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Library Bottlenecking Protocols V.1

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Protocol status: Working
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.

Abstract

Protocol for bottlenecking a variant library for downstream use in the Pooled, Growth-Based Assay pipeline using either a positive-displacement flow cytometer or basic microbial culture equipment.

Materials

Flow Cytometer Materials

Starting cultures:

- glycerol stock of variant library

Reagents:

- M9 Media (ThermoFisher A1374401)
- Attune Flow Cytometer Focusing Fluid (ThermoFisher 90039273)
- Glycerol (MilliporeSigma G5516)

Consumables:

- Six 15 mL snap cap tubes (Corning 352059)
- Three 250mL baffled flasks (ThermoFisher 4116-0250)
- Three 1.5 mL Microcentrifuge tubes (ThermoFisher 69715)
- Three Agar Plates with LB and Kanamycin-50 (MilliporeSigma L0543)
- Multiple 1.2 mL Cryogenic vials (Corning 430487)

Basic Microbial Equipment Materials

Starting cultures:

- glycerol stock of variant library

Reagents:

- M9 Media (ThermoFisher A1374401)
- Glycerol (MilliporeSigma G5516)

Consumables:

- Six 15 mL snap cap tubes (Corning 352059)
- Eight 250mL baffled flasks (ThermoFisher 4116-0250)
- Eighteen 1.5 mL Microcentrifuge tubes (ThermoFisher 69715)
- Eighteen Agar Plates with LB and Kanamycin-50 (MilliporeSigma L0543)
- Multiple 1.2 mL Cryogenic vials (Corning 430487)

Create the first overnight culture

1 Dilute a full 1 mL vial of the library glycerol stock into 50 mL of media in a 250 mL baffled flask.

2 Incubate the resulting culture at 37 C with shaking (300 rpm) for 12 - 24 hours (to reach stationary phase).
- This generates the **overnight flask**

STEP CASE

Flow Cytometer 27 steps

This protocol uses a flow cytometer to bottleneck variant libraries down to the desired size to run in the Pooled, Growth-Based Assay Protocol.

Prepare all flasks and tubes

3 Prepare one baffled flask (125 mL or 250 mL) with 49.9 mL media
- This is the **dilution flask**

4 One new 250-mL baffled flask with 50 mL media.
- This is the **final flask**

5 Six 15 mL snap-cap culture tubes, each with 2 mL media and number the tubes 1 - 6.

6 Three 1.5 mL microcentrifuge tubes and number the tubes 1, 2, 3
- Leave tube no. 1 empty
- Add 900 uL media to each of the other tubes

7 Three agar plates (with LB + kan) and number the plates 1, 2, 3

Calibrate the flow cytometer

8 Run the startup and performance test protocols for the flow cytometer.
- Also, run a blank sample, 1 mL of focusing fluid to make sure the system is well warmed up and running properly.

Make culture dilutions

9 Mix the 50 mL overnight culture well, then pipette 0.1 mL from **the overnight flask** culture into the **dilution flask** (with 49.9 mL media) and swirl the flask to mix well.

10 Make a 3-fold serial dilution series from the **dilution flask**:
- Pipette 1 mL from extra flask to culture tube no. 1
- Mix culture tube no. 1 well
- Pipette 1 mL from culture tube no. 1 to culture tube no. 2
- Mix culture tube no. 2 well
- Pipette 1 mL from culture tube no. 2 to culture tube no. 3
- Mix culture tube no. 3 well
- Continue this for the remaining tubes 4-6.

Measure samples using the flow cytometer

11 Measure a media blank sample with the flow cytometer.
- Use a 150 uL acquisition volume at 100 uL/min flow rate

12 Measure cultures tubes 1-6 with the flow cytometer:
- Mix each tube well by vortexing before measurement.
- Measure tubes in reverse order (i.e., start with tube 6).
- Use a 150 uL acquisition volume at 100 uL/min flow rate

Analyze the data

13 For the media blank and each culture tube, analyze the cytometry data to calculate the apparent number of cells per 100 uL.
- Ignore the first 30 s of data for each sample
- Adjust the flow cytometry gates based on the scattering signals for the culture tube samples, and use the same gate for all samples (including the media blank)

14 Record the “raw cell count” as the number of events within the scattering-signal gate

15 For the culture tube samples, subtract the number of events recorded with the media blank sample to calculate the number of cells per 100 uL for each sample

16 Choose the culture tube with closest to 20,000 cells per 100 uL.

17 Calculate the volume of the chosen culture required for 200,000 cells.
- This is the **input volume**

- 200,000 is used here instead of 100,000 because some variants will have multiple copies in the culture. So, the counted number of cells will be an overestimate of the resulting bottleneck diversity.

Dilute and plate cultures

18 Add the **input volume** of the chosen culture to the **final** 250-mL baffled flask with 50 mL media.

19 Mix the **final flask** by swirling several times.

20 Pipette 1 mL from the **final flask** to microcentrifuge tube 1 (the empty one).

21 Mix microcentrifuge tube 1

22 Pipette 100 uL from microcentrifuge tube 1 to microcentrifuge tube 2 and mix.

23 Mix microcentrifuge tube 2

24 Pipette 100 uL from microcentrifuge tube 2 to microcentrifuge tube 3

25 Mix microcentrifuge tube 3

26 Plate 150 uL from each microcentrifuge tube onto the corresponding agar plate.

27 Incubate the bottlenecked culture in the **final** 250 mL baffled flask at 37 C with shaking (300 rpm) for 16 - 24 hours (to reach stationary phase).

Count cultures to verify flow cytometry data

28 The following day, count the number of colonies on each plate and use the results to estimate the number of cells that were initially diluted into the bottlenecked culture.

Make stocks

29 Make several 1 mL glycerol stocks (0.5 mL cells + 0.5 mL 40% glycerol) from the bottlenecked culture, label appropriately, and store at -80C until use.