1. Characterization of the pSC101-dCas9-eGFP

Through the co-transformation of the pYB1a-eGFP plasmid and the pSC101-dCas9 plasmid into BWΔCD Escherichia coli competent cells, followed by separate inductions of eGFP fluorescent protein and dCas9 protein expression using arabinose and IPTG, we conducted an investigation to assess the inhibitory effect of dCas9 protein on the expression of eGFP fluorescent protein.

Transform the respective plasmids into DH5α and select suitable monoclonal colonies, insert them into liquid LB for a period of time. It was induced with Ara and IPTG in test tube, and after 48h of culture, the optical density at 600 nm (OD600) and eGFP fluorescence were measured (430 nm as excitation wavelength and 510 nm as emission wavelength), and the fluorescence response curve was plotted.

Experimental group (three groups in parallel):

5mL ZYM5052 medium + 5μL 100 mg/mL Amp + 5μL 50 mg/mL Kana + 50μL 20% Ara + 5μL 1mol/L IPTG + 50μL Bacterial solution (pYB1a-eGFP + pSC101-dCas9)

Control group (three groups in parallel):

5mL ZYM5052 medium + 5μL 100 mg/mL Amp + 5μL 50 mg/mL Kana + 50μL 20% Ara + 50μL Bacterial solution (pYB1a-eGFP + pSC101-dCas9)

5mL ZYM5052 medium + 5μL 100 mg/mL Amp + 50μL 20% Ara + 50μL Bacterial solution (pYB1a-eGFP)

5mL ZYM5052 medium + 5μL 100 mg/mL Amp + 50μL 20% Ara + 5μL 1mol/L IPTG + 50μL Bacterial solution (pYB1a-eGFP)

5mL ZYM5052 medium + 50μL Bacterial solution (Untransformed plasmid)

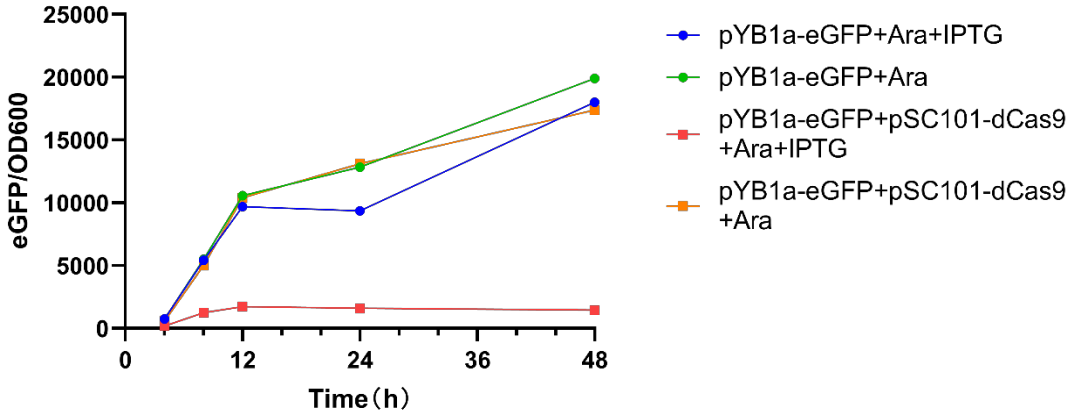

Figure 1 The temporal change in relative eGFP fluorescence intensity under the influence of dCas9 protein

2. Screening of the pSC101-dCas9-eGFP mutant library

The pSC101-dCas9-eGFP mutant library plasmids and pYb1a-eGFP plasmids were co-transformed into BWΔCD. After incubating at 37°C on the corresponding antibiotic selection plates for 12 hours, 72 randomly selected monoclonal colonies were transferred into 600 μL LB medium in deep-well plate for cultivation. The control group consisted of strains transformed solely with the pYb1a-eGFP plasmid. The experimental group was induced with Ara and IPTG in 96-well plate, and after 24h of culture, the optical density at 600 nm (OD600) and eGFP fluorescence were measured (430 nm as excitation wavelength and 510 nm as emission wavelength).

Experimental group:
200μL ZYM5052 medium + 0.2μL 100 mg/mL Amp + 0.2μL 50 mg/mL Kana + 2μL 20% Ara + 0.2μL 1mol/L IPTG + 2μL Bacterial solution (pYB1a-eGFP + pSC101-dCas9-eGFP-M)
Experimental group:
200μL ZYM5052 medium + 0.2μL 100 mg/mL Amp + 2μL 20% Ara + 2μL Bacterial solution(pYB1a-eGFP)
200μL ZYM5052 medium + 2μL Bacterial solution (Untransformed plasmid)

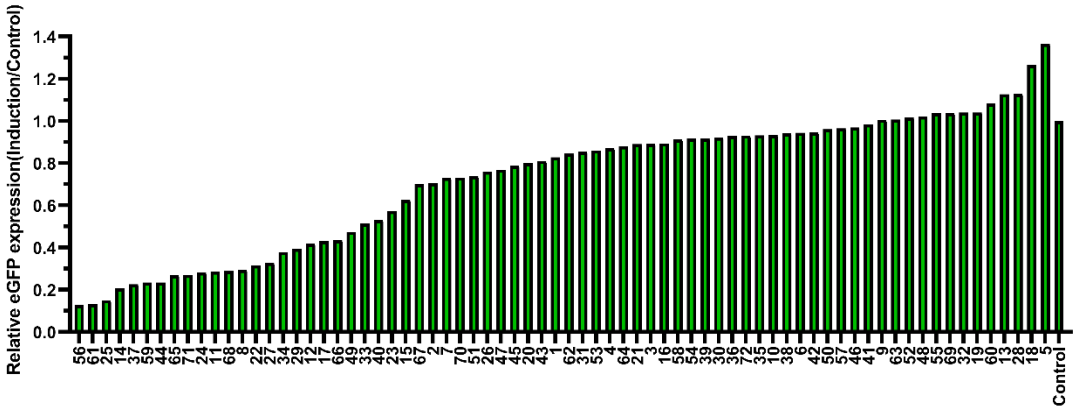

Figure 2 The temporal change in relative eGFP fluorescence intensity under the influence of dCas9 protein

3. Characterization of the R6K-dCas9-eGFP

Through the co-transformation of the pYB1a-eGFP plasmid and the R6K-dCas9 plasmid into BWΔCD Escherichia coli competent cells, followed by separate inductions of eGFP fluorescent protein and dCas9 protein expression using arabinose and IPTG, we conducted an investigation to assess the inhibitory effect of dCas9 protein on the expression of eGFP fluorescent protein. Transform the respective plasmids into DH5α and select suitable monoclonal colonies, insert them into liquid LB for a period of time. It was induced with Ara and IPTG in test tube, and after 48h of culture, the optical density at 600 nm (OD600) and eGFP fluorescence were measured (430 nm as excitation wavelength and 510 nm as emission wavelength), and the fluorescence response curve was plotted.
Experimental group (three groups in parallel):
5mL ZYM5052 medium + 5μL 100 mg/mL Amp + 5μL 50 mg/mL Kana + 50μL 20% Ara + 5μL 1mol/L IPTG + 50μL Bacterial solution (pYB1a-eGFP + R6K-dCas9)
Control group (three groups in parallel):
5mL ZYM5052 medium + 5μL 100 mg/mL Amp + 5μL 50 mg/mL Kana + 50μL 20% Ara + 50μL Bacterial solution (pYB1a-eGFP + R6K-dCas9)
5mL ZYM5052 medium + 5μL 100 mg/mL Amp + 50μL 20% Ara + 50μL Bacterial solution (pYB1a-eGFP)
5mL ZYM5052 medium + 5μL 100 mg/mL Amp + 50μL 20% Ara + 5μL 1mol/L IPTG + 50μL Bacterial solution (pYB1a-eGFP)
5mL ZYM5052 medium + 50μL Bacterial solution (Untransformed plasmid)

4. Screening of the R6K-dCas9-eGFP mutant library

The R6K-dCas9-eGFP mutant library plasmids and pYb1a-eGFP plasmids were co-transformed into BWΔCD. After incubating at 37°C on the corresponding antibiotic selection plates for 12 hours, 24 randomly selected monoclonal colonies were transferred into 600 μL LB medium in deep-well plate for cultivation. The control group consisted of strains transformed solely with the pYb1a-eGFP plasmid. It was induced with Ara and IPTG in 96-well plate, and after 24h of culture, the optical density at 600 nm (OD600) and eGFP fluorescence were measured (430 nm as excitation wavelength and 510 nm as emission wavelength).

Experimental group (three groups in parallel):

200μL ZYM5052 medium + 0.2μL 100 mg/mL Amp + 0.2μL 50 mg/mL Kana + 2μL 20% Ara + 0.2μL 1mol/L IPTG + 2μL Bacterial solution (pYB1a-eGFP + R6K-dCas9-eGFP-M)

Experimental group (three groups in parallel):

200μL ZYM5052 medium + 0.2μL 100 mg/mL Amp + 2μL 20% Ara + 2μL Bacterial solution (pYB1a-eGFP)

200μL ZYM5052 medium + 2μL Bacterial solution (Untransformed plasmid)

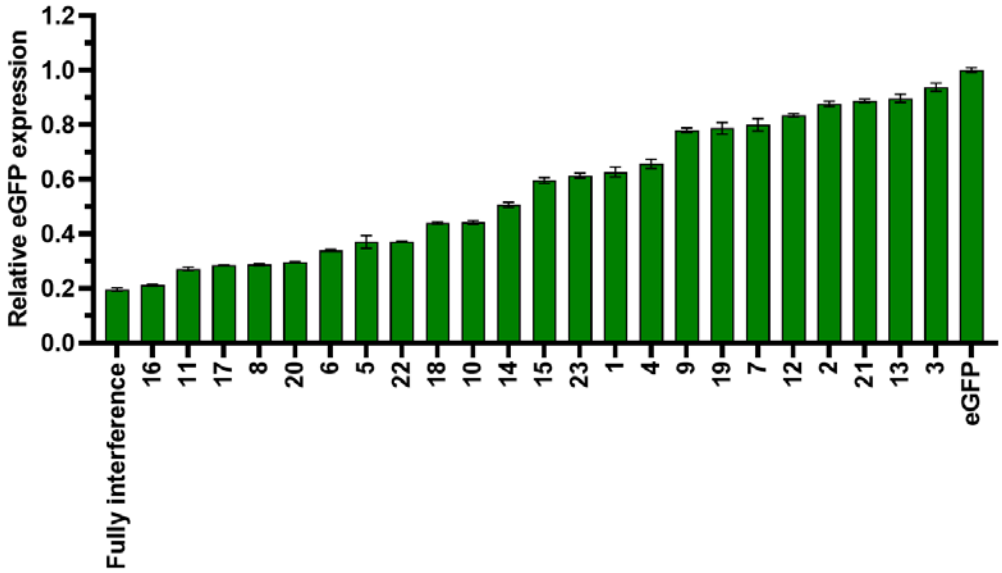

Figure 3 Fluorescence characterization of sgeGFP mutant library

5. Characterization of dCas9-mediated competitive gene inhibition

We selected four target genes, pykF, tyrB, tyrR, and pheA, from the E. coli genome for inhibition and constructed dCas9 plasmids containing their corresponding gRNAs. Additionally, we created a mutant library for these genes.

The R6K-dCas9-pykF/tyrB/tyrR/pheA/pykF-tyrB-tyrR-pheA mutant library plasmids, pSb1c-AroG-tKta-ppsa plasmid and Bio177 plasmid were co-transformed into BWΔCD. After incubating at 37°C on the corresponding antibiotic selection plates for 12 hours, 48-96 randomly selected monoclonal colonies were transferred into 600 μL LB medium in deep-well plate for cultivation. It was induced with Ara and IPTG in 96-well plate, and after 24h of culture, the optical density at 600 nm (OD600) and eGFP fluorescence were measured (430 nm as excitation wavelength and 510 nm as emission wavelength).

Experimental group:
200μL M9 medium + 0.2μL 100 mg/mL Str + 0.2μL 50 mg/mL Kana + 0.2μL 30 mg/mL Chl + 2μL 20% Ara + 0.2μL 1mol/L IPTG + 2μL Bacterial solution (R6K-dCas9-pykF-M + pSb1c-AroG-tKta-ppsa + Bio177)
200μL M9 medium + 0.2μL 100 mg/mL Str + 0.2μL 50 mg/mL Kana + 0.2μL 30 mg/mL Chl + 2μL 20% Ara + 0.2μL 1mol/L IPTG + 2μL Bacterial solution (R6K-dCas9-tyrB-M + pSb1c-AroG-tKta-ppsa + Bio177)
200μL M9 medium + 0.2μL 100 mg/mL Str + 0.2μL 50 mg/mL Kana + 0.2μL 30 mg/mL Chl + 2μL 20% Ara + 0.2μL 1mol/L IPTG + 2μL Bacterial solution (R6K-dCas9-tyrR-M + pSb1c-AroG-tKta-ppsa + Bio177)
200μL M9 medium + 0.2μL 100 mg/mL Str + 0.2μL 50 mg/mL Kana + 0.2μL 30 mg/mL Chl + 2μL 20% Ara + 0.2μL 1mol/L IPTG + 2μL Bacterial solution (R6K-dCas9-pheA-M + pSb1c-AroG-tKta-ppsa + Bio177)
200μL M9 medium + 0.2μL 100 mg/mL Str + 0.2μL 50 mg/mL Kana + 0.2μL 30 mg/mL Chl + 2μL 20% Ara + 0.2μL 1mol/L IPTG + 2μL Bacterial solution (R6K-dCas9-pykF-tyrB-tyrR-pheA-M + pSb1c-AroG-tKta-ppsa + Bio177)
Experimental group (three groups in parallel):
200μL M9 medium + 2μL Bacterial solution (Untransformed plasmid)
200μL M9 medium + 0.2μL 100 mg/mL Str + 2μL Bacterial solution (Bio177)
200μL M9 medium + 0.2μL 100 mg/mL Str + 0.2μL 30 mg/mL Chl + 2μL 20% Ara + 2μL Bacterial solution (pSb1c-AroG-tKta-ppsa + Bio177)
200μL M9 medium + 0.2μL 100 mg/mL Str + 0.2μL 50 mg/mL Kana + 0.2μL 30 mg/mL Chl + 2μL 20% Ara + 0.2μL 1mol/L IPTG + 2μL Bacterial solution (R6K-dCas9-pykF + pSb1c-AroG-tKta-ppsa + Bio177)
200μL M9 medium + 0.2μL 100 mg/mL Str + 0.2μL 50 mg/mL Kana + 0.2μL 30 mg/mL Chl + 2μL 20% Ara + 0.2μL 1mol/L IPTG + 2μL Bacterial solution (R6K-dCas9-tyrB + pSb1c-AroG-tKta-ppsa + Bio177)
200μL M9 medium + 0.2μL 100 mg/mL Str + 0.2μL 50 mg/mL Kana + 0.2μL 30 mg/mL Chl + 2μL 20% Ara + 0.2μL 1mol/L IPTG + 2μL Bacterial solution (R6K-dCas9-tyrR + pSb1c-AroG-tKta-ppsa + Bio177)
200μL M9 medium + 0.2μL 100 mg/mL Str + 0.2μL 50 mg/mL Kana + 0.2μL 30 mg/mL Chl + 2μL 20% Ara + 0.2μL 1mol/L IPTG + 2μL Bacterial solution (R6K-dCas9-pheA + pSb1c-AroG-tKta-ppsa + Bio177)
200μL M9 medium + 0.2μL 100 mg/mL Str + 0.2μL 50 mg/mL Kana + 0.2μL 30 mg/mL Chl + 2μL 20% Ara + 0.2μL 1mol/L IPTG + 2μL Bacterial solution (R6K-dCas9-pykF-tyrB-tyrR-pheA + pSb1c-AroG-tKta-ppsa + Bio177)

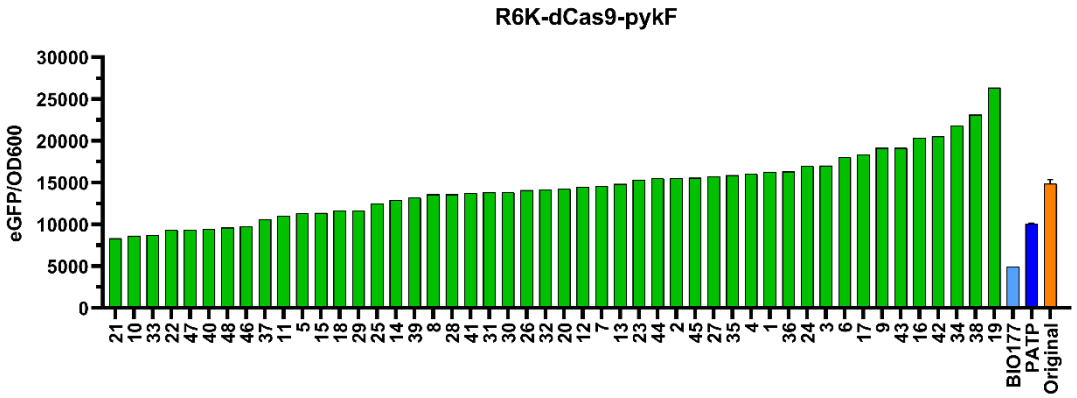

Figure 4

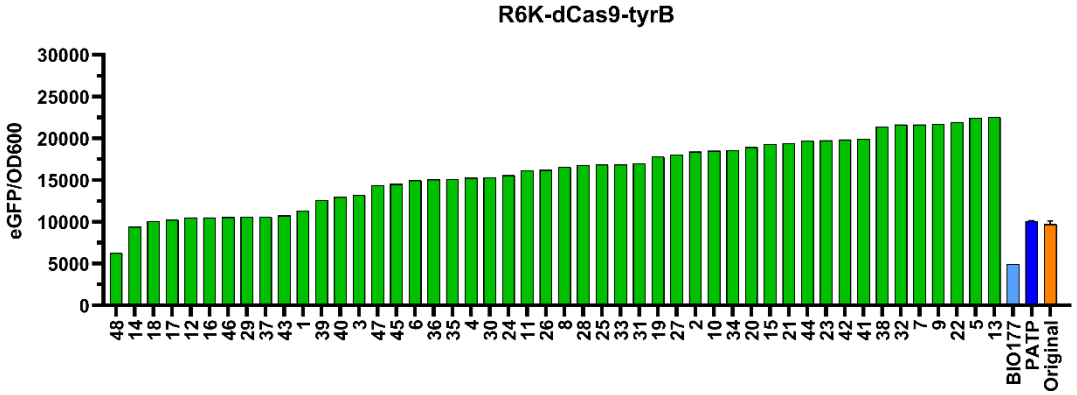

Figure 5

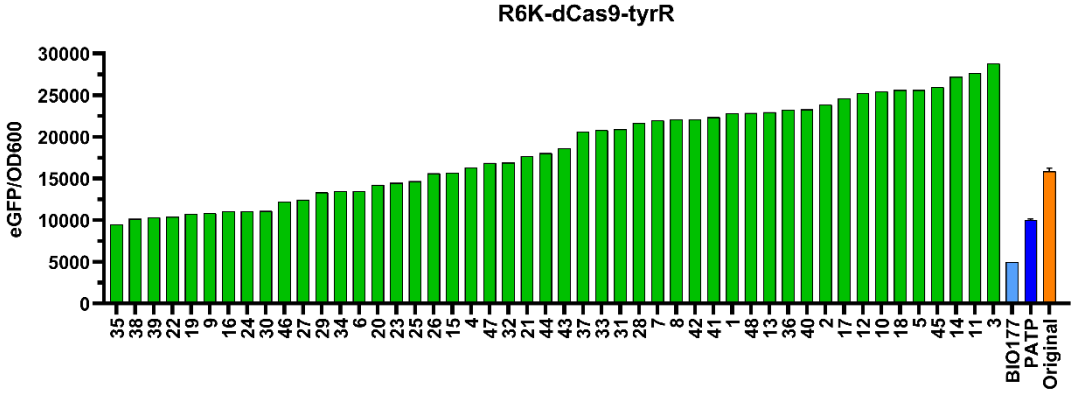

Figure 6

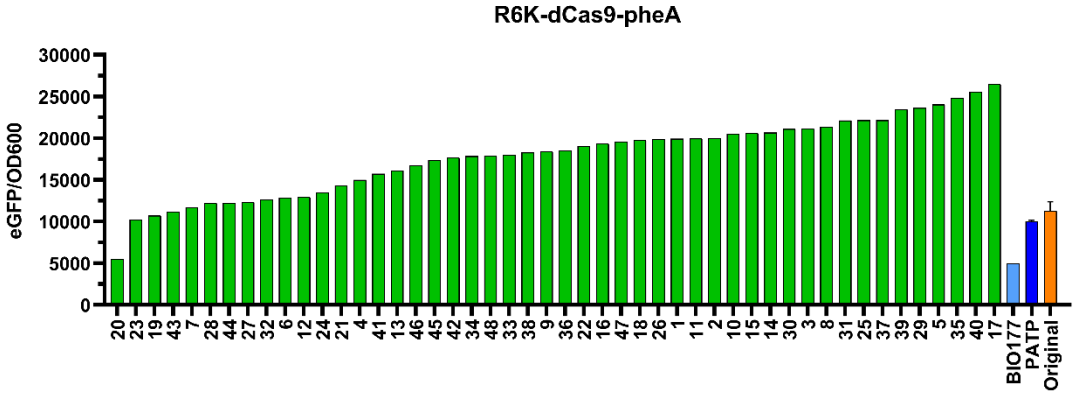

Figure 7

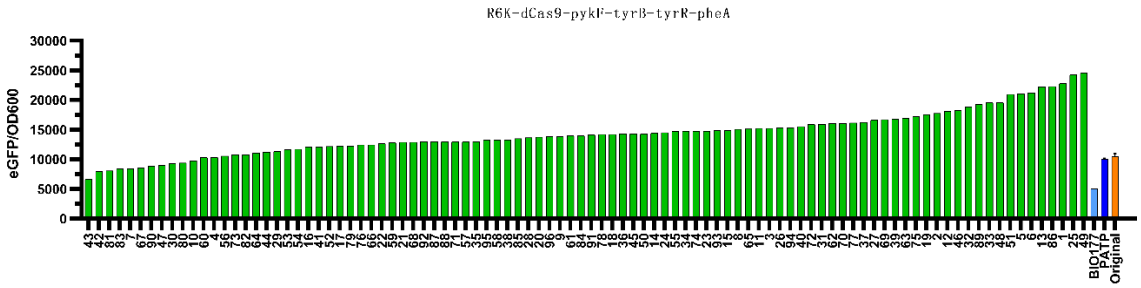

Figure 8

5. HPP characterization of sgRNA mutant library

The R6K-dCas9-pykF/tyrB/tyrR/pheA/pykF-tyrB-tyrR-pheA mutant library plasmids and pSb1c-AroG-tKta-ppsa plasmid were co-transformed into BWΔCD. After incubating at 37°C on the corresponding antibiotic selection plates for 12 hours, 48-96 randomly selected monoclonal colonies were transferred into 600 μL LB medium in deep-well plate for cultivation. Colonies of the generated bacteria harboring these two plasmids were grown in 5 mL LB medium containing Chl and Kana for 12 h. Next, 5 μL the bacterial culture was transferred into 5 ml ZYM5052 medium containing 0.2 g/L Ara, 1 mM IPTG, 30 mg/L Chl, 50 mg/L Kana, and cultured at 30℃ until OD600 reached 5. The bacteria were collected by centrifugation at 4,000 rpm for 10 min, and resuspended in 10 mL M9 medium containing 20 g/L glucose, 0.2 g/L Ara, 1 mM IPTG, 30 mg/L Chl and 50 mg/L Kana. After further cultivation at 30℃ for 24 h, HPP levels were detected by HPLC.

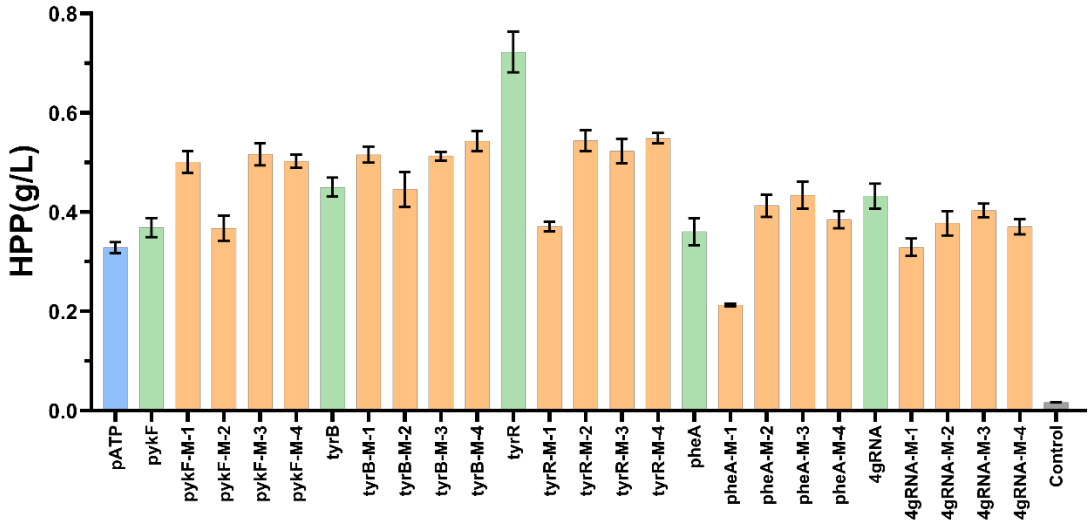

Figure9 HPP production of different sgRNA mutants