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Protocol for Transfection of *Bodo saltans* with SaCas9 RNP complex in conjunction with *eGFP-NEO* plasmid by electroporation

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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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MATERIALS TEXT

-Table 1: NEPA 21 electroporation parameters used in our study

	Poring pulse			Transfer pulse								
	V	PD (ms)	PI (ms)	N	decay rate	polarity	V	PD (ms)	PI (ms)	N	decay rate	polarity
1	200	25	0	1	10%	+	60	99	999	5	40%	+/-
2	99	5	50	7	10%	+	99	50	50	5	40%	+/-
3	150	5	50	3	10%	+	50	10	999	5	40%	+/-

-V: voltage strength; PD: pulse duration; PI: pulse interval; N: number of pulses

Figure 1: Illustration of results obtained from transfection of *B. saltans* with Cas9RNP complex along with DNA repair cassette. **A:** Schematic representation of the PFR-2 locus into the genome of *B. saltans.* the targeted locus of the Cas9RNP complex and the PCR primers annealing sites; **B:**Schematic representation of the double stranded DNA repair template and the PCR primers annealing sites; **C**: Schematic representation showing that the construct replace the endogenous *BsPFR2* with *eGFP-NEO* cassette by double cross-over homologous recombination, PCR#1 gel image and diagram showing the on-target integration of the plasmid in transfected cell at the 3′ flanking region PCR with a PCR product at 0.8 kb; PCR#3 image and diagram showing the integration of the plasmid at the 5′ flanking region with PCR product at 2.6 kb; **D:** Schematic representation showing that the construct replace the endogenous *BsPFR2* with *eGFP-NEO* cassette by double cross-over homologous recombination, with an insertion of additional 600 bp of the PFR-2 resulted from cross-over event, with PCR# 2 gel image showing a PCR product at 1.4 kb.

1 *Step 1: Plasmid construction to target the PFR-2 gene*

-A 2512 bp promoter-less cassette is designed to target and knock out the*B. saltans* 69 KDa paraflagellar rod protein 2C (PFR-2), (GenBank accession #CYKH01000743: scaffold1667, positions 3455 to 6406).

-This cassette is designed to replace the PFR-2 gene with a fusion of the *eGFP* (enhanced green fluorescent protein) and Neomycin genes. It contains 500 bp homologous arms at the 5' and 3' ends.

-The construct was linearized with the restriction enzyme XbaI (New England BioLabs) prior to electroporation. The

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plasmid sequence was deposited in GenBank under accession number (MZ522125).

2 *Step 2: Co-delivery of SaCas9 RNP complex and the DNA repair template for BsPFR2 disruption*

-10 ml of *B. saltans* cells from cultures at log phase (2-3 days old cultures) were filtered using a 7 micron nylon filter and washed 3 times in ddH2O by centrifugation at 950xg for 4 minutes.
-Total cell count used for electroporation was between $1x10^6$ to $2x10^6$
-2 µg of sgRNA were annealed with 4 µg of SaCas9 for 5 minutes at 37°C, then this mixture was combined with 50 µg of PFR-GFP-Neo plasmid and incubated 2 minutes at room temperature.
-*B. saltans* cells were electroporated using a square wave electroporator (NEPA21, Bulldog Bio, Inc.), with the electroporation parameters presented in Table 1.
-The cells were recovered immediately after electroporation and incubated in *B. saltans* growth media.
-G418 was added to transfected cultures 48 hours post-electroporation at concentration 2 µg/ml and increased gradually to 3 µg/ml over few weeks.
-Genotyping analysis using PCR primers sets targets different regions on the plasmid as well as on the genome of *B. saltans* to confirm the on-target plasmid integration are presented in **Figure 1**

2.1 -Table 1: NEPA 21 electroporation parameters used in our study

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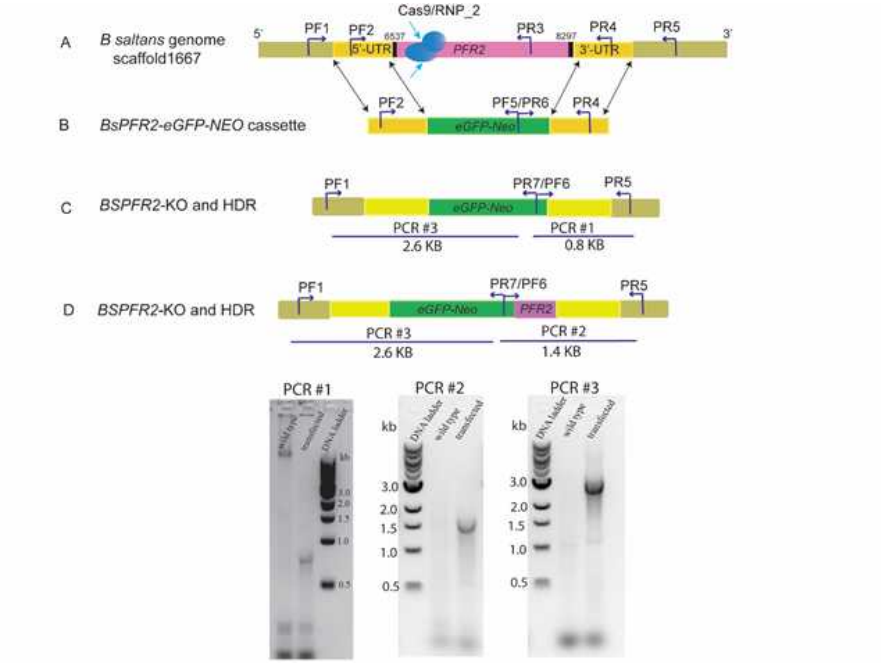
2.2



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