

A streamlined CRISPR/Cas9 approach for fast genome editing in *Toxoplasma gondii* and *Besnoitia besnoiti*

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Abbreviations used: ATP, adenosine 5'-triphosphate disodium salt; *B. besnoiti*, *Besnoitia besnoiti*; Cas, CRISPR-associated; CRISPR, clustered regularly interspaced short palindromic repeats; crRNA, crisprRNA; DBS, double-strand break; GOI, gene of interest; GSH, glutathione; IC, intracellular buffer; indel, insertion/deletion; KOD polymerase, DNA polymerase from *thermococcus kodakaraensis*; NHEJ, non-homologous end joining; nt, nucleotide; PAM, protospacer adjacent motif; PS, phosphorothioate bonds; RNP, ribonucleoprotein; RT, room temperature; sgRNA, single guide RNA; *T. gondii*, *Toxoplasma gondii*; *S. pyogenes*, *Streptococcus pyogenes*; TIDE, Tracking of Indels by DEcomposition; tracrRNA, trans-activating CRISPR RNA; UPRT, uracil phosphoribosyl transferase; 2'OMe, 2'-O-Methyl

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ABSTRACT

Toxoplasma gondii (*T. gondii*) and *Besnoitia besnoiti* (*B. besnoiti*) are closely related coccidian parasites belonging to the phylum Apicomplexa, which comprises many other important pathogens of humans and livestock. *T. gondii* is considered a model organism for studying the cell biology of Apicomplexa mainly due to the ease of propagation in diverse host cells and the availability of a wide range of genetic tools. Conversely, *B. besnoiti* *in vitro* culture systems currently exist only for the acute phase of infection, and genetic manipulation has proven much more challenging. In recent years, the targeted editing of chromosomal DNA by the programmable CRISPR-associated (Cas)9 enzyme has greatly improved the scope and accuracy of genetic manipulation in *T. gondii* and related parasites but is still lagging in *B. besnoiti*. The CRISPR/Cas9 technology enables the introduction of single point and insertion/deletion mutations, precise integration of in-frame epitope tags, and deletions of genes at reduced time and cost compared to previous methods. Current protocols for CRISPR-mediated genome editing in *T. gondii* rely on either constitutive or transient expression of Cas9 as well as target specific sgRNAs encoded separately or together on transfected plasmid vectors. Constitutively expressed Cas9 carries the risk of toxicity, whilst the transient approach is laborious and error-prone. Here we present a protocol for plasmid vector-independent genome-editing using chemically synthesized and modified sgRNAs. This protocol allows for rapid and cost-effective generation of mutant cell lines of *T. gondii* and *B. besnoiti*.

Keywords: chemical modifications, CRISPR/Cas9, genome editing, parasites, ribonucleoprotein

BACKGROUND

Toxoplasma gondii (*T. gondii*) and *Besnoitia besnoiti* (*B. besnoiti*) are both tissue cyst-forming, obligate intracellular parasites belonging to the phylum Apicomplexa. Whilst *T. gondii* is zoonotic and infects any warm-blooded animal including humans [1], Bovidae are intermediate hosts for *B. besnoiti* but details of the lifecycle are largely missing [2]. In contrast to tachyzoites of the acute stage, bradyzoites in tissue cysts of both species are not susceptible to pharmacological interventions. Especially chronic infections with *T. gondii* can become problematic in immunocompromised patients as the parasite reactivates and causes serious and potentially lethal tissue damage in the brain [1]. Chronic besnoitiosis on the other hand leads to high morbidity with reduced productivity and fertility of affected cattle in endemic areas. Besnoitiosis is considered an emerging disease of bovines, which continues to

expand in Europe and has a significant economic impact on the cattle industry [2]. To increase the understanding of the parasites' molecular mechanism of infection and persistence within their host, tools for rapid and efficient genome editing are necessary. In recent years, genome manipulation using the CRISPR system has largely facilitated efforts to investigate the molecular mechanisms of host-parasite interaction and pathogenesis. The CRISPR system is an adaptive immune response in bacteria and archaea, which functions by recognition and cleavage of foreign DNA. The type II CRISPR system from *Streptococcus pyogenes* (*S. pyogenes*) is the most commonly used for targeted genome editing in numerous organisms including *T. gondii* due to its simplicity and the well-known mode of action. The CRISPR RNA (crRNA) whose 5' end is complementary to the target DNA, partially hybridizes with the trans-activating CRISPR RNA (tracrRNA) to form an RNA complex, termed single guide RNA (sgRNA). Whereas the 20 nt part of the

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crRNA confers DNA target specificity, the tracrRNA plays a crucial role in Cas9 recruitment and binding [3]. In CRISPR/Cas9, recognition of self vs. non-self involves a short sequence motif referred to as the protospacer adjacent motif (PAM). In the *S. pyogenes* type II system, the PAM conforms to an NGG sequence downstream of the crRNA binding sequence [4]. The sgRNA:Cas9 ribonucleotide complex is guided to the PAM site adjacent to the target DNA in the genome, where a site-specific double-strand break (DSB) is introduced [5]. DSBs can be repaired either by nonhomologous end joining (NHEJ), non-homology dependent integration, or by homologous recombination [3].

Genome editing in *T. gondii* using CRISPR/Cas9 strategies benefits significantly from a ΔKU80 background favoring the homologous recombination pathway of DNA repair [6]. A plasmid encoding Cas9 and two specific sgRNAs (Fig. 1A) is co-transfected with a homology-directed resistance cassette (Fig. 1B). The resistance cassette is a PCR-generated

amplicon with ~30 bp flanking homology regions to the targeted locus of interest. Double homologous recombination at the locus of interest is triggered by the CRISPR/Cas9 mediated DSB. This strategy is well established in *T. gondii*, however, the frequency of site-specific disruption is reported to be 0.1%–0.2% [7]. In *B. besnoiti*, disruption of the uracil phosphoribosyltransferase (UPRT) has previously been achieved in our lab (C. Ramakrishnan, in preparation) using the same approach albeit only with very high plasmid concentrations carrying the risk of Cas9 toxicity and off-target effects. Importantly, homologous recombination in *B. besnoiti* was not successful using current protocols for *T. gondii* transfection. Whereas the generation of the linear resistance cassette with homologous arms to the excised gene of interest is purely PCR-based, the exchange of the target-specific sgRNAs in the Cas9 vector requires cloning for every single gene of interest (GOI), which is error-prone and labor-intensive.

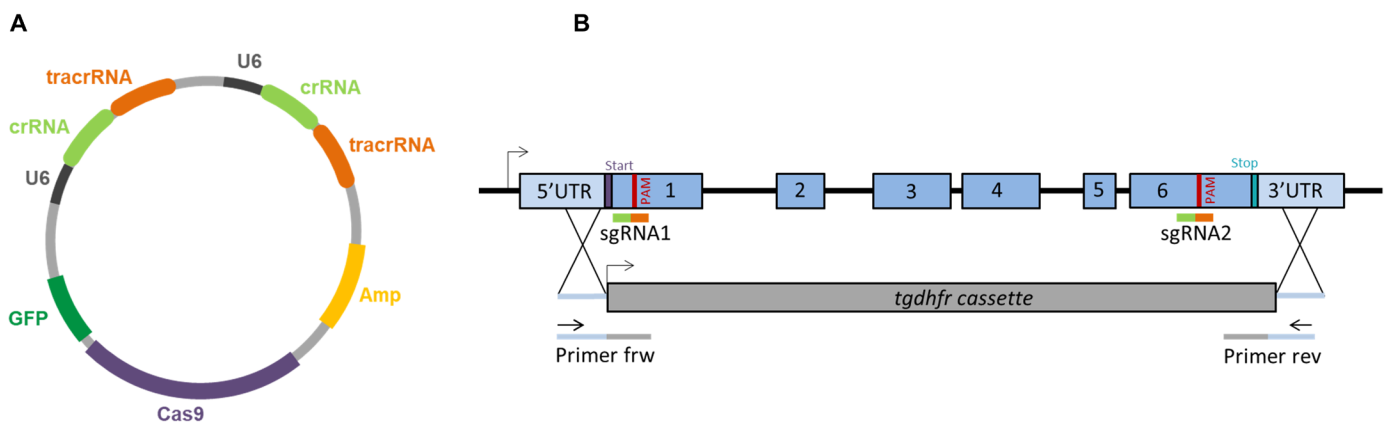


Figure 1. Conventional plasmid-based CRISPR/Cas9 approach. **A.** Plasmid expressing Cas9 and two target-specific sgRNAs, whereas one is targeting the first exon and the second is targeting the last exon, leading to a clean gene excision. **B.** The DHFR-TS* selection cassette [8] sequences derived from the parasite’s fused dihydrofolate reductase-thymidylate synthase (DHFR-TS contains homologous arms to the excised gene of interest and thus integrates *via* homologous recombination.

A key advance in CRISPR programmability came with the engineering of chimeric sgRNAs by hybridizing synthetically manufactured crRNAs with tracrRNAs to form a crRNA:tracrRNA complex with correct secondary structure. Cas9 is able to bind the fused RNA chimera, resulting in a ribonucleoprotein (RNP) complex [4]. RNPs are increasingly used for genome editing in mammalian cell lines that are difficult to transfect, *e.g.*, human primary cells, hematopoietic stem, and progenitor cells [9,10]. However, similar approaches have not been established for Apicomplexa or other protozoan parasites. A major contribution to the success of this method is the direct delivery of RNPs that are immediately active after nuclear import bypassing the requirement for heterologous Cas9 expression in target cells. Of note, the use of Cas9 in human cell lines was reported to induce DSB at the target site almost immediately after delivery, after which the enzyme is degraded rapidly, greatly reducing the chance of toxicity and off-target effects [9,10].

The crRNA used in this study consists of 20 nucleotides (nt) DNA recognition region and a 16 nt repeat region complementary to the 5’ tracrRNA (Fig. 2A). The tracrRNA consists of a region homologous to the 16 nt repeat region (anti-repeat) of the crRNA and therefore hybridizes efficiently to the corresponding region of the crRNA. It also

includes three stem-loops, which are required for Cas9 binding (Fig. 2B).

Native RNA molecules are prone to rapid degradation by ubiquitous nucleases in the context of cell culture systems [11]. Hence, the stability, potency, and therefore half-life [11–13] of synthetic RNAs used for transfection assays need to be increased by introducing specific chemical modifications originally developed in the oligotherapeutics field. Several studies report CRISPR/Cas9 approaches using chemically modified crRNA and tracrRNA resulting in enhanced stability, *in vitro* editing efficiency, as well as improved specificity [9,14]. The most frequently used modifications in synthetic RNAs are phosphorothioate (PS) bonds and 2’-O-Methyl (2’OMe) [11,15]. RNAs with 2’OMe (Fig. 3B) are conformationally more rigid and chemically more stable than natural RNA molecules. In PS linkages (Fig. 3C), one of the non-bridging oxygen atoms of the phosphodiester linkage between two ribonucleotides in the backbone is replaced with a sulfur atom. Replacement of three or more phosphodiester linkages with PS linkages improves overall nuclease resistance and antisense activity [13]. The effect of a single chemical modification varies based on the position of the nucleotide, the combination of chemical modifications introduced into a molecule as well as interactions with other modified nucleotides. Optimizing RNAs for stability and efficiency is empirical [16] but data from previous

- ✓ Chemically modified crRNA and tracrRNA
- ✓ p2854 DHFR-TS* [8] and HA-DHFR-TS*(unpublished) plasmids
- ✓ gDNA extraction kit (Thermo Fisher, Phire tissue direct PCR Master kit, Cat. # F-170S)
- ✓ Diagnostic primers
- ✓ Online tools:
 - <http://toxodb.org/toxo/>
 - <http://grna.ctegd.uga.edu/>
 - <https://tide.deskgen.com/>
- ✓ Glutathione (GSH): 100 mM in H₂O, pH to 7.0 with KOH.
- ✓ Annealing buffer: 30 mM HEPES, pH 7.5, 100 mM potassium acetate.
- ✓ Intracellular (IC)-buffer: 5 mM NaCl, 142 mM KCl, 1 mM MgCl₂, 2 mM EGTA, 5.6 mM Glucose, 25 mM HEPES, pH to 7.2 with KOH.
- ✓ Pyrimethamine (Lubio Science Selleckchem, Cat. # S2006), 10 mM stock solution in DMSO.
- ✓ 5-Fluoro-2'-deoxyuridine (FUDR, Sigma, Cat. # F0503), 10 mM stock solution in H₂O.

NOTES: (1) Filter-sterilize reagents designated for cell culture. (2) Store solutions at 4°C or freeze aliquots where applicable.

Recipes

- ✓ Dulbecco's Modified Eagle's Medium (DMEM, Sigma Cat. # D6429) supplemented with 10% heat inactivated fetal bovine serum (FBS, Bioswisstec, Cat. # S0613), 2 mM L-glutamine (Sigma, Cat. # G7513-100 ml) and 100 units/ml penicillin, 0.10 µg/ml streptomycin, 0.15 µg/ml amphotericin B (PSF, Gibco, Cat. # 15240062-100 ml).
- ✓ PBS: 0.137 M NaCl, 0.0027 M KCl, 0.01 M Na₂HPO₄, 0.0018 M KH₂PO₄, pH 7.4.
- ✓ Cytomix: 120 mM KCl, 0.15 mM CaCl₂, 10 mM K₂HPO₄/KH₂PO₄ pH 7.6, 25 mM HEPES pH 7.6, 2 mM EGTA, 5 mM MgCl₂ in H₂O.
- ✓ Adenosine 5'-triphosphate disodium salt (ATP): 100 mM in H₂O, adjusted to pH 7.0 with KOH.

Equipment

- ✓ Thermocycler (Biorad C1000 Touch, Thermal Cycler)
- ✓ Agarose Electrophoresis System (Biorad, PowerPac HC and gel chamber)
- ✓ Chemiluminescence Imager (Witec, Multi Wavelength Illuminator)
- ✓ Nanodrop (Thermo Scientific, NanoDrop One Microvolume UV-Vis Spectrophotometer)
- ✓ Electroporation system (Bio-Rad Gene Pulser X cell)
- ✓ Cooling centrifuge with 15 ml tube holders (Beckman Coulter) and Eppendorf tube holders (Eppendorf Centrifuge 5418R)

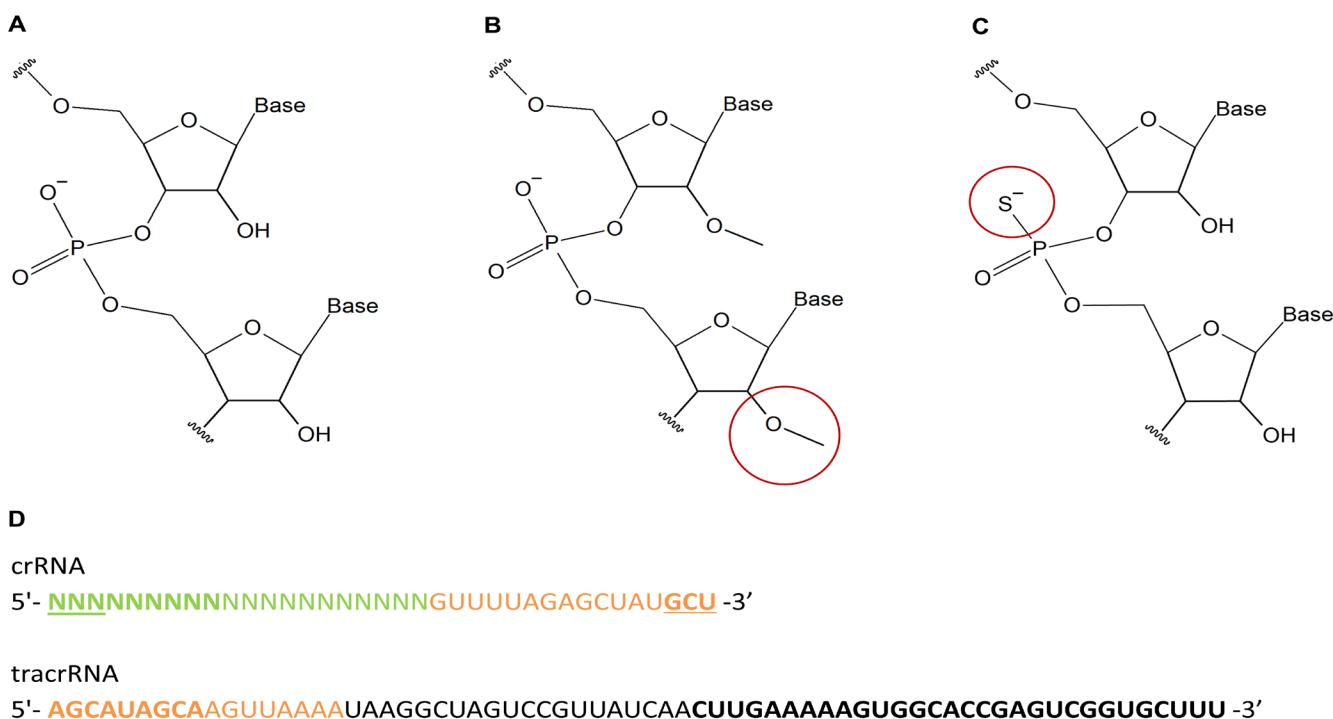


Figure 3. Chemical modifications that are introduced into the crRNA and tracrRNA design. **A.** Native RNA nucleotide. **B.** The hydroxyl group at the 2' position of the ribose is substituted with a methoxy group (2' OMe). **C.** The non-bridging oxygen of the phosphodiester linkage between two ribonucleotides of the native phosphate backbone is substituted with sulfur. **D.** Chemical modifications applied to the tracrRNA and crisprRNA used in this study. PS bonds are underlined, 2'O-Me are represented in bold. The Ns refer to the 20 nt crRNA sequence which is specific to each GOI.

9. Check the PCR product quality by agarose gel electrophoresis.
10. Determine the concentration of the PCR product by Nanodrop.
11. Precipitate 40 µg of the PCR product using 10% sodium acetate 3 M and 3× volumes of ice-cold 100% EtOH.
12. Incubate at −20°C for > 20 min.
13. Centrifuge at 16000 × *g* for 30 min at 4°C.
14. Discard the supernatant.
15. Wash the pellet by adding 1000 µl 70% ice-cold ethanol. Either store the pellet in 70% ethanol at −20°C or proceed directly for transfection.
16. Centrifuge the pellet at 16000 × *g* for 10 min at 4°C and discard the supernatant.
17. Air-dry the pellet for approximately 20 min in the laminar flow, and then dissolve in 100 µl cytomix supplemented with 2 mM ATP and 5 mM GSH.

HINTS: Purification of the KOD PCR reaction might reduce the risk of primer integration into the genome as they contain 30 bp of homologous regions to the genome.

Preparation of the RNP complex

18. Dissolve crRNA and tracrRNA in nuclease-free water each to 100 µM.
19. Dilute crRNA and tracrRNA in annealing buffer to 40 µM.
20. Mix crRNA and tracrRNA equimolar in a PCR tube.
21. Follow a temperature gradient protocol to anneal crRNA:tracrRNA (sgRNA formation, **Fig. 4A**). Start with 92°C for 2 min and gradually decrease the temperature over 30 min to RT.
22. Mix 5.8 µl IC buffer and 0.6 µl NEB Cas9 NLS.
23. Mix 4 µl of the cooled sgRNA with Cas9/IC-buffer and incubate at RT for at least 20 min, which results in the RNP complex formation (**Fig. 4B**).

NOTES: (1) Whereas in *T. gondii*, 12 pmol Cas9-NLS was the only of all tested conditions (4 pmol, 12 pmol, 24 pmol, 120 pmol) that led to successful gene disruption, in *B. besnoiti* all tested concentrations (12 pmol, 24 pmol, 120 pmol) were efficient. However, due to the possible toxic effects of Cas9, concentrations should be kept at a minimum. (2) Within the RNP complex, sgRNA and Cas9 are predicted to exist in a 1:1 molar ratio. However, several studies reported that an excess of sgRNA leads to higher knockout efficiencies [19,20].

HINTS: (1) Annealed crRNA:tracrRNA (step 21) can be stored at −20°C, however should be re-annealed before the next use. (2) IC buffer can be replaced by cytomix without ATP/GSH (step 22). (3) Use whenever possible nuclease-free reagents. Elimination of RNase from laboratory surfaces using *e.g.*, RNase AWAY (Sigma, 83931) might be considered.

Parasite transfection and drug selection

24. Grow parasites in a T25 cell culture flask on HFF for 3–4 d until big vacuoles and freshly egressed parasites are visible (**Fig. 4C**).
25. Scrape the infected HFF monolayers from the T25 flask and homogenize cells by syringe passage through 24 G and 22 G needles to fully release the parasites.
26. Filter the parasites with a 5 µm syringe filter into a falcon tube.
27. Centrifuge at 1000 × *g*, 5 min, 4°C and discard the supernatant.
28. Wash the parasites once in ice-cold PBS; use an aliquot for parasite count using a hemocytometer. One T25 flask typically results in 5 × 10⁷ parasites.
29. Centrifuge at 1000 × *g*, 5 min, 4°C and remove the supernatant.
30. Resuspend the parasites in 300 µl cytomix supplemented with 2 mM ATP and 5 mM GSH.
31. Mix 100 µl KOD PCR product, 300 µl parasites, and the RNP complex and transfer to pre-cooled 2 mm electroporation cuvette.

32. Electroporate the parasites with 2 pulses of a pulse length of 0.3 ms in an interval of 5 s with 1.5 kV.
33. After electroporation, let the cuvette stand for 5 min at RT.
34. Add the transfected tachyzoites to a confluent T25 flask with HFF cells and let grow at 37°C with 5% CO₂ in a humidified incubator.
35. 24 h post-transfection, drug selection is initiated with 1 μM pyrimethamine for resistance to DHFR-TS* in *T. gondii* or 10 μM FUDR for *B. besnoiti* for selection of *uprt* knockout mutants.
36. After 3–4 d, drug-resistant parasites emerge.
37. Single clones are isolated by limiting dilution cloning.
38. Amplify single clones by passaging to get enough material for genomic DNA extraction and the assessment for successful integration of the selection marker by diagnostic PCR and Sanger sequencing.

NOTES: For cell culture, parasite handling, passage as well as transfection, the same protocols apply to *B. besnoiti* as for *T. gondii*.

HINTS: (1) Transfection of a Cas9 expressing plasmid with the chemically modified sgRNA is possible as the latter is stable to reside within the parasite until the Cas9 is expressed. (2) Cas9-NLS protein can be produced from an expression vector *e.g.*, Adgene plasmid # 62933. (3) Further adaptations (*e.g.*, the incorporation of 2'-deoxy-2'-fluoro-ribose nucleotide) for the chemical stabilization of the crRNA and tracrRNA might be considered for enhanced transfection efficiency in *T. gondii* and *B. besnoiti*. Transfection efficiencies of up to 20%–30% that are reached in some cell lines [17] would be desirable to circumvent the need for co-transfecting selection markers.

TIDE analysis for efficiency control

39. Use the ME49 wild type strain instead of the ΔKU80 as only the wild type parasites have the capability of introducing indels that can be monitored by TIDE.
40. Transfection of the RNP is sufficient, as there is no need for a selection marker.
41. Perform a mock transfection with wild type parasites in absence of an RNP complex.
42. After 3–4 d, harvest the parasites (Steps 25–29).
43. Extract the genomic DNA.
44. Perform a standard PCR with genomic DNA as template from the mock and RNP transfection. Primers should be designed according to the instructions section in <https://tide.deskgen.com/>.
45. Purify the PCR products using *e.g.*, the Promega Wizard SV Gel and PCR Clean-Up System.
46. Sequence the PCR products using standard sequencing reactions.
47. Enter the 20 nt crRNA sequence as well as the chromatogram (.ab1) files from the sequencing reaction into TIDE. The chromatogram from the mock transfection is entered under “Control Sample Chromatogram (.ab1)” and the Chromatogram from the RNP transfection under “Test Sample Chromatogram (.ab1)”.
48. Pressing the “Update View” button will display the transfection efficiency.

NOTES: (1) PAGE purified primers for TIDE decrease the background from the Sanger sequencing and lead to more reliable efficiency predictions. However, if TIDE is only taken as means to compare the different methods or crRNAs, conventional primers are sufficient. (2) Editing efficiencies in *T. gondii* ME49 wild type and *B. besnoiti* wild type predicted by TIDE were between 1.7%–2.3% depending on the target and selected crRNA. Using PAGE purified primers resulted in predicted efficiencies of up to 5% due to the reduced background in the Sanger sequencing. (3) A direct comparison with the conventional approach is not appropriate, as the usual comparative criteria (*e.g.*, transfectants per μg plasmid DNA) cannot be applied to both methods. The closest possible approximation is the comparison of the new approach (one sgRNA) with the conventional 2 sgRNA plasmid-directed approach. The crRNA used in the former was identical either to the first or second crRNA sequence of the conventional approach. Targeting TGME49_219742 led to a clear increase of the efficiency (using TIDE) from 0.6% to 2.3% (**Fig. S4**). Targeting TGME49_309990, TIDE analysis showed comparable efficiencies (2.2 % and 2.3%) (**Fig. S5**). (4) The TIDE prediction error was estimated to lay between 0.2%–0.8% when comparing ab1. files of PCR sequences from separate mock transfections using wild type parasites (**Fig. S6**).

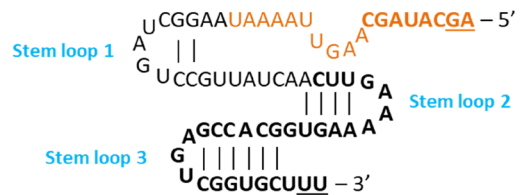
HINTS: TIDER (<https://tider.deskgen.com/>) is a more recent web tool version which allows the efficiency assessment of template-mediated genome editing.

A

crRNA



tracrRNA



B

sgRNA



Cas9



C

RNP

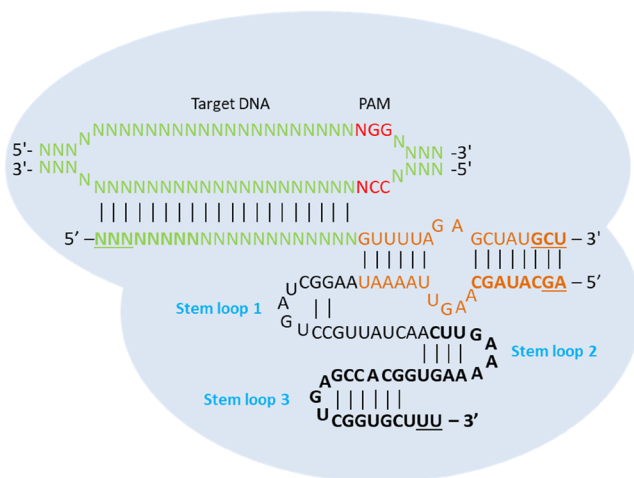


Figure 4. Streamlined workflow for genome manipulation in *T. gondii* and *B. besnoiti*. **A.** Annealing of the crRNA to the tracrRNA is carried out following a temperature gradient for 30 min, resulting in the formation of the sgRNA complex. **B.** The sgRNA is complexed to the Cas9-NLS by an incubation step of 20 min at RT. **C.** RNP transfection of the parasites by electroporation can be performed within approximately 60 min.

ANTICIPATED RESULTS

This streamlined CRISPR/Cas9 approach enables the introduction of single point and insertion/deletion mutations, precise integration of in-frame epitope tags, and deletions/replacements of genes at significantly

reduced time and costs compared to the method previously utilized in *T. gondii* and *B. besnoiti*. Furthermore, it decreases the risk of Cas9 toxicity, off-target modifications, and random integration of plasmid DNA into the genome. Synthetic, chemically modified sgRNAs allow for a modular and versatile system using either the conventional endo-

nuclease Cas9 but can be further combined with fluorescently labeled Cas9 for subsequent FACS sorting, dCas9 or Cas9 expressed from a plasmid. Therefore, this CRISPR/Cas9 method significantly increases the possibility of generating biological information on these complex, intracellular parasites.

TROUBLESHOOTING

Possible problems and their troubleshooting solutions are listed in **Table 1**.

Table 1. Troubleshooting.

Step	Problems	Causes	Suggestions
2	The efficiency of single crRNAs can vary widely	Efficiencies vary based on the nucleotide compositions and secondary structures of sgRNAs	It might be necessary to design more than one crRNA if first trials are not successful
11,17	Arcing during electroporation	An excess of salts present in the transfection reaction might cause arcing and lead to reduced viability of the parasites and transfection efficiency	PCR products should therefore be precipitated and IC buffer volume should be minimal
48	The transfection efficiencies reached in the two parasites is close to the minimal detection limit of TIDE	TIDE is designed for transfection efficiencies of up to 30%, reached in human cell lines	To monitor efficiencies in parasites, the experiment should be repeated three times; similar efficiency outcomes indicate a correct result

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Supplementary information

Figure S1. Knockout strategy.

Figure S2. C-terminal tagging strategy.

Figure S3. N-terminal tagging strategy.

Figure S4. TIDE Indel Spectrum of TGME49_219742.

Figure S5. TIDE Indel Spectrum of TGME49_309990.

Figure S6. TIDE Indel Spectrum of the wild type control.

Supplementary information of this article can be found online at <http://www.jbmethods.org/jbm/rt/suppFiles/343>.



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