Jul 25, 2024

Singleplex Assay for Fitness Measurements

DOI
dx.doi.org/10.17504/protocols.io.8epv5xye4g1b/v1

David Ross[1]

[1]NIST

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DOI: dx.doi.org/10.17504/protocols.io.8epv5xye4g1b/v1

Protocol Citation: David Ross 2024. Singleplex Assay for Fitness Measurements. **protocols.io** https://dx.doi.org/10.17504/protocols.io.8epv5xye4g1b/v1

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.

Created: February 28, 2024

Last Modified: July 25, 2024

Protocol Integer ID: 95877

Keywords: Deep mutational scanning, protein sequence-function relationships, fitness landscape, laboratory automation

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 the singleplex measurement the fitness of plasmid variants in E.coli.

This protocol is used during onboarding of a new protein function for the Pooled, Growth-Based Assay. In this protocol, clonal bacterial stocks are measured instead of a pooled mixture, and the end-point OD measured after each growth cycle is used as a proxy for the DNA barcode count data that is produced by the Pooled, Growth-Based Assay. The fitness estimates from the end-point OD measurements are used to fine-tune the plasmid circuit and the assay conditions in advance of running the Pooled, Growth-Based Assay.

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), with optical density (OD) and fluorescent measurements being taken every 5 minutes and at the end of each growth plate's incubation. After this point, the cultures are ready to act as a inputs for subsequent growth cycles.

The four subsequent growth cycles (i.e., timepoints 1-4) are all ~3 hours long, so that cells stay in mid-log phase. Small amount of the plated cultures at the end of each cycle acting as input for the subsequent growth cycle. The OD measured at the end of each growth cycle (i.e., the end-point OD) will be used in the downstream fitness calculation.

Contents of each growth plate for timepoints 1-4 (growth plates 2-5):

- Timepoint 1: This growth plate contains additives to initiate gene expression, **but no selection antibiotic.**
- Timepoint 2: This growth plate contains additives to initiate gene expression and the selection antibiotic.
- Timepoint 3: This growth plate contains additives to initiate gene expression and the selection antibiotic.
- Timepoint 4: This growth plate contains additives to initiate gene expression and the selection antibiotic.

Materials

Starting cultures:

- glycerol stocks of each of the variants to be tested

Reagents:

- M9 Media (ThermoFisher A1374401)
- Example Inducer: Isopropyl β-D-1-thiogalactopyranoside (IPTG) (MilliporeSigma I5502)
- Example Selection Antibiotic: Tetracycline (Tet) (MilliporeSigma T3258)

Consumables:

- One 15 mL snap cap tube per variant being tested (Corning 352059)
- Five 96-well growth plates (Agilent 204799-100)
- Five gas permeable seals (Azenta P98-712)

Culture Preparation

1 Use a scraping from the glycerol stock for each clonal variant to start 5 mL cultures in M9 media, each in a 15 mL snap-cap culture tube.

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

Quality control check 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, antibiotics, etc.) and growth-plates into automation system or liquid handler.

First Growth Plate: cells reach stationary phase

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

6 Apply gas-permeable seal to the 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).

7.1 Measure OD600 every 5 minutes throughout the incubation.
- Optionally, measure fluorescence if appropriate for the plasmid circuit.

7.2 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.

7.3 Approximately ten minutes before the end of the 12-hour incubation, pre-warm the second growth plate. 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 (timepoint 1)

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

9 Transfer 10 uL from each well in the first growth plate to the corresponding well in 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 second growth plate.

11 Incubate the second growth plate for approximately 3 hours, 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 (e.g., with zero antibiotic):

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 every 5 minutes throughout the incubation.
- Optionally, measure fluorescence if appropriate for the plasmid circuit.

11.2 During incubation, prepare the third growth plate.
- Total volume should be 450 uL per well. Each well contains the same additives as in the second growth plate, plus the addition of the selection antibiotic in some of the wells.

11.3 Approximately ten minutes before the end of the 3-hour incubation, pre-warm the third growth plate. Adjust the timing here so that the media in the plate is pre-warmed to 37C at the same time as the end of the end-point OD600 measurement (next step).

12 After ~3-hour incubation, remove the gas-permeable seal and take an end-point OD600 measurement of the second growth plate.

Third Growth Plate (timepoint 2)

13 Transfer 50 uL from each well in the second growth plate to the corresponding well in the third growth plate.

14 Apply gas-permeable seal to third growth plate.

15 Incubate the third growth plate for approximately 3 hours (same incubation time as second growth plate).
- 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).

15.1 Measure OD600 every 5 minutes throughout the incubation.
- Optionally, measure fluorescence if appropriate for the plasmid circuit.

15.2 During incubation, prepare the fourth growth plate.
- Total volume should be 450 uL per well. Each well contains the same additives and selection antibiotic as in the third growth plate.

15.3 Approximately ten minutes before the end of the 3-hour incubation, pre-warm the fourth growth plate. Adjust the timing here so that the media in the plate is pre-warmed to 37C at the same time as the end of the end-point OD600 measurement (next step).

16 After ~3-hour incubation, remove the gas-permeable seal and take an end-point OD600 measurement of the third growth plate.

Fourth Growth Plate (timepoint 3)

17 Transfer 50 uL from each well in the third growth plate to the corresponding well in the fourth growth plate.

18 Apply gas-permeable seal to fourth growth plate.

19 Incubate the fourth growth plate for approximately 3 hours (same incubation time as third growth plate).
- 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).

19.1 Measure OD600 every 5 minutes throughout the incubation.
- Optionally, measure fluorescence if appropriate for the plasmid circuit.

19.2 During incubation, prepare the fifth growth plate.

- Total volume should be 450 uL per well. Each well contains the same additives and selection antibiotic as in the third and forth growth plates.

19.3 Approximately ten minutes before the end of the 3-hour incubation, pre-warm the fifth growth plate. Adjust the timing here so that the media in the plate is pre-warmed to 37C at the same time as the end of the end-point OD600 measurement (next step).

20 After ~3-hour incubation, remove the gas-permeable seal and take an end-point OD600 measurement of the fourth growth plate.

Fifth Growth Plate (timepoint 4)

21 Transfer 50 uL from each well in the fourth growth plate to the corresponding well in the fifth growth plate (automated liquid handler).

22 Apply gas-permeable seal to fifth growth plate.

23 Incubate the fifth growth plate for approximately 3 hours (same incubation time as third and fourth growth plate).

- 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).

23.1 Measure OD600 every 5 minutes throughout the incubation.

- Optionally, measure fluorescence if appropriate for the plasmid circuit.

24 After the 3-hour incubation of the 5th growth plate, remove the gas-permeable seal and take an end-point OD600 measurements.

Data Analysis- Calculating end-point density Fitness

25 At the end of the protocol, you will have end-point OD measurements, corresponding to the density of cells, for plates 2-5 (i.e., timepoints 1-4).

25.1 The data for log(end-point density) vs. growth plate number is also used to adjust the length of incubation periods for growth plates 2-5 (i.e., timepoints 1-4). Ideally, the end-point density of the fastest growing cultures should remain constant or decrease slightly for each subsequent growth plate.

- If there is a slight increase in end-point density from timepoint 1 to 4, the incubation period should be reduced slightly.
- If the end-point density for the fastest growing culture decreases more than 2-fold from timepoint 1 to timepoint 4, the incubation period should be increased.

- Once the incubation period is adjusted to give constant or slightly decreasing end-point density for the fastest growing cultures, that incubation period should be used for all subsequent growth protocols (for the singleplex fitness, singleplex function, and pooled assays).

26 Plot log(end-point density) vs. timepoint

27 Use linear fits from growth plates 3-5 to get Fitness.
- Fitness = slope of fit line + log(10), where 10 is the dilution factor used to passage cells from one timepoint to the next.

Data Analysis- Plotting Fitness vs Function (along with Function measurements)

28 Plot a separate curve for each antibiotic concentration (and zero antibiotic).

29 The measurable range of function typically needs to span multiple orders of magnitude, so make the x-axis logarithmic (i.e., log(function))

30 Look at the difference between Fitness with and without antibiotic to choose the best selection plasmid for the assay (which combination of resistance gene and RBS strength) and the best concentration(s) to use for the selection antibiotic.
- If this Fitness difference has non-zero slope over the relevant range of function for one or more antibiotic concentrations and the zero antibiotic concentration is approximately constant across the functional range of the protein, then the selection plasmid is good.

Protocol references

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