LeishGEdit: a method for rapid gene knockout and tagging using CRISPR-Cas9

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Running Head

CRISPR-Cas9 gene editing method

Abstract

Post-genomic analyses of *Leishmania* biology benefit from rapid and precise methods for gene manipulation. Traditional methods of gene knockout or tagging by homologous recombination have limitations: they tend to be slow and require successive transfection and selection rounds to knock out multiple alleles of a gene. Clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 systems overcome these limitations. We describe here in detail a simple, rapid and scalable method for CRISPR-Cas9 mediated gene knockout and tagging in *Leishmania*. This method details how to use simple PCR to generate (i) templates for single-guide RNA (sgRNA) transcription in cells expressing Cas9 and T7 RNA polymerase and (ii) drug-selectable editing cassettes, using a modular set of plasmids as templates. pT plasmids allow for amplification of drug resistance genes for knockouts and pPLOT plasmids provide a choice of different tags to generate N- or C- terminally tagged proteins. We describe how to use an online platform (LeishGEdit.net) for automated primer design and how to perform PCRs and transfections in small batches or on 96-well plates for large-scale knockout or tagging screens. This method allows generation of knockout mutants or tagged cell lines within one week.

Keywords

LeishGEdit, Leishmania, kinetoplastids, CRISPR, Cas9, gene editing, T7 RNA polymerase, knockout, tagging

1. Introduction

Targeted genetic manipulation is a powerful approach for the study of *Leishmania* biology. Since the first report of gene replacement in *Leishmania* [1], manipulation of the genome has relied on the homologous recombination (HR) pathway to introduce DNA constructs, which were traditionally produced by step-wise cloning of DNA fragments in plasmids. This was necessary to produce the required length of homology arms, which needed to be longer than 300 nt for efficient integration as measured in *L. mexicana* [2]. This approach has been highly successful in elucidating the function of a small number of *Leishmania* genes in detail (see references in [3]) but it is very time consuming and knockout attempts have only been reported for 200 of a total of ca. 9000 *Leishmania* genes [3].

Post-genomic analyses of parasite biology call for high-throughput genetic tools to enable the study of larger cohorts of genes. Clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 systems are revolutionizing genome editing across eukaryotes [4], including protozoan parasites [5,6]. A variety of different approaches have been used in *Leishmania* spp. to supply the required single guide RNA (sgRNA) and Cas9 nuclease: Cas9 nuclease (typically derived from *Streptococcus pyogenes*) can be expressed in the target cell [7-9] or alternatively the smaller Cas9 protein from *Staphylococcus aureus* can be delivered by transfection of recombinant protein [10]. Precise cleavage of double-stranded DNA is achieved upon formation of a ribonucleoprotein complex, composed of Cas9 and a sequence-specific sgRNA. sgRNA molecules can be synthesized [11] or transcribed from template DNA [12,7,9], using a variety of promoters, including ribosomal RNA promoters [12,13,8], U6 [12,14,15] or T7 [7,9,10]. Repair of the resulting double strand break (DSB) occurs in kinetoplastids by a mechanism called microhomology-mediated end joining (MMEJ) [8] rather than non-homologous end joining (NHEJ), which is the dominant pathway in mammalian cells. MMEJ relies on short stretches of sequence identity on either side of the

DSB. Providing repair templates with matching homology flanks (which can be as short as 24 nt [7]) enables precise editing of a gene locus.

We designed a streamlined protocol for gene editing in *Leishmania* spp. and other kinetoplastids [16,17,7] which we termed LeishGEdit. The key advantages of this robust system are that it does not require any gene-specific cloning procedures or *in vitro* transcription prior to transfections making it rapid, scalable and economical. Our system relies on a cell line that expresses Cas9 nuclease and T7 RNA polymerase (RNAP) constitutively. This allows *in vivo* transcription of sgRNAs from transfected short 124 nt PCR fragments made by using two overlapping single oligonucleotides and incorporating a T7 promoter [18]. Donor DNA constructs, containing 30 nt homology flanks identical to the target locus and drug-selectable marker genes, can be amplified from a plasmid template using long primers. A modular set of template plasmids allows the re-use of the same primer pairs for generation of a variety of tagging or knockout (KO) constructs (Figure 1A). Applying drug-selection to transfected cells eliminates non-edited cells from the population enabling the generation of *Leishmania* null mutants in a single transfection in one week. To facilitate high-throughput projects, an online platform www.leishgedit.net has been generated to design the required primers for a variety of different kinetoplastid species [7].

Here we describe the standard protocol for gene editing using the LeishGEdit system to insert a tag at the 5' or 3' end of a gene or knock out both alleles of a gene in a single step. The protocol offers a choice of performing the PCRs and transfections in individual tubes or on 96-well plates.

2. Materials

PCR reagents and primers should be handled at room temperature, unless otherwise stated, and stored at -20°C. Genomic DNA can be stored at 4°C or -20°C. Transfection reagents are

stored at room temperature. Follow local rules for handling and disposal of hazardous chemicals and for safe handling and containment of genetically modified *Leishmania* spp.

2.1 PCR amplification of sgRNA template and donor DNA

1. Primers (Figure 1B):
    a. Target-specific sgRNA primers containing the T7 promoter, the 20 nt sgRNA target sequence and sequence complementary to the sgRNA scaffold can be designed manually (see **3.1 Primer design**) or downloaded from www.leishgedit.net and ordered from a suitable manufacturer.
    b. Donor DNA primer sequences containing target-specific 30 nt homology flanks and recognition sequence for the pT and pPLOT template plasmids (Figure 1A) [7] can be designed manually or downloaded as above.
    c. G00 primer (sgRNA scaffold):

    5′aaaagcaccgactcggtgccacttttcaagttgataacggactagccttattttaacttg
    ctatttctagctctaaaac3′

    d. Dilute primers to 100 μM in ultrapure water (see **Note 1**).
2. PCR reagents: Expand™ High Fidelity PCR System (Roche; contains 10x reaction buffer supplemented with 15 mM $MgCl_2$, 10 mM deoxynucleotide (dNTP) mix, Expand High Fidelity enzyme mix and 25 mM $MgCl_2$ solution), dimethyl sulfoxide (DMSO).
3. Template plasmids for PCR amplification of donor DNA: pT and pPLOT plasmids [7] diluted to 30 ng/μl in ultrapure water.
4. Multichannel pipette
5. Standard or 96-well PCR thermal cycler
6. PCR tubes (we recommend using strips of eight 0.2 ml thin-walled tubes) or 96-well PCR plates (half skirted, polypropylene)
7. PCR sealing film.

2.2 Agarose gel electrophoresis

1. 50x stock of Tris-Acetate-EDTA (TAE) buffer: 2 M Tris acetate, 0.05 M EDTA. Dissolve 242 g Tris base in 500 ml distilled water (dH_2O), add 57.1 ml glacial acetic acid, and 100 ml of 500 mM EDTA (pH 8.0) solution. Bring up solution to 1 l final volume using dH_2O.
2. Agarose
3. Ethidium bromide solution (10 mg/ml in water)
4. DNA ladder.
5. Equipment: Gel tank, casting tray, gel combs, power supply, trans-illuminator.

2.3 Transfection and selection

1. 1 M sucrose solution. Add 250 ml dH_2O to a 500 ml glass bottle and add 171 g sucrose while stirring. Fill up to 500 ml and incubate at 100 rpm at 50°C until completely dissolved.
2. 1 M HEPES. Add 250 ml dH_2O to a 500 ml glass bottle and add 10 solid NaOH pellets while stirring. When the pellets have dissolved add 119 g HEPES, titrate to pH 7.4 while mixing, using 1 M NaOH solution, and fill up to 500 ml with dH_2O.
3. 1 M Na_2HPO
4. 1 M NaH_2PO_4
5. 1 M KCl
6. 200 mM $CaCl_2$
7. 3x Tb-BSF buffer [19]: 200 mM Na_2HPO_4, 70 mM NaH_2PO_4, 15 mM KCl, 150 mM HEPES pH 7.4. For 500 ml buffer mix stock solutions as follows: 100 ml of 1 M Na_2HPO_4, 35 ml of 1 M NaH_2PO_4, 7.5 ml of 1 M KCl and 75 ml of 1 M HEPES pH 7.4. Add dH2O to bring to 500 ml.
8. 3x modified Tb-BSF buffer (based on observations made in [20]): 22.3 mM Na_2HPO_4, 7.67 mM NaH_2PO_4, 45 mM KCl, 75 mM, HEPES pH 7.4. For 500 ml buffer mix stock solutions as follows: 11.2 ml of 1 M Na_2HPO_4, 3.8 ml of 1 M NaH_2PO_4, 22.5 ml of 1 M

KCl, 37.5 ml of 1 M HEPES pH 7.4 and 225 ml of 1 M sucrose. Add dH2O to bring to 500 ml.

9. 1.5 mM $CaCl_2$. Make 500 ml by adding 3.75 ml of 200 mM $CaCl_2$ to 496.25 ml dH_2O.
10. Transfection mix for knockouts: 25 μl 1.5 mM $CaCl_2$, 83 μl 3x Tb-BSF, 42 μl ddH2O, 100 μl pooled PCR products (sgRNA template and donor DNA). Just before transfection, make a transfection buffer master mix for the required number of transfections.
11. Transfection mix for tagging: 25 μl 1.5 mM $CaCl_2$, 83 μl 3x Tb-BSF, 92 μl ddH2O, 50 μl pooled PCR products (sgRNA template and donor DNA). Just before transfection, make a transfection buffer master mix for the required number of transfections.
12. M199 medium. Dissolve 9.5 g M199 powder in 500 ml ddH_2O and add 2.2 g $NaHCO_3$. Add 100 ml foetal bovine serum (FBS) (10% v/v final concentration), 40 ml of 1 M HEPES pH 7.4 (prepared as described in **step 1** in **2.3 Transfection and selection**) and 2 ml of 2.5 mg/ml Haemin solution (dissolved in dH_2O). Fill up with ddH_2O to 1 l and sterilise by passage through a 0.22 μm filter. Batches of FBS should be tested before use.
13. MM199 medium [2]. Dissolve 9.5 g M199 powder in 500 ml ddH_2O and add 2.2 g $NaHCO_3$. Add 200 ml FBS (20% v/v final concentration), 40 ml of 1 M HEPES pH 7.3 (prepared as described in **step 1** in **2.3 Transfection and selection**), 1 ml of 2.5 mg/ml Haemin solution (dissolved in dH_2O), 4 ml of 0.3 mg/ml biopterin (dissolved in DMSO) and 20 ml of 5 mM Adenine hemisulphate (dissolved in ddH_2O). Fill up with ddH_2O to 1 l and sterilise by passage through a 0.22 μm filter.
14. 20 mg/ml Puromycin Dihydrochloride, sterilised.
15. 10 mg/ml Blasticidin S Hydrochloride, sterilised.
16. 45 mg/ml G-418 Disulphate, sterilised.
17. 100 mg/ml Nourseothricin Sulphate, sterilised.
18. 10 mg/ml Phleomycin, sterilised.
19. 100 mg/ml Hygromycin B Gold (see **Note 2)** sterilised.

20. Amaxa Nucleofector 2b (Lonza)
21. 2 mm gap Electroporator Cuvettes (MBP) or 2 mm gap BTX Electroporation Cuvettes Plus (see **Note 3**).
22. BTX ECM 830 Electroporation System with HT-200 plate handler and 96-well disposable electroporation plates, 4 mm gap, 250 μl (BTX).

2.4 Genomic DNA extraction and diagnostic PCR for knockout validation

1. Lysis buffer (200 mM NaCl, 0.5% SDS, 5 mM EDTA, 100 µg/ml Proteinase K, 10 mM Tris–HCl, pH 8.0): Make 50 ml by mixing 1 ml of 0.5 M Tris–HCl pH 8.0, 0.5 ml of 0.5 M EDTA pH 8.0, 1.25 ml of 20% SDS solution, 10 ml of 1 M NaCl and 0.5 ml of 10 mg/ml proteinase K solution. Add dH_2O to bring to 50 ml. Aliquot and store at -20°C. Defrost in 37°C water-bath before use.
2. Primers: Design primers as described in section **3.7 Validation of transfected cell lines** and prepare 100 μM stock in ddH_2O.
3. PCR reagents: Expand™ High Fidelity PCR System (Roche; contains 10x reaction buffer supplemented with 15 mM $MgCl_2$, 10 mM deoxynucleotide (dNTP) mix, Expand High Fidelity enzyme mix and 25 mM $MgCl_2$ solution).
4. Standard or 96-well PCR thermal cycler
5. PCR tubes (we recommend using strips of eight 0.2 ml thin-walled tubes).

3. Methods

An overview of the workflow is shown in Figure 2. The protocols detailed below using PCR tubes and individual transfection cuvettes are designed for generating a small number of cell lines at a time; protocols using 96-well plates are designed for higher throughput gene editing projects.

3.1 Primer design

1. Manual design of sgRNA primers: the sequence consists of (i) a T7 promoter sequence, (ii) 20 nt sgRNA target site ([sgN_{20}]) (see **Note 4**) which must be next to a protospacer adjacent motif (PAM) "NGG" at the target locus and (iii) sequence complementary to the sgRNA backbone (G00 primer) (Figure 1B). For designing the [sgN_{20}] manually, use the EuPaGDT CRISPR gRNA Design Tool [9] (http://grna.ctegd.uga.edu/).
2. Manual design of donor DNA primers: the sequence consists of 30 nt homology flanks ([HFN_{30}] at either end of the donor DNA (corresponding to target locus sequence next to the sgRNA target) and sequences binding to the pT or pPLOT template plasmids (Figure 1B).
3. Steps 1-2 can be automated for genomes available on www.leishgedit.net. To get sequences, go to the website and navigate to the primer design tab.
4. Enter the target gene GeneID (from TritrypDB.org) in the primer design box and chose "pT and pPLOT plasmids" for gene editing in *Leishmania*. Choose your desired gene editing strategy (tagging, knockout or both).
5. Press the button "Design primers".
6. A new tab in your browser will appear showing the primer sequences. You should first validate your search result by checking the sgRNA target count for each primer, which represents the number of total matches for the sgRNA target sequence (including a PAM site) within your chosen reference genome. For most genes the sgRNA target sequence is unique, i.e. a sgRNA target count of 1. If the sgRNA target count is >1 this means the same sequence is found elsewhere in the genome; this could result in a lower efficiency of editing the gene of interest and there may be a higher chance of off-target modifications.

7. Once validated, primers can be exported as .csv file. All primer sequences are written in a 5' to 3' orientation and are ready for ordering from a suitable manufacturer without further modifications.

3.2 PCR amplification of sgRNA templates

Gene tagging requires preparation of only one PCR to produce one sgRNA template. For tagging a protein at the N-terminus, design the sgRNA to direct a DSB at the 5' end of the target gene (Figure 3a), for a C-terminal tag, place the DSB at the 3' end (Figure 3b). A gene knockout requires preparation of two PCRs for each target gene (one sgRNA directing a DSB upstream of the target CDS and one downstream, (Figure 4). The following protocol is suitable for individual PCR tubes (strips of eight tubes are convenient) or 96-well PCR plates.

1. Dilute 100 μM sgRNA primer stock to 4 μM by placing 4 μl of 100 μM primer stock and 96 μl ddH_2O into a PCR tube or well of a 96-well plate.
2. Mix your 4 μM primer dilutions well and pipette 10 μl of this dilution into new PCR tubes or 96-well plate. Briefly spin down to collect contents at the bottom. Freeze at -80°C for 30 minutes (ideally in a cold rack) (see **Note 5**).
3. During the freezing process, calculate the required number of reactions and prepare a PCR master mix. For each reaction add 0.4 μl of 100 μM G00 primer, 0.4 μl of 10 mM dNTP mix, 2 μl of 10x reaction buffer supplemented with 15 mM $MgCl_2$ and 7 μl of ddH_2O. Add 0.2 μl High-fidelity polymerase last (see **Note 6**).
4. Start the following program on a PCR thermo cycler:

   Step 1: First denaturation 98°C 30 seconds

   Step 2: Denaturation 98°C 10 seconds

   Step 3: Annealing 60°C 30 seconds

   Step 4: Elongation 72°C 15 seconds

   Step 5: 35x back to Steps 2-4

Step 6: 72°C 10 minutes

Step 7: keep reaction at 4°C (until removal from the PCR machine)

5. As soon as 98°C is reached, pause the program and remove your primer dilutions from the freezer (see **Note 5**).
6. Add PCR master mix to primer dilutions: When processing a small number of samples (less than 24) add 10 µl of the PCR master mix to each primer dilution (total reaction volume 20 µl). For a larger number of samples pipet the entire master mix into a reagent reservoir and add 10 µl of the PCR master mix from the reservoir to each primer dilution by using a multichannel pipette. Use a new tip every time and work quickly before reactions reach room temperature.
7. Briefly centrifuge tubes or plate and place in hot PCR block. Resume the PCR program.

3.3 PCR amplification of donor DNA

Gene tagging requires preparation of one PCR product encoding the tag and drug resistance marker. A gene knockout requires preparation of two PCR products (one for each drug resistance marker). Alternatively, knockouts can be generated using only one drug resistance marker followed by cloning out cell lines after transfection (see **Note 7**). Transfection efficiencies may depend on the chosen drug resistance markers (see **Note 8**). The following protocol is suitable for PCR tubes or 96-well plates.

1. Dilute 100 µM stocks of donor PCR primers to 10 µM by adding 5 µl of 100 µM forward primer stock, 5 µl of corresponding 100 µM reverse primer stock and 40 µl ddH_2O to PCR tube or well of a 96-well plate.
2. Mix your 10 µM primer dilutions well and pipette 8 µl of this dilution into new PCR tubes or 96-well plate. Briefly centrifuge tubes to collect liquid at the bottom. Freeze tubes at -80°C for 30 minutes (ideally in a cold rack).
3. During the freezing process, calculate the required number of reactions and prepare the PCR master mix accordingly. For each reaction add 0.5 µl of 30 ng/µl plasmid template,

0.8 µl of 10 mM dNTP mix, 1.2 µl of 100% (v/v) DMSO, 3 µl of 25 mM $MgCl_2$ solution, 4 µl of 10x reaction buffer supplemented with 15 mM $MgCl_2$ and 22.1 µl of ddH_2O. Add 0.4 µl High-fidelity polymerase last (see **Note 6**).

4. Start the following program on a PCR thermo cycler:

   Step 1: First denaturation 94°C 5 minutes

   Step 2: Denaturation 94°C 30 seconds

   Step 3: Annealing 65°C 30 seconds

   Step 4: Elongation 72°C 2 minutes and 15 seconds

   Step 5: 40x back to Step 2-4

   Step 6: 72°C 7 minutes

   Step 7: keep reaction at 4°C (until removal from the PCR machine)

5. As soon as 94°C is reached, pause the program and remove primer dilutions from the freezer (see **Note 5**).
6. Add PCR master mix to primer dilutions: When processing a small number of samples (less than 24) add 32 µl of the PCR master mix to each primer dilution (total reaction volume 40 µl). For a larger number of samples pipet the entire master mix into a reagent reservoir and add 32 µl of the PCR master mix from the reservoir to each primer dilution by using a multichannel pipette. Use a new tip every time and work quickly before reactions reach room temperature.
7. Briefly centrifuge tubes or plate and place in hot PCR block. Resume the PCR program.

3.4 Agarose gel electrophoresis

1. Make a 1% (w/v) agarose gel by dissolving 1 g agarose in 100 ml 1x TAE buffer. Bring to the boil until agarose is dissolved then cool to ca. 50°C and add 1 µl ethidium bromide solution. Pour gel into tray, insert comb and leave to set at room temperature (see **Note 9**).

2. Remove comb and load 2 µl of each reaction into the wells of the gel without loading dye and before submerging the gel in the buffer tank. Loading is easiest using a multichannel pipette. Carefully and slowly place your gel into the gel tank containing 1x TAE buffer, then load DNA ladder.
3. Run the agarose gel for 20 minutes at 120 V and verify product yield by using a UV trans-illuminator.

3.5 Transfection of *Leishmania* promastigotes

Prepare an exponentially growing culture of a *Leishmania* promastigote cell line expressing Cas9 and T7 RNAP (e.g. *L. mexicana Cas9 T7* [7]). This parental cell line should be cultured with selection drugs for Cas9 and T7 RNAP construct over several passages until the day of transfection. For each transfection you will need 1 x 10^7 cells.

3.5.1 Pooling of PCR reactions

1. For each new cell line to be generated, pool sgRNA PCR reaction(s) together with the corresponding donor PCR reaction(s) to combine all PCR reactions for the same target gene in a single tube or well of a plate. If applicable use a multichannel pipette. For a knockout you will end up with approximately 100 µl total PCR reaction (2 x 40 µl donor DNA PCR plus 2 x 20 µl sgRNA DNA template PCR minus amount loaded for gel electrophoresis minus evaporation and sample loss during handling); for a tagging transfection the yield is approximately 50 µl total PCR reaction (40 µl donor DNA PCR plus 20 µl sgRNA DNA template PCR minus amount loaded for gel electrophoresis minus evaporation and sample loss during handling).
2. Close PCR tubes, or if pooled on 96-well plates, seal plates with adhesive foil. Heat-sterilize pooled PCR products at 94°C for 5 minutes. Proceed to transfection protocol.

3.5.2 Single cuvette transfection

1. Pre-warm medium: Fill the required number of 25 cm^2 flasks with 3-5 ml M199 medium or fill the required number of wells of a 24-well plate with 1 ml M199 and warm up in 28°C incubator. If 24-well plates are used, it is recommended to incubate them in an incubator with a humid 5% CO_2 atmosphere (see **Note 10**).
2. Collect the required number of cells (1 x 10^7 cells per transfection) by centrifugation at 800*g* for 10 min.
3. During the centrifugation step, prepare appropriate amount of transfection buffer master mix (as described in **2.3 Transfection and selection**) for the required number of transfections and volume needed for the washing step below (see **step 5**).
   For 50 knockout transfections, mix 1.25 ml 1.5 mM $CaCl_2$, 4.15 ml 3x Tb-BSF, 2.1 ml ddH_2O (7.5 ml total).
   For 50 tagging transfections, mix 1.25 ml 1.5 mM $CaCl_2$, 4.15 ml 3x Tb-BSF, 4.6 ml ddH_2O (10 ml total).
4. Sterilise transfection mix by passaging though a 0.22 *µ*m filter.
5. Following centrifugation of cells, discard supernatant and resuspend cells in 1 ml transfection buffer. Do not exceed 9 x 10^7 cells per ml. If the cell number is > 9 x 10^7 cells scale up the buffer volume.
6. Transfer cell suspension to 1.5 ml Eppendorf tube and centrifuge at 800*g* for 5 min. If volume exceeds 1 ml, aliquot into multiple Eppendorf tubes.
7. Resuspend cells in desired final volume of transfection buffer as follows:
   a. For knockouts add 150 *µ*l transfection buffer per 1 x 10^7 cells
   b. For tagging add 200 *µ*l transfection buffer per 1 x 10^7 cells
8. Mix the cell suspension with the PCR products in electroporation cuvettes as follows:
   a. For knockouts: 150 *µ*l cells in transfection buffer (from **step 7.a** above) + 100 *µ*l heat-sterilized pooled PCR reactions (from **3.5.1 Pooling of PCR** reactions).

b. For tagging: 200 μl cells in transfection buffer (from **step 7.b** above) + 50 μl heat-sterilized pooled PCR reactions (from **3.5.1 Pooling of PCR** reactions).

9. Leave the cells in the cuvettes for the shortest possible time. When doing a big batch of transfections, do no more than five cuvettes at a time and keep the remaining cell suspension in the Eppendorf tube until use.
10. Place cuvette in Amaxa Nucleofector 2b and apply one pulse with program X-001 (see **Note 11**).
11. Quickly and carefully transfer cells from the cuvette into pre-warmed M199: dilute either into 1 ml medium in a well of a 24-well plate or into 3-5 ml in a 25 cm^2 flask. Rinse cuvette once with medium to flush out any remaining cells (see **Note 10**).
12. Leave cells to incubate at 28°C for 8-16 h, then add the required selection drugs for the repair cassettes (see **3.6 Selection**) and incubate until drug resistant populations emerge.

3.5.3 96-well plate transfection

1. Pre-warm M199 medium to 28°C (see **Note 10**).
2. Collect 52 x 10^7 cells expressing Cas9 and T7 RNAP (1 x 10^7 cells per reaction) by centrifugation at 800*g* for 15 min (see **Note 12**).
3. Whilst waiting for the centrifugation step to complete, label two 24-well plates and fill each well with 1 ml of M199 medium.
4. Prepare transfection buffer as follows:
    a. For knockouts: 2000 μl $CaCl_2$, 6500 μl **<u>modified</u>** 3x Tb-BSF, 1500 μl ddH2O.
    b. For tagging: 2000 μl $CaCl_2$, 6500 μl **<u>modified</u>** 3x Tb-BSF, 6500 μl ddH2O.
5. Sterilise transfection buffer by passaging though a 0.22 μm filter.
6. Following centrifugation of cells, remove supernatant and resuspend cells in 3 ml transfection buffer.
7. Centrifuge again as above.

8. During the second spin, program the BTX ECM 830 Electroporation System with the following settings: 1500 V, 24 pulses, 2 counted pulses, 500 ms interval, unipolar, 100 μs.
9. Pipet pooled and heat-sterilized PCR products (from **3.5.1 Pooling of PCR** reactions) into wells of 96-well transfection plate.
10. After the second spin remove supernatant and resuspend cells in transfection buffer:
    a. For knockouts, in 5200 μl transfection buffer.
    b. For tagging, in 7800 μl transfection buffer.
11. Transfer cell suspension from **step 10** into a sterile reservoir and pipet 100 μl (for knockouts) or 150 μl (for tagging) of cell suspension to each well containing the pooled PCR products.
12. Do not pipet up or down, just add the cell suspension to the wells with the pipette tip touching the wall on the bottom corner.
13. Quickly seal the plate with foil (do not press foil too tightly).
14. Transfer plate to BTX unit and apply electroporation pulses using the settings defined above (see **step 8**).
15. After electroporation, remove the foil and transfer cells to 24-well plates (from **step 3** above). Rinse wells with medium to flush out any remaining cells.
16. Place plates in 28°C / 5% CO_2 incubator and leave for 8-16 h, then add the required selection drugs for the repair cassettes (see **3.6 Selection**) and incubate until drug resistant populations emerge.

3.6 Selection

1. For selection on 24-well plates, add 1 ml M199 with double the concentration of the desired drug. For selection in flasks, add an equal volume of M199 with double the concentration of the desired drug (or add the required volume of drug stock solution directly to each flask). For selection of *L. mexicana* or *L. major* transfectants, we use the following concentrations: 32 μg/ml Hygromycin B, 20 μg/ml Puromycin Dihydrochloride, 5

μg/ml Blasticidin S Hydrochloride, 40μg/ml G-418 Disulphate, 50 μg/ml Nourseothricin Sulphate or 25 μg/ml Phleomycin (see **Note 13**). If desired, cells can be cloned at this point (see **Note 14**). Do not add selection drugs for Cas9 and T7 RNAP constructs anymore.

2. Place plates in 28°C / 5% CO_2 incubator.
3. Depending on the type of genetic modification, drug resistant populations will have emerged and be ready for splitting between 4 and 10 days after transfection. Exact timings will depend on the growth rate of the chosen species and strain. Putative knockout populations should be passaged twice (first time dilute 1:10, second time dilute 1:100) before testing for loss of the open reading frame (ORF). Tagged cell lines should be passaged at least once (dilute 1:20) before imaging.

3.7 Validation of transfected cell lines

To verify loss of the target ORF in drug-resistant transfectants, perform a diagnostic PCR, amplifying a short PCR product (100 – 300 bp) within the ORF of the target gene, using primers that are unique to the target gene. As a positive control for detection of the target ORF, run the same diagnostic PCR on genomic DNA from the parental cell line. A further technical control PCR is required, amplifying a different gene from the genomic DNA of the putative knockout cell line to demonstrate presence of DNA (e.g. amplify a short fragment from the phosphomannomutase (LmxM.36.1960) ORF) (see **Note 15**).

3.7.1 DNA isolation

Genomic DNA can be isolated quickly and cost-effectively from a large number of samples using a simplified DNA isolation procedure described in [21]:

1. Collect cells from 400 to 800 μl of exponentially growing *Leishmania* by centrifugation at 800*g* for 5 minutes in a 1.5 ml Eppendorf tube. Extract DNA from each of the mutant cell lines to be tested and from the parental cell line.
2. Remove supernatant and resuspend cells in 100 μl pre-warmed lysis buffer.

3. Incubate samples for 30 minutes at 65°C.
4. After incubation add 250 µl 100% (v/v) EtOH and spin at 17,000g for 20 minutes at 4°C.
5. Discard supernatant (see **Note 16**).
6. Add 50 µl of ddH20.
7. Allow genomic DNA to dissolve overnight at 4°C.
8. Vortex genomic DNA after overnight incubation for 5 seconds and spin Eppendorf tubes briefly to collect liquid from lid and walls.
9. Determine the DNA concentration of your samples and calculate the necessary dilution to reach a concentration of about 30 ng/µl (see **Note 17**)
10. Dilute DNA into new PCR tubes (see **Note 16**).
11. Repeat steps 1 to 8 from above to isolate genomic DNA from the parental cell line separately (scale up number of collected cells if DNA is required for a large number of PCRs). Dilute DNA to 60 ng/µl.

3.7.2 Knockout verification – test mutant DNA for loss of ORF

1. Prepare 100 µM stocks of ORF PCR primers then dilute corresponding forward and reverse primers to 10 µM by adding 5 µl of 100 µM forward primer stock, 5 µl of 100 µM reverse primer stock and 40 µl ddH_2O to a PCR tube.
2. Mix your 10 µM primer dilutions well and pipet 4 µl of this dilution into a new PCR tube.
3. Add 2 µl of diluted genomic DNA (as prepared in **step 10** in **3.7.1 DNA isolation**) from the corresponding cell line. Centrifuge tubes to collect liquid at the bottom. Freeze tubes at -80°C for 30 minutes (ideally in a cold rack).
4. During the freezing process, calculate the required number of reactions and prepare the PCR master mix accordingly. For each reaction add 0.4 µl of 10 mM dNTP mix, 2 µl of 10x reaction buffer supplemented with 15 mM $MgCl_2$ and 11.4 µl of ddH_2O. Add 0.2 µl High-fidelity polymerase last (see **Note 6**).

5. Start the following program on a PCR thermo cycler

   Step 1: First denaturation 94°C 5 minutes,

   Step 2: Denaturation 94°C 30 seconds

   Step 3: Annealing 60°C 5 seconds

   Step 4: Elongation 72°C 30 seconds

   Step 5: 35x back to Step 2-4

   Step 6: 72°C 7 minutes

   Step 7: keep reaction at 4°C (until removal from the PCR machine)
6. As soon as it reaches 94°C, pause the program and remove your primer dilutions from -80°C (see **Note 5**).
7. Add PCR master mix to primer dilutions: When processing a small number of samples (less than 24) add 14 µl of the PCR master mix to each primer dilution (total reaction volume 20 µl). For a larger number of samples pipet the entire master mix into a reagent reservoir and add 14 µl of the PCR master mix from the reservoir to each primer dilution by using a multichannel pipette. Use a new tip every time and work quickly before reactions reach room temperature.
8. Briefly centrifuge tubes and place in hot PCR block. Resume the PCR program.

3.7.3 Knockout verification – ORF detection in parental cell line

1. Use the 10 µM primer dilutions prepared for ORF detection in mutant DNA and pipette 4 µl of this dilution into new PCR tubes. Centrifuge tubes to collect liquid at the bottom. Freeze tubes at -80°C for 30 minutes (ideally in a cold rack).
2. During the freezing process, calculate the required number of reactions and prepare the PCR master mix accordingly. For each reaction add 1.0 µl of 60 ng/µl parental genomic DNA, 0.4 µl of 10 mM dNTP mix, 2 µl of 10x reaction buffer supplemented with 15 mM $MgCl_2$ and 12.4 µl of ddH_2O. Add 0.2 µl High-fidelity polymerase at last (see **Note 6**).

3. Start the PCR thermo cycler as described in **Step 5** in **3.7.2 Knockout verification – test mutant DNA for loss of ORF**. As soon as it reaches 94°C, pause the program and remove your primer dilutions from -80°C (see **Note 5**).
4. Add PCR master mix to primer dilutions: When processing a small number of samples (less than 24) add 16 µl of the PCR master mix to each primer dilution (total reaction volume 20 µl). For a larger number of samples pipet the entire master mix into a reagent reservoir and add 16 µl of the PCR master mix from the reservoir to each primer dilution by using a multichannel pipette. Use a new tip every time and work quickly before reactions reach room temperature.
5. Briefly centrifuge tubes and place on hot PCR block. Resume the PCR program.

3.7.4 Knockout verification – control gene PCR

1. Pipette 2 µl of diluted mutant genomic DNA (as prepared in **step 10** in **3.7.1 DNA isolation**) into new PCR tubes. Centrifuge tubes to collect liquid at the bottom. Freeze tubes at -80°C for 30 minutes (ideally in a cold rack).
2. During the freezing process, calculate the required number of reactions and prepare the PCR master mix accordingly. For each reaction add 0.4 µl of each 100 µM housekeeping gene forward and reverse primer, 0.4 µl of 10 mM dNTP mix, 2 µl of 10x reaction buffer supplemented with 15 mM $MgCl_2$ and 14.6 µl of ddH_2O. Add 0.2 µl High-fidelity polymerase last (see **Note 6**).
3. Start the PCR thermo cycler as described in **Step 5** in **3.7.2 Knockout verification – test mutant DNA for loss of ORF**. As soon as it reaches 94°C, pause the program and remove your primer dilutions from -80°C (see **Note 5**).
4. Add PCR master mix to primer dilutions: When processing a small number of samples (less than 24) add 18 µl of the PCR master mix to each primer dilution (total reaction volume 20 µl). For a larger number of samples pipet the entire master mix into a reagent reservoir and add 18 µl of the PCR master mix from the reservoir to each primer dilution

by using a multichannel pipette. Use a new tip every time and work quickly before reactions reach room temperature.

5. Briefly spin tubes or plate down and place on hot PCR block. Resume the PCR program.

3.7.5 Knockout verification – Agarose gel electrophoresis

Assess all PCR products by running 4 µl of each PCR reaction on a 2% agarose gel **3.4 Agarose gel electrophoresis**.

4. Notes

1. Avoid too many freeze and thaw cycles of primers. Especially for the frequently used G00 primer, we recommend storing the stock in smaller aliquots, which shouldn't be thawed more than about four times.
2. We found that there are quite large batch-to-batch differences with hygromycin B from various suppliers. Therefore, we recommend using hygromycin B Gold from InvivoGen.
3. We have not found any notable differences between 2 mm gap electroporation cuvettes from various suppliers.
4. LesihGEdit sgRNA target sequences were designed with the EuPaGDT CRISPR gRNA Design Tool [9] (http://grna.ctegd.uga.edu/) and represent the highest scoring 20 nt sgRNA sequence identified within 105 bp upstream or downstream of the target gene open reading frame.
5. Freezing of primers or template DNA at -80°C before addition of other PCR reagents enables a physical separation of polymerase, oligos and template DNA. This reduces non-specific primer annealing and avoids oligo degradation, which can be introduced by unspecific exonuclease activity at low temperatures (this is similar to a PCR hot-start, in which the polymerase is added once the PCR reaction is heated up to 94°C). This is particularly important when handling a larger number of samples (e.g. 96-well plates).

1. When taking out frozen PCR tubes for addition of PCR master mix, wipe ice off tubes or plate foils with facial tissue before opening. Otherwise lids and foils are liable to crack.
6. Always include at least one spare reaction in your PCR master mix to avoid running out of mix due to inaccuracies in pipetting. When using reservoirs for pipetting PCR master mix include multiple spare reactions, about 100 – 200 μl spare volume.
7. The advantage of using two drug resistance markers is that typically all cells in a non-clonal population of drug resistant cells will be knockouts (given the gene is non-essential for promastigote viability in culture). When only a single drug resistance marker is used, typically about half of the cells in a population are full knockouts whilst the others have retained an allele of the target gene.
8. For single allele mutations, e.g. in tagging experiments, our preferred selection drug is puromycin. For knockouts in populations our preferred selection drug combination (using pT plasmid knockout cassettes) is blasticidin and puromycin, followed by a combination of puromycin and neomycin. Try to avoid generation of knockout populations using the combination blasticidin and neomycin. For generation of knockouts using only one selection drug (one pT knockout cassette), we recommend using neomycin.
9. Diluting PCR amplicons in DNA gel loading buffer can be very time-consuming for a large number of samples. To load amplicons without loading dye, it is best to pipette reactions into a dry gel. To get sharp bands and good resolution, prepare 1x TAE buffer fresh from 50x stock and make the gels relatively thin (ideally 0.3 to 0.5 cm).
10. We routinely recover cells after transfections in M199 medium either in flasks in an incubator without CO_2 or on 24-well plates in an incubator with a humid 5% CO_2 atmosphere. However, for some slow growth phenotypes, we have found that culturing in MM199 (see **2.3 Transfection and selection**; [2]) and incubation in a humid 5% CO_2 atmosphere appears to increase transfection success rates. Plates allow efficient handling of transfectants (especially for more than 10 transfections), while flasks are ideal for a smaller samples sizes.

11. For single cuvette electroporation we have also successfully used the BTX ECM 830 Electroporation System using 1600 V, 3 pulses, 500 ms interval, unipolar, 100 μs. Please note, the total reaction volume for a BTX single cuvette electroporation needs to be 500 μl. Please scale up the transfection mix appropriately. Other electroporation systems that have been used for transfection of *Leishmania* are also likely to work.
12. To avoid cross-contamination between wells, we only fill a maximum of 48 wells on a 96-well plate. We use either columns 1 to 12 in rows A, C, E and G or in rows B, D, F and H, leaving always one entire row empty between wells containing cells for a transfection.
13. The optimal selection drug concentration may vary between different *Leishmania spp*. and strains, therefore it is important to establish suitable drug concentrations before starting an experiment.
14. For generation of clonal cell lines after transfection, we recommend diluting cells and plating on 96-well plates immediately after the addition of drugs.

2. **Selection with one drug:** To select using puromycin or blasticidin resistance markers, make a 1:50 and 1:500 dilution (10 ml each) of the culture containing ~$1 \cdot 10^7$ electroporated cells. Dispense into sterile reagent reservoirs and use a multichannel pipette to aliquot dilutions on half of a 96-well plate (200 μl per well). For neomycin resistance markers, dilute cells 1:10 and 1:100 (10 ml each). For phleomycin resistance markers, plate cells undiluted. Always keep the remaining undiluted cell cultures from each transfection to select transfectants in the original flask.
3. **Selection with two drugs:** To select using puromycin and blasticidin resistance markers, aliquot undiluted culture containing ~$1 \cdot 10^7$ electroporated cells on half of a 96-well plate and a 1:10 dilution on the other half. For a combination of puromycin and neomycin plate cells undiluted. We do not recommend using any other drug combinations.

15. The diagnostic PCRs described in **3.7 Validation of transfected cell lines** are suited to high-throughput screening. Further PCR tests can be performed using primers in the

5'UTR and/or the ORF to validate correct integration of drug resistance genes or successful deletion of the target gene. Depending on the nature of the modification, other tests may be required such as Southern blotting or DNA sequencing. This may be of particular relevance for validation of larger deletions such as gene arrays.

16. It is extremely important to avoid cross-contamination at any stage during isolation and handling of genomic DNA for knockout validation. Try to minimize cross-contamination by not leaving multiple tubes open at any given time and do not evaporate remaining ethanol from tubes at the end of DNA precipitation.
17. This DNA isolation protocol works very reliably. It will typically yield about 10 - 20 μg total DNA per cell line (depending on cell density when harvested), which means that samples are typically diluted about 1:8 for the knockout verification PCR. The dilution increases also the likelihood of PCR success, since PCR inhibitors will be reduced.

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Figure Captions

Figure 1. A modular plasmid system for streamlined genetic editing.

(**a**) Strategy for donor DNA amplification from pT and pPLOT plasmids to repair a target locus after a double-strand break has been introduced by CRISPR-Cas9. Numbers 1-6 denote oligos used for gene editing: Oligos 1, 2, 4 and 5 contain 30 nt homology flanks specific to the target locus for integration of donor DNA fragments; 3 and 6 specify the sgRNA target sites, directing Cas9 to cut immediately upstream (5') or downstream (3') of the target ORF. Knockout constructs encoding a drug-selectable marker gene with endogenous UTRs from *L. mexicana* are amplified from pT plasmids with primer pair 1 and 5 and integrated in the target locus after cutting with sgRNAs 3 and 6. Tagging constructs encode a protein-tag for in-frame fusions to the target gene and a selectable marker. Examples of

available tags are listed. Tagging constructs are amplified from pPLOT plasmids with primer pair 1 and 2 to a tag protein at its N-terminus (cutting target locus at 5' end with sgRNA 3) or primer pair 4 and 5 for C-terminal tagging (cutting target locus at 3' end with sgRNA 6). (**b**) Primer sequences. Oligos 1, 2, 4 and 5 contain a target-gene specific 30 nt homology flank ([HFN30]) followed by primer binding sites for pT and pPLOT plasmids (underlined in red). Oligos 3 and 6 contain a T7 promoter (underlined in blue) followed by 20 nt sgRNA target sequence ([sgN20]) and sequence complementary to the sgRNA backbone (underlined in green) (reproduced from ref. 2 with permission).

Figure 2. Gene editing workflow using the LeishGEdit toolkit.

Each step is indicated in a single frame with approximate times for each step indicated below each box in green. Time required for ordering of oligos is not indicated as it will vary; the selection time depends on the type of experiment and may vary too. (**a**) Primer design can be automated with the LeishGEdit.net primer design tool. (**b**) Primers are used to generate sgRNAs and donor DNAs. (**c**) PCR products from (b) can be mixed without further purification with a Cas9 and T7 RNAP expressing cell line followed by electroporation. (**d**) Cells are subjected to drug selection to obtain populations of edited cells. Genomic DNA is extracted from knockout candidates and tagged cell lines are assessed by microscopy. (**e**) Knockout candidates are screened using diagnostic PCRs to test genomic DNA for (1) ORF presence in mutant DNA, (2) ORF presence in parental DNA and (3) control gene presence in mutant DNA. Finally, the phenotype of validated cell lines can be studied in detail e.g. by measuring growth rate, motility, analysing cell shape or testing infectivity (**f**).

Figure 3. Insertion of protein tag into endogenous locus

Strategy for generating cell line expressing protein tagged at the N-terminus (**a**), or C-terminus (**b**). **Left panels**, Locus map of *PF16* (LmxM.20.1400) showing position of homology flanks (HFN30) used for integration of donor DNA, 20 nt sgRNA target sequence (sgN20), the PAM site (highlighted in red) and the site of the double-strand break (DSB, red

line). The stop codon of PF16 ORF is indicated with asterisk. **Right panels**, Micrographs showing the result of tagging PF16 with eYFP at the N-terminus **(a)** or at the C-terminus (**b**). Cells were imaged live and images are a composite of phase contrast and fluorescence channels. Both PF16 fusion proteins localize to the flagellum (eYFP fluorescence shown in green). DNA is stained with Hoechst (red). Scale bar, 5 μm. Sequence maps were generated with SnapGene (www.snapgene.com).

Figure 4. Knocking out ORF by replacement with donor DNA cassette.

(**a**) Locus map of *PF16* (LmxM.20.1400) showing upstream and downstream region. The dotted line denotes *PF16* ORF internal sequence not shown in figure. Yellow arrows indicate the position of the homology flanks (HFN30), sgRNA target sequence (sgN20), PAM site (red) and DSB site (red line) for targeting the 5' end of the donor DNA to a site immediately upstream of the *PF16* ORF. Grey arrows indicate the position of the same elements, targeting the 3' end of the donor DNA to a site immediately downstream from the *PF16* ORF (stop codon indicated with asterisk). Insertion of the donor DNA will result in loss of the *PF16* ORF. (**b, c**) Strategy and results of diagnostic PCRs for knockout verification. PCR products were run on agarose gels, the numbers indicate band sizes for DNA ladder. The *PF16* ORF is detected in the parental DNA (P) but missing from the knockout (K). (**c**) Control PCR amplifying a control gene from the parental and the PF16 knockout DNA. The cartoons show the gene loci with small arrows indicating primer binding sites. Sequence maps were generated with SnapGene (www.snapgene.com).

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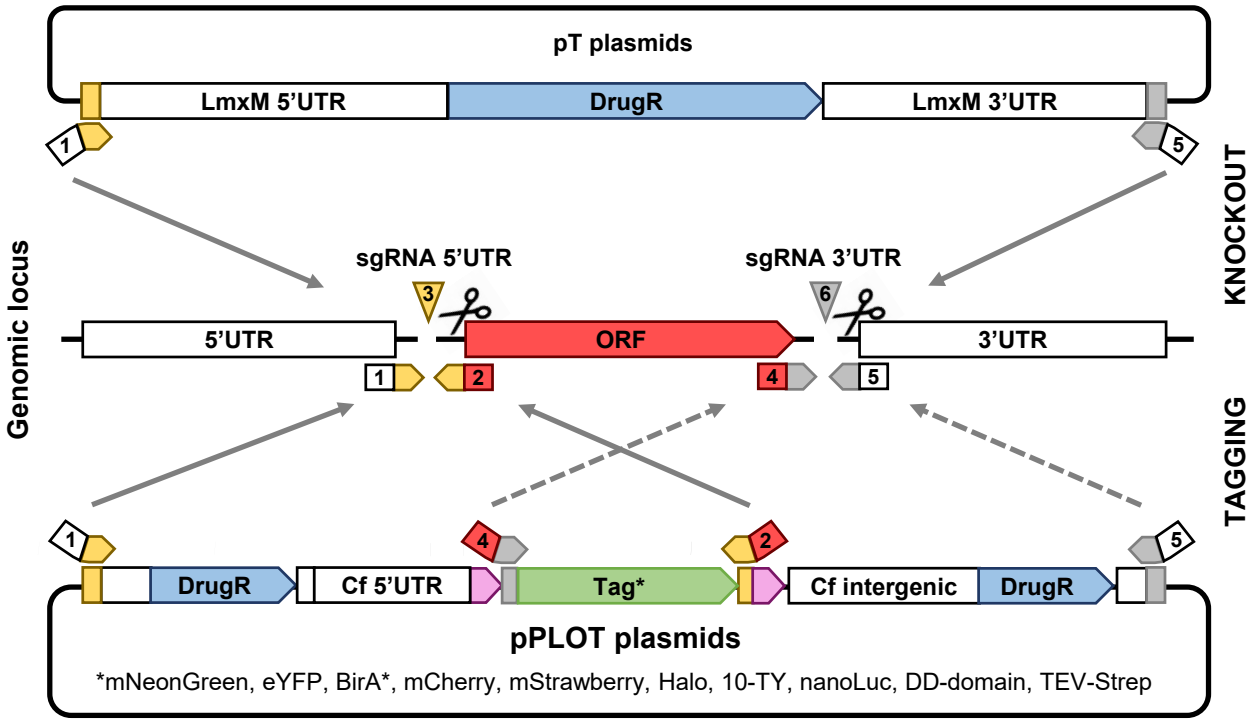
a



b

Upstream forward primer:	5′[HFN30]gtataatgcagacctgctgc 3′	1
Upstream reverse primer:	5′[HFN30]actacccgatcctgatccag 3′	2
Downstream forward primer:	5′[HFN30]ggttctggtagtggttccgg 3′	4
Downstream reverse primer:	5′[HFN30]ccaatttgagagacctgtgc 3′	5
5'sgRNA primer:	5′gaaattaatacgactcactatagg[sgN20]gttttagagctagaaatagc 3′	3
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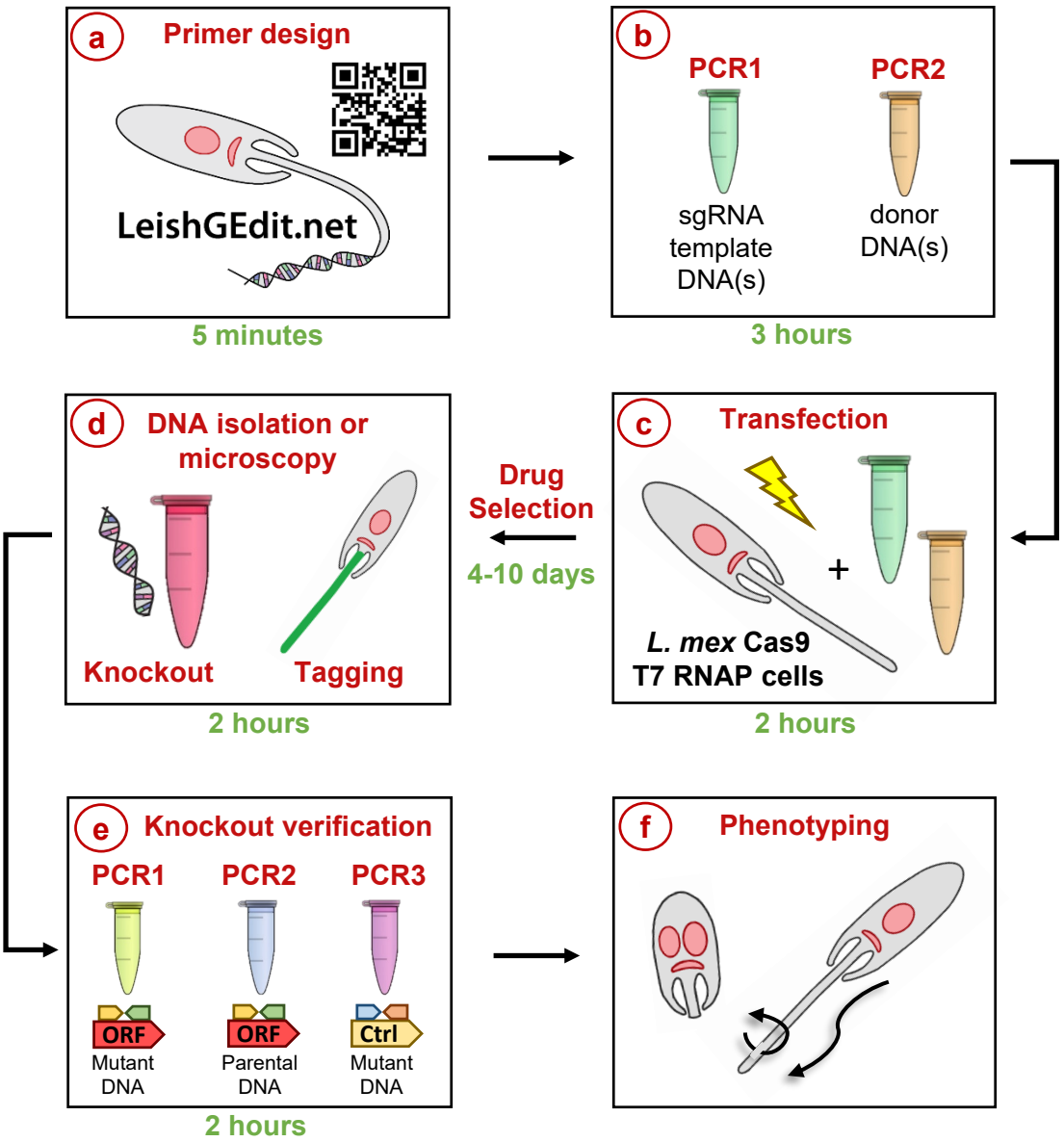
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LeishGEdit.net
5 minutes
b
PCR1
PCR2
sgRNA template DNA(s)
donor DNA(s)
3 hours
c
Transfection
L. mex Cas9
T7 RNAP cells
2 hours
Drug Selection
4-10 days
d
DNA isolation or microscopy
Knockout
Tagging
2 hours
e
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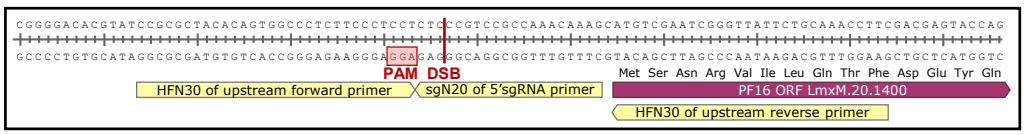
2 hours
f
Phenotyping

a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PAM
DSB
Met Ser Asn Arg Val Ile Leu Gln Thr Phe Asp Glu Tyr Gln
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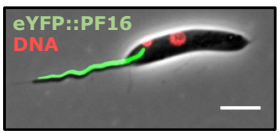
sgN20 of 5'sgRNA primer
PF16 ORF LmxM.20.1400
HFN30 of upstream reverse primer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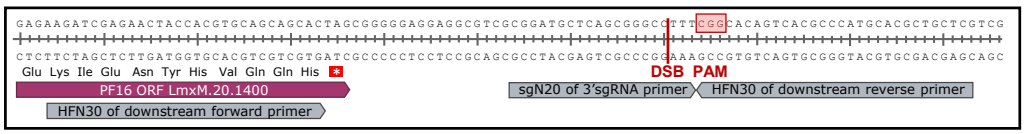
DNA

b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Glu Lys Ile Glu Asn Tyr His Val Gln Gln His
DSB
PAM
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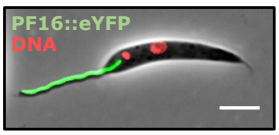
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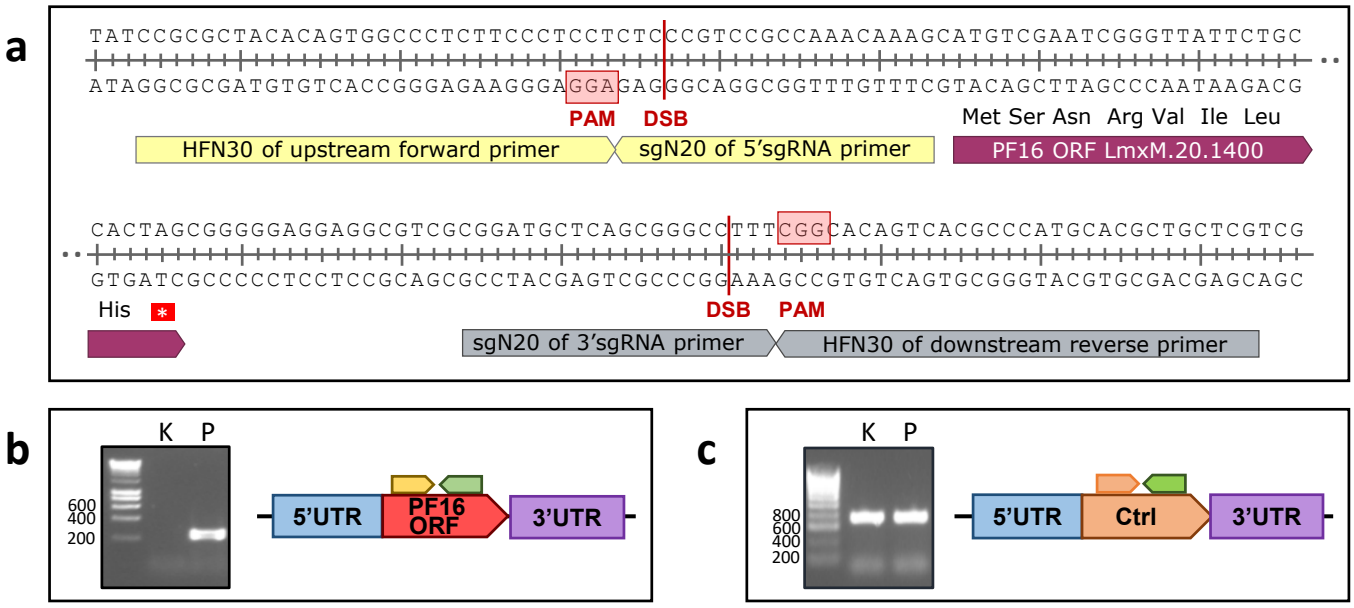
DNA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PAM
DSB
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HFN30 of upstream forward primer
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His
DSB
PAM
sgN20 of 3'sgRNA primer
HFN30 of downstream reverse primer

b
K
P
600
400
200
5'UTR
PF16 ORF
3'UTR

c
K
P
800
600
400
200
5'UTR
Ctrl
3'UTR