Jul 25, 2024

Pooled, Growth-Based Assays

DOI
dx.doi.org/10.17504/protocols.io.5qpvokq1bl4o/v1

David Ross[1]

[1]NIST

Align to Innovate | Pooled, Growth-Based As…

Open Datasets Initiative
Align to Innovate

OPEN ACCESS

DOI: dx.doi.org/10.17504/protocols.io.5qpvokq1bl4o/v1

Protocol Citation: David Ross 2024. Pooled, Growth-Based Assays. **protocols.io** https://dx.doi.org/10.17504/protocols.io.5qpvokq1bl4o/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: July 16, 2024

Last Modified: July 25, 2024

Protocol Integer ID: 103516

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 a pooled, growth-based assay for measuring the fitness of a library of variants in E.Coli. This protocol defines paths for either:

- **only measuring a pool of control variants**
- **or an entire library of variants pooled in addition to the controls.**

The inputs for this protocol are: 1) barcoded normalization variants, 2) barcoded calibration variants, and *depending on the branch taken* 3) a pooled library of barcoded variants containing 100,000-500,000 members as well. The protocol begins with several growths which convert the separate glycerol stocks into pooled cultures that have reach stationary phase in a 96-well plate. The glycerol stocks are first grown overnight in separate tubes and flasks. The next morning, the optical density (OD) of each culture is measured, after which all cultures are pooled into a single flask and grown for 4-5 hours to achieve one doubling. The OD of this pooled culture is then measured again before distributing it 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). These cultures are then used as an input for the next growth cycle (i.e., timepoint 1), explained in further detail in the next section.

The 4 subsequent growth cycles (i.e., timepoints 1-4) will produce culture samples that will eventually be sequenced and used in the fitness calculation. These growth cycles (i.e., timepoints 1-4) are all ~3 hours long, so that cells stay in mid-log phase. At the end of each growth cycle, a small amount of each culture acts as input for the subsequent growth cycle, while the remaining culture is processed for downstream measurement (see the Notes section below for details on subsequent steps). The media for the first of these 4 growth cycles (i.e., timepoint 1) contains only additives to initiate gene expression (i.e., inducers), but no selection antibiotic. The media for the following 3 growth cycles contain both the additives and the selection antibiotic.

Notes:

- Throughout all growths involving 96-well plates, OD and fluorescent measurements are recommended to be taken every 5 minutes and at the end of each growth plate's incubation. The OD measured at the end of each ~3 hour growth should be constant or just slightly decreasing across timepoints 1-4.
- Immediately after each growth plate is done incubating and a sample from each well has been transferred to the next growth plate, perform a **DNA extraction protocol** on the remaining culture in each well.
- After DNA extraction is complete for each timepoint, you can proceed to the Automated Bar-Seq Library Preparation and Pooling protocol

Attachments

PNG

Pooled, Growth-Based...

375KB

Materials

Measuring Control Variants Only

Starting cultures:

- glycerol stock of each of the two normalization variants
- glycerol stock of pooled calibration variants

Reagents:

- M9 Media (ThermoFisher A1374401)
- Example Inducer: Isopropyl β-D-1-thiogalactopyranoside (IPTG) (MilliporeSigma I5502)
- Example Inducer: Tetrahydropapaverine Hydrochloride (THP) (Tokyo Chemical Industry N0918)
- Example Selection Antibiotic: Tetracycline (Tet) (MilliporeSigma T3258)

Consumables:

- Two 15 mL snap cap tubes (Corning 352059)
- Two 250 mL baffled flasks (ThermoFisher 4116-0250)
- Five 96-well growth plates (Agilent 204799-100)
- Five gas permeable seals (Azenta P98-712)

Materials for measuring a Variant Library

Starting cultures:

- glycerol stock of each of the two normalization variants
- glycerol stock of pooled calibration variants
- glycerol stock of variant library

Reagents:

- M9 Media (ThermoFisher A1374401)
- Example Inducer: Isopropyl β-D-1-thiogalactopyranoside (IPTG) (MilliporeSigma I5502)
- Example Inducer: Tetrahydropapaverine Hydrochloride (THP) (Tokyo Chemical Industry N0918)
- Example Selection Antibiotic: Tetracycline (Tet) (MilliporeSigma T3258)

Consumables:

- Three 15 mL snap cap tubes (Corning 352059)
- One 250 mL baffled flask (ThermoFisher 4116-0250)
- Five 96-well growth plates (Agilent 204799-100)
- Five gas permeable seals (Azenta P98-712)

1
- If you are running a pooled, growth based assay for the first time (i.e., only using control variants pooled together) then follow the 'Controls Only' branch below.
- If you are running a pooled, growth-based assay on an entire library of variants pooled together (including controls as well), then use the 'Variant Library' branch below.

STEP CASE

Assay for Measuring Variant Libraries 46 steps

This protocol is for measuring pooled variants libraries with control variants. All plasmids need to be barcoded.

Culture Preparation & Overnight Growth

2 Start separate cultures of the two “best” normalization variants, each in their own 15 mL snap-cap culture tubes.
- Take a scraping from the glycerol stock for each of the normalization (always-on) variants and put it in 5 mL of M9 Media to start cultures

3 Start a culture of the mixed calibration variants in a 15 mL snap-cap culture tube.
- Take a scraping from the glycerol stock of the mixed calibration variants and put it in 5 mL of M9 Media to start culture.

4 Start a culture of your variant library in a 250 mL baffled culture flask.
- Use one aliquot of glycerol stock and put it in 100 mL of M9 media.

5 Incubate all cultures overnight
- 37 C, with shaking at 300 rpm
- Approximately 18 hours

First QC check: Overnight OD600 measurement

6 Measure the OD600 of all four cultures.

Pool cultures & grow for one doubling

7 In a new 250 mL baffled flask, combine the following:
- 50 mL M9 Media
- 0.5 mL of each normalization variant culture's overnight tube
- 1 mL of mixed calibration variant culture's overnight tube
- 48 mL of the variant library culture

8 Incubate for 4-5 hours to allow for approximately one doubling.

- 37 C, w/ shaking at 300 rpm

Prepare the automation system or liquid handler

9 Load the reagents and labware required for the 96-well growth plate prep into automation system or deck of automated liquid handler:
- The combined cell culture
- M9 Media
- Selection Antibiotic stocks, typically at 10x higher concentration than the final concentration to be used in the wells of the growth plates
- Additional additives needed for the experiment, e.g., inducers for control of selection circuit; ligand stocks for sensor protein assay; inhibitors (i.e., drugs) for protease assay, maintenance antibiotics
- Growth plates: square-well polystyrene plate with 1.0 mL per well max volume, made by Agilent, part no. 204799-100 (only fill cultures up to 0.5 mL per well).

The first growth plate: cells reach stationary phase

10 Pipette 450 uL M9 Media into each well being used in the growth plate
- Each condition should be represented by 4 replicate wells.

11 Pipette 50 uL of the combined cell culture into each well

12 Apply gas-permeable seal to plate

13 Incubate first growth plate for 12 hours
- 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)

13.1 Measure OD600 and fluorescence every 5 minutes
- Start next steps during the last hour of the 12-hour incubation

13.2 During incubation, prepare the second growth plate with 490 uL mixed media per well, where the mixed media now includes:
- M9 Media
- additives (e.g., inducers, ligands, maintenance antibiotics, or inhibitors if they are used in the assay)

13.3 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.

The second growth plate (timepoint 1)

14 After 12-hour incubation, remove gas-permeable seal from the first growth plate.

15 Transfer 10 uL from each well in the first growth plate to the corresponding well in the second growth plate
- If using 96 channel head, there are some subtleties required to get a reproducible transfer. Details are described in the supplemental information of **this paper**.

16 Apply gas-permeable seal to second growth plate.

17 Incubate the second growth plate for approximately 3 hours and prepare the 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)
- 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 selection antibiotic) fulfill the following conditions:

1. The cells are always in mid-log phase or lower.
2. The cell density at the end point of each subsequent incubation step is constant or slightly decreasing.

17.1 Measure OD600 and fluorescence every 5 minutes
- Start next steps during the last hour of the 12-hour incubation

17.2 During incubation, prepare the third growth plate with 450 uL mixed media per well, where the mixed media now includes:
- M9 Media
- The same additives as the second growth plate (e.g., inducers, ligands, maintenance antibiotics, or inhibitors if they are used in the assay)
- Selection antibiotic in some of the wells (can be at varying concentrations, depending on the pooled assay design)

17.3 Approximately ten minutes before the end of the 3-hour incubation, pre-warm the third growth plate.

The third growth plate (timepoint 2)

18 After 3-hour incubation, remove gas-permeable seal from the second growth plate.

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

20 Apply gas-permeable seal to third growth plate and place into incubator.

20.1 Immediately after the third growth plate goes into the incubator, combine each set of 4 replicate wells from each condition of the *second* growth plate into a single well of a new polypropylene deep well plate (suitable for centrifugation). Run the plasmid **DNA extraction protocol** with the combined cultures in the deep well plate.

- Store the resulting extracted DNA until ready to run the BarSeq library prep protocol. If barcode sequencing prep is to be performed immediately after DNA extraction, nuclease-free water can be used for the final plasmid DNA elution (as in the last two steps of the protocol on protocols.io). Otherwise, substitute “EB buffer” or “TE-4” for the final elution step to avoid possible DNA degradation from uncontrolled (low) pH.

21 Incubate the third growth plate for approximately 3 hours (or the same 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)

21.1 Measure OD600 and fluorescence every 5 minutes

- Start next steps during the last hour of the 12-hour incubation

21.2 During incubation, prepare the fourth growth plate with 450 uL of the same mixed media as the third growth plate:

- M9 Media
- The same additives (e.g., inducers, ligands, maintenance antibiotics or inhibitors if they are used in the assay)
- Selection antibiotic in some of the wells (at the same concentrations as the third growth plate)

21.3 Approximately ten minutes before the end of the 3-hour incubation, pre-warm the fourth growth plate.

The fourth growth plate (timepoint 3)

22 After 3-hour incubation, remove gas-permeable seal from the second growth plate.

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

24 Apply gas-permeable seal to third growth plate and place into incubator.

24.1 Immediately after the third growth plate goes into the incubator, combine each set of 4 replicate wells from each condition of the *second* growth plate into a single well of a new polypropylene deep well plate (suitable for centrifugation). Run the plasmid **DNA extraction protocol** with the combined cultures in the deep well plate.
- Store the resulting extracted DNA until ready to run the BarSeq library prep protocol. If barcode sequencing prep is to be performed immediately after DNA extraction, nuclease-free water can be used for the final plasmid DNA elution (as in the last two steps of the protocol on protocols.io). Otherwise, substitute “EB buffer” or “TE-4” for the final elution step to avoid possible DNA degradation from uncontrolled (low) pH.

25 Incubate the third growth plate for approximately 3 hours (or the same 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)

25.1 Measure OD600 and fluorescence every 5 minutes
- Start next steps during the last hour of the 12-hour incubation

25.2 During incubation, prepare the fourth growth plate with 450 uL of the same mixed media as the third growth plate:
- M9 Media
- The same additives (e.g., inducers, ligands, maintenance antibiotics or inhibitors if they are used in the assay)
- Selection antibiotic in some of the wells (at the same concentrations as the third growth plate)

25.3 Approximately ten minutes before the end of the 3-hour incubation, pre-warm the fourth growth plate.

The fifth growth plate (timepoint 4)

26 After 3-hour incubation, remove gas-permeable seal from the fourth growth plate.

27 Transfer 50 uL from each well in the fourth growth plate to the corresponding well in the fifth growth plate.

28 Apply gas-permeable seal to fifth growth plate and place in incubator.

28.1 Immediately after the fifth growth plate goes into the incubator, combine each set of 4 replicate wells from each condition of the *fourth* growth plate into a single well of a new polypropylene deep well plate (suitable for centrifugation). Run the plasmid **DNA extraction protocol** with the combined cultures in the deep well plate.

- Store the resulting extracted DNA until ready to run the BarSeq library prep protocol. If barcode sequencing prep is to be performed immediately after DNA extraction, nuclease-free water can be used for the final plasmid DNA (as in the last two steps of the protocol on protocols.io). Otherwise, substitute “EB buffer” or “TE-4” for the final elution step to avoid possible DNA degradation from uncontrolled (low) pH.

29 Incubate the fifth growth plate for approximately 3 hours (or the same 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)

29.1 Measure OD600 and fluorescence every 5 minutes

- Start next steps during the last hour of the 12-hour incubation

30 After 3-hour incubation, remove gas-permeable seal from the fourth growth plate.

31 Combine each set of 4 replicate wells from each condition of the fifth growth plate into a single well of a new polypropylene deep well plate (suitable for centrifugation). Run the plasmid **DNA extraction protocol** with the combined cultures in the deep well plate.

- Store the resulting extracted DNA until ready to run the BarSeq library prep protocol. If barcode sequencing prep is to be performed immediately after DNA extraction, nuclease-free water can be used for the final plasmid DNA (as in the last two steps of the protocol on protocols.io). Otherwise, substitute “EB buffer” or “TE-4” for the final elution step to avoid possible DNA degradation from uncontrolled (low) pH.

Protocol references

Tack, D. S., Tonner, P. D., Pressman, A., Olson, N. D., Levy, S. F., Romantseva, E. F., Alperovich, N., Vasilyeva, O., & Ross, D. (2021). The genotype-phenotype landscape of an allosteric protein. Molecular Systems Biology, 17(12). https://doi.org/10.15252/msb.202110847