

Tools for CRISPR/Cas9-mediated gene editing in *Babesia duncani*

Standwell C. Nkhoma[†], Amel O.A. Ahmed, Abigail Min, Sharmeen Zaman, Sujatha Rashid, Rebecca Bradford, and Robert E. Molestina
ATCC Federal Solutions, ATCC, 10801 University Boulevard, Manassas, VA 20110-2209, USA

Correspondence: snkhoma@atcc.org



CRISPR/Cas9 technology is a valuable tool for gene editing in various apicomplexan parasites, including *Plasmodium falciparum*, the causative agent of human malaria. However, there is a dearth of CRISPR-based genetic modification tools for *Babesia duncani*, a related apicomplexan parasite responsible for human babesiosis, an emerging tick-borne disease. Such tools could play a pivotal role in probing gene function and identifying new targets for drug and vaccine development. The availability of an established *in vitro* culture system for *B. duncani* and a published genome offers a promising opportunity to develop an efficient gene editing system in this parasite. We leveraged advances in genome modification protocols for bovine *Babesia* parasites to create *B. duncani* lines that stably express GFP or mCherry for various downstream applications, including compound screening. To achieve this, we developed a Cas9/gRNA expression plasmid that is codon-optimized for *Babesia* parasites. This plasmid expresses Cas9 from *Streptococcus pyogenes* and a guide RNA scaffold into which target-specific gRNAs can be cloned. To test the practical utility of this Cas9/gRNA expression vector, gRNAs specific for the parasite apical membrane antigen 1 (AMA-1) or thioredoxin peroxidase 1 (TPX-1) were cloned between its *Bsbl* sites. The resulting plasmid and a donor DNA plasmid expressing either mCherry-tagged AMA-1 or GFP-tagged TPX-1 were co-transfected into *B. duncani* parasites. Results from these transfections highlight the practical utility of this gene editing system. Through this work, our efforts will contribute towards the identification of novel targets for drug and vaccine development using reverse genetics, and a better understanding of the biology of *Babesia* parasites that affect humans.

Background

- ❖ Babesiosis is a malaria-like disease caused by *Babesia* parasites that are transmitted to humans through bites from infected ticks (**Figure 1**).
- ❖ There are over 100 known *Babesia* species, but the major causative agents of human babesiosis are *microti*, *divergens*, and *duncani*.

www.cdc.gov

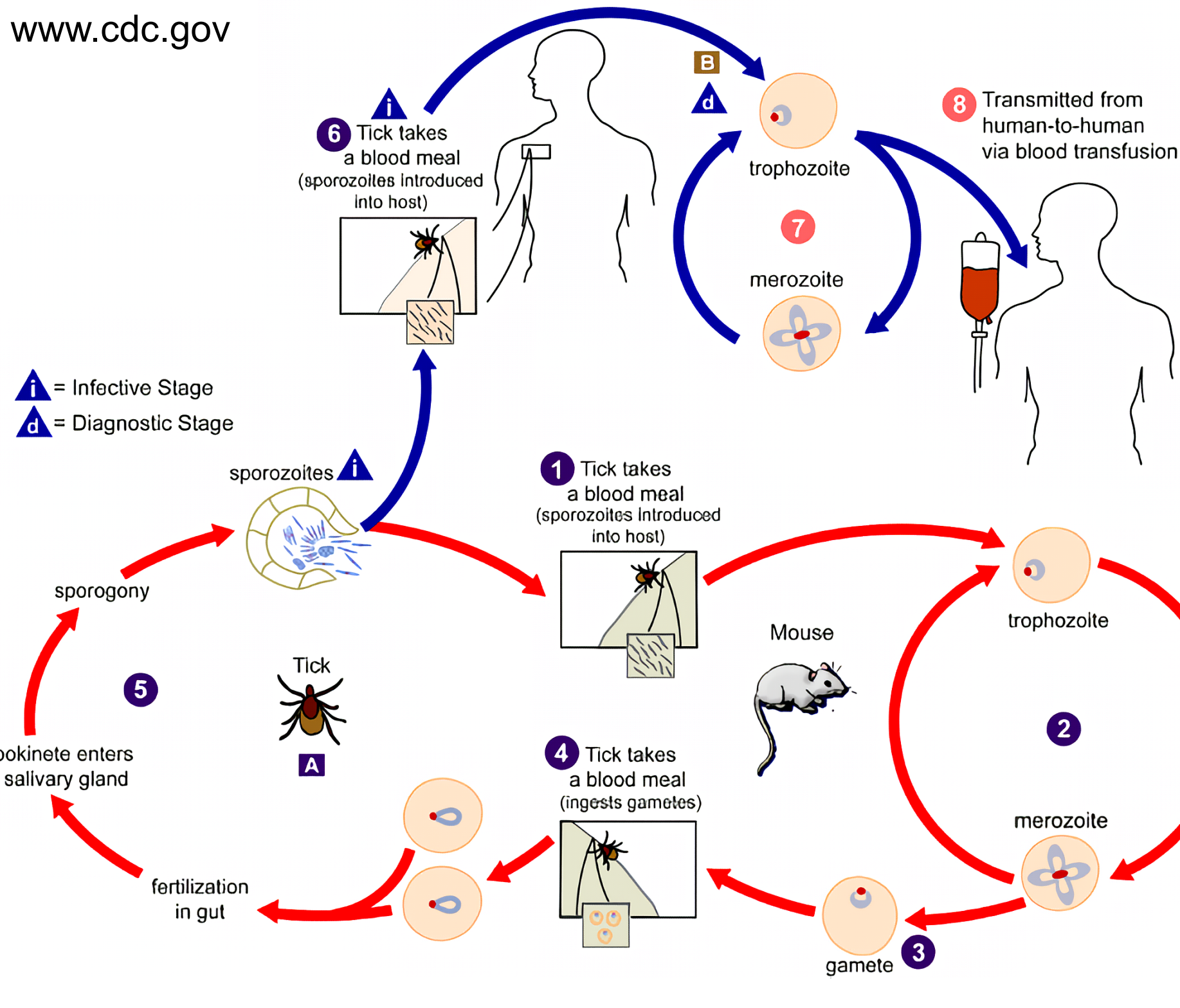


Figure 1. Life cycle of human babesia parasites. Humans are incidental hosts; primary hosts include a rodent and a tick of the genus, *Ixodes*. A human being is infected through a bite from an infected tick, for example, while hiking. During a blood meal, the tick inoculates babesia parasites (sporozoites) into erythrocytes (6), where they undergo differentiation and continuous rounds of asexual replication (7), leading to clinical symptoms such as fever, chills, fatigue, and hemolytic anemia. Humans are dead-end hosts. Adapted from CDC: <https://www.cdc.gov/dpdx/babesiosis/index.html>

- ❖ A limited number of drugs are available for the treatment of human babesiosis & there is no suitable vaccine for it.
- ❖ Understanding gene function is critical for developing new drugs and vaccines for human babesiosis, but there are no efficient tools to do so.

Project Goals & Objectives

Project Goal: To develop CRISPR/Cas9 genome modification tools for human *Babesia* parasites that can be used to probe gene function.

Specific Objective: To adapt genetic manipulation protocols routinely used for *P. falciparum* and bovine *Babesia* parasites to create *Babesia duncani* reporter lines expressing the Green Fluorescent Protein (GFP) or the red fluorescent protein, mCherry, which could be used to screen for *in vitro* activity of candidate drug compounds.

Technical Approach

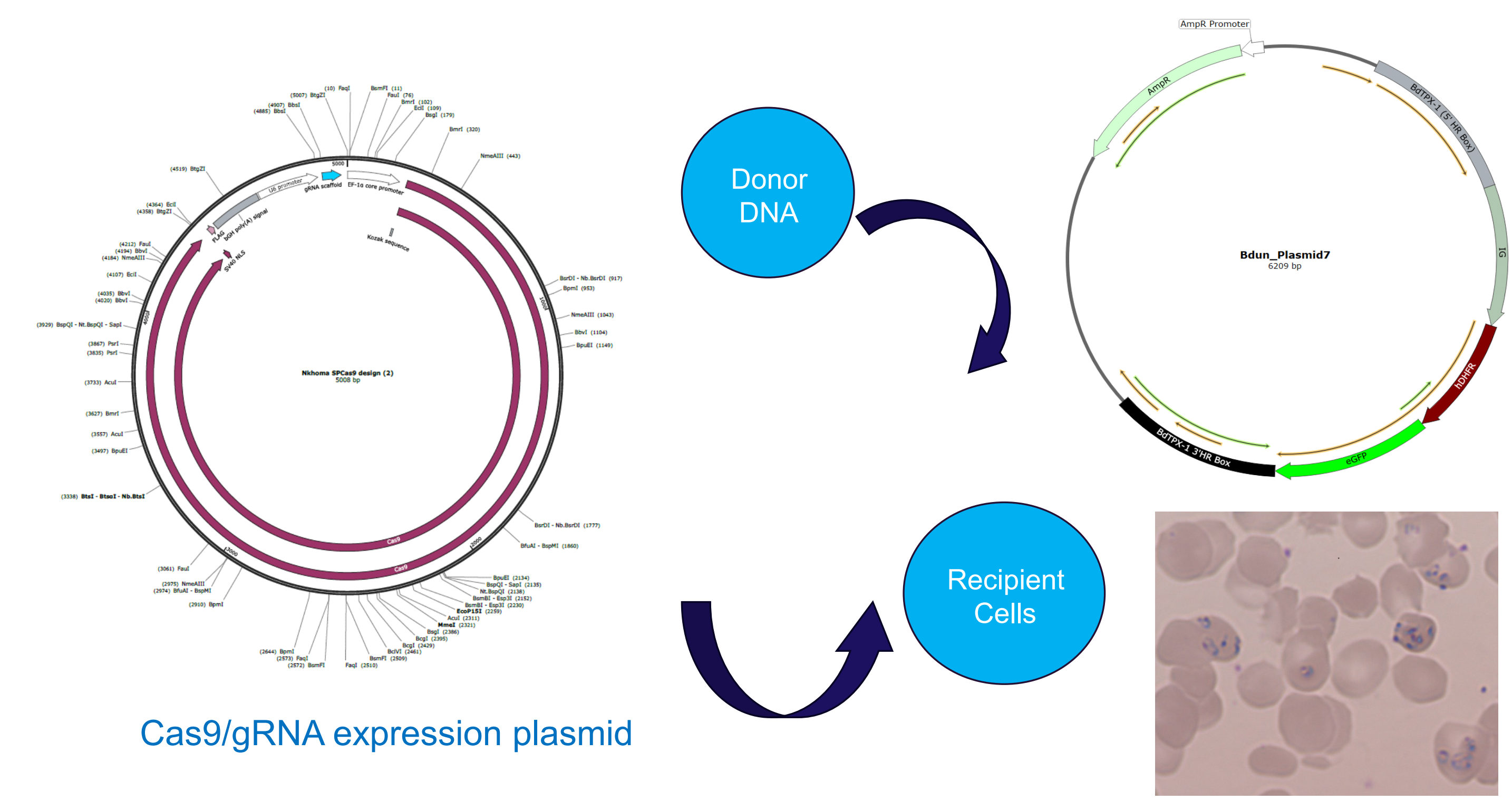


Figure 2. Genome modification in *Babesia duncani*. To introduce a GFP-tagged or mCherry-tagged gene into *B. duncani* parasites, gRNAs specific for either the thioredoxin peroxidase 1 (TPX-1) or apical membrane antigen 1 (AMA-1) were cloned between the two *Bsbl* sites in the Cas9/gRNA expression plasmid (left panel). The resulting plasmid was expanded using chemically competent cells and validated using next-generation sequencing. Donor plasmids containing GFP-tagged TPX-1 (right panel) and mCherry-tagged AMA-1 were obtained from our collaborators. Both plasmids were introduced into a cloned *B. duncani* parasite NR-59103 (BEI Resources) by electroporation, followed by 10 days of selection with the antifolate drug, WR-99210. Transfected parasites were recovered three days after the cessation of drug selection and cultivated in drug-free media.

Parasites transfected with a donor plasmid containing a GFP-tagged TPX-1 exhibit green fluorescence

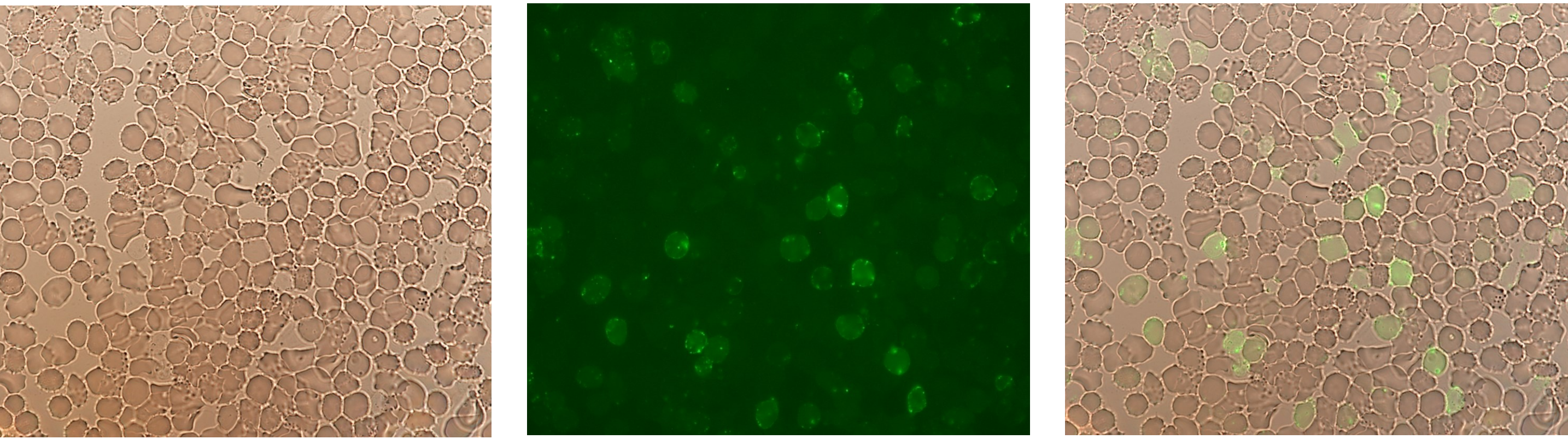


Figure 3. Successful transfection of a GFP-tagged gene into the *B. duncani* genome. Thin blood smears were prepared from transfected cells 14 days after the cessation of drug selection. Slides were visualized using a fluorescence microscope (wet mount, 100x magnification). A bright field image (left panel) was merged with the GFP image (middle panel) to better visualize the parasitized cells. Parasitized cells expressing GFP were visible. The GFP signal in these parasites remained stable after several cycles in culture and following cryopreservation of the cells and subsequent re-growth from frozen vials.

Parasites transfected with the donor plasmid containing an mCherry-tagged AMA-1 show red fluorescence

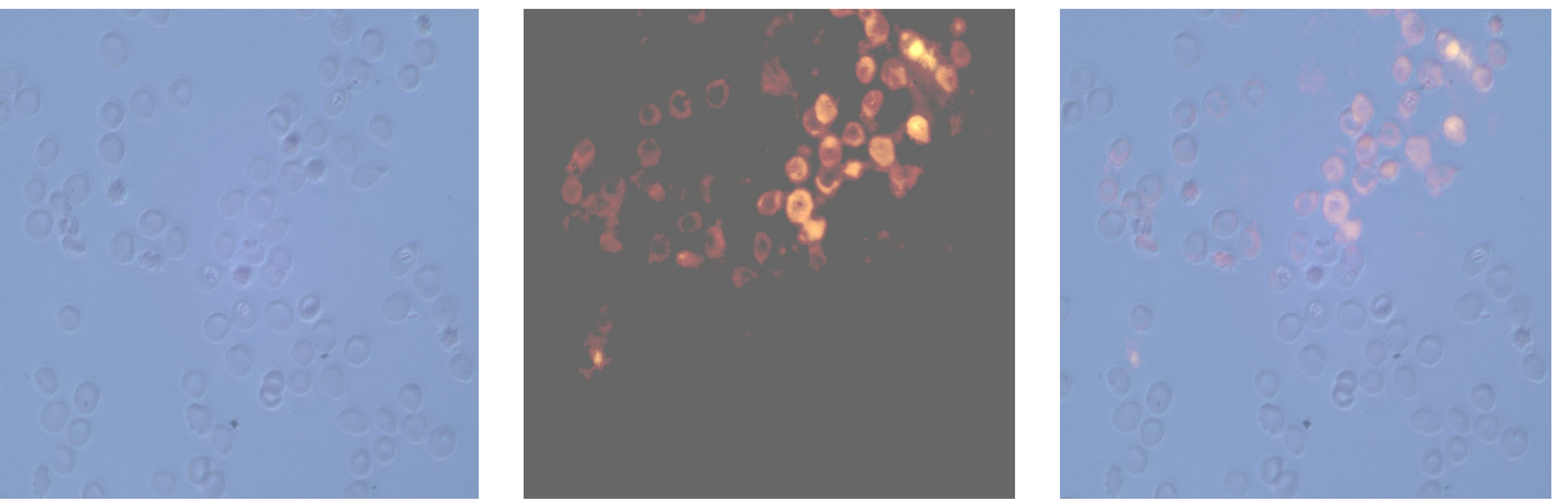


Figure 4. Visualization of parasites transfected with an mCherry donor plasmid. A bright field image (left panel) was merged with the GFP image (middle panel) to better visualize the parasites. The merged image (right panel) shows parasitized erythrocytes with red fluorescence signal, indicating successful transfection of mCherry-tagged AMA-1 into the progenitor parasites.

Concluding Summary

- ❖ We have developed a codon-optimized Cas9/gRNA expression plasmid that can be utilized for editing different *B. duncani* genes to probe their functions
- ❖ We have successfully adapted gene modification protocols previously used in *B. bovis* and *P. falciparum* to perform gene editing in *B. duncani*
- ❖ We have used these protocols to create *B. duncani* GFP and mCherry-expressing lines, which can be used for various applications, including compound screening.

Planned Future Work

- ❖ Immunofluorescence Assays using anti-GFP and anti-mCherry antibodies to amplify the fluorescence signal and enhance visualization of parasitized cells.
- ❖ Whole genome sequencing of both the progenitor *Babesia* parasite, NR-59103, & transfected parasite lines to confirm integration and examine off-target effects.
- ❖ Examine the potential use of GFP and mCherry-expressing lines for compound screening.

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