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Singleplex Assay for Function Measurements V.2

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Protocol status: Working
This protocol is meant to be a template. Each onboarded function in the Pooled, Growth-Based Assay for Function Measurement group should use this template to create their exact protocol, including growth times, volumes, and concentrations for reagents and additives.

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Disclaimer

The protocol outlined in this document was created as a part of the *Pooled, Growth-Based Assays for Protein Function Measurements* pipeline for Align to Innovate's Open Dataset Initiative. Align to Innovate is a non-profit research organization operating under open science principles with the goal of improving science research with programmable experiments. The Open Datasets Initiative is working to accelerate community-driven science with the use of automated labs to pioneer robust data collection methods and curated, high-fidelity, public biological datasets amenable to machine learning. This work was supported by Align to Innovate's Open Datasets Initiative which receives philanthropic funding in part from Griffin Catalyst.

Abstract

This protocol outlines an assay for measuring the function of plasmid variants in singleplex.

The inputs include separate E. coli glycerol stocks for each variant. The protocol begins with several growths which convert the separate glycerol stocks into cultures that have reach stationary phase in a 96-well plate. The glycerol stocks are first grown overnight in separate tubes. The next morning, the optical density (OD) of each culture is measured, and then each culture is distributed into a 96-well growth plate. This plate is placed in a plate reader/incubator to grow to stationary phase (~12 hours) without antibiotics or additives (except those required for plasmid maintenance). After this point, the cultures are ready to act as an inputs for next growth cycle. Throughout the subsequent growths, optical density (OD) and fluorescent measurements are recommended to be taken every 5 minutes and at the end of each growth plate's incubation. The growth cycles are all ~3 hours long, so that cells stay in mid-log phase. At the end of the last growth cycle, a small amount of the plated cultures act as input for quantification using a flow-cytometer and fluorescent beads.

Note that unlike the **related fitness assays**, this assay does not use a selection antibiotic.

Contents of each growth plates 2-3:

- Growth plate 2: This growth plate contains additives to initiate gene expression, **but no selection antibiotic.**
- Growth plate 3: This growth plate contains additives to initiate gene expression, **but no selection antibiotic.**

Materials

Starting cultures:

- glycerol stocks of each of the variants to be tested

Reagents:

- M9 Media (ThermoFisher A1374401)
- Isopropyl β-D-1-thiogalactopyranoside (IPTG) (MilliporeSigma I5502)
- Phosphate Buffered Saline (PBS) (MilliporeSigma 806544)
- Chloramphenicol (Fisher Scientific BP904-100)
- Fluorescence Calibration Beads (Spherotech RCP-30-20A)

Consumables:

- One 15 mL snap cap tube per variant being tested (Corning 352059)
- Three 96-well growth plates (Agilent 204799-100)
- Three gas permeable seals (Azenta P98-712)
- 96-well plate for flow cytometry (Fisher Scientific 08-772-54)

Culture Preparation

1 For each variant to be tested, fill a 15 mL snap-cap culture tube with 5 mL of M9 media.
- Use a scraping from the glycerol stock for each clonal variant and place into its culture tube.

2 Incubate cultures overnight (18-24 hours) at 37°C with shaking at 300 rpm.

Quality control the overnight culture

3 After incubation, measure the OD600 of each overnight culture as a growth check.

Prepare the automation system or liquid handler

4 Load required reagents (M9 media, additives, PBS, etc.) and growth-plates into automation system or liquid handler.

First Growth Plate: cells reach stationary phase

5 Prepare first growth plate by pipetting 450 µL M9 media and 50 µL cell culture into each well.

6 Apply gas-permeable seal to the first growth plate.

7 Incubate for 12 hours at 37°C, with fastest shaking possible in the plate reader (e.g., in Biotek Neo2SM reader: double orbital shaking at 807 cpm and 1 mm shaking diameter).
- Measure OD600 every 5 minutes throughout the incubation.
- Optionally, measure fluorescence if appropriate for the plasmid circuit.

7.1 During incubation, prepare the second growth plate by adding 490 µL mixed media (media, additives like inducers, maintenance antibiotics, etc but no selection antibiotic) per well to the second growth plate.

7.2 Approximately ten minutes before the end of the 12-hour incubation, pre-warm the second growth plate. Note: Adjust pre-warming temperature and timing so that the media temperature in the plate is 37C at the end of the pre-warming, and so that the pre-warming step ends at the same time as the 12-hour incubation.

Second Growth Plate

8 After 12-hour incubation, remove gas-permeable seal.

9 Transfer 10 µL from each well of the first growth plate to corresponding wells of the second growth plate.
- There are some subtleties in the 96-channel pipetting required to get a reproducible transfer, details are described in the SI of this paper: https://academic.oup.com/synbio/article/7/1/ysac013/6659220

10 Apply gas-permeable seal to the second growth plate.

11 Incubate the second growth plate for approximately 3 hours at 37°C, with fastest shaking possible in the plate reader (e.g., in Biotek Neo2SM reader: double orbital shaking at 807 cpm and 1 mm shaking diameter).
- The exact incubation time for this step needs to be worked out during the testing phase. The time needs to be adjusted so that in the wells with the fastest growing cultures:
1. The cell density always remains in mid-log phase or lower.
2. The cell density at the end of each subsequent incubation step is constant or slightly decreasing.

11.1 Measure OD600 and fluorescence every 5 minutes throughout the incubation.
- Use excitation and emission wavelengths appropriate for the fluorescent protein used in the plasmid system.

11.2 During incubation, prepare the third growth plate by adding 450 µL mixed media (media, additives like inducers, maintenance antibiotics, etc but no selection antibiotic) per well to the third growth plate.

11.3 Approximately ten minutes before the end of the 12-hour incubation, pre-warm the third growth plate. Note: Adjust pre-warming temperature and timing so that the media temperature in the plate is 37C at the end of the pre-warming, and so that the pre-warming step ends at the same time as the 12-hour incubation.

12 After incubation, remove gas-permeable seal from the second growth plate and measure end-point OD600.

Third Growth Plate

13 Transfer 50 µL from each well of the second growth plate to corresponding wells of the third growth plate.

14 Apply gas-permeable seal to the third growth plate.

15 Incubate the third growth plate for approximately 3 hours at 37°C, with fastest shaking possible in the plate reader (e.g., in Biotek Neo2SM reader: double orbital shaking at 807 cpm and 1 mm shaking diameter).
- Use the same timing as calculated for the second growth plate.

15.1 Measure OD600 and fluorescence every 5 minutes throughout the incubation.
- Use excitation and emission wavelengths appropriate for the fluorescent protein used in the plasmid system.

16 After incubation, remove gas-permeable seal from the third growth plate and perform the quantitative function measurements using one of the following options (Flow Cytometer or Plate Reader).

STEP CASE

Flow cytometer fluorescent measurement 6 steps

Using a flow cytometer and fluorescent standard beads, perform a quantitative function measurement.

Prep for Flow Cytometry

17 Transfer 5 µl of each cell sample to a well in a round-bottom 96-well plate containing 195 µl of PBS supplemented with 170 µg/mL chloramphenicol.
- Note: Chloramphenicol is added to the PBS to halt production of new proteins in the E. coli cells to give a more stable fluorescence measurement, since it can take over one hour to measure all of the samples.

18 Measure the fluorescence of the cells containing the selection plasmid on a flow cytometer equipped with a 96-well plate autosampler using an excitation wavelength and emission filter appropriate for the fluorescent protein in the plasmid.
- Use fluorescence calibration beads with each batch of samples to facilitate the calibration of flow cytometry data to molecules of equivalent fluorophore.
- Use automated gating to distinguish cell events and singlet cell events from other background events for each sample measured with cytometry, with the same gating strategy applied to fluorescent and non-fluorescent control cells.

19 Measure the fluorescence background usin a non-fluorescent control (i.e., E. coli cells of the same strain with a plasmid that is similar to the selection plasmid, but with no fluorescent protein).

Data analysis - Calculating Function

20 Calculate the mean fluorescence signal of the singlet cell events for all measurements of the non-fluorescent control cells. This is the background signal.

21 Calculate the mean fluorescence signal of the singlet cell events for each sample measured with the (fluorescent) selection plasmid.

22 For each sample, the quantitative Function is the mean fluorescence for that sample with the background signal subtracted.

Protocol references

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