sfGFP Reporter Assay for tRNA Promoter Activity

Materials

E. coli strains carrying pUC57-based sfGFP reporter plasmids with wild-type or mutated tRNA promoters
LB broth
Ampicillin, 50 mg/mL stock solution
SacI and BamHI restriction enzymes
Synthetic oligonucleotides containing the corresponding promoter sequences
DNA ligase and competent *E. coli* cells

Consumables

Sterile 96-well microplates suitable for absorbance and fluorescence measurements
Sterile culture tubes
Sterile pipette tips

Equipment

Shaking incubator
Microplate reader capable of measuring OD_{600} and sfGFP fluorescence
Micropipettes

Procedure

Construction of Reporter Plasmids

1. Digest the pUC57-based sfGFP reporter backbone with SacI and BamHI.
2. Anneal complementary oligonucleotides containing the selected wild-type or mutated tRNA promoter and approximately 10 bp of flanking sequence.
3. Ligate each annealed promoter insert into the digested reporter backbone and incubate the ligation reaction overnight.
4. Transform the ligation products into competent E. coli cells and select transformants on LB agar containing ampicillin.

Preparation of Bacterial Cultures

1. Prepare three independent bacterial cultures for each reporter construct as biological replicates.
2. Grow all cultures under the same conditions in LB medium containing the appropriate antibiotic.
3. Mix each bacterial culture thoroughly before preparing the dilution series.

Preparation of the Dilution Series

1. For each culture, prepare four samples corresponding to 1×, 5×, 10× and 50× dilution factors using fresh LB medium.
2. Use the original culture for the 1× sample. Prepare the 5×, 10× and 50× samples at final culture-to-total-volume ratios of 1:5, 1:10 and 1:50, respectively.
3. Mix each dilution thoroughly and keep the dilution factors consistent across all strains and biological replicates.

Preparation of the 96-Well Plate

1. Transfer equal volumes of the four dilutions of each bacterial culture into designated wells of a 96-well microplate.
2. Arrange samples so that promoter variants, dilution factors and biological replicates can be identified separately.
3. Add fresh, uninoculated LB medium to blank wells using the same volume as the bacterial samples.

OD_{600} and sfGFP Fluorescence Measurement

1. Place the prepared microplate in the microplate reader and mix briefly before measurement.
2. Measure the optical density of every sample and blank well directly at 600 nm (OD_{600}), without pre-adjusting all cultures to the same OD_{600}.
3. For sfGFP reporter analysis, record fluorescence from the corresponding wells using the same instrument settings for every promoter variant and dilution.
4. Export the OD_{600} values and, where measured, the fluorescence intensity (RFU) together with the strain identity, biological replicate and dilution factor.

Data Processing

5. Subtract the LB blank absorbance from each OD_{600} measurement. Apply the corresponding blank correction to fluorescence measurements when available.
6. Inspect the dilution series and identify readings within the reliable measurement range of the microplate reader; exclude saturated or near-background readings from quantitative comparisons.
7. For samples with fluorescence data, normalize blank-corrected sfGFP fluorescence to the corresponding blank-corrected OD_{600}, or compare the fluorescence–OD_{600} relationship across valid dilution points.
8. Calculate the relative reporter signal of each mutated promoter using the wild-type promoter as a reference.
9. Summarize the independent biological replicates as mean ± standard deviation (SD) (n = 3) and compare the experimentally observed reporter activities with the model predictions.

Data Processing

1. Subtract the corresponding LB blank OD_{600} value from each bacterial sample measurement.
2. Calculate the mean OD_{600} of the six technical replicates at each time point to obtain one growth curve for each biological replicate.
3. Calculate the mean and standard deviation (SD) across the three independent biological replicates for each strain.
4. Plot the blank-corrected OD_{600} values against cultivation time to generate growth curves, presenting the results as mean ± SD (n = 3 biological replicates).
5. Compare the growth profiles of engineered strains with those of the wild-type control to evaluate potential effects of tRNA pool engineering on bacterial growth.