15 min.

4.2.3.4. Wash three times with 1x PBS and resuspend cells in 500 µL of 1x PBS.

4.2.3.5. Spread cells on poly-L-lysine coated slides and incubate for 10 min.

4.2.3.6. Wash three times with 1x PBS and incubate with a DNA intercalant as Hoechst 33342 (10 µg/mL) or DAPI (5 µg/mL). Incubate for 15 min.

4.2.3.7. Wash three times with 1x PBS and mount the slides with PPD solution (Glycerol 90%+10% Tris HCl 30 mM, pH 8.0)

4.2.3.8. Analyze slides in an epifluorescence microscope.

REPRESENTATIVE RESULTS

The first step to generate knockout or *in situ* tagged cell lines of the GOI is to design the primers that will allow the preparation of the DNA fragments to be transfected for T7 RNAPol-based expression of sgRNAs in vivo, and the repair templates containing the desired tag and/or the selectable marker gene, to enable *in situ* tagging or gene deletion **(Figure 1A)**, respectively. **Figure 1B** shows the PCR products of two DNA fragments for sgRNA expression, targeting the 5' and 3' UTR region of GOI. **Figure 1C** represents the amplicon product of the repair template for N-terminus tagging the GOI with mNeonGreen fluorescent protein, obtained using the pPLOTv1 blast-mNeonGreen-blast plasmid as template. **Figure 1D**, shows two repair templates for gene deletion bearing the blasticidin (1.7 kb) and puromycin (1.8 kb) resistance genes, generated using pTBlast and pTPuro plasmids as template, respectively.

To demonstrate the efficiency of this method, we chose one of the genes we have been working in our lab, which is non-essential for procyclic viability in culture to obtain knockout and fluorescent tagged parasites. After transfection of the specific sgRNA and repair template fragments, we selected some parasites to confirm the generation of the desired cells. For gene deletion attempts, genomic DNA was extracted and used to perform diagnostic PCRs, using oligonucleotides annealing sequences upstream (Fwd – P1) and inside (Rev – P2) the GOI. The DNA obtained was used in PCR reactions to confirm the gene deletion mutants. There are different strategies to check for deleted mutants: 1) A single diagnostic PCR using primers binding upstream and downstream the coding sequence (CDS) and distinguish among wildtype, single allele deletion mutants and null mutants, based on the size of the fragment amplified; 2) Two diagnostic PCRs to verify the presence or absence of the wildtype gene and correct integration of the repair template containing the resistance gene. In our lab, we always give preference to use the second strategy, since it avoids complications as lengthy extension periods of PCR reaction, which may be required for long genes, and similar fragment sizes between the integrated repair template and the GOI.

For this strategy, at least two sets of primers are necessary, depending on the decision made during the transfection step (single or double marker). The first one is a forward primer (Fwd) upstream the region of recombination, usually at 5' UTR of GOI, and one reverse primer (Rev) for

any internal region of the wild-type gene. The second set will use the same Fwd primer from set 1, and a Rev primer annealing at the 5' end region of the selectable marker gene (*BSD*, *PAC* or *NEO*). **Figure 2A**, shows the primer design for the second strategy, which we chose to confirm the mutants presented here.

The first set of primers is expected to produce a positive PCR for the parental and single allele-knockout strains, and a negative PCR for null mutants. On the other hand, for the second set of primers, the PCRs will be negative for parental cell line and positive for single and double allele-knockouts. Using this approach, we were able to confirm the generation of single allele-knockouts or null mutants (both alleles replaced), as demonstrated in **Figure 2B**. The fluorescent tagged parasites were confirmed using two approaches, flow cytometry and Western blotting. The flow cytometry allows to determine the percentage of the mutant parasites that are fluorescent-positive after selection, and to quantify the fluorescence intensity of the population. After drug selection we obtained a population of about 90% of fluorescent parasites **(Figure 3A)**. To guarantee that the fluorescent tagged protein corresponds to the expected size, wildtype and fluorescent tagged cells were used to prepare protein extracts used to perform Western blotting with anti-c-myc antibody, since 3 copies of the c-myc epitope are also expressed in fusion with the fluorescent and the target proteins after endogenously tagging the GOI. As observed in **Figure 3B**, fluorescent tagged cell lines produced a single band corresponding to 93 kDa when membranes were probed with c-myc antibody.

We used the tagged cell lines to determine the protein subcellular localization in *Leishmania* procyclic promastigotes by confocal microscopy **(Figure 4)**. A clear nuclear signal was observed for the tagged protein and no signal was detected in the wildtype cells.

FIGURE AND TABLE LEGENDS

Figure 1. Generation of DNA cassettes of sgRNA and repair fragments of GOI for CRISPR-Cas9. **A.** Schematic representation of the sgRNA cassette (1) for transfection and *in vivo* expression of sgRNA by T7RNAPol to be recognized by SpCas9 and introduce double strand breaks (DSB) in the 5' and 3' UTR of the GOI. Here we also show the example of a repair template (2) for gene deletion containing the resistance marker, flanked by *Leishmania* sp. regulatory regions to guarantee robust expression of the drug resistance gene used for selection, and 30 nt for homologous recombination (HR) upstream and downstream the DSB introduced. **B-D**. Representative agarose gel of DNA fragments prepared for transfection. PCR-generated sgRNA templates targeting the 5' and 3' UTR regions of the GOI (B). PCR products of repair templates for *in situ* gene tagging with mNeonGreen fluorescent protein (C) and for the generation of double allele replacements (i.e. target gene deletion) with blasticidin (BSD) and puromycin (PAC) resistance marker genes (D).

Figure 2. Confirmation of gene knockout parasites generated using CRISPR-Cas9. **A.** Schematic representation of the GOI locus showing the oligonucleotides annealing regions used in diagnostic PCR experiments. **B.** Illustrative agarose gel of PCR products for knockout cell lines. As expected, for single allele-knockout parasites, both the GOI and selectable markers were amplified (-/+ BSD or -/+ PAC), indicating the GOI has a duplicated copy or may be refractory to

deletion (due to its suspected essential function) and the parasites managed to generate an extra copy of it to survive. In null mutants, both chromosomal alleles of the target gene were replaced (-/-) and only the drug resistance markers were detected.

Figure 3. *In situ* gene tagging confirmation using flow cytometry and Western blotting. **A**. Schematic representation of flow cytometry analyses of GOI in situ tagged with mNeonGreen fluorescent protein. The fluorescence intensity was measured comparing wildtype and tagged parasites, and higher fluorescent levels were observed in the tagged parasites (green) compared to wildtype (red), as shown in the left panel. About 90% of parasites were fluorescent in the mutant population (right panel). **B.** Western blotting detected the correct protein expressed in fusion with mNeonGreen and confirmed the expression of the tagged protein with the correct size (93 kDa – green band). No signal was observed in the wildtype protein samples. Anti-aldolase was used as loading control (red band). M1 and M2 represent the selected populations for analyses of fluorescent intensity in the flow cytometer.

Figure 4. Subcellular localisation of GOI *in situ* tagged with mNeonGreen protein using CRISPR-Cas9 methodology. Wild-type and mNeonGreen tagged promastigote parasites were used on fluorescence microscopy analysis to determine the subcellular localisation of the protein of interest. High nuclear signal from mNeonGreen protein (green) was detected in the nucleus. As expected, no signal was detected in the wildtype parasites. DAPI (blue). Scale bar: 5 µm.

DISCUSSION

Leishmaniasis is a global health problem affecting millions of people every year, but despite the availability of the genome sequence of several *Leishmania* species has been available for years, genetic manipulation of this parasite was restricted to time-consuming and low efficient methods. The emergence of CRISPR-Cas9 technology changed this scenario and is contributing substantially to the better understanding of *Leishmania* biology, and potentially allow the development of new treatments for leishmaniasis.

The step-by-step method presented here allows investigators to genetically manipulate different species of *Leishmania* in a short period of time, but as with any experimental procedure, has limitations and several critical steps that require careful execution. The first thing that can affect the time and efficiency to obtain mutants is the *Leishmania* species the investigator will use. Based on our experience, *L. mexicana* and *L. major* are the most prone species for genetic manipulation, while *L. braziliensis* is the most difficult to generate knockouts. The difficulty found in *L. braziliensis* can be explained by the presence of an active RNAi machinery in this parasite[26] that may have an effect on ectopic expression of integrated and episomal transgenes, as the Cas9.

After transfection, antibiotics for selection are added on the next day after microscopic evaluation to confirm cells are motile. Up to 50% of cell death is expected and samples must be compatible to the negative control to discard electroporation failure. As previously stated, different species and strains may require optimization of selection antibiotic concentration and it is recommended to establish the concentration that kills the *Leishmania* Cas9 T7Pol

promastigotes within 7 days, which must be tested prior to transfections. For *in situ* tagging one antibiotic will be used according to the repair template plasmid used, while deleted mutants (knockouts; KOs) can either be transfected for one or two repair templates with different resistance markers to insure concomitant two alleles deletion. In case a knockout wants to be obtained using one or two resistance marker single cell cloning is recommended to identify null mutants.

Another factor that will affect the efficiency of the method is the gene copy number and the number of alleles (allele dosage) of the GOI. The presence of trisomic and tetrasomic chromosomes has been reported in several species of *Leishmania*[27]. Previously, using deletion by homologous recombination-based gene replacement, reports of gene duplication after attempting to delete the first allele were frequent, and considered an evidence of essentiality of the gene[4]. Using CRISPR-Cas9 the deletion of *Leishmania* genes present in supernumerary chromosomes, were achieved in a single transfection step, using two selectable markers, but extra steps of transfection may be required[13]. We recommend limiting dilution and clone expansion specially for those cases. Also relevant are the differences in the genome sequence of reference strains compared to other strains, especially considering sgRNA design for specific DSB.

The generation of null mutants of essential genes is not possible with this method. However, after transfection with repair templates containing resistance genes, it is possible to split the transfected culture and select the cells in the presence of one antibiotic that will enable the generation of single allele-knockout strains for further phenotypic analyses. To investigate loss of function of essential genes it is possible to use the inducible deletion system (CRISPR-Cas9+DiCre) mentioned earlier[19].

The success of in *situ gene* tagging can be affected by the region, N- or C-terminus, that will be used for the insertion of the repair template containing the tag, and will vary considerably depending on the GOI function. If this information is not available in the literature, or predicted domains (e.g.: transmembrane; nuclear localization signal, etc) at either terminus are not informative, it is recommended to perform *in situ* tagging at both N- and C-terminus, which will increase the cost to obtain the mutants. The *T. brucei in situ* tag collection (http://tryptag.org) is also a valuable tool to obtain information about your GOI homologue, when available, and decide which region to tag.

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DISCLOSURES

The authors declare they have no competing financial interests.

REFERENCES

1. Website. at <http://www.who.int/leishmaniasis/en/>.
2. De Pablos, L.M., Ferreira, T.R., Walrad, P.B. Developmental differentiation in Leishmania lifecycle progression: post-transcriptional control conducts the orchestra. *Current opinion in microbiology*. **34**, 82–89, doi: 10.1016/j.mib.2016.08.004 (2016).
3. Cruz, A., Beverley, S.M. Gene replacement in parasitic protozoa. *Nature*. **348** (6297), 171–173, doi: 10.1038/348171a0 (1990).
4. Jones, N.G., Catta-Preta, C.M.C., Lima, A.P.C.A., Mottram, J.C. Genetically Validated Drug Targets in Leishmania: Current Knowledge and Future Prospects. *ACS infectious diseases*. **4** (4), 467–477, doi: 10.1021/acsinfecdis.7b00244 (2018).
5. Cruz, A.K., Titus, R., Beverley, S.M. Plasticity in chromosome number and testing of essential genes in Leishmania by targeting. *Proceedings of the National Academy of Sciences of the United States of America*. **90** (4), 1599–1603, doi: 10.1073/pnas.90.4.1599 (1993).
6. Sterkers, Y., Crobu, L., Lachaud, L., Pagès, M., Bastien, P. Parasexuality and mosaic aneuploidy in Leishmania: alternative genetics. *Trends in parasitology*. **30** (9), 429–435, doi: 10.1016/j.pt.2014.07.002 (2014).
7. Dumetz, F. *et al.* Modulation of Aneuploidy in during Adaptation to Different and Environments and Its Impact on Gene Expression. *mBio*. **8** (3), doi: 10.1128/mBio.00599-17 (2017).
8. Beneke, T. *et al.* Genetic dissection of a Leishmania flagellar proteome demonstrates requirement for directional motility in sand fly infections. *PLoS pathogens*. **15** (6), e1007828, doi: 10.1371/journal.ppat.1007828 (2019).
9. Damianou, A. *et al.* Essential roles for deubiquitination in Leishmania life cycle progression. *PLoS pathogens*. **16** (6), e1008455, doi: 10.1371/journal.ppat.1008455 (2020).
10. Burge, R.J., Damianou, A., Wilkinson, A.J., Rodenko, B., Mottram, J.C. Leishmania differentiation requires ubiquitin conjugation mediated by a UBC2-UEV1 E2 complex. *PLoS pathogens*. **16** (10), e1008784, doi: 10.1371/journal.ppat.1008784 (2020).
11. Grewal, J.S., Catta-Preta, C.M.C., Brown, E., Anand, J., Mottram, J.C. Evaluation of clan CD C11 peptidase PNT1 and other Leishmania mexicana cysteine peptidases as potential drug targets. *Biochimie*. **166**, 150–160, doi: 10.1016/j.biochi.2019.08.015 (2019).
12. Martel, D., Beneke, T., Gluenz, E., Späth, G.F., Rachidi, N. Characterisation of Casein Kinase 1.1 in Using the CRISPR Cas9 Toolkit. *BioMed research international*. **2017**, 4635605, doi: 10.1155/2017/4635605 (2017).
13. Baker, N. *et al.* Systematic functional analysis of Leishmania protein kinases identifies regulators of differentiation and survival. *Cold Spring Harbor Laboratory*. 2020.09.06.279091, doi: 10.1101/2020.09.06.279091 (2020).
14. Beneke, T., Madden, R., Makin, L., Valli, J., Sunter, J., Gluenz, E. A CRISPR Cas9 high-throughput genome editing toolkit for kinetoplastids. *Royal Society open science*. **4** (5), 170095, doi: 10.1098/rsos.170095 (2017).
15. Beneke, T., Gluenz, E. LeishGEdit: A Method for Rapid Gene Knockout and Tagging Using CRISPR-Cas9. *Methods in molecular biology* . **1971**, 189–210, doi: 10.1007/978-1-4939-9210-2_9 (2019).

16. Adaui, V. *et al.* Application of CRISPR/Cas9-Based Reverse Genetics in Leishmania braziliensis: Conserved Roles for HSP100 and HSP23. *Genes*. **11** (10), 1159, doi: 10.3390/genes11101159 (2020).
17. Dean, S., Sunter, J., Wheeler, R.J., Hodkinson, I., Gluenz, E., Gull, K. A toolkit enabling efficient, scalable and reproducible gene tagging in trypanosomatids. *Open biology*. **5** (1), 140197, doi: 10.1098/rsob.140197 (2015).
18. Yagoubat, A., Corrales, R.M., Bastien, P., Lévêque, M.F., Sterkers, Y. Gene Editing in Trypanosomatids: Tips and Tricks in the CRISPR-Cas9 Era. *Trends in parasitology*. **36** (9), 745–760, doi: 10.1016/j.pt.2020.06.005 (2020).
19. Yagoubat, A. *et al.* Universal highly efficient conditional knockout system in Leishmania, with a focus on untranscribed region preservation. *Cellular microbiology*. **22** (5), e13159, doi: 10.1111/cmi.13159 (2020).
20. Damasceno, J.D. *et al.* Conditional knockout of RAD51-related genes in Leishmania major reveals a critical role for homologous recombination during genome replication. *PLoS genetics*. **16** (7), e1008828, doi: 10.1371/journal.pgen.1008828 (2020).
21. Beneke, T., Gluenz, E. Bar-seq strategies for the LeishGEdit toolbox. *Molecular and biochemical parasitology*. **239**, 111295, doi: 10.1016/j.molbiopara.2020.111295 (2020).
22. Shrivastava, R., Tupperwar, N., Drory-Retwitzer, M., Shapira, M. Deletion of a Single LeishIF4E-3 Allele by the CRISPR-Cas9 System Alters Cell Morphology and Infectivity of Leishmania. *mSphere*. **4** (5), doi: 10.1128/msphere.00450-19 (2019).
23. Jesus-Santos, F.H. *et al.* LPG2 Gene Duplication in Leishmania infantum: A Case for CRISPR-Cas9 Gene Editing. *Frontiers in Cellular and Infection Microbiology*. **10**, doi: 10.3389/fcimb.2020.00408 (2020).
24. Schumann Burkard, G., Jutzi, P., Roditi, I. Genome-wide RNAi screens in bloodstream form trypanosomes identify drug transporters. *Molecular and biochemical parasitology*. **175** (1), 91–94, doi: 10.1016/j.molbiopara.2010.09.002 (2011).
25. Robinson, K.A., Beverley, S.M. Improvements in transfection efficiency and tests of RNA interference (RNAi) approaches in the protozoan parasite Leishmania. *Molecular and biochemical parasitology*. **128** (2), 217–228, doi: 10.1016/s0166-6851(03)00079-3 (2003).
26. Lye, L.-F. *et al.* Retention and loss of RNA interference pathways in trypanosomatid protozoans. *PLoS pathogens*. **6** (10), e1001161, doi: 10.1371/journal.ppat.1001161 (2010).
27. Rogers, M.B. *et al.* Chromosome and gene copy number variation allow major structural change between species and strains of Leishmania. *Genome research*. **21** (12), 2129–2142, doi: 10.1101/gr.122945.111 (2011).

Figure 1

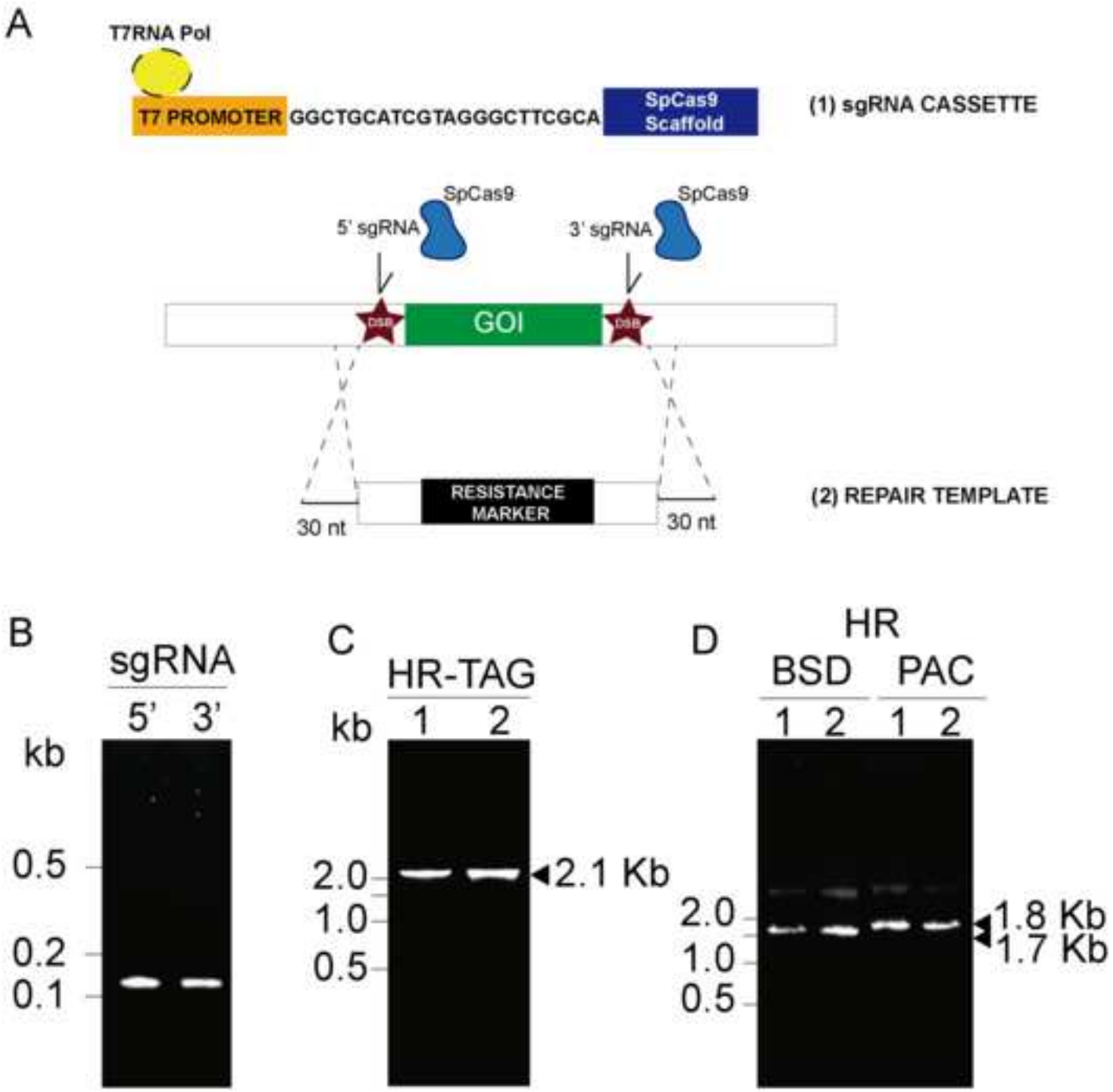


Figure 2

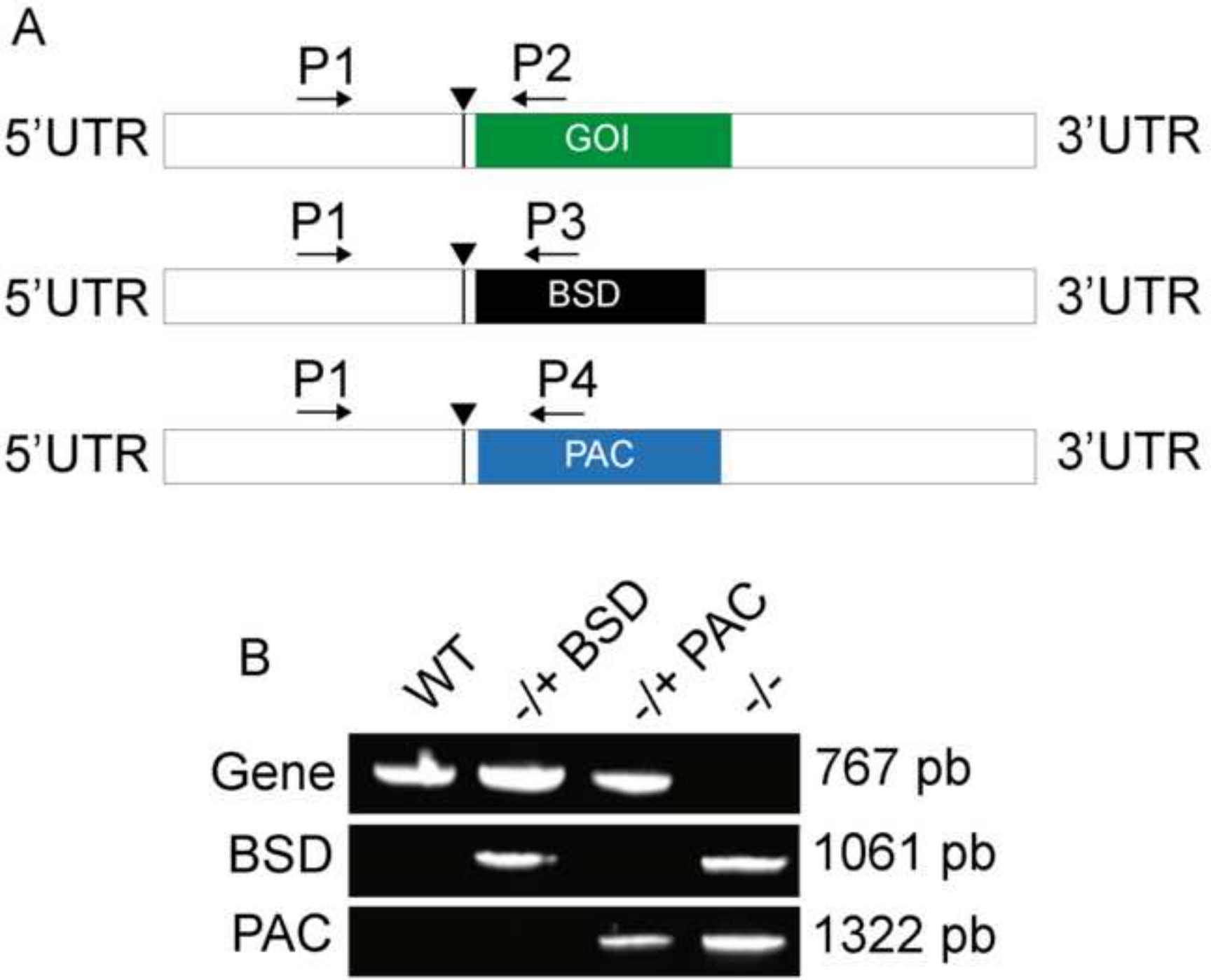


Figure 3

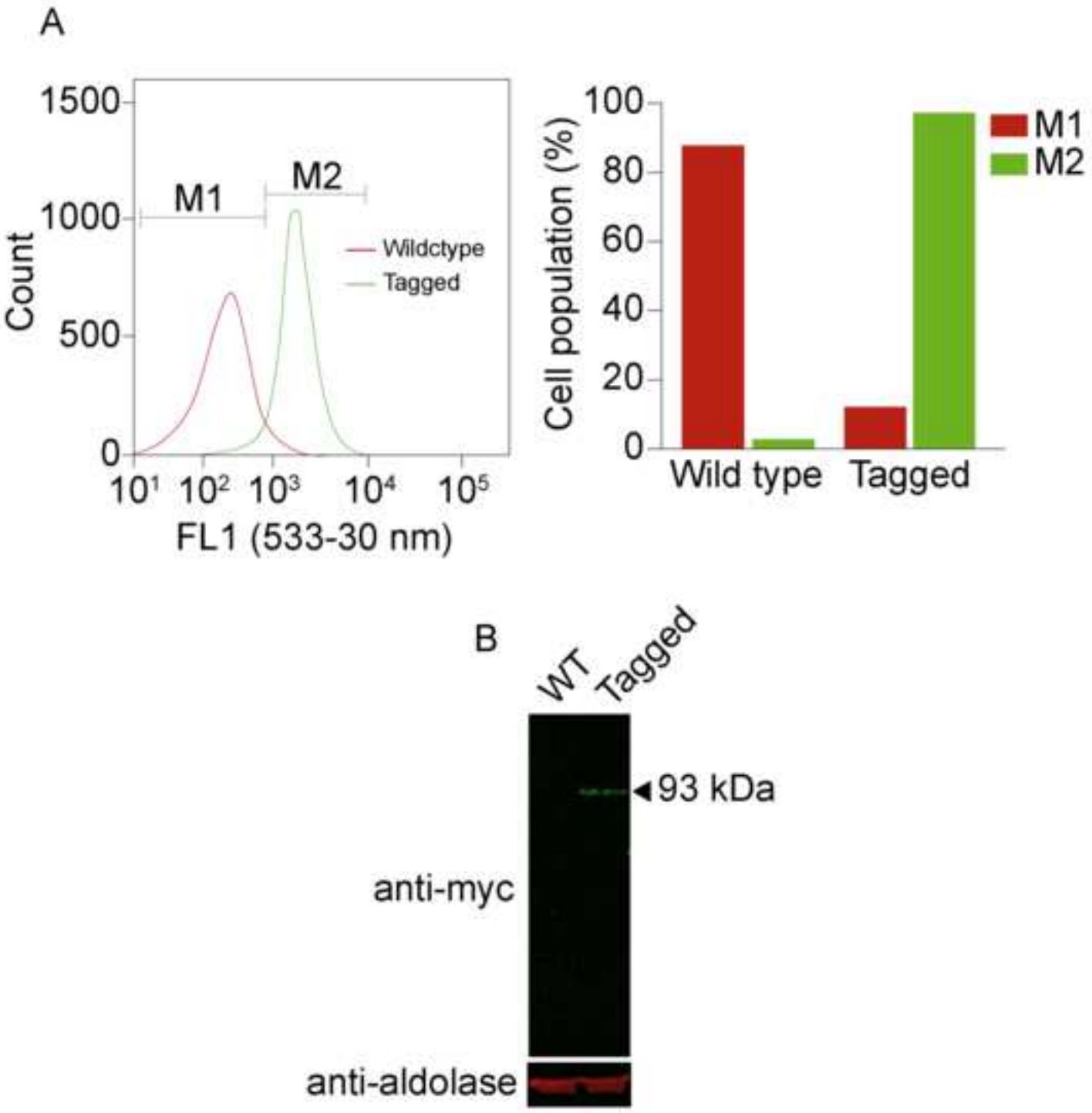
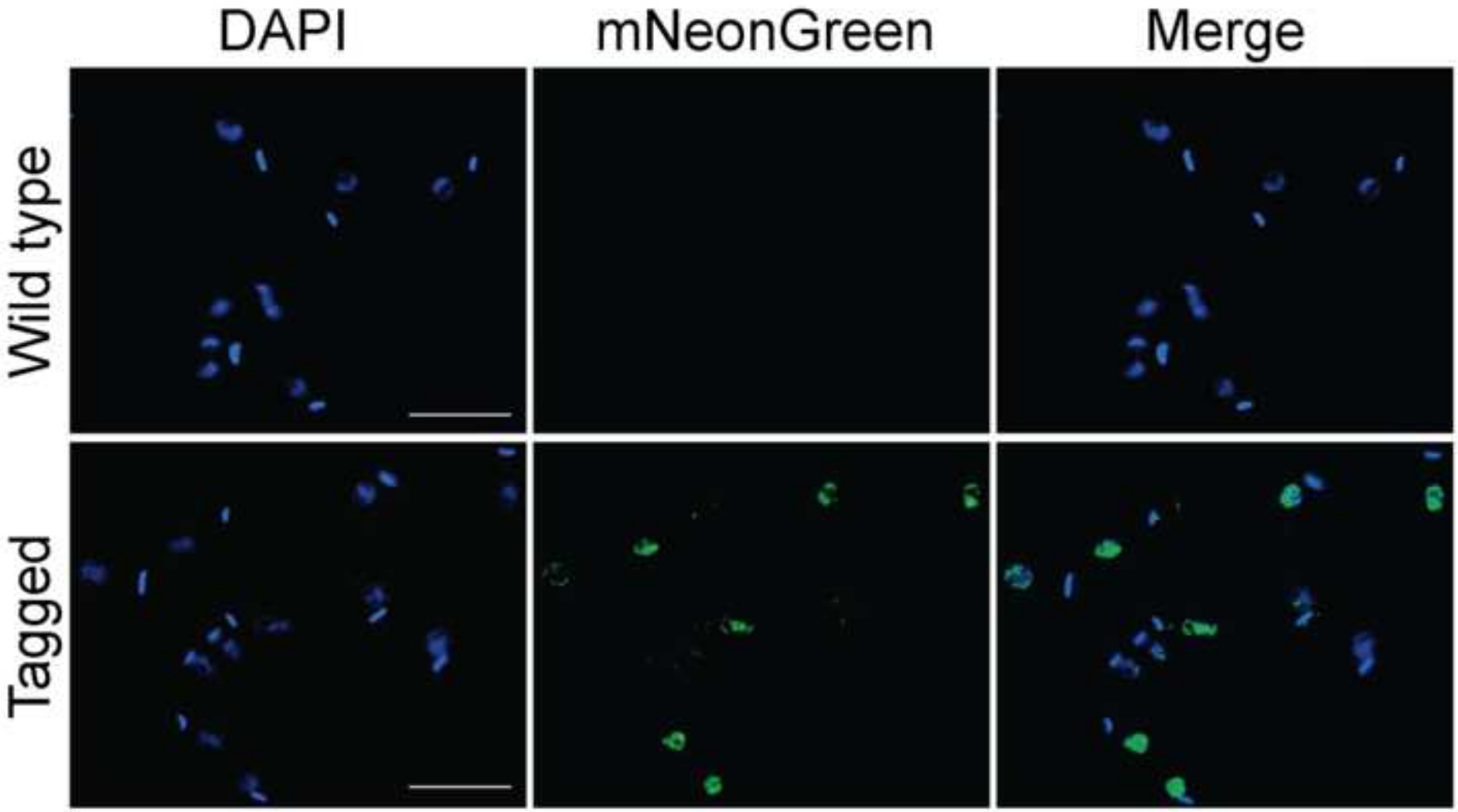


Figure 4



Name of Material/ Equipment	Company	Catalog Number
Part I - Primer design for knockout and *in situ* tagging		
pPLOT and pT plasmids		
Primers	ThermoScientific	
Universal sgRNA reverse primer (G00)	ThermoScientific	
Part II - Transfection DNA preparation		
1 kb Plus DNA Ladder	ThermoScientific	Cat: 10787018
Agarose	ThermoScientific	Cat: 16500500
Ethidium bromide	Sigma Aldrich	Cat: E8751
Disodium Salt dihydrate (EDTA)	Honeywell	Cat: 34549
dNTPs	ThermoScientific	Cat: 10297018
Glacial acetic acid	Anidrol	Cat: A-8684
Horizontal Electrophoresis Systems	Bio-Rad	Mini-Sub cell GT
Magnesium Chloride Anhydrous	Merck	Cat: 7786-30-3
PCR tubes	Sarstedt	Cat: 72.737002
pH meter	Oakton	75233
Platinum Taq Polymerase High Fidelity	ThermoScientific	Cat: 11304011
Potassium chloride	Sigma Aldrich	Cat: 31248
Thermocycler	Bio-Rad	#1861096
Tris Base	Fisher Bioreagents	Cat: BP152-1
Part III - Transfection and cloning		
70% Ethanol	Honeywell	Cat: 02860
96 well cell culture plate	Greiner bio-one	Cat: 655180
Adenine	Interlab	Cat: 321-30-2
Amaxa Nucleofector IIb	Lonza	AAB-1001
Biotin	Sigma Aldrich	58-85-5
Blasticidin S hydrochloride	Invivogen	Cat: ant-bl-1
Bottle Top Filter 0.22 µmm	Kasvi	Cat: K16-1500
Cell culture flask - 25 cm2	Sarstedt	Cat: 833910
Centrifuge	Thermo Electron Corporation	75004333
Conical tubes 50 mL	Corning	Cat: 352070
Conical tubes 15 mL	Corning	Cat: 430766

di-Sodium Hydrogen Phosphate	AppliChem	Cat: 131678.1210
Electroporation Cuvettes 0.2 cm gap	Bio-Rad	Cat: 1652086
Fetal Bovine Serum (FBS)	ThermoScientific	12657029
Glass Pasteur pipets	Corning	Cat: 13-678-4A
Geneticin (G418)	Invivogen	Cat: ant-gn-5
HEPES	Fisher Bioreagents	Cat: BP310-500
Hygromycin B	Invivogen	Cat: 10687010
Incubator	Tecnal	TE-371
Inverted microscope	Labomed	TCM 400
Medium 199	ThermoScientific	Cat: 31-100-019
Microcentrifuge	Eppendorf	5417C
Microtube 1.5 mL	Sarstedt	Cat: 72.690001
Multichannel Pipette (p200)	HTL Lab Solutions	6283
Muse Cell Analyzer	Merck Millipore	0500-3115
Penicillin G	Interlab	Cat: 69-57-1
Puromycin dihydrochloride	Invivogen	Cat: ant-pr-5b
Serological pipette 10 mL	Sarstedt	Cat: 861254001
Serological pipette 5 mL	Sarstedt	Cat: 861253001
Single Channel Pipette (p1000)	HTL Lab Solutions	LMP-1000
Single Channel Pipette (p200)	HTL Lab Solutions	LMP-200
Single Channel Pipette (p10)	HTL Lab Solutions	LMP-10
Single Channel Pipette (p2)	HTL Lab Solutions	LMP-2
Sodium bicarbonate	Fisher Bioreagents	Cat: 144-55-8
Sodium Phosphate Monobasic	USB Corporation Cleveland	Cat: 20233
Streptomycin sulfate salt	Gibco	Cat: 11860-038
Syringe Filter 0.2 µmm	ForlabExpress	Cat: K18-230
Syringe 10 mL	Interlab	Cat: 990173
Part IV - Cell line confirmation and phenotyping		
Accuri C6	BD Biosciences	-
Ammonium persulfate (APS)	Sigma-Aldrich	Cat: A3678
Confocal fluorescence microscope	Leica	TCS SP5 II Tandem Scanner
Coverslip	Glasstecnica	Lot: 44888/08
Digital Shaker	Labnet	S2030-1000-B

Goat Anti-Mouse 800CW antibody	LI-COR Biosciences	Cat: 926-32210
Goat Anti-Rabbit 680RD antibody	LI-COR Biosciences	Cat: 926-68071
Hoechst 33342	Invitrogen	Cat.: H3570
Imaris software	Imaris	Version: 6.0
LiCl	Sigma-Aldrich	L4408
Microscope slides	Tekdon Incorporated	Cat: 258-041-120
Monoclonal c-Myc epitope antibody	EMD Millipore	Cat: 05-724
Nitrocellulose membrane	Bio-Rad	#1620115
Non-fat milk	Molico	-
Odyssey Fc Imaging System	LI-COR Biosciences	Model number 2800
Paraformaldehyde (PFA)	Sigma Aldrich	Cat: P6148
PBS 1X	house made	house made
Poly-L-lysine	Sigma Aldrich	Cat: P8920
Polyacrylamide	Invitrogen	Cat: 15512023
Polyclonal Aldolase antibody	house made	house made
Protein Ladder	LI-COR Biosciences	928-60000
Sample Buffer	Sigma-Aldrich	S3401-1VL
Sodium dodecyl sulfate (SDS)	Sigma-Aldrich	Cat: L3771
TEMED	Life Technologies	Cat: 15524-010
Triton X-100	Sigma-Aldrich	X-100
Wet blotting system	Bio-Rad	1703930

Comments/Description
(www.leishgedit.net)
PCR primer design (www.leishgedit.net)
5' AAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAAC 3'
Molecular weight standards for gel electrophoresis of DNA
Agarose gels
Agarose gel
TAE Buffer
PCR mix
TAE Buffer
Gel electrophoresis
PCR mix
Plastic material
Calibrate pH solution
For amplification of DNA using PCR
Buffer 10x
PCR amplification
Buffer 10x
Sterilize
Cell culture
Cell culture medium supplement
Cell transfection
Cell culture medium supplement
Antibiotics for selection
Culture medium filter
Plastic material
Centrifugation
Plastic material
Plastic material

Transfection buffer
Transfection
Cell culture medium supplement
Glass material
Antibiotics for selection
Transfection buffer
Antibiotics for selection
Cells maintenance
Microscope
Cell culture medium
Centrifugation
Plastic material
Pipette reagents
Cell counter
Cell culture medium supplement
Antibiotics for selection
Plastic material
Plastic material
Pipette reagents
Pipette reagents
Pipette reagents
Pipette reagents
Cell culture medium supplement
Transfection buffer
Cell culture medium supplement
Filter transfection buffer
Plastic material

Flow cytometer
Casting polyacrylamide gel
Microscopy
Glass material
Agitation

Western blot antidoby
Western blot antidoby
Fluorescence antidoby
Data analysis
TELT solution preparation
Glass material
Western blot antidoby
Protein Blotting
Blocking solution for Western Blot
Antibodies detection
Fixation for fluorescence
Neutral Buffer
Adhesion for fluorescence
Casting denaturing polyacrylamide gel
Western blot antidoby
Ladder
Lysis solution
Casting polyacrylamide gel
Casting polyacrylamide gel
TELT solution preparation
Gel transfer cell

Escola Paulista de Medicina
Departamento de Microbiologia, Imunologia e Parasitologia

Sao Paulo, March 8th 2021

Dr **Nam Nguyen**, Ph.D.
Manager of Review - JoVE
Cambridge, MA - USA

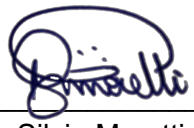
Dear **Dr. Nam Nguyen**,

We would like to submit the new version of our manuscript entitled "**Use of CRISPR-Cas9 technology to genetic manipulation of protozoan parasite *Leishmania"*** to be evaluated for the especial issue "Current methods to study parasite-host interactions and immunomodulation" in the JoVE.

In this new version we revised the manuscript based on the editor suggestions. We believe that this version is now suitable for acceptance in JoVE.

Regarding the video, we are working in the production and we intend to submit a first version for evaluation within 30 days.

Best regards,



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