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Transcription Factors: Pooled, Growth-Based Assays

Forked from Pooled, Growth-Based Assays

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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 **Gro**wth-based **Q**uantitative **Seq**uencing-GROQ-Seq) Platform. The GROQ-Seq platform was created under **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 fitness of transcription factor variants for LacI, RamR, and VanR in E.Coli. This protocol defines paths for either:

- **only measuring a pool of control variants**
- **measuring a 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) pooled libraries 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 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 as a quality control check 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 (10%) 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

final TF pooled plat...
232KB

inducer and antibiot...
57KB

Pooling method.png
305KB

Materials

Note 1: The M9-gly Media reagent should be created using **this protocol**.

Note 2: Please note the following **important reagent handling considerations**:

- **Trimethoprim (TMP) is extremely sensitive to freeze/thaw cycles.** Due to this, make sure to 1) thaw the TMP freezer stocks gently (either at room temperature or on a heat block at 30C), and 2) use each TMP freezer stock aliquot only once. Throw away any unused thawed TMP.
- **Vanillic Acid is light sensitive.** Due to this, make sure to 1) keep all Vanillic acid aliquots in opaque containers, 2) cover all Vanillic acid stocks/solutions in foil when not in use, 3) the 6-column reservoir and lid that holds the Vanillic acid solution during the automated liquid handling steps should be opaque or be covered in black electrical tape.

Note 3: The following reagents should be pre-aliquoted at the following concentrations:

- 1S-TIQ should be prepared in ~0.5 mL aliquots at 0.1 mol/L in DMSO and stored in the freezer.
- IPTG should be prepared in ~0.5 mL aliquots at 1 mol/L in water stored in the freezer.
- Vanillic acid should be prepared in ~0.5 mL aliquots at 0.1 mol/L in ethanol stored in the freezer.
- TMP should be prepared in ~50 uL aliquots at 25 mg/mL in DSMO and stored in the freezer. TMP stocks in DMSO are sensitive to freeze-thaw cycles. So, aliquots should only be used once.
- Kan should be prepared in ~0.5 mL aliquots at 50 mg/mL in water and stored in the freezer.

Reagents:

	A	B	C	D
	Reagent	Supply and cat. #	Controls only	Variant Library + Controls
	M9-gly Media	Prepared using the M9-gly Media Protocol	590 mL	1080 mL
	kanamycin (kan)		590 µL	1080 µL
	Dimethyl sulfoxide (DMSO) anhydrous	ThermoFisher, part no. D12345	2.3 mL	4.4 mL
	(S)-1-Phenyl-1,2,3,4-tetrahydroisoquinoline (1S-TIQ)	Ambeed, # A236973	125 µL	125 µL
	Trimethoprim (TMP)	Millipore-Sigma 92131-1G	30 µL	30 µL
	Isopropyl β-D-1-thiogalactopyranoside (IPTG)	ThermoFisher R0392	100 µL	100 µL
	Vanillic Acid	Millipore-Sigma, 94770-10G	50 µL	50 µL
	tap water		100 mL	100 mL

Consumables:

	A	B	C	D
	Consumable	Supply and cat. #	Controls only	Variant Library + Controls
	500 mL sterile media bottles	ThermoFisher 10340001	1	2
	14 mL snap cap tubes	Corning 352057	24	27
	250 mL baffled flasks	ThermoFisher 4116-0250	4	9
	1 cm cuvettes for OD600 measurements		3	3
	50mL falcon tubes	ThermoFisher 339652	7	5
	single cavity media reservoirs, sterile	Agilent 204093-100	2	2
	lid for a single cavity media reservoir	Agilent 202497-100	1	1
	6 column reservoir plate*	Agilent 204284-100*	1	1
	lid for 6 column reservoir, black	Agilent 200858-100**	1	1
	96-well growth plates	Agilent 204799-100	5	5
	gas permeable seals	Azenta P98-712	5	5
	lids for growth plates	Agilent 202497-100	5	5
	60 mL reagent reservoirs	Hamilton 56694-01	3	3

*The 6-column reservoir must be opaque to protect the Van reagent from light. The listed Agilent part number is a clear polypropylene plate that can be used if it is covered with black tape (e.g., electrical tape); black 6-column reservoirs are also available as a custom order.
**The listed Agilent part number for the black reservoir lids is not for sterile lids. Non-sterile black lids can be sterilized with 70% ethanol before use; sterile black lids are also available as a custom order.

Input samples:

1. The following control variant glycerol stocks (see table below)

2. If measuring a variant library in addition to the controls, than you also need glycerol stalks for each library
Control Variant Glycerol Stocks:

	A	B	C
	Stock Name	**Purpose**	**Library Used in**
	pLacI-norm-02-A	Normalization Variant	LacI, RamR, and VanR
	pRamR-norm-02-A	Normalization Variant	LacI, RamR, and VanR
	pLacI-WT-O1_ecDHFR-A	Calibration Variant	LacI
	pLacI-WT-O2_ecDHFR-A	Calibration Variant	LacI
	pLacI-WT(V52A)_ecDHFR-A	Calibration Variant	LacI
	pLacI-WT(V52Q)_ecDHFR-A	Calibration Variant	LacI
	pLacI-157(S70R)_ecDHFR-A	Calibration Variant	LacI
	pLacI-WT(D88N)-Oid_ecDHFR-A	Calibration Variant	LacI
	pLacI-WT(V80L/S279T)_ecDHFR-A	Calibration Variant	LacI
	pLacI-002(S69T/V80L)-Oid_ecDHFR-A	Calibration Variant	LacI
	pRamR-WT_ecDHFR-A	Calibration Variant	RamR
	pRamR-S_v8_2.3k_ecDHFR-A	Calibration Variant	RamR
	pRamR-S(S7F)_ecDHFR-A	Calibration Variant	RamR
	pRamR-S(D9H)_ecDHFR-A	Calibration Variant	RamR
	pRamR-D2-1(R103G)_ecDHFR-A	Calibration Variant	RamR
	pRamR-D2-1(L98I)_ecDHFR-A	Calibration Variant	RamR
	pRamR-D2-1(M141T)_ecDHFR-A	Calibration Variant	RamR
	pRamR-7753_ecDHFR-A	Calibration Variant	RamR
	pRamR-WT-v2_ecDHFR-A	Calibration Variant	RamR
	pRamR-WT-fin_ecDHFR-A	Calibration Variant	RamR
	pVanR-WT_ecDHFR-A	Calibration Variant	VanR
	pVanR-am_ecDHFR-A	Calibration Variant	VanR
	pVanR-am_t2a_ecDHFR-A	Calibration Variant	VanR
	pVanR-am_a1t_ecDHFR-A	Calibration Variant	VanR

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 with libraries of variants pooled together (including controls as well), then use the 'Variant Libraries' branch below.

Important Note: All preparation steps performed outside of the automated liquid handler should use Aseptic Technique, and a biosafety cabinet is recommended. Also, to maintain sterility, the growth plates should be lidded except when noted in the protocol steps.

This protocol is the beginning of the GROQ-Seq pipeline, which includes the following protocols run sequentially:
1. Starting with this protocol, the Pooled, Growth-Based Assay Protocol.
2. The **DNA Extraction Protocol** (must be run for growth plates 2-5 immediately after the finish incubating)
3. The **BarSeq Library Preparation and Pooling Protocol** (run on the extracted plasmid DNA from growth plates 2-5).
4. Follow by Illumina Sequencing and Nanopore Sequencing

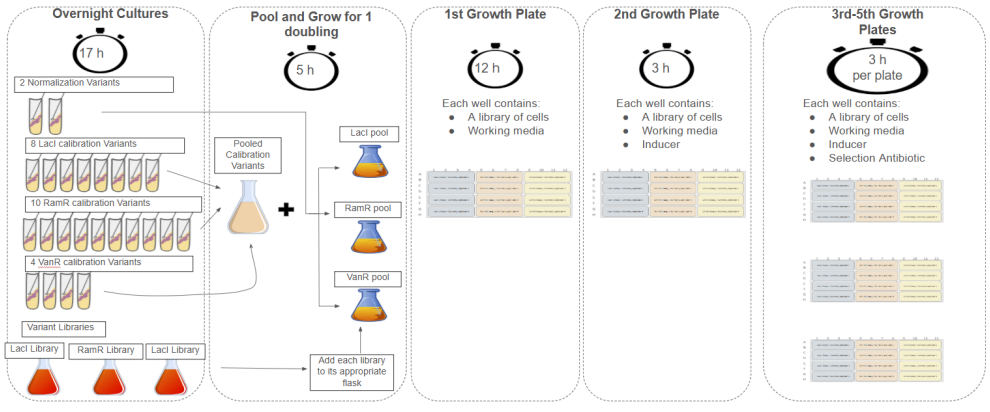
STEP CASE

Measuring Variant Libraries 144 steps

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

Overview of protocol for measuring variant libraries + controls

2



Prepare Overnight Growth Media

3 Gather a sterilized bottle or flask with at least 500 mL capacity and label it as "Overnight Media"

4 Add 500mL of M9-gly Media to the flask.

5 Add 500µL of kanamycin stock (50 mg/mL) to get M9-gly-kan Media with a concentration of 50 µg/mL kanamycin.

6 Mix well.

Culture Preparation & Overnight Growth

7 Start separate cultures of the two normalization variants: ‘pLacI-norm-02’ and ‘pRamR-norm-02’.

7.1 Gather two 14mL snap-cap culture tubes and label one for each variant.

7.2 Add 5mL of M9-gly-kan Media to each tube.

7.3 For each variant, take a scraping from its glycerol stock and put its corresponding tube to start the culture.

7.4 Loosely cap the tubes (leave in the upper/loose position for incubation).

8 Start separate cultures for each of the calibration variants. This protocol uses the following calibration variants:
For LacI:
1. pLacI-WT-O1_ecDHFR-A
2. pLacI-WT-O2_ecDHFR-A
3. pLacI-WT(V52A)_ecDHFR-A
4. pLacI-WT(V52Q)_ecDHFR-A
5. pLacI-157(S70R)_ecDHFR-A

6. pLacI-WT(D88N)-Oid_ecDHFR-A
7. pLacI-WT(V80L/S279T)_ecDHFR-A
8. pLacI-002(S69T/V80L)-Oid_ecDHFR-A

For RamR:

1. pRamR-WT_ecDHFR-A
2. pRamR-S_v8_2.3k_ecDHFR-A
3. pRamR-S(S7F)_ecDHFR-A
4. pRamR-S(D9H)_ecDHFR-A
5. pRamR-D2-1(R103G)_ecDHFR-A
6. pRamR-D2-1(L98I)_ecDHFR-A
7. pRamR-D2-1(M141T)_ecDHFR-A
8. pRamR-7753_ecDHFR-A
9. pRamR-WT-v2_ecDHFR-A
10. pRamR-WT-fin_ecDHFR-A

For VanR:

1. pVanR-WT_ecDHFR-A
2. pVanR-am_ecDHFR-A
3. pVanR-am_t2a_ecDHFR-A
4. pVanR-am_a1t_ecDHFR-A

8.1 Gather 22 of the 14mL snap-cap culture tubes and label one for each variant.

8.2 Add 5mL of M9-gly-kan Media to each tube.

8.3 For each variant, take a scraping from its glycerol stock and put its corresponding tube to start the culture.

8.4 Loosely cap the tubes (leave in the upper/loose position for incubation).

9 Start a culture for each of your three variant libraries (LacI, RamR, VanR).

9.1 For each of the three libraries, prepare a 250 mL baffled culture flask and label it with the library name. e.g., "Variant Library for LacI"

9.2 Add 100 mL of M9-gly-kan media to each flask.

9.3 Add one aliquot of glycerol stock for each library to its corresponding flask.

9.4 Close the flasks with an appropriate cap for aerobic incubation.

10 Incubate all normalization, calibration, and library cultures overnight:
- 37 C, with shaking at 300 rpm
- 16-17 hours

Pool cultures and grow for one doubling

11 Pool together all of the calibration variant cultures.

11.1 Gather one 250mL baffled flask and label it with "Pooled Calibration Variants"

11.2 Pour each of the calibration variant cultures into the baffled flask
- 8 LacI calibration cultures
- 10 RamR calibration cultures
- 4 VanR calibration cultures
- 22 cultures total
- Mix well

12 Prepare working media for pooled cultures. Create 180mL M9 Media with 1% DMSO.
Note: Use DMSO handling precautions.

12.1 Gather a sterile media bottle with at least 100 mL capacity and label it with "Working Media for Pooled Culture".

12.2 Add 75 mL of M9-gly Media to the media bottle.

12.3 Add 75 µL of kanamycin stock (50 mg/mL) to get M9-gly-kan media with a concentration of 50 µg/mL kanamycin.

12.4 Add 0.758 mL of DMSO to the media bottle to get M9-gly-kan with 1% DMSO.

12.5 Mix the media with DMSO well. This is now the working media.

13 Create the final pooled cultures for each library.

13.1 Prepare one 250 mL sterile baffled flask for each transcription factor library, and label it with the name of the library (e.g., "Final Pooled Library for LacI").

13.2 To each flask, add 25 mL of the working media (i.e., the M9-gly-kan Media with 1% DMSO).

13.3 To each flask, add 0.25 mL of each normalization variant culture from their overnight tubes. The two normalization variants are:
- ‘pLacI-norm-02’
- ‘pRamR-norm-02’

Note: These normalization variants are the same for all libraries, i.e., they are both added to the LacI, RamR and VanR flasks.

13.4 For each library, add 0.5 mL of the pooled calibration variant culture.

Note: The calibration variants are the same for all libraries, i.e., they are added to the LacI, RamR and VanR flasks.

13.5 For each library, add 24 mL of the corresponding variant library culture.
e.g., add the "Variant Library for LacI" to the "Final Pooled Library for LacI" flask.

14 Incubate the flasks containing the pooled library cultures for 5-7 hours to allow for approximately one additional doubling.
- 37 C, w/ shaking at 300 rpm

Note: While the growth is occurring, prepare the working media, inducer solutions, and selection antibiotic solutions as described in the following section.

Prepare working media, inducer solutions, and selection antibiotic solutions

15 **Notes: Important reagent handling considerations.**
- Use DMSO handling precautions for ligand and antibiotic stocks.
- TMP is sensitive to freeze/thaw cycles, so do not use a TMP stock aliquot for more than one experiment. Throw away any leftover TMP after creating the TMP solutions described in this section.

- Vanillic acid is light sensitive, so cover any Vanillic acid solution in foil when not in use.

Final concentrations of inducers created in this section (2x higher than used in the growth plates):
- LacI's inducer: 4000 umol/L IPTG
- RamR's inducer: 500 umol/L 1S-TIQ
- VanR's inducer: 200 umol/L Vanillic acid

Final concentrations of selection antibiotic (Trimethoprim or TMP) created in this section (10x higher than used in the growth plates) for all transcription factors:
- Low TMP: 3 ug/mL
- Medium TMP: 10 ug/mL
- High TMP: 30 ug/mL

16 Thaw the following freezer stocks gently, either at room temperature or on a heat block at 30C:
- TMP
- IPTG
- 1S-TIQ
- Vanillic Acid

17 Create M9-gly-Kan media using the following sub-steps.

17.1 Add 400 mL M9-gly into a 500mL sterile media bottle.

17.2 Add 400 uL kanamycin (kan) to the M9-gly media and mix well.

18 Create 25 mL of 1S-TIQ at 500 umol/L using the following steps:

18.1 Label a 50mL falcon tube "1S-TIQ at 500 umol/L "

18.2 Pipette 24.875 mL M9-gly-kan (without DMSO) into the Falcon tube.

18.3 Mix the contents of the 1S-TIQ freezer stock either by vortexing or with a pipette.

18.4 Add 125 uL 1S-TIQ freezer stock (100 mmol/L in DMSO) to the falcon tube

18.5 Mix by pipetting up and down

19 Create 25mL of TMP at 30 ug/mL stock using the following sub-steps.
Note: Use only new TMP freezer stocks; multiple freeze-thaw cycles will degrade the TMP.

19.1 Label a 50mL falcon tube "TMP at at 30 ug/mL - High TMP solution "

19.2 Pipette 24.875 mL M9-gly-kan (without DMSO) into a Falcon tube

19.3 Add 95 uL DMSO to the flacon tube.

19.4 Mix by pipetting up and down.

19.5 Mix the contents of the TMP freezer stock either by vortexing or with a pipette.

19.6 Add 30 uL TMP freezer stock (previously prepared at 25 mg/mL in DMSO)

19.7 Mix and rinse pipette tip by pipetting up and down.

Note: This solution is the highest TMP concentration used in the experiment and will be used in subsequent steps to create the medium and low TMP solutions.

20 Create the working media by adding DMSO to the remaining M9-gly-kan media using the following sub-steps.

20.1 Add 1.76 mL DMSO to M9-gly-kan media bottle.

20.2 Mix well.

Note: This solution is now the working media that will be referred to throughout the rest of this protocol.

21 Prepare 15 mL of the low and medium TMP solutions using the following sub-steps.

21.1 Create the low TMP solution (3 ug/mL TMP):
1. Label a 50 mL falcon tube 'Low TMP solution'
2. Add 13.5 mL working media media into the flacon tube.
3. Add 1.5 mL of the 30 ug/mL TMP stock (i.e., the high TMP solution).
4. Mix by pipetting up and down.

21.2 Create the medium TMP solution (10 ug/mL TMP):
1. Label a 50 mL falcon tube 'Medium TMP solution'
2. Add 10 mL working media media into the flacon tube.
3. Add 5 mL of the 30 ug/mL TMP stock (i.e., the high TMP solution).
4. Mix by pipetting up and down.

22 Prepare 25 mL of 4000 umol/L of IPTG.

22.1 Labeled a 50mL falcon tube "IPTG at 4000 umol/L"

22.2 Pipette 24.9 mL M9-gly-kan (with DMSO) into Falcon tube

22.3 Mix the contents of the IPTG freezer stock either by vortexing or with a pipette.

22.4 Add 100 uL IPTG freezer stock (1 mol/L in water).

22.5 Mix and rinse pipette tip by pipetting up and down.

23 Prepare 25 mL of 200 umol/L Vanillic Acid
- Reminder: Vanillic acid is light sensitive, so cover any Vanillic acid solution in foil when not in use.

23.1 Labeled a 50mL falcon tube "Vanillic acid at 200µmol/L"

23.2 Pipette 24.95 mL of the working media into the Falcon tube

24 Mix the contents of the Vanillic Acid freezer stock either by vortexing or with a pipette.

24.1 Add 50 uL Vanillic acid freezer stock (100 mmol/L in ethanol)

24.2 Mix and rinse the pipette tip by pipetting up and down.
- **Note:** Some of the Vanillic Acid may crash out of solution onto the inner walls of the pipette tip at the beginning of the mixing/rinsing step. This will appear as a slight cloudy film on the inside of the tip. Continue rinsing the tip until this precipitate has re-dissolved (until the tip is completely clear again).

Perform a QC check for growth

25 Perform an OD600 measurement on a sample of all three pooled cultures.

25.1 To measure, dilute a small sample of each of the pooled cultures 10x into a 1cm cuvette (e.g., 100 uL culture + 900 uL M9-gly-kan).
- Alternately, dilute each pooled culture sample 10x into one or more wells in a 96-well plate for the OD600 measurement.

25.2 Measure and record the OD600 for each pooled culture using a spectrophotometer (with 1cm cuvettes) or plate reader (with 96-well plate).
- When measuring the 10x dilute plate culture, expect the OD600 to be be between 0.3 to 0.8. If the OD is lower than this range, then you will need to troubleshoot the reason for lack of growth.
- Note: if a plate reader is used, the OD600 values will need to be corrected to the 1-cm pathlength equivalent using a suitable calibration between the plate reader and spectrophotometer formats.

Automation System: Load the automation system

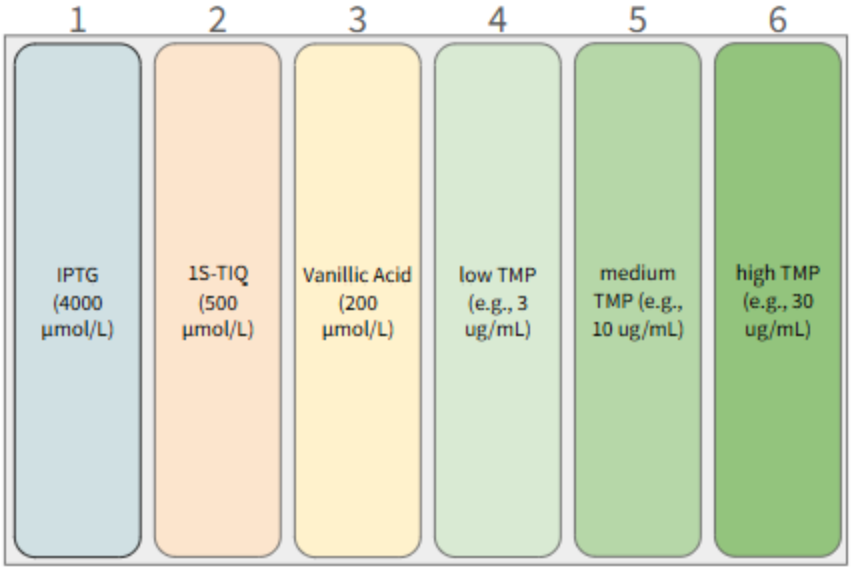
26 Load a single cavity media reservoir with a lid, filled with ~200 mL of the M9-gly-kan-DMSO final working media.

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

28 Fill a 6 column reservoir plate using the following steps.

28.1 Fill each column according to the instructions below (also shown in image).

1. Fill column 1 with the IPTG at 4000µmol/L solution.
2. Fill column 2 with the IS-TIQ at 500µmol/L solution.
3. Fill column 3 with the Vanillic acid at 200µmol/L solution.
4. Fill column 4 with the low TMP solution.
5. Fill column 5 with the medium TMP solution.
6. Fill column 6 with the high TMP solution.



29 Place lid on the 6 column reservoir plate and load into liquid handler.

30 Load the first growth plate (Growth Plate 1).

31 Load the three pooled library cultures (for LacI, RamR, and VanR):

31.1 Put each pooled library culture into a separate, labeled Hamilton 60 mL reagent reservoir

- Transfer cultures from shake flasks or snap-cap tubes in a biosafety cabinet or other sterile working space, and place lids on the Hamilton reservoirs.
- Move reservoirs to the appropriate position on the liquid handler deck.

31.2 Remove lids from the library culture reservoirs just before running the Hamilton method. Note: It is important to have the lids on the growth plates, the media reservoir and the reagent reservoir when they are not in use to prevent evaporation and to avoid contamination. A critical step is to make sure the lids are on the media and reagent reservoirs during the cell culture pipetting. Pipetting can generate small aeresols, and those could transfer cells into the media or reagents if they are left open.

Automation System: The first growth plate (cells reach stationary phase)

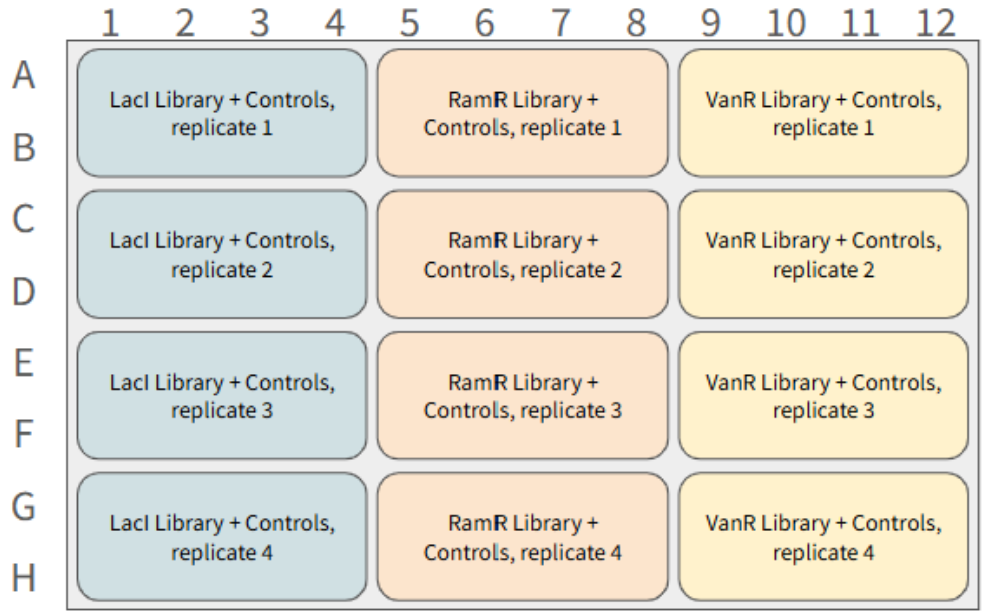
32 Pipette 450 uL working Media into each well being used in the growth plate

32.1 Remove the lids from the media reservoir and the first growth plate.

32.2 Pipette the working Media from the media reservoir to the first growth plate.

32.3 Replace the lid on the media reservoir.

33 Pipette 50 uL of the appropriate pooled library + control culture into each well following the plate map below.



Note: the four replicates for each library are pipetted from the same pooled library culture.

34 Replace the lid on the first growth plate to move it to the location used to apply the gas-permeable seal.

35 Apply gas-permeable seal to plate.

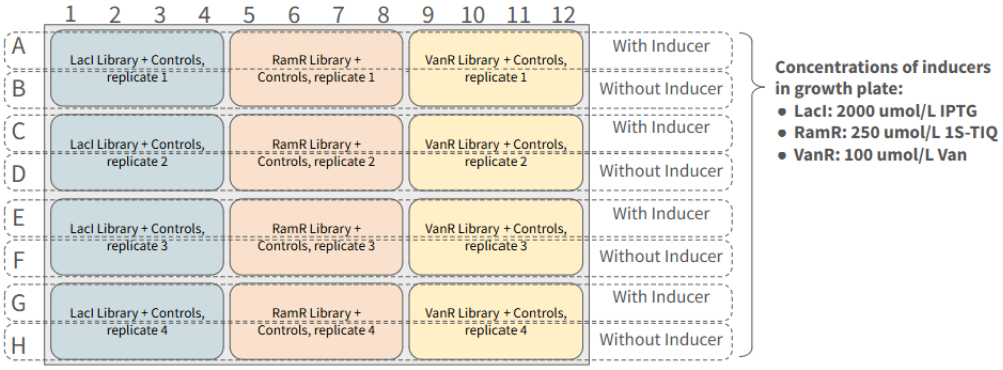
35.1 Remove the lid first

36 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)

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

36.2 During incubation, move the second growth plate to the pipetting position, remove its lid, and prepare the second growth plate with 490 uL mixed media per well, where the mixed media now includes working media and the required inducers for each library following the plate map below.
Note: No TMP is added to this plate.

Note 2: The volume of inducer added to rows A, C, E, and G should be set to give the desired final concentration *after* 10 uL of cells are added (in the Automation System: The second growth plate (timepoint 1) section). The cell cultures in the first growth plate had zero inducer. So, to set the correct final concentration, media and inducers should be added to the wells of the second growth plate with the following volumes:
A1-A4: 240.0 uL media + 250.0 uL IPTG solution
A5-A8: 240.0 uL media + 250.0 uL 1S-TIQ solution
A9-A12: 240.0 uL media + 250.0 uL Vanillic Acid solution
B1-B12: 490.0 uL media
The wells in rows C, E, and G should be the same as in row A.
The wells in rows D, F, and H should be the same as in row B.
Note 3: Remove the lids from the media and reagent reservoirs only during the relevant pipetting steps, i.e., remove lid from Media reservoir, pipette Media, replace lid on Media reservoir, remove lid from Reagent reservoir, pipette inducers, replace lid on Reagent reservoir.



36.3 Replace the lid onto the second growth plate.

36.4 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.
- Approximate temperature and timing: on a Hamilton Heater-Cooler with a flat adapter, use a set-point temperature of 67 C. Then, ten minutes before the end of the 12-hour incubation, move the growth plate to the Hamilton Heater-Cooler, wait ten minutes,

then move the growth plate back to the pipetting position. Proceed with the following steps immediately after.

Automation System: The second growth plate (timepoint 1)

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

38 Remove the lid from the second growth plate.

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

Note: The preferred transfer method uses a 96-channel head on the automated liquid handler. If using a 96 channel head, there are some important details required to get a reproducible transfer:

1. Using 300 µL tips, insert tips into the source culture to a depth 1 mm from the bottom of the wells.
2. At that position, mix by aspirating and re-dispensing 200 µL of culture at a flow rate of 250 µL/s, then aspirate 200 µL at a flow rate of 100 µL/s and withdraw the pipette tips from the culture.
3. Reinsert the tips back into the same culture wells, to a depth 2 mm below the liquid surface, and dispense 200 µL at a flow rate of 120 µL/s, then withdraw the tips from the culture.
4. Reinsert the tips back into the same culture wells, to a depth 1 mm from the bottom of the wells.
5. At that position, aspirate the volume to be transferred to the destination plate (at 100 µL/s) and withdraw the tips from the culture.
6. Move the 96-channel pipetting head to the destination plate and dispense the volume to be transferred at a depth of 3 mm below the liquid surface (at 120 µL/s).

40 Replace the lid on the second growth plate to move it to the location used to apply the gas-permeable seal.

41 Apply gas-permeable seal to second growth plate.

41.1 Remove lid first.

42 Incubate the second growth plate for **2 hours and 45 minutes**, 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:

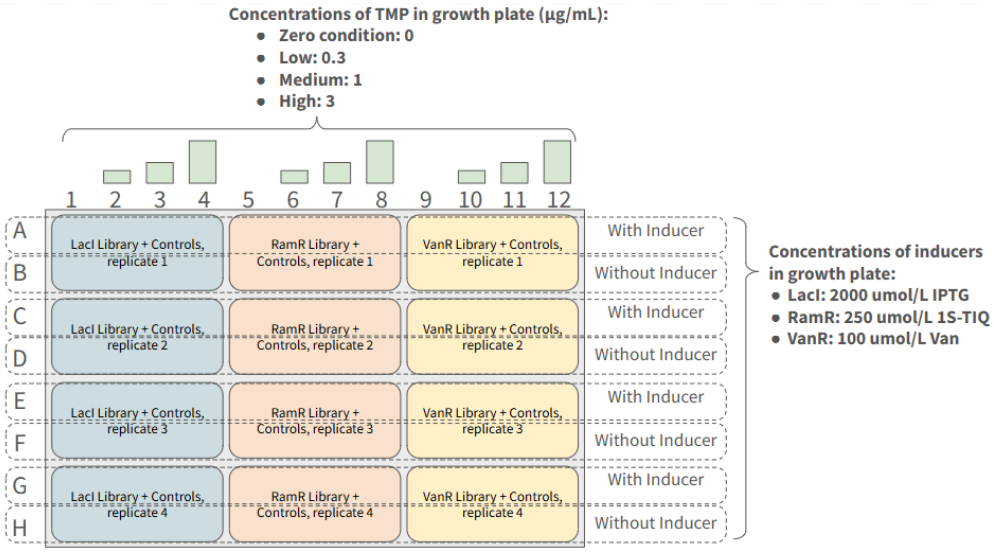
1. The cells are always in mid-log phase or lower.
2. The cell density at the end point of each subsequent incubation step is constant or slightly decreasing.

42.1 Measure OD600 and fluorescence every 5 minutes

- Start next steps during the last hour of the incubation

42.2 During incubation, move the third growth plate to the pipetting position, remove its lid, and prepare the third growth plate with 450 uL mixed media per well, where the mixed media now includes working media, inducers, and TMP following the plate map below. Note: The volume of inducer added to rows A, C, E, G and the volume of TMP solution added to columns 2-4, 6-7, and 10-12 should be set to give the desired final concentration *after 5*0 uL of cells are added (in the Automation System: The third growth plate (timepoint 2) section). The cell culture solutions in the second growth plate already have inducer (in the appropriate wells) but zero TMP. So, to set the correct final concentration, media, inducers, and TMP should be added to the wells of the third growth plate with the following volumes:
A1: 225.0 uL media + 225.0 uL IPTG solution
A2: 175.0 uL media + 50.0 uL low TMP solution + 225.0 uL IPTG solution
A3: 175.0 uL media + 50.0 uL medium TMP solution + 225.0 uL IPTG solution
A4: 175.0 uL media + 50.0 uL high TMP solution + 225.0 uL IPTG solution
A5: 225.0 uL media + 225.0 uL 1S-TIQ solution
A6: 175.0 uL media + 50.0 uL low TMP solution + 225.0 uL 1S-TIQ solution
A7: 175.0 uL media + 50.0 uL medium TMP solution + 225.0 uL 1S-TIQ solution
A8: 175.0 uL media + 50.0 uL high TMP solution + 225.0 uL 1S-TIQ solution
A9: 225.0 uL media + 225.0 uL Vanillic Acid solution
A10: 175.0 uL media + 50.0 uL low TMP solution + 225.0 uL Vanillic Acid solution
A11: 175.0 uL media + 50.0 uL medium TMP solution + 225.0 uL Vanillic Acid solution
A12: 175.0 uL media + 50.0 uL high TMP solution + 225.0 uL Vanillic Acid solution
B1: 450.0 uL media
B2: 400.0 uL media + 50.0 uL low TMP solution
B3: 400.0 uL media + 50.0 uL medium TMP solution
B4: 400.0 uL media + 50.0 uL high TMP solution
B5: 450.0 uL media
B6: 400.0 uL media + 50.0 uL low TMP solution
B7: 400.0 uL media + 50.0 uL medium TMP solution
B8: 400.0 uL media + 50.0 uL high TMP solution

B9: 450.0 uL media
B10: 400.0 uL media + 50.0 uL low TMP solution
B11: 400.0 uL media + 50.0 uL medium TMP solution
B12: 400.0 uL media + 50.0 uL high TMP solution
The wells in rows C, E, and G should be the same as in row A.
The wells in rows D, F, and H should be the same as in row B.
Note 3: Remove the lids from the media and reagent reservoirs only during the relevant pipetting steps, i.e., remove lid from Media reservoir, pipette Media, replace lid on Media reservoir, remove lid from Reagent reservoir, pipette TMP and inducers, replace lid on Reagent reservoir.



42.3 Replace the lid onto the third growth plate.

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

Automation System: The third growth plate (timepoint 2)

43 After the incubation is done, remove gas-permeable seal from the second growth plate.

44 Remove the lid from the third growth plate.

45 Transfer 50 uL from each well in the second growth plate to the corresponding well in the third growth plate.
- The preferred transfer method uses a 96-channel head on the automated liquid handler. If using a 96 channel head, use the previously recommended steps for cell transfer from the Automation System: The second growth plate (timepoint 1) section.

46 Replace the lid on the third growth plate to move it to the location used to apply the gas-permeable seal.
Replace the lid on the second growth plate to move it to the location used for the DNA extraction protocol.

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

47.1 Remove lid first.

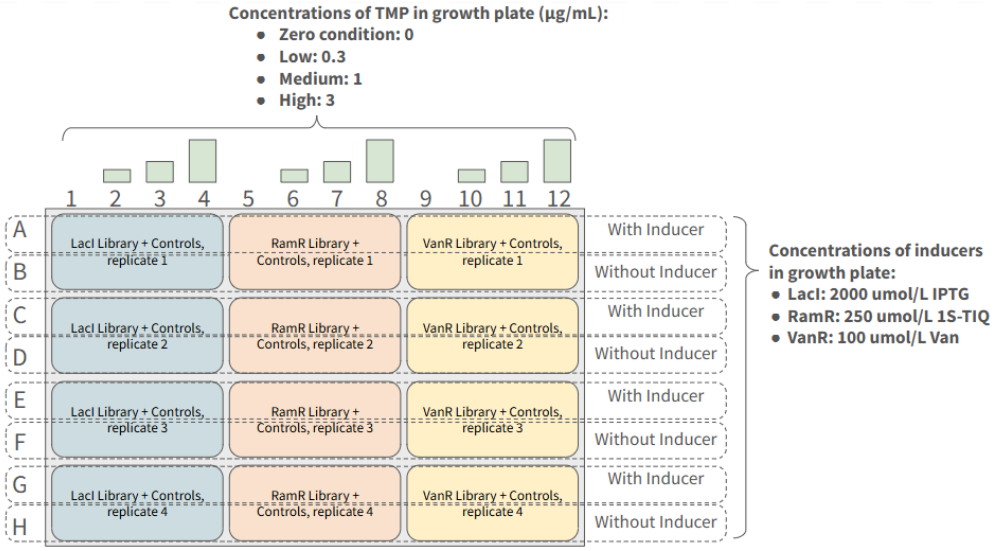
47.2 Immediately after the third growth plate goes into the incubator, remove the lid from the second growth plate and combine each set of 4 replicate wells from each condition of the *second* growth plate into a single well of a new polypropylene deep well plate (suitable for centrifugation). Run the plasmid **DNA extraction protocol** with the combined cultures in the deep well plate.
- Label the plate with the extracted DNA with the experiment identifier and "time point 1."
- Cover the second growth plate with a lid when when preparing for the DNA extraction protocol or if transferring to any other system.
- Store the resulting extracted DNA at 4C 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 **DNA extraction protocol**). Otherwise, substitute “EB buffer” or “TE-4” for the final elution step to avoid possible DNA degradation from uncontrolled (low) pH.

48 Incubate the third growth plate for **2 hours and 45 minutes** 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)

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

48.2 During incubation, move the fourth growth plate to the pipetting position, remove its lid, and prepare the fourth growth plate with 450 uL mixed media per well, where the mixed media now includes working media, inducers, and TMP following the plate map below.

Note: The volume of inducer added to rows A, C, E, G and the volume of TMP solution added to columns 2-4, 6-7, and 10-12 should be set to give the desired final concentration *after 5*0 uL of cells are added (in the Automation System: The fourth growth plate (timepoint 3) section). The cell culture solutions in the third growth plate already have inducer and TMP (in the appropriate wells). So, to set the correct final concentration, media, inducers, and TMP should be added to the wells of the fourth growth plate with the following volumes:
A1: 225.0 uL media + 225.0 uL IPTG solution
A2: 180.0 uL media + 45.0 uL low TMP solution + 225.0 uL IPTG solution
A3: 180.0 uL media + 45.0 uL medium TMP solution + 225.0 uL IPTG solution
A4: 180.0 uL media + 45.0 uL high TMP solution + 225.0 uL IPTG solution
A5: 225.0 uL media + 225.0 uL 1S-TIQ solution
A6: 180.0 uL media + 45.0 uL low TMP solution + 225.0 uL 1S-TIQ solution
A7: 180.0 uL media + 45.0 uL medium TMP solution + 225.0 uL 1S-TIQ solution
A8: 180.0 uL media + 45.0 uL high TMP solution + 225.0 uL 1S-TIQ solution
A9: 225.0 uL media + 225.0 uL Vanillic Acid solution
A10: 180.0 uL media + 45.0 uL low TMP solution + 225.0 uL Vanillic Acid solution
A11: 180.0 uL media + 45.0 uL medium TMP solution + 225.0 uL Vanillic Acid solution
A12: 180.0 uL media + 45.0 uL high TMP solution + 225.0 uL Vanillic Acid solution
B1: 450.0 uL media
B2: 405.0 uL media + 45.0 uL low TMP solution
B3: 405.0 uL media + 45.0 uL medium TMP solution
B4: 405.0 uL media + 45.0 uL high TMP solution
B5: 450.0 uL media
B6: 405.0 uL media + 45.0 uL low TMP solution
B7: 405.0 uL media + 45.0 uL medium TMP solution
B8: 405.0 uL media + 45.0 uL high TMP solution
B9: 450.0 uL media
B10: 405.0 uL media + 45.0 uL low TMP solution
B11: 405.0 uL media + 45.0 uL medium TMP solution
B12: 405.0 uL media + 45.0 uL high TMP solution
The wells in rows C, E, and G should be the same as in row A.
The wells in rows D, F, and H should be the same as in row B.
Note 3: Remove the lids from the media and reagent reservoirs only during the relevant pipetting steps, i.e., remove lid from Media reservoir, pipette Media, replace lid on Media reservoir, remove lid from Reagent reservoir, pipette TMP and inducers, replace lid on Reagent reservoir.



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

Automation System: The fourth growth plate (timepoint 3)

49 After the incubation is done, remove gas-permeable seal from the third growth plate.

50 Remove the lid from the fourth growth plate.

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

- The preferred transfer method uses a 96-channel head on the automated liquid handler. If using a 96 channel head, use the previously recommended steps for cell transfer from the Automation System: The second growth plate (timepoint 1) section.

52 Replace the lid on the fourth growth plate to move it to the location used to apply the gas-permeable seal.
Replace the lid on the third growth plate to move it to the location used for the DNA extraction protocol.

53 Apply gas-permeable seal to fourth growth plate and place into incubator.

53.1 Remove lid first.

53.2 Immediately after the fourth growth plate goes into the incubator, remove the lid from the third growth plate and combine each set of 4 replicate wells from each condition of the *third* 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.

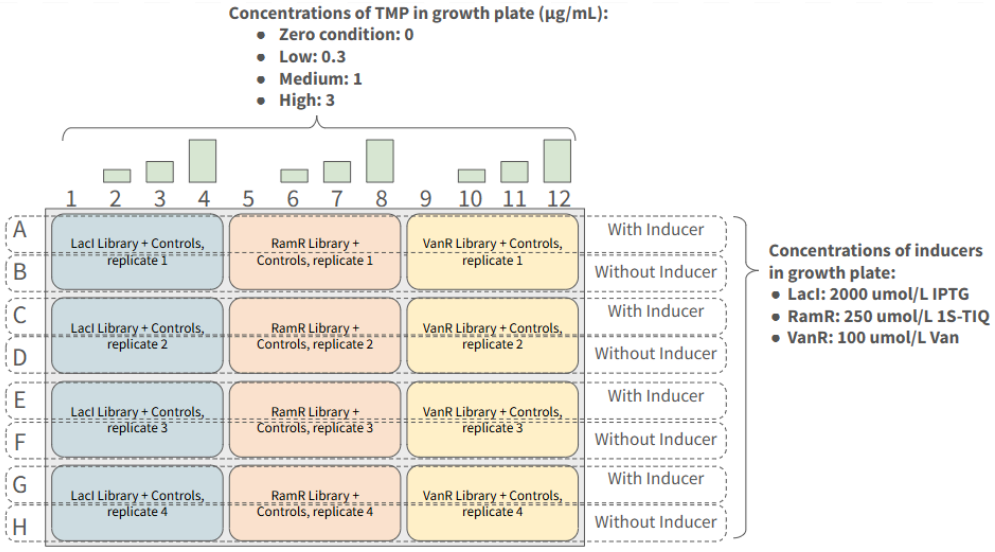
- Label the plate with the extracted DNA with the experiment identifier and "time point 2."
- Cover the third growth plate with a lid when when preparing for the DNA extraction protocol or if transferring to any other system.
- Store the resulting extracted DNA at 4C until ready to run the BarSeq library 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.

54 Incubate the fourth growth plate for **2 hours and 45 minutes** 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)

54.1 Measure OD600 and fluorescence every 5 minutes

- Start next steps during the last hour of the incubation

54.2 During incubation, move the fifth growth plate to the pipetting position, remove its lid, and prepare the fifth growth plate with 450 uL mixed media per well, where the mixed media now includes working media, inducers, and TMP following the plate map below. The cell culture solutions in the fourth growth plate already have inducer and TMP (in the appropriate wells). So, to set the correct final concentration, media, inducers, and TMP should be added to the wells of the fifth growth plate with the same volumes as for the fourth growth plate.
Note 3: Remove the lids from the media and reagent reservoirs only during the relevant pipetting steps, i.e., remove lid from Media reservoir, pipette Media, replace lid on Media reservoir, remove lid from Reagent reservoir, pipette TMP and inducers, replace lid on Reagent reservoir.



54.3 Approximately ten minutes before the end of the incubation, pre-warm the fifth growth plate.

Automation System: The fifth growth plate (timepoint 4)

55 After the incubation is done, remove gas-permeable seal from the fourth growth plate.

56 Remove the lid from the fifth growth plate.

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

- The preferred transfer method uses a 96-channel head on the automated liquid handler. If using a 96 channel head, use the previously recommended steps for cell transfer from the Automation System: The second growth plate (timepoint 1) section.

58 Replace the lid on the fifth growth plate to move it to the location used to apply the gas-permeable seal.
Replace the lid on the fourth growth plate to move it to the location used for the DNA extraction protocol.

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

59.1 Remove lid first.

59.2 Immediately after the fifth growth plate goes into the incubator, remove the lid from the fourth growth plate and 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.

- Label the plate with the extracted DNA with the experiment identifier and "time point 3."
- Cover the fourth growth plate with a lid when when preparing for the DNA extraction protocol or if transferring to any other system.
- Store the resulting extracted DNA at 4C 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 **DNA extraction protocol**). Otherwise, substitute “EB buffer” or “TE-4” for the final elution step to avoid possible DNA degradation from uncontrolled (low) pH.

60 Incubate the fifth growth plate for approximately **2 hours and 45 minutes**, 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)

60.1 Measure OD600 and fluorescence every 5 minutes.

61 After the incubation is done, remove gas-permeable seal from the fifth growth plate.

62 Replace the lid on the fifth growth plate to move it to the location used for the DNA extraction protocol.

63 Remove the lid from the fifth growth plate and 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.

- Label the plate with the extracted DNA with the experiment identifier and "time point 4."
- Cover the fifth growth plate with a lid when when preparing for the DNA extraction protocol or if transferring to any other system.
- Store the resulting extracted DNA at 4C 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 **DNA extraction protocol**). 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

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