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Version 1

VECTOR^L V.1

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Protocol status: Working

We use this protocol and it's working

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Abstract

Our protocol lays out a high-throughput microbial engineering platform for the screening of microbial populations *en masse*. This approach employs a pooled conjugation approach, relying on fluorescent protein expression and growth in presence of antibiotic as measures of success. This protocol aims to provide a streamlined, scalable approach to engineering numerous strains simultaneously.

The expectation of this process is to produce a library of engineered strains, utilizing the variable plasmid constructs to provide each recipient strain with its desired makeup. The downstream implication of this protocol is to be able to identify the success of each plasmid by utilizing NGS techniques to separate reads by their unique barcode. This protocol can be adjusted to include a single plasmid variant or a single recipient strain.

Attachments



[VECTORL.png](#)

1.5MB

Guidelines

Troubleshooting Guide

- **Cultures Splashing on Cover of Plate** (Days 3-8)

To avoid splashing during the agitation of culture plates, gently shake the plates back and forth on a flat surface in a fluid motion. Do not stop and start abruptly. If the liquid cultures are too disturbed and are found on the lid of the plate, carefully remove the cover and replace it. Failure to replace the cover may result in contamination between wells.

- **No Bacterial Growth in Wells After Incubation of Recipient Cells** (Prep, Day 1)

If no bacterial growth is observed after incubation, this may be due to improper transfer during inoculation. Be sure that enough freezer stock is on loop/needle or liquid culture is being extracted in each pipette tip when inoculating.

- **No Bacterial Growth in Wells After Incubation of Donor Cells** (Day 1)

If no bacterial growth is observed after incubation, this is most likely due to the concentration of antibiotic being too high, the wrong antibiotic being added to the media, or an absence of the necessary selection supplement. Fix by restarting the process, making sure all additives are be due to improper transfer during inoculation. Be sure that enough freezer stock is on loop/needle or liquid culture is being extracted in each pipette tip when inoculating.

- **Parafilm Degradation** (Prep-Day 6)

Parafilm on the edges of plates are likely to tear and fall apart as they incubate. It is important to replace these wraps as exposure to air can lead to evaporation of bacterial cultures.

- **Echo 525 Exceptions Report Showing Errors** (Prep, Day 2)

Should there be exceptions during the acoustic handling process, indicating that the proper amount of liquid was transferred to the destination plate, the cause is often that the volume in the source plate well was too high or that the well ran out of sufficient stock. To remedy this, add or remove the desired liquid to the source plate well. The Echo source plates may have an issue transferring any liquid should the volume in the well fall below 12 μL or above 48 μL . Another issue may be the presence of bubbles inside the source plate wells. Use sterile 20 μL pipette tips to manually move or "pop" bubbles.



Materials

Biologic materials

- Donor *E. coli* (Frozen stock)
- Recipient cells (Frozen stock)

Reagents

- Select yeast extract (Sigma-Aldrich, product no. Y0875)
- Tryptone (Sigma-Aldrich, product no. T2559)
- Luria Bertani broth (LB) (Sigma-Aldrich, product no. L3022)
- Gentamicin sulfate salt (Sigma-Aldrich, product no. G1264)
- Neomycin trisulfate salt hydrate (Sigma-Aldrich, product no. N6386)
- Tetracycline hydrochloride (Sigma-Aldrich, product no. T7660)
- Magnesium chloride hexahydrate ($\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, Sigma-Aldrich, cat. no. M2670-100G)
- Sodium chloride (NaCl , VWR cat. no. X190-1kg)
- Glycerol (Thermo Scientific, cat. no. A16205.0F)
- Agar (Thermo Scientific Agar powder cat. no. AAA107520E)
- Ethanol (Fisher cat. no. BP2818-500)
- Nuclease-free water (Qiagen, cat. no. 129114)

Equipment

- White reservoirs (Southern Labware cat. no. P8050-5)
- Reagent reservoir PP (Sigma-Aldrich, product no. BR703409)
- Standard microcentrifuge tubes (1.5 and 5 ml; Eppendorf, cat. no. 0030125.150, 30119401)
- 96 U-bottom deep well plate (VWR, cat. no. 7323803)
- 96-well cell culture plate (Amazon, NEST, cat. no. 701001)
- 96-well PCR plate (Hard-Shell, BIORAD, cat. no. HSS9601)
- 96-well EIA/PIA plate (Costar, cat. no. 3590)
- Echo Mass Spectrometry (MS) Qualified 384-well Polypropylene (PP) Microplate (Beckman Coulter, product no. C74290)
- 2.0-ml cryogenic vial (NEST, cat. no. 607001)
- Multi-channel pipettes (Rainin cat. no. 17013803, 17013805, 17014496)
- Single channel pipettes (Rainin cat. no. 17014393, 17014388, 17014384 and 17014382)
- Pipette tips (nuclease-free, sterile filter tips; 20, 200 and 1,000 μl ; Rainin cat. no. 17014961, 30389239, and 30389213)
- Dry bath metal beads (Thermo Fisher, cat. no. A1254301)
- Dry bath (Sigma-Aldrich, product no. Z742509)
- Inoculating loops and needles, (10 μl capacity, VWR cat. no. 612-9358)
- Breathe-Easy sealing membrane (Sigma-Aldrich, product no. Z380059)
- Parafilm (VWR, cat. no. 291-1213))
- Plate seals (VWR cat. no. 732-3212)
- Adhesive foil seals (VWR, cat. no. 60941-074)
- Class II biological safety cabinet (Nu-Aire model NU425-400 series 30)

- Vortex mixer (VWR cat. no. 10027-194)
- Electronic balance (Mettler Toledo, cat. no. AL104)
- Incubator-shaker (Eppendorf, cat. no. M1299-0090)
- -80 °C Upright Ultra-Low Temperature Freezers (Thermo Fisher Scientific, model no. 907)
- -20 °C freezer (VWR Lab HC Manual Def -20 CF, cat. no. 75836-238)
- Echo 525 Acoustic Handler (Beckman Coulter, product no. 001-10080)
- BioTek Cytation 5 Cell Imaging Multimode Reader (Agilent)

Computer requirements

- Operating system requirements: The bioinformatic pipeline was developed and tested on a 64-bit RedHat Enterprise Linux version 8+ system, but any operating system capable of running a modern version of the R language – Linux, MacOS, or Windows – should work.
- Hardware requirements: 16+ GB of RAM is recommended for most datasets. RAM requirements increase with number of plates per analysis. If analyzing only a few plates, 4-8 GB of RAM may be sufficient.

Software

- Echo Plate Reformat Software (Beckman Coulter)
- Echo Cherry Pick Software (Beckman Coulter)

Safety warnings



1. Risk of Contamination

- Cross-contamination between wells and plates can affect the accuracy of microbial isolation and plate readings.
- Bacterial cultures may splash onto plate covering.
- Work in a laminar flow hood when handling cultures.
- Always use sterile pipette tips, nuclease-free water, and reagents to minimize contamination risks.

2. Handling of Microbial Cultures

- Some environmental bacteria may pose biosafety risks, especially opportunistic pathogens.
- Always wear appropriate personal protective equipment (PPE) (lab coat, gloves, eye protection) when handling cultures.
- Dispose of biohazardous waste properly according to institutional guidelines.

3. Storage and Freezing Precautions

- Bacterial glycerol stocks at -80°C must be handled carefully to prevent repeated freeze-thaw cycles, which can reduce viability.
- Ensure proper mixing of glycerol and bacterial culture before freezing to maintain even distribution.

Start Recipient Cultures (Prep)

1w

- 1 Inoculate TY Broth:
 - Add 150 μ L of TY broth to each well of a 96-well flat-bottom plate.
 - Using a loop, inoculate one of the wells directly from recipient freezer stock. Repeat for each recipient strain.
 - Include replicates of inoculations to ensure successful growth.
- 2 Plate Layout:
 - Use a pattern that facilitates easy transfer to a 384-well source plate.
 - Include replicates to ensure successful growth.
- 3 Control Wells:
 - Add at least one blank well containing uninoculated TY broth to act as a control.
- 4 Storage:
 - Place the lid on the plate and seal the edges with parafilm.
 - Store the plate in a drawer at room temperature.
- 5 Check Growth:
 - Monitor the plate daily for successful growth (3-7 days).

Prepare Conjugation Mating Spot Plates (Prep)

- 6 Prepare TY Agar:
 - Make TY agar and autoclave as required.
 - Allow agar to cool slightly before proceeding.
 - Add the appropriate volumes of 0.5M CaCl_2 and other nutrients to the TY agar.
- 7 Heat Bead Bath:
 - Set the bead bath to "high".
 - Allow time for the bead bath to heat up, adjusting as needed.
- 8 Dispense Agar into Wells:
 - Place the reservoir containing the media into the beads to keep the agar warm.
 - Place the 96-well flat-bottom plate on the heating block, moving it occasionally to prevent plastic melting.
 - Add 100 μ L of TY + ALA agar to each desired well in the plate.
 - While still on the heating block, use pipette tips to carefully remove any bubbles from the agar.
 - Remove the plate from the heating block to allow agar to cool and solidify inside the hood.



Develop Echo Protocol (Prep)

- 9 Create Mating Spots Protocol:
 - Use Echo plate reformat software to develop a protocol for creating mating spots with donor and recipient cell cultures.
- 10 Test Protocol:
 - Run a test using water to verify the protocol is successful.
- 11 Save and Export:
 - Save the transfer report and convert it to a .xlsx file.
 - In the .xlsx file, add commands to calculate the required transfer volume from each source well based on cell counts (to be measured later).
 - Include calculations to ensure the final volume in each mating spot is 5 μ L.

Start Overnight Cultures (Day 1)

1d

- 12 Recipient Cultures:
 - Add 15 μ L of each recipient culture to 135 μ L of TY broth in a new 96-well flat-bottom plate.
 - Follow the same inoculation pattern as before.
 - Cover the plate with Breath-Easy film.
 - Incubate overnight at 28°C in a shaking incubator at 225 rpm.
- 13 Donor Cultures:
 - Inoculate a 96-well flat-bottom plate with *E. coli* donor cells, directly from freezer stock, in a similar pattern to the recipient cells.
 - Each well should contain:
 - LB broth + required nutrients and antibiotic corresponding to the resistance module in the donor cells.
 - Cover the plate with Breath-Easy film.
 - Incubate overnight at 37°C in a shaking incubator at 225 rpm.

Echo Setup and Conjugation (Day 2)

1d

- 14 Prepare for Measurement:
 - Replace the Breath-Easy film with clear plastic film.
 - Measure OD600 and RFU using a Cytation5 instrument.
- 15 Verify Cell Growth:
 - Remove the plates from the incubators and confirm the cultures are normalized in density.
 - If needed, return the plates to the incubators and recheck later that day.
- 16 Input Cell Counts:



- Use the previously saved Excel protocol file to input OD600-derived cell counts for donor and recipient cultures.
- Assume OD600 = 1.0 $\approx$ 1 million cells/mL.

17 Adjust Volume:

- Calculate the required culture dilutions to ensure that total volume in each mating spot is $\leq 5 \mu\text{L}$.
- Prepare donor and recipient cultures accordingly.

18 Prepare Source Plate:

- Ensure volumes in each source well are between 12 μL and 48 μL .

19 Convert File:

- Convert the updated .xlsx file to .csv.

20 Run Cherry-Pick Protocol:

- Add the required culture volumes and media to the source plate.
- Load the cherry-pick program on the Echo-525 instrument.
- Select the appropriate source and destination plates and input the .csv file.

21 Handle Exceptions:

- Review the exceptions report.
- Rerun the protocol with the exceptions report or adjust volumes manually as needed.

22 Conjugation Incubation:

- Allow the conjugation plate to dry inside sterile hood.
- Seal the edges with parafilm.
- Place the plate upside down and incubate overnight at 28°C.

Prepare Selection Plates (Day 3)

1d

23 Add Media:

- Add 142.5 μL of TY broth + antibiotic to each well of a new 96-well plate, mirroring the layout of the conjugation plate.
- Add 150 μL of media to a blank well (uninoculated) as a control.

24 Resuspend Mating Spots:

- Wash the bottom of each well containing mating spots with 150 μL TY broth.
- Be careful not to scrape the agar with the pipette tip.
- Transfer the resuspended cells to a new 96-well flat-bottom plate.

25 Transfer to Selection Plates:

- Add 7.5 μL of each resuspension to the corresponding well in the selection plates.

26 Measure Cultures:

- Seal the plates with parafilm.



- Measure OD600 and RFU (485/20nm excitation, 528/20nm emission) using a Cytation5.

27 Store Plates:

- Place plates in a dark drawer for incubation.

Daily Monitoring (Day 4-7)

4d

28 Measure Cultures Daily:

- Shake plates gently to resuspend cells before reading. Avoid splashing cultures onto the plate covers.
- Measure OD600 and RFU (485/20nm excitation, 528/20nm emission) daily.

29 Storage:

- Replace parafilm as needed.
- Return plates to the dark drawer after measurements.

Final Measurements and Sample Storage (Day 8)

1d

30 Measure OD600 and RFU:

- Shake plates and record final readings.

31 Prepare PCR Samples:

- For each well containing transconjugants, remove 10 μ L and transfer to a PCR plate.
- Seal the PCR plate and store at -20°C.

32 Prepare Glycerol Stocks:

- Add an equal volume (e.g., 140 μ L) of 80% glycerol to each remaining culture in the selection plates.
- Mix thoroughly to ensure proper distribution.
- Seal the glycerol stock plates with adhesive foil strips.
- Store at -80°C.