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Single-step assembly of double guide plasmid (pCas9-Duo) for gene-editing in Plasmodium

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Protocol status: Working
We use this protocol and it's working

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Abstract

This protocol describes a one-pot GoldenGate assembly of a new dual-guide targeting plasmid (pCas9-Duo) expressing two distinct guide RNAs in order to enhance the chances of a successful Cas9-mediated modification at a target region in *Plasmodium falciparum*. Designed as part of SHIFTiKO (frameshift-based trackable inducible knockout) system[1] (based on[2]).

Attachments

FILE

pCas9-Duo.gb

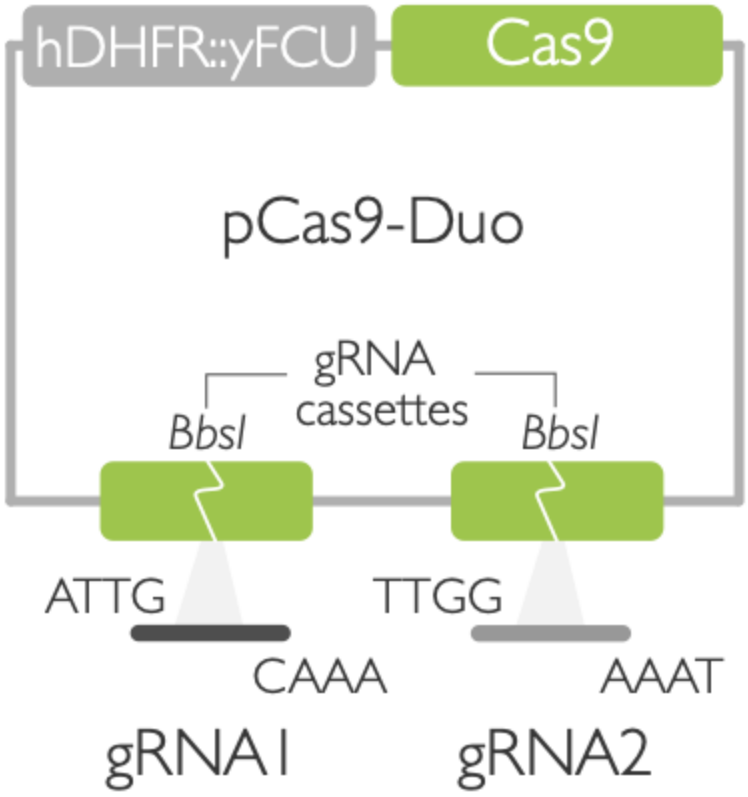
20KB

Protocol materials

- M13 Reverse — Step 10
- Nuclease-free Water — In 3 steps
- T4 DNA Ligase Reaction Buffer - 6.0 ml **New England Biolabs Catalog #**B0202S — Step 2
- T4 Polynucleotide Kinase - 500 units **New England Biolabs Catalog #**M0201S — Step 2
- UltraPure ATP 10mM **Promega Catalog #**NC2683865 — Step 5
- BbsI-HF - 300 units **New England Biolabs Catalog #**R3539S — Step 5
- Cutsmart Buffer — Step 5
- LB plates with 100 µg/ml ampicillin — Step 9
- T4 DNA Ligase **Roche Catalog #**11635379001 — Step 5
- XL-10 Gold Ultracompetent cells **Agilent Technologies Catalog #**200314 — Step 7
- M13Forward_reverse — Step 10

gRNA oligo design

1 Add the overhangs "ATTG" and "AAAC" to forward and reverse oligos of gRNA1, and "TTGG" and "TAAA" to gRNA2 respectively.



Double guide plasmid containing two gRNA expression cassettes with distinct insertion sites.

Anneal gRNA oligos

1h 10m

2 Set up annealing reactions.

10m

1.0 µL gRNA.F [M] 100 micromolar (µM)

1.0 µL gRNA.R [M] 100 micromolar (µM)

1.0 µL

T4 DNA Ligase Reaction Buffer - 6.0 ml **New England Biolabs Catalog #**B0202S

0.5 µL
T4 Polynucleotide Kinase - 500 units **New England Biolabs Catalog #**M0201S
6.5 µL Nuclease-free Water **Contributed by users**

3 Step 1 37 °C 00:30:00 1h
Step 2 94 °C 00:05:00
Step 3 90 °C to 25 °C RAMP 5 °C per minute
Step 4 4 °C Hold

Single-step GoldenGate assembly 1h 15m

4 Make 1:200 dilution mixture of annealed gRNA1 and gRNA2.

1 µL gRNA1 annealed reaction
1 µL gRNA2 annealed reaction
198 µL Nuclease-free Water **Contributed by users**

5 Set up assembly reaction

2 µL pCas9 [M] 200 ng/µL
1 µL 1:200 diluted oligo
0.5 µL BbsI-HF - 300 units **New England Biolabs Catalog #**R3539S
0.5 µL T4 DNA Ligase **Roche Catalog #**11635379001
2 µL Cutsmart Buffer **Contributed by users**
2 µL UltraPure ATP 10mM **Promega Catalog #**NC2683865
12 µL Nuclease-free Water **Contributed by users**

6 Step 1 37 °C 00:05:00 1h
Step 2 16 °C 00:05:00
Repeat Step 1-2 for 6 cycles
Step 3 4 °C Hold

Transform, plate and screen colonies 20m 40s

7 Add 2 µL of assembly reaction to 15 µL ultracompetent cells (like XL-10 Gold Ultracompetent cells **Agilent Technologies Catalog #**200314).

8 Place On ice for 00:20:00 . 20m

9 Heat shock at 42 °C for 00:00:40 and spread transformed cells on LB plates with 100 µg/ml ampicillin **Contributed by users** . 40s

10 Pick colonies, miniprep-isolate plasmids and screen for gRNA1 and gRNA2 insertions by Sanger sequencing using M13 Reverse (CAGGAAACAGCTATGAC) and M13Forward_reverse (ACTGGCCGTCGTTTTAC), respectively.

Protocol references

Protocol developed as part of
1. Ramaprasad, Abhinay, and Michael J. Blackman. 2024. 'A Scaleable Inducible Knockout System for Studying Essential Gene Function in the Malaria Parasite'. bioRxiv. https://doi.org/10.1101/2024.01.14.575607.
Based on
2. Adikusuma, Fatwa, Chandran Pfitzner, and Paul Quinton Thomas. 2017. 'Versatile Single-Step-Assembly CRISPR/Cas9 Vectors for Dual gRNA Expression'. *PLoS ONE* 12 (12): e0187236. https://doi.org/10.1371/journal.pone.0187236.