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Transcription Factor Binding: Singleplex Assay for Function Measurements

Forked from Singleplex Assay for Function Measurements

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Pooled, Growth-Based As…

Open Datasets Initiative
Align to Innovate

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We use this protocol and it's working

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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. This protocol was typeset by Dana Cortade and David Ross.

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 of 3 variants, distributed according to the provided function plate map (see attachments). The protocol begins with several growths which convert the separate glycerol stocks into cultures that have reached 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 the next growth cycles where a 2-fold dilution series of the corresponding inducer is introduced. 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, the cultures in the growth plate act as input for quantification using either a flow-cytometry or a plate reader.

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

Attachments

PNG **Flow cytometer plate...** 84KB

PNG **Plate reader varaint...** 60KB

PNG **plate reader fluores...** 74KB

Materials

Starting cultures:

- a glycerol stock of each of the variants to be tested
- a glycerol stock of a non-fluorescent 'blank' plasmid for background control

Reagents:

- 250 mL of media, M9 with glycerol (M9-gly)
- Kanamycin (kan) stock, 250 uL, 50 mg/mL in water
- DMSO, anhydrous (ThermoFisher, part no. D12345)
- Tap water
- Inducer stock, use either 1 or 2 depending on which transcription factor is being measured:

1. For LacI plasmids, use IPTG stock at 120 uL, 1 mol/L in water (pre-prepared ~0.5 mL aliquots at 1 mol/L in water stored in the freezer).
2. For RamR plasmids, use 1S-TIQ stock at 40 uL, 0.1 mol/L in DMSO (**Ambeed**, Part no. A236973) (pre-prepared ~0.5 mL aliquots at 0.1 mol/L in DMSO stored in the freezer).

For plate reader measurements, materials needed **to calibrate plate reader FL/OD to molecules of equivalent fluorophore** including:

- Phosphate Buffered Saline (PBS) (MilliporeSigma 806544)
- Sterile Water
- 950nm **Silica microspheres**
- Sulforhodamine 101 (**ThermoFisher S359**)

For flow cytometry measurements:

- Chloramphenicol (Fisher Scientific BP904-100)
- 1x phosphate-buffered saline (PBS) with 170 ug/mL chloramphenicol
- **Fluorescent calibration beads for flow cytometry** (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)
- One additional 96-well growth plate (Agilent 204799-100) for plate reader calibration
- Two single-cavity reservoir plates (Agilent 204093-100)
- One 6-column reservoir plate (Agilent 204284-100)

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Note

To edit these fields, click on the edit symbol (a small square with a pencil in it) located to the right of this step's boarder. Enter the experiment ID generated when the Experiment Requester (or Lead) filled out the Experiment Registration excel. If samples generated using this protocol are used as inputs samples in another experiment/protocol, use the same Experiment ID throughout the pipeline. The operator is the person carrying out the experiment. If several operators will work on the same protocol, enter in their names and indicate which sections of steps each operator will carry out.

Experiment ID:
Operator Name:

Next, select the step-case below that matches the type of transcription factor variants you are measuring. Below, you will see step cases for each of the transcription factor genes of interest. Please select the gene of interest you are investigating to ensure you follow the protocol with the correct reagents.

After the third growth plate finishes incubating, you will see another step-case, where you can choose to perform final calibrated Function measurements by using a flow cytometer or plate reader.

See the Attachments section for suggested plate map layouts for growth plates depending on if you choose flow cytometry or plate reader measurements. The plate reader measurements have one plate map for the variant measurement and a seprarate plate map for the fluorescent/OD calibration measurement.

STEP CASE

LacI control variants From 45 to 47 steps

Follow these steps for testing LacI control variants. You will need IPTG as an inducer.

Culture Preparation & Overnight Growth

2 Mix 30 mL M9-gly media with 30 µL of kanamycin stock (50 mg/mL) to get working media with 50 µg/mL kanamycin, M9-gly-kan.

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

- Each run should contain a non-fluorescent variant as a background control.

4 Incubate cultures overnight (Overnight) at 37°C with shaking at 300 rpm.

Quality control the overnight culture

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

Prepare media and inducer working solutions

6 Prepare the final working media: M9-gly-kan-DMSO

6.1 Add 250 mL M9-gly to a sterile media bottle.

6.2 Add 250 µL kanamycin (kan) to the media bottle, creating M9-gly-kan at 50 µg/mL kanamycin.

6.3 Add 1.256 mL DMSO to the media bottle, creating M9-gly-kan-DMSO at 0.5% DMSO

6.4 Mix the media bottle well

7 Prepare the inducer working solution: 4 mmol/L IPTG in M9-gly-kan-DMSO

7.1 Transfer 19.92 mL M9-gly-kan-DMSO media to a 50mL Falcon tube

7.2 Add 80 µL IPTG stock (see Materials: stock at 1 mol/L in water) to Falcon tube

7.3 Mix the inducer working solution well.

Prepare the automation system or liquid handler

8 Load a single cavity media reservoir with a lid, filled with the remaining M9-gly-kan-DMSO final working media.

9 Load a single cavity waste reservoir without lid, filled with ~100 mL of tap water

10 Load a 6-reservoir plate with a lid.

10.1 Fill column 1 of the 6-reservoir plate with the IPTG inducer working solution.

10.2 For downstream measurements performed by flow-cytometry, fill column 5 with focusing fluid and column 6 with PBS.

11 Load in the first growth plate (Growth Plate 1).

12 Load in the bacterial cultures in 14 mL snap-cap culture tubes (with lids removed).

First Growth Plate: cells reach stationary phase

13 Prepare first growth plate:

13.1 Add 450 µL of the final working media (M9-gly-kan-DSMO) to the appropriate wells.

13.2 Add 50 µL cell culture into the appropriate wells.

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

15 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, OD700, and fluorescence (569 nm excitation, 593 nm emission for the mScarlet-I3 fluorescent protein) every 5 minutes throughout the incubation.

15.1 Approximately 45 minutes before the end of the 12-hour incubation, prepare the second growth plate.

- Create a 2-fold dilution series of IPTG across the columns of the plate using the working media (M9-gly-kan-DMSO) as a dilutor. Note: the exception is that all wells in column one should have no inducer (490 µL of working media only).
- Each well should end with 490µL of mixed media (working media + inducer).
- The highest final concentration of IPTG is 2000µmol/L, which is a 2-fold dilution from the IPTG working solution (4000µmol/L)
- Final IPTG concentrations (µmol/L) in columns 1-12 should be:

0, 1.953, 3.908, 7.813, 15.625, 31.25, 62.5, 125, 250, 500, 1000, 2000

15.2 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

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

17 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

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

19 Incubate the second growth plate for 3 hours and 5 minutes 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 OD and fluorescent readings take a total of 5 extra minutes - this is why the incubation in 3 hours and 5 minutes.

19.1 Measure OD600, OD700, and fluorescence (569 nm excitation, 593 nm emission for mScarlet-I3 fluorescent protein) every 5 minutes throughout the incubation.

19.2 Approximately 45 minutes before the end of the 12-hour incubation, prepare the third growth plate. Note: it is similar to the second growth plate, but each well has 450µL of mixed media instead of the previous 490µL.

- Create a 2-fold dilution series of IPTG across the columns of the plate using the working media (M9-gly-kan-DMSO) as a dilutor. Note: the exception is that all wells in column one should have no inducer (450 µL of working media only).
- Each well should end with 450µL of mixed media (working media + inducer).
- The highest final concentration of IPTG is 2000µmol/L, which is a 2-fold dilution from the IPTG working solution (4000µmol/L)
- Final IPTG concentrations (µmol/L) in columns 1-12 should be:

0, 1.953, 3.908, 7.813, 15.625, 31.25, 62.5, 125, 250, 500, 1000, 2000

19.3 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.

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

Third Growth Plate

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

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

23 Incubate the third growth plate for 3 hours and 5 minutes 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 OD and fluorescent readings take a total of 5 extra minutes - this is why the incubation in 3 hours and 5 minutes.

23.1 Measure OD600, OD700, and fluorescence (569 nm excitation, 593 nm emission for mScarlet-I3 fluorescent protein) every 5 minutes throughout the incubation.

24 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).

25 Here we provide a step-case to chose between using flow cytometry or a plate reader to perform the final calibration function measurements using a fluorescent standard.

STEP CASE

Plate Reader 4 steps

For measurements with a plate reader

Plate Reader Fluorescent Measurement

26 Put the third growth plate back into the plate reader (without the gas-permeable seal) and perform a single additional (i.e., end-point) measurement of OD600, OD700 and fluorescence.

27 Calibrate the OD600 and fluorescence measurements using silica microspheres (950μm) and a fluorescent dye (Sulforhodamine 101). This requires using a new plate, following a fluorescent calibration protocol: **The reference for performing this measurement**

Note: For additional instruction see References Beal 2018, Beal 2020, and Beal 2022.

Data Analysis

28 Using the calibrated data, calculate the mean fluorescence per cell in molecules of equivalent fluorophore (MEF) for each sample, including the non-fluorescent control samples, and the standards.

29 Calculate the quantitative Function by subtracting the mean fluorescent signal of the non-fluorescent control from the measured variants.

Protocol references

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