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Stop codon reversal assay

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We use this protocol and it's working

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Abstract

This protocol is used to characterize viral RNA polymerase based directed evolution tools via fluctuation analysis to calculate the mutation rate. It is specific to the stop codon reversal of the aadA gene, which we use to characterize the mutational power of the tool.

DNA verification

1 Ensure your constructs are sequenced correctly using Plasmidsaurus, paying particular attention to your stop codon and other mutations in promoter or CDS sequences.

Transformation

2 Transform your plasmid(s) into E. coli BL21 (DE3) line, selecting for the plasmid backbone (kan) using the high efficiency transformation protocol

Thaw a tube of BL21 Competent *E. coli* cells on ice for 10 minutes. Mix gently and carefully pipette 50 µl of cells into a transformation tube on ice.

Add 1 µl of plasmid DNA to the cell mixture. Carefully flick the tube 4-5 times to mix cells and DNA. **Do not vortex.**
Place the mixture on ice for 30 minutes. Do not mix.
Heat shock at exactly 42°C for exactly 30 seconds. Do not mix.
Place on ice for 5 minutes. Do not mix.
Pipette 950 µl of room temperature SOC into the mixture..
Place at 37°C for 45 minutes. Shake vigorously (250 rpm) or rotate.
Spin down cells and remove ~80% of supernatant. Resuspend in remaining supernatant.
Warm selection plates to 37°C.
Mix the cells thoroughly by flicking the tube and inverting.
Plate 50-200 uL on appropriate selections

3 Grow on a plate overnight at 37C

Outgrowth

4 Pick n colonies into separate 15 mL tubes containing 3 mL of LB kan and grow to saturation. This is passage 0 (P0)

Aim for a minimum of 5 biological replicates in each experiment

I typically do passages at 8 AM and 5 PM

Passaging

5 At the end of P0, dilute 1:1000 the culture in fresh LB kan supplemented with filter sterilized arabinose (final concentration 0.2%). This is now passage 1 (P1). Grow to saturation until it is

time to do the next passage. This can be repeated any number of times based on your experiment.

For the specR stop codon assay, do up to P8.

Challenge

6 For the challenge part of the assay, create serial dilutions at 10^-2 , 10^-4 , and 10^-6 of each of your strains and replicates.

7 Spot 5-10 uL of the serial dilutions of each strain and replicate unto 2 different plates:

- Non-selective plate (LB agar kan, selects for plasmid backbone)
- Selective plate (LB agar spec, selects for reversal of stop codon)

8 Incubate overnight at 37C

9 Count colonies:

r = cfu / mL in selective plates (mutants/reversants)
N = cfu / mL in non-selective plates

Make sure to account for the dilution factor when calculating cfu/mL.

10 Suppressor frequency is calculated as r/N and plotted.

11 Use the FALCOR tool with the MSS option to estimate mutation rate

https://lianglab.brocku.ca/FALCOR/

Sequencing

12 For each strain of interest, you can pick 3 random colonies at different cycles, grow in LB kan and prep, then send for sequencing to see mutation frequency downstream and upstream. This needs to be calculated by hand based on generation time estimates.