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Protocol for sgRNA in vitro transcription and screening for effective SaCas9 RNP complex cleavage assay

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Protist Research to Optimize Tools in Genetics (PROT-G)

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ABSTRACT

Developing transfection protocol for *Bodo saltans*, using SaCas9/sgRNA ribonucleoprotein (RNP) complex in conjunction with DNA repair template to disrupt the Paraflagellar rod 2 gene (*BsPFR2*) and increase the efficiency of targeted homologous recombination when a repair template DNA is provided. The exogenous repair template is double stranded DNA and it consists of *eGFP* fused with the drug selection gene *nptII/neo* and flanked by 500 bp of the untranslated regions (UTRs) upstream and downstream of the targeted *BsPFR2* as homologous repair arms.

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Primer ID	Forward sgRNA primers including the sgRNA sequences	PAM
SaCas9gRNA_PFRF1_KO	GAGAATTGTAATACGACTCACTATAGGGAGAGCGACGTCTTCAGCTTCAGATTGTTTTAGTACTCTGTAATTTTAGGTATGAGGTAGACGAAAATTGTA	GTGGAT
SaCas9gRNA_PFRF3_KO	GAGAATTGTAATACGACTCACTATAGGGAGAGCCAGTCGGACACGTGCAGGTCGTTTTAGTACTCTGTAATTTTAGGTATGAGGTAGACGAAAATTGTA	CTGGAT
SaCas9gRNA_PFRF4_KO	GAGAATTGTAATACGACTCACTATAGGGAGAGTTGCCGTGCTGAAGAACCTCGGTTTTAGTACTCTGTAATTTTAGGTATGAGGTAGACGAAAATTGTA	AGGAGT
SaCas9_sgRNA_R	AAAAAAATCTCGCCAACAAGTTGACGAGAT	
SaCas9_scaff_F	GTTTTAGTACTCTGTAATTTTAGGTATGAGGTAGACGAAAATTGTACTTATACCTAAAATTACAGAATCTACTAAAACAAGGCAAAATGCCGTGTTTA	
SaCas9_scaff_R	AAAAAAATCTCGCCAACAAGTTGACGAGATAAACACGGCATTTTGCCTTGTTTTAGTAGATTCTGTAATTTTAGGTATAAGTACAATTTTCGTCT	

Table 1. List of the primers that were used for constructing sgRNAs with target sequences included in the forward primers in blue. PAM sequences in red color.

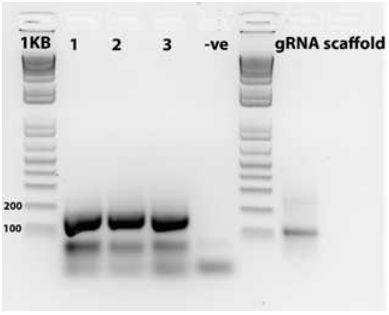

Figure 1: Gel electrophoresis image of the three sgRNAs in lane 1 to 3 and the gRNA scaffold used to prepare these sgRNA in this study.

Figure 2: Gel electrophoresis image showing that the three synthesized gRNAs directed Cas9-mediated cleavage of the target *BsPFR2*gene amplified fragment. Lanes S1 and S2 (on the right) are the amplified fragment of the *BsPFR2* with primers PF1-PR3 at 2kb. The differences between both S1 and S2 is the concentration of the amplified products that was used.Lanes 1 to 4 are the products of the S1 samples post the in vitro cleavage assay with SaCas9 RNP1, 2, 3 and all the 3 SaCas9 RNPs complex combined, respectively; showing that the 2kb band is “digested”. Lanes 5 to 8 are the products of the S2 samples post the in vitro cleavage assay with SaCas9 RNP1, 2, 3 and all the 3 SaCas9 RNPs complex combined, respectively, also showing that the 2 kb is digested into lower fragments.

1 Step 1: *PCR amplification of the targeted gene*
-The targeted gene in our study is the 69 KDa paraflagellar rod protein 2C (PFR-2) from extracted DNA of wild *Bodo saltans*.
1. The PCR amplification reaction consists of Onetaq® 2x master mix with standard buffer (New England BioLabs)

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and specific primers that we designed: PF1-GATTCAAGATCGATCTTCGAAC and PR3-TGCGCCTTGATGTAGAACTGCTC to amplify a 2 kb fragment of the PFR-2 gene (**Figure 2, lanes S1 and S2**)
2. Following the PCR amplification, we purified the PCR products using the E.N.Z.A. cycle pure kit following the manufacturer's instructions (Omega, BIO-TEK).

2 Step 2: *Single guide RNA (sgRNA) design and preparation.*

1. sgRNAs targeting the69 kDa paraflagellar rod protein 2C (PFR-2) (scaffold1667 WGS), were designed using the Eukaryotic Pathogen CRISPR Guide RNA/DNA Design Tool (http://grna.ctegd.uga.edu) with the SaCas9 option (21-bp target sequence preceding an NNGRRT PAM site. We designed three forward PCR primers to generate DNA templates for each of the three sgRNAs **(Table 1)**.
2. Each primer is 99 bp long and consists of the T7 promotor sequence, the target sgRNA sequence that does not include the PAM sequence, and the Scaffold Template annealing sequence. The reverse primer consists of the Guide-it Scaffold Template (sequences of primers and the sgRNA are provided in **Table 1**.
3. The sgRNA DNA templates were amplified by PCR using the Onetaq® 2x master mix with standard buffer (New England BioLabs), 30 ul of the sgRNA-specific forward primer at a concentration of 10 uM and 18 ul of the SaCas9_sgRNA_R reverse primer at concentration of 10 uM, and 2ul of the sgRNA scaffold template (ds DNA from annealed and extended oligos the SaCas9_scaff_F and SaCas9_scaff_R) with amplification conditions that consist of the following: 94°C for 2 min, 35X (94°C for 30 sec, 66.5°C for 30 sec, 68°C for 25 sec) and final elongation step 68°C for 5 mins. The PCR products were purified using the E.N.Z.A. cycle pure kit following the manufacturer's instructions (Omega, BIO-TEK).
4. The purified PCR product was used for in vitro transcription (IVT) using the TranscriptAid T7 High Yield Transcription Kit (ThermoFisher). The transcription reaction mix consisted of 500 to 2000 ng of the purified sgRNA DNA template in 6 ul, 2 ul of each NTPs (ATP, UTP, CTP, and GTP), 4ul of 5x in vitro transcription buffer and 2 ul of TranscriptAid Enzyme Mix.
5. The reactions were incubated for 5 hours at 37°C in a preheated thermal cycler with a heated lid. The reaction was terminated by adding 15 ul of 3M sodium acetate and 115 ul of nuclease-free water. The sgRNA was precipitated by adding 2.5 volume of 100% ethanol (375 ul) and placing the tube at -20°C for overnight, followed by centrifugation at maximum speed (>12000 g) at 4°C for 20 minutes.
6. The supernatant was discarded, and the sgRNA pellet was washed by adding 75% ethanol and centrifuging again for 20 minutes at maximum speed (> 12000 g). All supernatants were removed carefully and the sgRNA pellet was air dried for > 20 minutes.
7. The sgRNA was dissolved in 40 ul of nuclease-free water and stored at -80°C. One µl of each sgRNA mixed with 18 µl of nuclease-free water was used to visualize the sgRNAs on 2% gel agarose **(Figure 1).**

3 *Step 3: SaCas9 and sgRNA assembly (RNP complex)* and *in vitro cleavage assay*

1. In a 200 ul PCR tube, we combined 1 µg of each of three sgRNAs with 100 ng of SaCas9. We then incubated this mix in a thermo cycler at 37°C for 5 minutes. Following this incubation, we added 5 µl of the amplified and purified PCR product from above (amplified 2kb of the PFR-2 gene) to the sgRNA/SaCAS9 mix at a concentration of (300-600 ng/ul), plus 1 µl of 15x BSA, and 1 µl of Nuclease-Free Duplex Buffer (Cat#11-05-01-12, Integrated DNA Technologies (IDT)) and adjusted the volume to 15 µl using RNase free water (Zymo Research).
2. The tubes were incubated at 37°C for 1 hour, followed by 5 minutes at 80°C to terminate the reaction and inactive the RNP complex.
3. To visualize the cleaved DNA fragments by the sgRNA/Cas9 complex, we ran the entire 15 µl of each sample on 2% agarose gel along with an appropriate DNA ladder and control samples (PCR products that was not treated by the RNP complex) (**Figure 2).**

4

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SaCas9gRNA_PFRF3_KO	GAGAATTGTAATACGACTCACTATAGGGAGAGCCAGTCGGACACGTGCAGGTCGTTTTAGTACTCTGTAATTTTAGGTATGAGGTAGACGAAAATTGTA	CTGGAT
SaCas9gRNA_PFRF4_KO	GAGAATTGTAATACGACTCACTATAGGGAGAGTTGCCGTGCTGAAGAACCTCGGTTTTAGTACTCTGTAATTTTAGGTATGAGGTAGACGAAAATTGTA	AGGAGT
SaCas9_sgRNA_R	AAAAAAATCTCGCCAACAAGTTGACGAGAT	
SaCas9_scaff_F	GTTTTAGTACTCTGTAATTTTAGGTATGAGGTAGACGAAAATTGTACTTATACCTAAAATTACAGAATCTACTAAAACAAGGCAAAATGCCGTGTTTA	
SaCas9_scaff_R	AAAAAAATCTCGCCAACAAGTTGACGAGATAAACACGGCATTTTGCCTTGTTTTAGTAGATTCTGTAATTTTAGGTATAAGTACAATTTTCGTCT	

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