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Version 1

Single-step assembly of double guide plasmid (pCas9-Duo) for gene-editing in Plasmodium V.1

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Abhinay Ramaprasad¹

¹Francis Crick Institute



Abhinay Ramaprasad

University of Glasgow

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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) system¹ (based on²).

Attachments



[pCas9-Duo.gb](#)

20KB

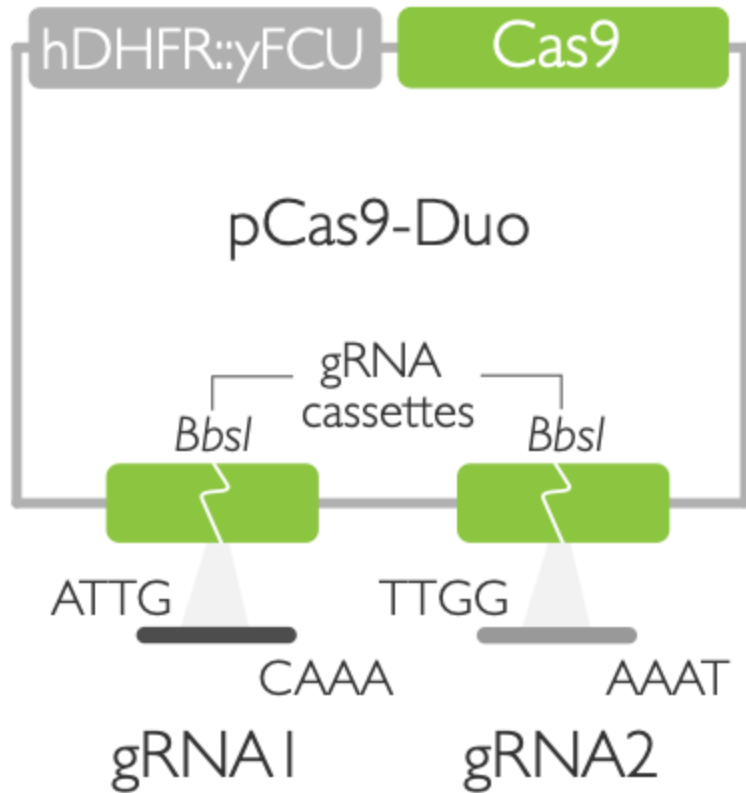


Protocol materials

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

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 μ L gRNA.F [1M] 100 micromolar (μ M)

1.0 μ L gRNA.R [1M] 100 micromolar (μ M)

1.0 μ L

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

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

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

🧪 2 µL 🧬 UltraPure ATP 10mM **Promega Catalog #NC2683865**

🧪 12 µL 🧬 Nuclease-free Water

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 . 40s
- 10 Pick colonies, miniprep-isolate plasmids and screen for gRNA1 and gRNA2 insertions by Sanger sequencing using M13 Reverse (CAGGAAACAGCTATGAC) and M13Forward_reverse (ACTGGCCGTCGTTTAC), 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>.