**5. Utilization of negative selection at the *SNR1* locus for genetic complementation or transgenic strain construction.**

Note: Inactivation of *SNR1* leads to sinefungin resistance without compromising the growth or fitness of the parasites. This makes *SNR1* an ideal locus for genetic complementation or transgene insertion, particularly in cases when drug selection options are limited. These protocols provide detailed steps for using CRISPR/Cas9 mediated site-specific integration at the *SNR1* locus for such applications (Figure 5). Insertion of a mini gene (*DHFR\**, a mutant allele of *DHFR-TS*) conferring pyrimethamine resistance into *SNR1* is used for demonstration.

5.1. Transgenic construct preparation.

Note: In order to express gene of interest (GOI) from the *SNR1* locus, it should be flanked by a promoter and a terminator to drive its expression. Such mini gene (promoter-GOI-terminator) can be constructed on a plasmid vector by classic cloning methods or by multi-fragment cloning. Multi-fragment cloning is recommended since it is efficient, cost- and time-effective. For example, to construct a transgenic parasite line expressing *DHFR\** from the *SNR1* locus, the promoter and terminator sequences of the *DHFR-TS* gene is used to drive the expression of *DHFR\**.

5.1.1. PCR amplify each fragment (promoter, GOI coding sequence, terminator and vector backbone) using primers with proper overlaps according to the instructions from the multi-fragment cloning kits.

5.1.2. Purify these PCR products by agarose gel electrophoresis and determine their concentrations by spectrophotometry.

5.1.3. Perform ligation reaction according to manufacturer's instructions.

Note: A control reaction lacking one or more of the inserts should be done in parallel.

5.1.4. Transform ligation products into *E. coli* competent cells using a chemical transformation protocol 1 and grow the transformants on LB plates containing corresponding antibiotics (determined by the cloning vector used).

5.1.5. Pick 4 – 6 colonies from the selection plate and individually inoculate them into 5 mL LB medium with corresponding antibiotics. Grow the cultures at 37 °C for 12 – 16 h with shaking.

5.1.6. Isolate plasmids from the cultures using a miniprep kit and verify the plasmids by restriction enzyme digestion and DNA sequencing.

5.1.7. Once positive clones are identified, store the plasmids at -20 °C for further use.

5.1.8. Obtain the promoter-GOI-terminator fragment from a positive plasmid by PCR amplification or enzyme digestion. Determine the concentration of the obtained fragment by spectrophotometry and use it for parasite transfection.

Note: In the demonstration experiment, the *DHFR\** mini gene is PCR amplified from the plasmid pUPRT::DHFR-D using the following primers: 5'-DHFR: CAGGCTGTAAATCCCGTGAG and 3'-DHFR: GATTCCGTCAGCGGTCTGTC. If the genetic complementation or transgenesis is to be done in a *Δku80* strain, the mini gene should be flanked by sequences homologous to the *SNR1* locus, since NHEJ activity is extremely low in *Δku80* strains. In such cases, the homologous sequences can be as short as 40 nt and are derived from the sequences near the CRISPR targeting site. They can be incorporated into the primers that amplify the mini gene for transfection 2.

5.2. Parasite transfection

5.2.1. In a cuvette, mix (1:1 molar ratio) 1.5 μg purified *DHFR\** PCR product and 4.5 μg CRISPR plasmid (pSAG1::CAS9-U6::sg290860-6) with corresponding parasites. Follow protocol section 4.2 for parasite transfection. In parallel, perform a transfection with no DNA included as a negative control.

Note: The total amount of DNA (CRISPR plasmid plus transgenic construct) used for transfection is suggested to be between 5 and 10 μg. However, the ratio between these two DNA molecules needs to be adjusted based on the subsequent selection strategies (see the Discussion).

5.3. Drug selection

5.3.1. Two days post transfection, replace the growth medium in the T25 flask containing transfected parasites with 5 mL selection medium according to the experimental design.

Note: D10 medium containing final concentrations of 1 μM pyrimethamine and 0.3 μM sinefungin is used to select *snr1::DHFR\** parasites.

5.3.2. Keep the parasites in selection medium for 2 - 3 passages (about 2 weeks) to obtain stable drug resistant parasites.

Note: Pyrimethamine is often used to treat human patients with active/acute *Toxoplasma* infection, so care should be taken when working with pyrimethamine resistant parasites to prevent accidental exposure, such as a needle stick (use blunt needles), as pyrimethamine will not be useful as a treatment in situations where infection occurs with drug resistant parasites. While pyrimethamine is used in the treatment of *Toxoplasma* infection in humans, because resistance to this drug occurs naturally via mutations in the DHFR-TS gene, selection using pyrimethamine is not subject to NIH Guidelines Section III-A. Additionally, other effective drug treatments are available for human use if pyrimethamine is not an option, such as clindamycin/sulfonamide or atovaquone.

5.3.3. When the drug resistant pool becomes stable (indicated by the absence of parasite growth in the control experiment), take an aliquot (≈ 2 mL) of naturally egressed parasite solution and extract genomic DNA for diagnostic PCR.

5.3.4. Perform PCR-F1R2 (Figure 5A) to check whether the pool contains transgenic parasites. A positive pool produces a larger sized PCR-F1R2 product than the WT strain due to the transgene integration.

5.4. Parasite subcloning

5.4.1. Once a positive pool is obtained, follow the procedures described in steps 4.4.2 – 4.4.3 to subclone the pool.

5.5. Screen individual clones by diagnostic PCR.

5.5.1. Prepare genomic DNA from single clones as in step 4.4.4.

5.5.2. Perform PCR-F1R2 (Figure 5A) to check whether the transgene is integrated at the *SNR1* locus.

Note: If GOI is indeed inserted into *SNR1*, then four sets of PCRs can be done to examine the integration orientation of GOI. These include PCR-F1R1/-F2R2, positive products of which suggest forward integration, and PCR-F1F2/-R1R2, positive products of which suggest reverse integration (Figure 5B). In many applications where the inserted transgenes cannot be directly selected with drugs, its expression from the *SNR1* locus needs to be examined by Western blotting 3, reverse transcription-polymerase chain reaction (RT-PCR) 1, fluorescent microscopy, or immunofluorescent microscopy 4. For example, to insert a fluorescent gene into *SNR1* to construct a reporter strain, selected clones can be examined by fluorescent microscopy to confirm the expression of the fluorescent protein.

5.6. Make frozen stocks for positive clones.

5.6.1. Once positive clones are identified, expand them in T25 flasks and make frozen stocks for long-term storage. See 5 for detailed protocols for parasite freezing.

Note: The methods and techniques in Protocol 5 can be built upon to use the CRISPR/Cas9 system for homologous integration of a construct to create allelic replacements or knockouts (Figure 6) 6-8.

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