MINI Interlab - Experiment 2: Using the three color calibration protocol: Does the order of transcriptional units influence their expression strength?

In this experiment, your team will measure the fluorescence of six devices that encode two fluorescence proteins in two transcriptional units. The devices differ in the order of the transcriptional units. You will calibrate the fluorescence of these devices to the calibrant dyes and the optical density of the culture to the cell density calibrant.

This experiment aims to assess the lab-to-lab reproducibility of the three color calibration protocol when two fluorescent proteins are expressed in the same cell. Besides this technical question, it also adresses a fundamental synthetic biology question: does the order of the transcriptional units (that encode for the two different fluorescent proteins) on the devices influence their expression levels?

Protocol summary: You will transform the eight devices listed in Table 1 into E. coli K-12 DH5-alpha cells. The next day you will pick two colonies from each transformation (16 total) and use them to inoculate 5 mL overnight cultures (this step is still in tubes). Each of these 16 overnight cultures will be used to inoculate four wells in a 96-well plate (200uL each, 4 replicates) for measurement and one test tube (12 mL) for growth. You will measure how fluorescence and optical density develops over 6 hours by taking measurements at time point 0 hour and at time point 6 hours. Follow the protocol below and the visual instructions in Figure 1 and Figure 2.

Protocol Outputs:

- `baseline absorbance of culture (day 2) measurements of cultures (0 hr timepoint)`
- `0 hr absorbance timepoint measurements of plate 1`
- `0 hr green fluorescence timepoint measurements of plate 1`
- `0 hr blue fluorescence timepoint measurements of plate 1`
- `0 hr red fluorescence timepoint measurements of plate 1`
- `6 hr absorbance timepoint measurements of plate 2`
- `6 hr green fluorescence timepoint measurements of plate 2`
- `6 hr blue fluorescence timepoint measurements of plate 2`
- `6 hr red fluorescence timepoint measurements of plate 2`

Protocol Materials:

- *E. coli* DH5 alpha competent cells
- Negative control 2018
- Positive control 2018
- Test Device 1 Exp 2 (Dual construct Green and Blue)
- Test Device 2 Exp 2 (Dual construct Green and Red)
- Test Device 3 Exp 2 (Dual construct Blue and Green)
- Test Device 4 Exp 2 (Dual construct Blue and Red)
- Test Device 5 Exp 2 (Dual construct Red and Green)
- Test Device 6 Exp 2 (Dual construct Red and Blue)

- LB Broth + Chloramphenicol (34 ug/mL)
- LB Agar + Chloramphenicol (34 ug/mL)
- Chloramphenicol stock solution (34 mg/mL)
- Ice
- Plate reader
- Shaking incubator
- Petri dish (x 8)
- culture tube (x 32)
- 1.5 mL microfuge tube (x 32)
- 50 ml conical tube (x 16)
- 96 well microplate (x 2)

Table 1: Part Locations in Distribution Kit

Part	Coordinate
BBa_R0040	Mini-interlab Plate Well G1
BBa_I20270	Mini-interlab Plate Well A1
H3A3_Green_Blue	Mini-interlab Plate Well A4
H3B6_Green_Red	Mini-interlab Plate Well B4
F2H6_Blue_Green	Mini-interlab Plate Well C4
F2B6_Blue_Red	Mini-interlab Plate Well D4
C3H6_Red_Green	Mini-interlab Plate Well E4
C3A3_Red_Blue	Mini-interlab Plate Well F4

Protocol Steps:

1. Obtain 8 x Petri dish containing LB Agar + Chloramphenicol (34 ug/mL) growth medium for culturing `transformant strains`
2. Transform `Negative control 2018` DNA into *`E. coli` DH5 alpha competent cells*. Repeat for the remaining transformant DNA: `Positive control 2018`, `Test Device 1 Exp 2 (Dual construct Green and Blue)`, `Test Device 2 Exp 2 (Dual construct Green and Red)`, `Test Device 3 Exp 2 (Dual construct Blue and Green)`, `Test Device 4 Exp 2 (Dual construct Blue and Red)`, `Test Device 5 Exp 2 (Dual construct Red and Green)`, and `Test Device 6 Exp 2 (Dual construct Red and Blue)`. Plate transformants on LB Agar + Chloramphenicol (34 ug/mL) `transformant strains` plates. Incubate overnight (for 16 hour) at 37.0°C.
3. Obtain 16 x culture tubes to contain `culture (day 1)`
4. Pick 2 colonies from each `transformant strains` plate.
5. Inoculate 2 colonies of each transformant strains, for a total of 16 cultures. Inoculate each into 5.0mL of LB Broth + Chloramphenicol (34 ug/mL) in culture (day 1) and grow overnight (for 16.0 hour) at 37.0°C and 220 rpm.
6. Obtain 16 x culture tubes to contain `culture (day 2)`
7. Dilute each of 16 `culture (day 1)` samples with LB Broth + Chloramphenicol (34 ug/mL) into the culture tube at a 1:10 ratio and final volume of 5.0mL. Maintain at 4.0°C while performing dilutions.

(This can be also performed on ice).

8. Obtain 16 x 1.5 mL microfuge tubes to contain `cultures (0 hr timepoint)`
9. Hold `cultures (0 hr timepoint)` on ice. This will prevent cell growth while transferring samples.
10. Transfer 1.0mL of each of 16 `culture (day 2)` samples to 1.5 mL microfuge tube containers to contain a total of 16 `cultures (0 hr timepoint)` samples. Maintain at 4.0°C during transfer. (This can be also performed on Ice).
11. Measure baseline absorbance of culture (day 2) of `cultures (0 hr timepoint)` at 600.0nm.
12. Obtain 16 x 50 ml conical tubes to contain `back-diluted culture` The conical tube should be opaque, amber-colored, or covered with foil.
13. Back-dilute each of 16 `culture (day 2)` samples to a target OD of 0.02 using LB Broth + Chloramphenicol (34 ug/mL) as diluent to a final volume of 12.0mL. Maintain at 4.0°C while performing dilutions.

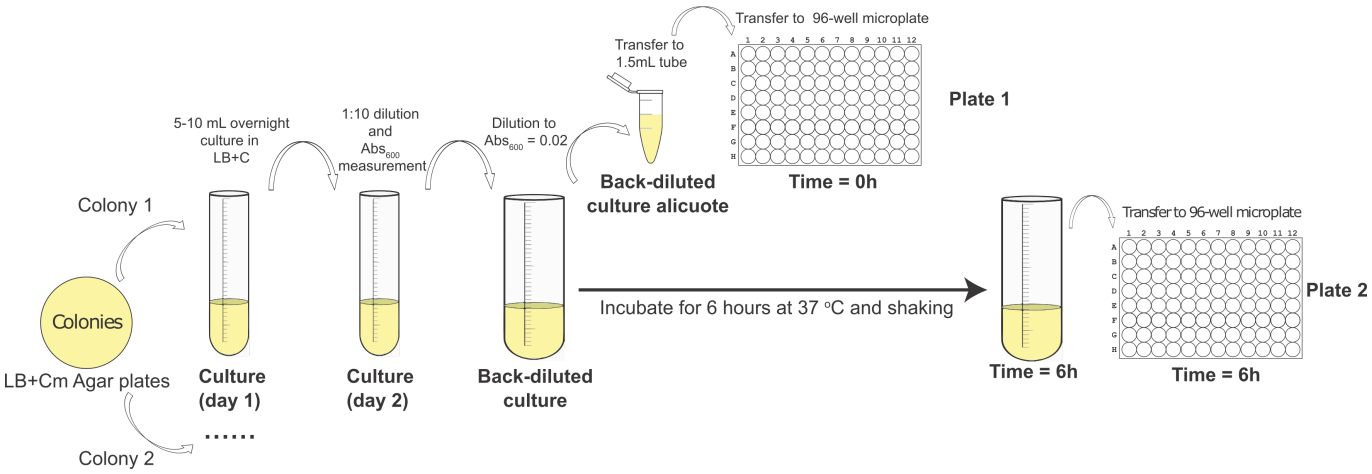

Fig 1: Visual representation of protocol

14. Obtain 16 x 1.5 mL microfuge tubes to contain `back-diluted culture aliquots`
15. Hold `back-diluted culture aliquots` on ice. This will prevent cell growth while transferring samples.
16. Transfer 1.0mL of each of 16 `back-diluted culture` samples to 1.5 mL microfuge tube containers to contain a total of 16 `back-diluted culture aliquots` samples. Maintain at 4.0°C during transfer. (This can be also performed on Ice).
17. Obtain a 96 well microplate to contain `plate 1`
18. Hold `plate 1` on ice.
19. Transfer 200.0uL of each `back-diluted culture aliquots` sample to 96 well microplate `plate 1` in the wells indicated in the plate layout. Maintain at 4.0°C during transfer.
20. Transfer 200.0uL of `LB Broth + Chloramphenicol (34 ug/mL)` sample to wells A1:H1, A10:H10, A12:H12 of 96 well microplate `plate 1`. Maintain at 4.0°C during transfer. These samples are blanks.

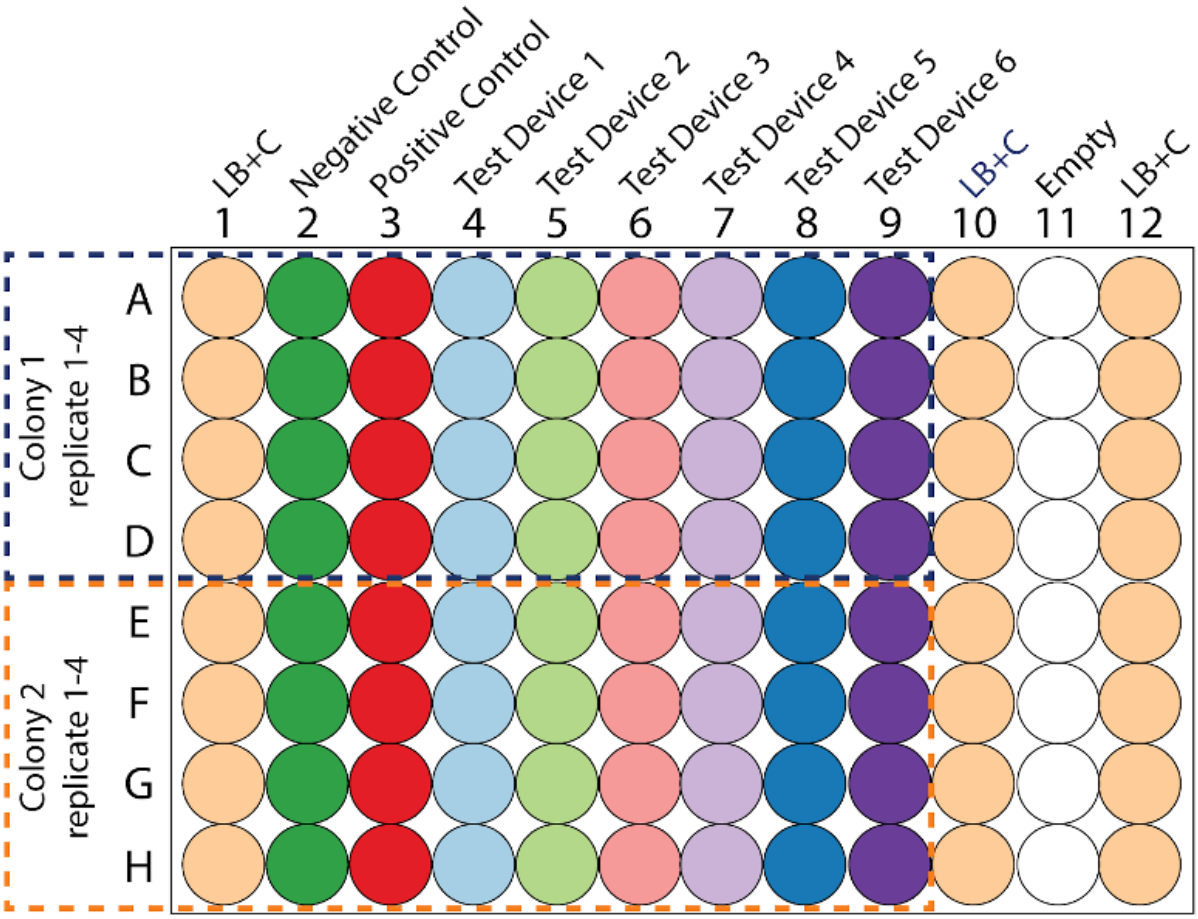

Fig 2: Plate layout

21. Measure 0 hr absorbance timepoint of `plate 1` at 600.0nm.
22. Measure 0 hr green fluorescence timepoint of `plate 1` with excitation wavelength of 488.0nm and emission filter of 530.0nm and 30.0nm bandpass.
23. Measure 0 hr blue fluorescence timepoint of `plate 1` with excitation wavelength of 405.0nm and emission filter of 450.0nm and 50.0nm bandpass.
24. Measure 0 hr red fluorescence timepoint of `plate 1` with excitation wavelength of 561.0nm and emission filter of 610.0nm and 20.0nm bandpass.
25. Incubate all `back-diluted culture` samples for 6.0 hour at 37.0°C at 220 rpm.
26. Hold all `back-diluted culture` samples on ice. This will inhibit cell growth during the subsequent pipetting steps.
27. Obtain a 96 well microplate to contain `plate 2`
28. Hold `plate 2` on ice.
29. Transfer 200.0uL of each `back-diluted culture` sample to 96 well microplate `plate 2` in the wells indicated in the plate layout. Maintain at 4.0°C during transfer.
30. Transfer 200.0uL of `LB Broth + Chloramphenicol (34 ug/mL)` sample to wells A1:H1, A10:H10, A12:H12 of 96 well microplate `plate 2`. Maintain at 4.0°C during transfer. These are the blanks.
31. Measure 6 hr absorbance timepoint of `plate 2` at 600.0nm.
32. Measure 6 hr green fluorescence timepoint of `plate 2` with excitation wavelength of 485.0nm and emission filter of 530.0nm and 30.0nm bandpass.
33. Measure 6 hr blue fluorescence timepoint of `plate 2` with excitation wavelength of 405.0nm and emission filter of 450.0nm and 50.0nm bandpass.

34. Measure 6 hr red fluorescence timepoint of `plate 2` with excitation wavelength of 561.0nm and emission filter of 610.0nm and 20.0nm bandpass.
35. Import data for `baseline absorbance of culture (day 2) measurements of cultures (0 hr timepoint)`, `0 hr absorbance timepoint measurements of plate 1`, `0 hr green fluorescence timepoint measurements of plate 1`, `0 hr blue fluorescence timepoint measurements of plate 1`, `0 hr red fluorescence timepoint measurements of plate 1`, `6 hr absorbance timepoint measurements of plate 2`, `6 hr green fluorescence timepoint measurements of plate 2`, `6 hr blue fluorescence timepoint measurements of plate 2`, `6 hr red fluorescence timepoint measurements of plate 2` into provided Excel file.

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