

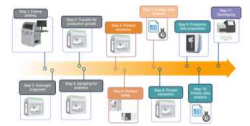


Apr 24, 2025

Version 2

A Modular High-Throughput Pipeline for Automated Microbial Strain Engineering: From Colony Picking to Phenotypic and Genotypic Analysis V.2

Forked from a private protocol



DOI

<https://dx.doi.org/10.17504/protocols.io.261gerqkdl47/v2>

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Joint BioEnergy Institute...



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We use this protocol and it's working

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Abstract

Microbial strain engineering has evolved to incorporate guide RNA, oligo pools, and random insertion libraries with millions of combinations to decipher mechanisms of bioproduct production in host organisms. However, effective high-throughput screening of individual colonies for both genotypic and phenotypic traits remains challenging.

We present an integrated high-throughput automation pipeline capable of handling over 5,000 samples daily for comprehensive strain engineering tasks. This system bridges the gap from initial colony selection through genotyping and phenotypic analysis using multiple analytical platforms.

Our modular workflow consists of several integrated processes:

1. **High-throughput colony picking** using automated colony picking systems
2. **Media preparation and distribution** with customizable volumes and plate formats
3. **Glycerol stock and production plate preparation** with flexible options for culture transfer, replication, and preservation
4. **Culture transfer for scaled production** allowing for additional stamping operations with customizable media compositions
5. **Sample preparation for genotyping and phenotyping** for specialized assays (proteomics, RapidFire) and NGS phenotyping with configurable transfer parameters

This pipeline offers significant flexibility, allowing researchers to employ individual modules independently or reconfigure workflows according to specific experimental requirements. The system's standardization enhances reproducibility while accelerating strain engineering cycles for synthetic biology and bioproduction applications.

Image Attribution

Created in Biorender Petzold, C. J. 2025.

Guidelines

This protocol is designed for processing high-throughput samples ($n > 1,000$) for applications such as glycerol stocks, phenotyping (e.g., Plate reader, RapidFire assays, and/or proteomics), and genotyping (e.g., Colony PCR, barcode sequencing). Potential applications include RB-Tn libraries, oligo pool libraries, Cas9 editing libraries, and more.



Materials

Instruments

Hamilton Vantage with Plate Sealer, Plate Pealer and Plate Reader (Hamilton Company, Reno, NV, United States)

Qpix 460 Colony Picker (Molecular Devices, CA, United States)

Eppendorf Centrifuge 5810R and (Eppendorf, CT, United States)

Agilent PlateLoc (Agilent, CA, United States)

Vortex-Genie 2 (Scientific Industries, Inc. NY, United States)

Reagents (dependent on experimental conditions)

Growth media (e.g., LB, M9)

Inducers (e.g., IPTG)

Selection Markers (e.g., Kanamycin)

Consumables

Tips (Hamilton Company, Reno, NV, United States)

96 deep-well Plates (Beckman Coulter, Brea, California, United States)

96-well round-bottom Nunc Plates (Thermo Fisher Scientific, Waltham, Massachusetts, United States)

Shallow or Deep Reservoirs (Thermo Fisher Scientific, Waltham, Massachusetts, United States)

Corning Coast Assay Plates, Black with Flat Bottom (Corning Inc. Maine, United States)

Safety warnings

- ! - Wear proper PPE (gloves, safety goggles, and lab coat), and prepare solvents in a chemical fume hood.
- Store organic solvents in a flammable storage cabinet when not in use.
- Discard used materials, solvents, and buffers in appropriate waste containers according to your institution's procedures.

Before start

Sample tracking: It is important to establish proper sample tracking before initiating any set of high-throughput experiments. We recommend barcoding according to standard LIMS guidelines to minimize sample tracking issues.

Plate sealer: If you want to seal your plates, configure and test the sealer parameters for optimal sealing. The ideal temperature and time settings vary depending on the specific plate material and seal type. It is recommended to consult the manufacturer's guidelines and conduct preliminary tests to determine the optimal settings. The goal is to achieve a seal that is easy to peel while maintaining a firm seal for each well. Monitor the sealing process to ensure the desired balance between peelability and seal integrity.

Grow Colonies on Agar plates

1d

1 Scale: 5-10 agar plates

30m

Before you start:

Sterilize your bench and equipment before handling the cultures.



Input materials: Glycerol stocks that contain mutant libraries and agar plates

Output: Agar plates with well-separated colonies

Steps:

1. Thaw your cultures on ice and perform serial dilution (1X, 10^{-2} X, 10^{-3} X, 10^{-4} X, 10^{-5} X) in 1.5 mL tubes.
2. Transfer 5 μ L of the culture dilutions to the Petri dish and spread the culture gently using glass beads. For Q-trays, use 20 μ L of the culture dilutions and spread the culture gently using glass beads.
3. Place the plates in a sterilized environment to let it dry if necessary.
4. Once dry, place the plates upside down at the optimal growth temperature to let the culture grow until the colonies reach a diameter of at least 1 mm.

Note

1. Do not over dry the plate. This requires careful monitoring of the process multiple times until the drying rate is well established. Utilization of a controlled drying environment is highly recommended.
2. Use the appropriate growth media for your strain when preparing culture dilutions.
3. Colony outgrowth time may vary from 12 hours to several days, depending on the strain.

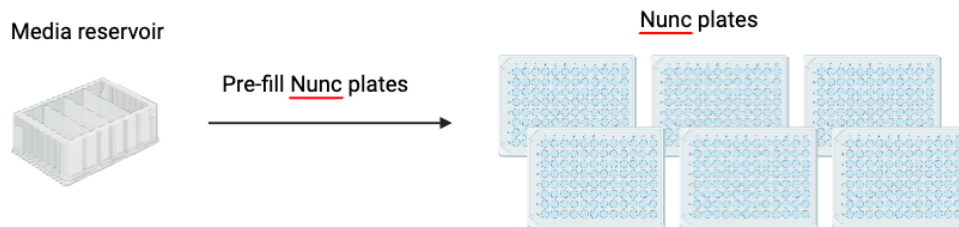
Pick Colonies and Grow Cultures in Nunc Plates

3h

2 Pre-fill Nunc plates with media

30m

Scale: 16 plates

**Before you start**

Perform a pilot test to determine culture outgrowth conditions (e.g., temperature, shaking speed, incubation time) in round-bottom Nunc plates.

Prepare Nunc plates on Hamilton Vantage

Source plates: Deep-well reservoir

Destination plates: 96-well Nunc Plates (round bottom)

Tip type: 1000 μ L Filtered Tips

Input materials: Nunc Plates and growth media

Output: Barcoded Nunc Plates with 150 μ L of growth media per well

Steps:

1. Place barcodes or labels on the Nunc Plates
2. Transfer 150 μ L of growth media per well from the reservoir to the 96-well Nunc plates using the same set of 300 μ L tips for all destination plates.
3. Examine the plates and briefly centrifuge those plates that contain bubbles.



4. Proceed with colony picking immediately to minimize evaporation and chances of contamination.

Note

1. If antibiotics or other methods of selection are needed, be sure to include the necessary components in the media.
2. If you are starting from a diverse genomic library of strains it is good practice to validate your library at this step to identify any potential bias in library composition.

3 Prepare positive controls

30m

Scale: 16 X 96-well Nunc plates

Before you start:

1. Grow 5 mL of positive control strains (or your base strains) until the culture reaches an OD₆₀₀ of approximately 0.6.
2. Mix well, then aliquot 150 uL of the culture into 96-well Nunc plates, ensuring at least three wells are used for each strain.

Prepare positive controls for each Nunc plate using the Hamilton Vantage

Source plates: A 96-well Nunc plate containing positive control strains in growth media, with at least 3 different replicates for each strain

Destination plates: 96-well Nunc Plates containing growth media

Tip type: 50 µL Filtered tips

Input materials: A 96-well Nunc plate containing positive control strains in growth media and 96-well Nunc plates containing growth media

Output: 96-well Nunc plates containing transferred positive control strains

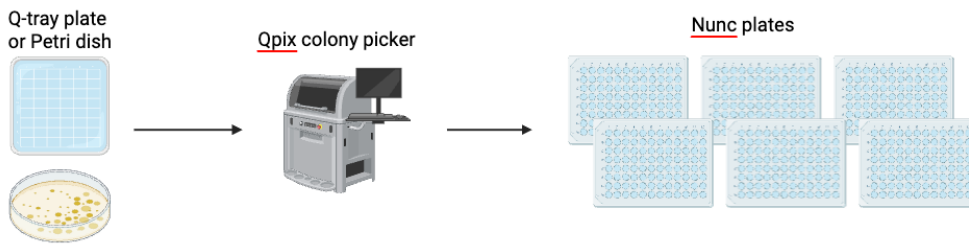
Steps:

1. Mix the plate containing positive controls by pipetting 50 µL of the culture mixture up and down at least 10 times using 50 µL tips.
2. Lift the tip and dip it into the corresponding well of a Nunc plate containing growth media.
3. Mix the growth media by pipetting 50 µL up and down 5 times.
4. Repeat steps 1–3 for each set of positive control and growth media plates until all Nunc plates are processed.

4 Pick colonies with Qpix automated colony picker

2h

Scale: 16 X 96-well Nunc plates containing up to 1,536 microbial strains



Before you start

Design plate maps on Qpix to reserve wells for positive controls (e.g., baseline strains) and negative controls (e.g., wells containing only growth media).

Pick colonies using Qpix

Source plates: Q-trays or Petri dishes

Destination plates: 96-well Nunc Plates

Input materials: Agar Petri dishes or Q-trays containing colonies; barcoded Nunc plates with 150 uL of growth media per well

Output: Nunc plates with a single colony in each well

Steps:

Set up Qpix

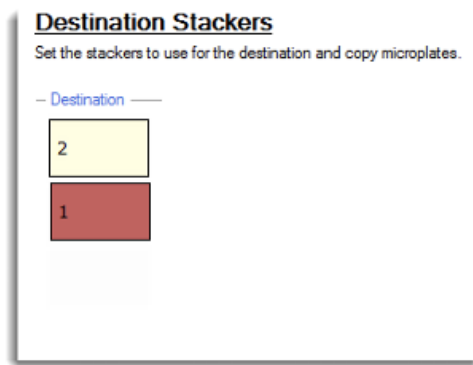
1. Fill each corresponding wash tray with 0.1% bleach, nuclease free water and 80% Ethanol.
2. Set the UV light on the QPix to run for 10 minutes using the QPix Colony Picking Software.
3. Check if the 96-picking pin head is installed. If not, proceed with the following step.
4. Open the Qpix control program. Go to "Instrument Utilities", click "Change head" to install picking head. Screw the knob tightly to hold the head in place.
5. Perform "Camera Alignment Process" to calibrate the imaging camera with the newly mounted picking head.

Pick colonies

1. Use the QPix Colony Picking Software. In the navigation window, double-click the "Picking" icon to begin.
2. Follow the instructions to set up your picking routine or select a saved file.



3. Select destination stackers by clicking the yellow rectangle on the screen and click Next.



4. Set up the head and sanitization conditions in the Head/Sanitise window. It is recommended to use at least 5 wash cycles, with 5 seconds of dry time and wait time each.



— Picking Head Options —

Picking Head: 96PinPicking ▾

Pin Columns Before Inoculation: All ▾

Wash Between Partial Inoculations: ☐

— Inoculation Heights (mm above well bottom) —

Destination: 1.45 ▴ ▾

Sanitise

Sanitise Profile: ecoliwash ▾

First and Main Wash Cycle

Wash Bath	Wash Cycles	Dry Time (s)	Wait Time (s)	
WashBath3	5	0	0	
WashBath2	5	0	0	
WashBath1	5	5	5	

5. Choose your picking source in the "Source" window and set the picking depth into the agar. *(The number of plates may vary depending on the number of pickable colonies and the total number of colonies you wish to pick.)*

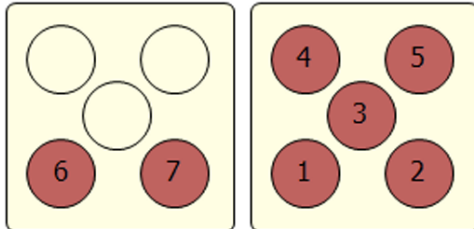
Source

Set the source receptacles and options to use.

Holder:

Receptacle:

Positions: Offset:



Picking Depth Into Agar (mm):

Limit Max. Number Of Features Per Position: ☐

Max. Number Of Features Per Position:

6. In the Destination Options, set the number of dips as needed. Then, under the Destination Microplate Template, click "Edit" to deselect the wells assigned to your negative controls and baseline strains. QPix will skip the wells that have been opted out during colony picking.



Destination Options

Set the destination microplate and options.

— Microplate Selection —

Select Destination Microplate: NUNC (268200) 96 Well ▾

☒ Multi Dip Number of Dips: 5 ▴ ▾

☐ Stir Destination

Make Copy: ☐

Select Copy Microplate: NUNC (268200) 96 Well ▾

— Deposit Order —

☒ By Columns

☐ By Rows

— Deposit Strategy —

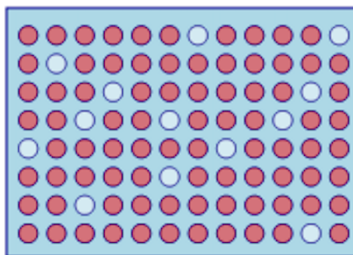
☒ Fill All Microplates

☐ New Microplate For Each Position

☐ New Microplate For Each Batch

☐ New Microplate Per Number of Positions 5 ▴ ▾

— Destination Microplate Template —



Edit

7. Review the setting summary after the Settings Summary window appears.
8. Load the Nunc culture plates with 150 μ L of LB containing suitable antibiotics per well into the stacker holder.
NOTE: Destination plates must be oriented with A1 in the front right corner; otherwise, the picking logs will misrecord the actual well mappings.
9. Place the stacker holder into position and turn knob to lock.
10. Place Q-trays or Petri dishes on the holder.
11. Run the program.
13. After the picking process is done, remove Nunc plates from the holder.

14. Seal the Nunc plates with air permeable seals and incubate the plates to let the culture grow to at least $OD_{600} \sim 0.6$.

Clean up Qpix

1. Take the wash trays 1-3 out of the picker and empty wash tray one and two into the sink, wash tray with 80 % ethanol must be poured into the SAA for flammable solvents.
2. Rinse all three wash trays with water and place back into the picker.
3. Set the UV decontamination to run for 10 minutes using the QPix Colony Picking Software.

Note

Key parameters to adjust on Qpix:

- A. Number of dips
- B. Inoculation height
- C. Picking depth into agar
- D. Number of source plates (This depends on the number of pickable colonies per plate and the target number of colonies for your experiment)
- E. Plate source (Petri dish or Q-tray)

Key parameters to adjust on culture outgrowth

- A. Incubation time
- B. Incubation temperature
- C. Shaker speed

It is recommended to grow cultures in an incubator with humidity control to minimize evaporation.

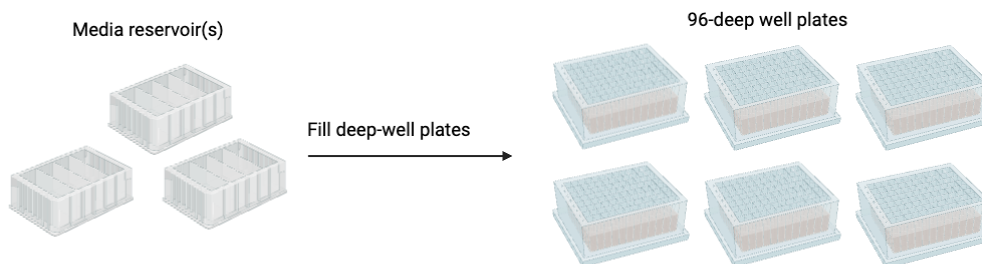
Prepare 96 Deep-Well Plates for Production Outgrowth

40m

5 Pre-fill deep-well plates with media

Scale: 64 X 96-deep well plates

40m



**Before you start**

If you want to seal the induction plate for short-term storage, configure the sealer parameters for optimal sealing.

Fill the 96 deep-well plates with production media on the Hamilton Vantage

Source plates: Reservoir

Destination plates: 96 deep-well plates (2 mL)

Tip types: 1000 µL Filtered tips

Input materials: induction media and 96 deep-well plates

Output: Induction plates containing growth media only

Steps:

1. Place barcodes or labels on the south side of the plate.
2. Transfer 800 µL of induction media per well from reservoir to deep-well plates with the same set of tips.
3. Optional: seal the plate for short-term storage

Note

1. Include antibiotics and other required inducers as needed for your experiment.
2. It is recommended to use 800–1000 µL of induction media in 96 deep-well plates. This volume helps minimize evaporation while avoiding splashing and cross-contamination during incubation.
3. Prepare extra plates and media to ensure flexibility during setup and to accommodate any last-minute changes or sample loss during the process.

Prepare Glycerol Stocks

1h 30m

6 Scale: 16X 96-well Nunc plates

1h 30m

Before you start

1. Examine the Nunc plates to ensure culture outgrowth; an OD₆₀₀ ≥ 0.6 is recommended.
2. Configure the sealer parameters for optimal sealing.

Reservoir with
60% Glycerol



Nunc plates



Prepare glycerol stock on the Hamilton Vantage

Source plates: A deep-well reservoir that contains 240 mL of 60 % glycerol

Destination plates: Nunc Plates with a single colony outgrowth in each well

Tip types: 300 μ L tips

Input materials: 60 % glycerol; Nunc Plates with culture outgrowth, foil seals for -80 °C storage

Output: Sealed Nunc Plates with glycerol stocks

Steps:

1. Perform a quick spin (<10 seconds) to remove any liquid from the seal, then carefully peel the Nunc plates to avoid cross-contamination (this step is optional for the 96-deep-well plates described in the previous step).
2. Using 300 μ L tips, transfer 50 μ L of 60% glycerol to each well of the Nunc plate containing 150 μ L of culture.
3. Using the same tips, mix 100 μ L of the glycerol-culture mixture by pipetting up and down 10 times.
4. Discard used tips and use a fresh set of tips for each new source plate.
5. Seal the plates with a foil seal—unless you are proceeding directly to induction plate preparation.
6. Store the plates at -80 °C or continue with induction plate preparation.

Note

Key parameters to adjust on the sealer:

- A. Seal temperature
- B. Seal time

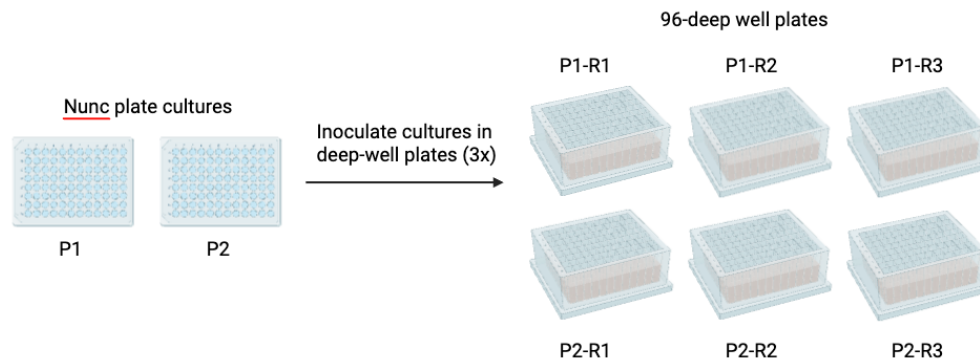
Transfer Cultures for Production Cultures

2h 30m

7 Scale: 64 X 96-deep well plates containing 4 replicates for each strain (6,144 samples)

2h 30m

Inoculate culture from Nunc plate to 96-deep well plates with replicates



Before you start

Configure the sealer parameters for optimal sealing.

Note

Key parameters to adjust on the sealer:

- A. Seal temperature
- B. Seal time

Prepare induction plates on the Hamilton Vantage

Source plates: 96-well Nunc Plates

Destination plates: 96 deep-well plates

Tip type: 50 μ L Filtered tips

Input materials: Glycerol stock plates (96-well Nunc Plates), induction plates containing growth media only

Output: Induction plates with single strain in each well

Steps:

1. Label the 96-well induction plates with barcodes or plate IDs.
2. Transfer 5 μ L of stock culture per well into the induction plates.
3. Repeat step 2 until all replicate plates are prepared.



4. Discard used tips and use a fresh set of tips for each new source plate.
5. Seal the induction plates with permeable seals.
6. Incubate the induction plates under appropriate conditions.
7. Store the stock culture plates at -80 °C for future use.

Note

1. It is recommended to keep the transfer volume between 5–10 uL to reduce the risk of contamination and to maintain better control over the growth phase for downstream experiments. Transferring a large amount of bacteria may cause some strains to reach stationary phase within a few hours.
2. For high-throughput work, at least three replicates are important to produce reliable phenotyping data.

Key parameters to adjust on induction:

It is good practice to perform a pilot test on a single plate to validate the following conditions:

- A. OD₆₀₀ of your stock culture
- B. Transfer volume on the robot
- C. Culture outgrowth time (some experiments collect samples during the log phase, while others require stationary phase cultures)

OD₆₀₀ Measurement or Fluorescent Detection

3h 30m

- 8 Scale: 64 X 96-well plates (6,144 samples)

3h 30m

Before you start

Set up parameters on Soft Max Pro for your project.

Note

1. It's important to determine the linear range of the measurement. Performing a pilot test before setting up high-throughput work is recommended.
2. Consider to include standards for a standard curve for better quantification in your run as needed.

Key parameters to consider for culture outgrowth:

1. Data collection timing
2. Optimal cell density or fluorescence intensity

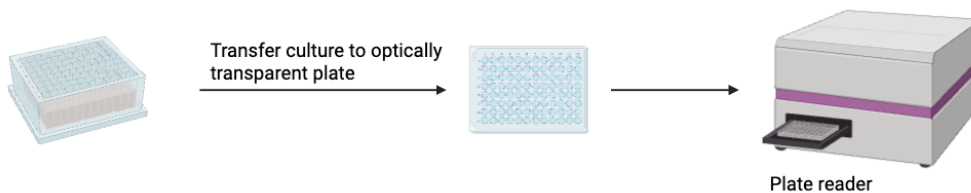
Key parameters to adjust on the plate reader via SoftMax Pro:

1. For OD₆₀₀ measurements :

- A. Read mode: ABS (absorbance)
- B. Read type: Endpoint
- C. Wave length: 600 nm
- D. Shaking before first read: enable and set for at least 10 seconds

2. For fluorescence detection:

- A. Read mode: FL (fluorescence)
- B. Read type: Endpoint
- C. Excitation and emission wavelengths: users' choice
- D. Shaking before first read: enable and set for at least 10 seconds



Perform automatic plate reading on the Hamilton Vantage

Source plates: 96-deep-well induction plates containing single-strain culture outgrowths

Destination plates: Clear-bottom plates compatible with plate readers

Tip types: 50 μ L and 300 μ L Filtered tips

Input materials: 96-deep-well induction plates containing single-strain culture outgrowths and clear-bottom plates compatible with plate readers

Output: OD₆₀₀ or fluorescent data

Option 1: Without Dilution



(Only 300 μ L tips are required)

1. Label the 96-well reading plates with barcodes or plate IDs.
2. Mix the induction plates by pipetting 100 μ L of the culture mixture up and down at least 10 times using 300 μ L tips.
3. Transfer 180 μ L of the culture mixture per well to the reading plates using the same tips.
4. Discard used tips and use a fresh set of tips for each new source plate.
5. Place the reading plate into the plate reader.
6. Measure OD₆₀₀ or fluorescence on plate reader.

Option 2: With Dilution

(May require both 50 μ L and 300 μ L tips depending on your dilution factor)

1. Label the 96-well reading plates with barcodes or plate IDs.
2. Add diluent per well to the reading plates according to your dilution factor using the same set of tips for all the destination plates.
3. Discard the used tips after diluents have been added to all plates.
4. Mix the induction plates by pipetting 100 μ L of the culture mixture up and down at least 10 times using 300 μ L tips.
5. Transfer the appropriate volume of culture mixture per well to the reading plates using the same set of tips.
6. Discard used tips and use a fresh set of tips for each new source culture plate.
7. Place the reading plate into the plate reader.
8. Measure OD₆₀₀ or fluorescence on plate reader.

Note

1. If you don't have a plate reader integrated into your automation system, consider preparing plates with your liquid handler and reading them on a standalone plate reader.
2. You may want to dilute your samples prior plate reading if the cell density or fluorescent is too high. In many cases, it's good to keep OD₆₀₀ between 0.1 -1.
3. It's more efficient if you can avoid diluting your samples. This can be achieved by performing a pilot test to identify the appropriate cell density or fluorescent intensity under certain growth conditions. However, in some cases—such as when collecting samples in stationary-phase cells is desired—the culture may exceed the optimal range for measurement and dilution will be necessary before plate reading.

Key parameters to adjust on your liquid handler:

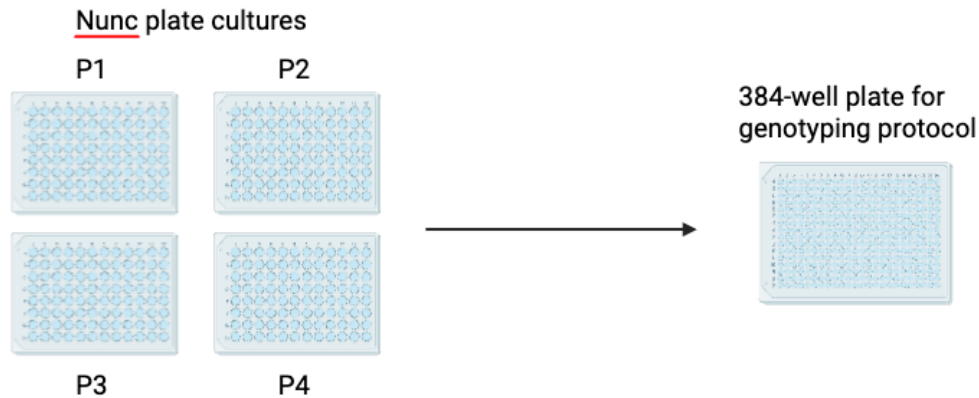
Dilution factor for your samples

Process Plates for Genotyping and Phenotyping

6h 30m

9 Scale: 16 X 96-well plates

Collect samples for genotyping



Source plates: 96 deep-well plates or 96-well Nunc plates

Destination plates: 384-well plates

Tip type: 50 μ L Filtered tips

Input materials: 96 deep-well plates or 96-well Nunc plates containing single-strain culture mix and 384-well plates

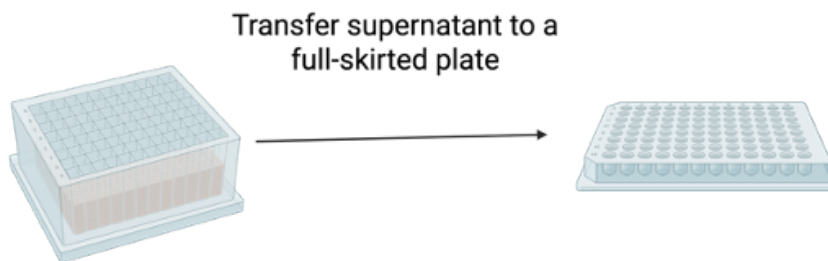
Output: 384-well plates containing single-strain cell mixtures

Steps:

1. Label the 384-well plates with barcodes or plate IDs.
2. Transfer 20 μ L of cell mixtures from each well of four 96-well plates and combine them into a single 384-well plate.
3. Discard used tips and use a fresh set of tips for each new source culture plate.
4. Repeat Step 2 and 3 until all plates are processed.
5. Seal the 384-well plates and store them at -20°C for preservation.

10 Collect samples for RapidFire-MS assays

Scale: 64X 96-well plates





Source plates: 96 deep-well plates

Destination plates: 96-well-full-skirted plates

Tip type: 300 µL Filtered tips

Input materials: 96-deep-well induction plates containing single-strain culture outgrowths and 96-well-full-skirted plates

Output: 96-well-full-skirted plates with supernatant that contain bioproducts

Steps:

1. Label the 96-well full-skirted plates with barcodes or plate IDs.
2. Centrifuge the 96-deep-well induction plates at $3,000 \times g$ for 5-30 minutes to pellet the cells (see Note below).
3. Transfer 10-100 µL of the supernatant from the 96-deep-well plate into 96-well full-skirted plate (see Note below).
4. Discard used tips and use a fresh set of tips for each new source plate.
5. Seal the full-skirted plates with pierceable foil seals.
6. Store the sealed plates at -20°C for future use.

Follow RapidFire-MS sample preparation protocol for the target molecule of interest, for example:

High-throughput quantitative analysis of isoprenyl acetate (IPA) by using an Agilent RapidFire-QqQ system

Note

You may want to consider adding an additional filtration step before performing the RapidFire assays to ensure removal of particulates that could interfere with MS analysis.

Key parameters

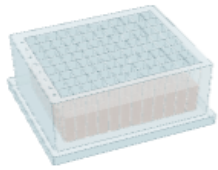
A. Centrifugation time: This is dependent on the organism and the growth conditions of your cultures.

B. Transfer volume: This is dependent on the amount of the target molecule in your cultures and the sensitivity of your HTS assay. Additional dilution steps may be required for target molecules that are present at high concentration in the culture (e.g., $>1.0 \text{ g/L}$).

11 Collect samples for proteomics on Vantage

2h 30m

Scale: 16X 96-well plates



Transfer cell pellets to an
non-skirted PCR plate



Source plates: 96 deep-well plates

Destination plates: 96-well unskirted plates

Tip types: 300 μ L and 1000 μ L Filtered tips

Input materials: 96-deep-well induction plates containing single-strain culture outgrowths and 96-well unskirted plates

Output: 96-well non-skirted plates with cell pellets

Steps:

1. Label the 96-well non-skirted plates with barcodes or plate IDs.
2. Centrifuge the 96-deep-well induction plates at 3,000 g for 5 minutes.
3. Use 1000 μ L tips to remove 600 μ L of the supernatant per well.
4. Mix the culture at least 10 times with the following parameters set up on the robot:
 - Mix volume: 200 μ L
 - Aspirate height: 0.2 mm
 - Dispense volume: 150 μ L
 - Dispense height: 1 mm
5. Discard used tips and use a fresh set of tips for each new source culture plate.
6. Centrifuge the non-skirted plates at 4000 rpm for 3 minutes.
7. Remove the supernatant from the non-skirted 96-well plate by inverting or aspirating.
8. Seal the 96-well non-skirted plates with foil seals.
9. Store the stock culture plates at -80°C for future use.

Note

It is recommended the culture reach OD_{600} at least 0.8. You may want to test a few samples to verify minimum OD_{600} and the volume of cell culture for proteomics. You will need enough quantity cell pellets in order to proceed downstream work.

Key parameters to adjust:

- A. Cell density (OD_{600})
- B. Transfer volumes from the 96 deep-well induction plates to 96-well unskirted plates

Follow proteomics sample preparation protocol:



Alkaline-SDS cell lysis of microbes with acetone protein precipitation for proteomic sample preparation in 96-well plate format