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Project Page Link: <https://review.jove.com/account/file-uploader?src=21794738>

Title: High-Throughput Screening of L-Lactic Acid-Hyperproducing *Bacillus coagulans* Mutants Using an Automated Droplet Microfluidic Platform

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Author Questionnaire

1. **Microscopy:** Does your protocol require the use of a dissecting or stereomicroscope for performing a complex dissection, microinjection technique, or something similar? **No**
2. **Software:** Does the part of your protocol being filmed include step-by-step descriptions of software usage? **Yes, all done!**
3. **Filming location:** Will the filming need to take place in multiple locations? **No**
4. **Testimonials (optional):** Would you be open to filming two short testimonial statements **live during your JoVE shoot**? These will **not appear in your JoVE video** but may be used in JoVE's promotional materials. **No**

Current Protocol Length

Number of Steps: 23

Number of Shots: 62 (26 SC)

Note: No response from authors on postshoot email or reminder. The newly added SCREEN shots were provided by the author on the day of filming and added as per the author's instructions.

Interviews

Videographer: Obtain headshots for all authors available at the filming location.

INTRODUCTION:

~~REQUIRED: Summarize your research focus and the main questions it aims to answer.~~

- 1.1. **Xin Da:** Our research focuses on developing a droplet microfluidic platform-based workflow for the rapid screening of high-yield microbial mutants.

1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

~~Describe the research contexts or systems in which this protocol can be applied.~~

- 1.2. **Xin Da:** This protocol is for industrial microbial breeding, especially for culturable bacteria that secrete small-molecule metabolites or enzymes and can be adapted for strict anaerobes.

1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

CONCLUSION:

~~What does this protocol allow researchers to study or measure?~~

- 1.3. **Xin Da:** This protocol measures secreted products and enriches the best producer strains from large mutant libraries via droplet sorting.

1.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

~~Describe the most important consideration or challenge when performing this protocol.~~

- 1.4. **Xin Da:** The key challenges include choosing a biosensor with high specificity for the target metabolite and optimizing culture conditions for both droplet stability and strong signals.

1.4.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

~~Are there any additional methods or analyses that can be performed following this procedure?~~

- 1.5. **Xin Da:** After sorting, microplate primary screening, shake-flask validation, HPLC-based L-lactic acid quantification, and evaluation of genetic stability across four generations can be performed.

- 1.5.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

Videographer: Obtain headshots for all authors available at the filming location.

Protocol

***Videographer:** Please always record an audio slate while filming each shot mentioned in the shotlist.*

2. High-Efficiency Single Cell Encapsulation of Mutant *Bacillus coagulans*

Demonstrator: Xin Da

- 2.1. To begin, pipette 300 microliters of fluorinated oil into the oil phase inlet vial [1] and 500 microliters of the bacterial suspension into the aqueous phase inlet vial prior to setting up in the microfluidic platform [2].
 - 2.1.1. WIDE: Talent pipetting fluorinated oil into the oil phase inlet vial. ***Videographer:** Please obtain CU shot as well for this shot.*
 - 2.1.2. Talent pipetting the bacterial suspension into the aqueous phase inlet vial.
- 2.2. After peeling off the tape on the droplet generation chip surface, connect the oil vial, sample vial, and collection vial to the chip's corresponding wells in sequence [1].
 - 2.2.1. Talent connecting the vials to the corresponding chip wells.
- 2.3. After securing the chip on the microfluidic instrument stage, connect the air tubes to the inlet vials one by one according to the gas path labels [1]. Open the automated Droplet Entrapping Microfluidic Cell-sorter, or the **DREM (Drem) Cell** software [2]. Adjust the stage movement knob according to the arrow directions on the chip [3] to move the field of view to the droplet generation site [4].
 - 2.3.1. Talent connecting the air tubes to the inlet vials.
 - 2.3.2. SCREEN: 71458_screening_1.mp4. 00:01–00:05; 00:14–00:16
 - 2.3.3. SCREEN: 71458_screening_16.mp4. 01:20–01:25 ***Videographer:** Please capture the arrow directions on the chip being displayed on the screen as backup.*
 - 2.3.4. Talent adjusting the stage movement knob.
- 2.4. Click on **Imaging** in the interface of the **DREM Cell** software to open the **Imaging** window [1]. Next, open the **Camera MV** window with a grid calibration panel to visualize the focal plane [2]. In the imaging window, drag the **Brightness Control** and **Z-axis** sliders to adjust image brightness and focus [3]. To initiate droplet generation, click **Start** under the **Generation** tab, then select **Yes** in the notification window [4]. Click the **plus button** next to the **Speed** and **Size controls** to increase both the generation speed and droplet size [5].
 - 2.4.1. SCREEN: 71458_screening_2.mp4.00:00–00:04
 - 2.4.2. SCREEN: 71458_screening_2.mp4.00:05–00:08
 - 2.4.3. SCREEN: 71458_screening_2.mp4.00:09–00:12; 00:15–00:21

- 2.4.4. SCREEN: 71458_screening_3.mp4. 00:00–00:04
- 2.4.5. SCREEN: 71458_screening_3.mp4. 00:06–00:16
- 2.5. Once the droplet generation state remains consistent [1], quickly replace the collection vial for harvesting the water-in-oil samples [2]. To stop the droplet collection after 30 minutes, click **Stop**, then select **Yes** in the pop-up window [3].
 - 2.5.1. SCREEN: 71458_screening_14.mp4. 00:00–00:06
 - 2.5.2. Talent replacing the collection vial.
 - 2.5.3. SCREEN: 71458_screening_3.mp4. 00:28–00:36
- 2.6. Gently remove the collection tube from the chip [1]. Place the tube and collection vial vertically and wait for the droplets in the tube to enter the collection vial [2].
 - 2.6.1. Talent removing the collection tube from the chip.
 - 2.6.2. Talent placing the tube and collection vial vertically.

3. *B. coagulans* Micro Cultivation and Lactic Acid Sensor Pico-Injection

- 3.1. Add 1 milliliter of mineral oil to the collection tube [1]. Place the loosely capped collection tube upright inside a 15-milliliter centrifuge tube containing sterile water and incubate at 45 °C for 10 hours. [2].
 - 3.1.1. Talent adding mineral oil to the collection tube.
 - 3.1.2. Talent placing the collection tube upright in the 15-milliliter centrifuge tube.
- 3.2. For droplet pico-injection, pipette 300 microliters of fluorinated oil into the oil phase inlet vial in the laminar flow hood [1]. Replace the inlet vial cap on the collection tube with a new one [2]. Trim the new inlet vial tip to reach below the liquid level of the droplets in the sample vial [3]. Tighten the cap to prevent leakage [4].
 - 3.2.1. Talent pipetting fluorinated oil into the oil phase inlet vial in the laminar flow hood.
 - 3.2.2. Talent replacing the inlet vial cap with a new cap.
 - 3.2.3. Talent trimming the tip of the new inlet vial.
 - 3.2.4. Talent tightening the cap.
- 3.3. Pipette 500 microliters of the lactic acid sensor into the reagent inlet vial [1], then add 500 microliters of sterile phosphate-buffered saline buffer [2].
 - 3.3.1. Talent pipetting the lactic acid sensor into the reagent inlet vial.
 - 3.3.2. Talent adding sterile PBS buffer to the reagent inlet vial.

- 3.4. Use a blade to cut through the tape covering the electrode wells to expose the electrodes [1]. Set the multimeter to continuity mode and test the circuit connection and the chip's short-circuit status [2].
 - 3.4.1. Talent cutting through the tape covering the electrode wells.
 - 3.4.2. Talent testing the circuit connection with the multimeter.
- 3.5. After peeling off the tape on the chip surface, connect the oil vial, sample vial, reagent vial, and collection vial to the corresponding wells of the chip in sequence [1]. Secure and set up the chip on the stage as demonstrated previously [2].
 - 3.5.1. Talent connecting the vials to the corresponding chip wells.
 - 3.5.2. Talent placing and securing the chip on the stage.
- 3.6. After opening the software, adjust the stage movement knob [1] according to the arrow directions on the chip to move the field of view to the electrode area of the droplet chip [2]. Under the **Picoinjection** tab, click **Start**, select **Yes** in the pop-up window, then click **Start Picoinjection** [3]. Click on **Imaging** to access and adjust the droplet **Speed** and spacing controls [4].
 - 3.6.1. Talent adjusting the stage movement knob. *Videographer: Please also capture the arrow directions on the chip being displayed on screen.*
Added shot: SCREEN: 71458_screening_17.mp4. 00:43–00:48
 - 3.6.2. SCREEN: 71458_screening_4.mp4. 00:00–00:10
 - 3.6.3. SCREEN: 71458_screening_4.mp4. 00:11–00:20
- 3.7. Wait for droplets to enter the field of view after initial air discharge [1]. Adjust the droplet injection speed, spacing between individual droplets, and injection volume by clicking the **plus** and **minus** buttons of **Speed**, **Distance**, and **Reagent Volume** controls [2].
 - 3.7.1. SCREEN: 71458_screening_15.mp4. 00:00–00:06
 - 3.7.2. SCREEN: 71458_screening_5.mp4. 00:00–00:06; 00:11–00:17; 00:26–00:30
- 3.8. Double-click the MindVision software icon to open the camera panel for observing the stable droplet injection into the sensor [1]. After stabilization, quickly replace the collection vial to start droplet collection [2]. After 2 hours, click **Stop** to end the droplet pico-injection [3]. Gently remove the collection tube from the chip [4]. Place the tube and collection vial vertically and wait for the droplets in the tube to enter the collection vial [5].
 - 3.8.1. SCREEN: 71458_screening_6.mp4. 00:00–00:07
 - 3.8.2. Talent replacing the collection vial.
 - 3.8.3. SCREEN: 71458_screening_5.mp4. 01:01–01:11

- 3.8.4. Talent removing the collection tube from the chip.
- 3.8.5. Talent placing the tube and collection vial vertically.

4. Fluorescence-Activated Droplet Sorting of Lactic Acid-Hyperproducing Mutants

Demonstrator: Xin Da

- 4.1. In the laminar flow hood, pipette 300 microliters of fluorinated oil into the oil-phase inlet vial [1]. Replace the inlet vial cap of the collection tube containing microinjected droplets with a new cap [2].
 - 4.1.1. Talent pipetting fluorinated oil into the oil phase inlet vial.
 - 4.1.2. Talent replacing the inlet vial cap of the collection tube.
- 4.2. After exposing the electrodes and confirming normal circuit connection with the multimeter, peel off the tape on the chip surface [1]. Connect the oil vial, sample vial, and sorting collection vial to the corresponding wells of the chip in sequence [2].
 - 4.2.1. Talent peeling tape from the chip surface.
 - 4.2.2. Talent connecting the vials to the corresponding chip wells.
- 4.3. After securing the chip and connecting the air tubes to the inlet vials, open both **MindVision** and **DREM Cell Imaging** softwares [1]. Adjust the stage movement knob [2] according to the arrow directions on the chip to move the field of view to the electrode area of the droplet chip [3]. Under the **Sorting** tab, click **Start**, select Yes in the pop-up window, then click **Start Sorting** [4].
 - 4.3.1. SCREEN: 71458_screening_7.mp4. 00:04–00:16
 - 4.3.2. Talent adjusting the stage movement knob. *Videographer: Please also capture the arrow directions on the chip being displayed on screen.*
Added shot: SCREEN:71458_screening_18.mp4. 00:32–00:38
 - 4.3.3. SCREEN: 71458_screening_8.mp4. 00:00–00:09
- 4.4. After completion of initial air discharge and setting the detection channel to 488 nanometers, toggle **On** to select the laser [1]. Click **Record**, then, under **Real-time Signal Graph**, select the **dot plot and histogram** icons to visualize the acquisition of droplet fluorescence signals [2]. To set the sorting threshold, click the rectangle tool in the dot plot window to select the top 0.1 percent of the total fluorescence signals, then set the deflection force to medium, and toggle the sorting switch to **On** [3]. Collect droplets with fluorescence signal values above this threshold [4].
 - 4.4.1. SCREEN: 71458_screening_8.mp4. 00:10–00:16
 - 4.4.2. SCREEN: 71458_screening_8.mp4. 00:17–00:26
 - 4.4.3. SCREEN: 71458_screening_8.mp4. 00:55–01:10

- 4.4.4. SCREEN: 71458_screening_9.mp4. 00:28–00:35. *Video Editor: Please emphasize the text “Sorted” and the dynamic values beside it in the given timestamp.*
- 4.5. Click **Data Recording** again to re-record the fluorescence signals from the remaining droplets and sort the target droplets based on the set threshold [1]. After selecting the target droplet region, turn on the **Sorting Switch** and set the **deflection force** value [2]. Allow the equipment to start sorting droplets [3].
- 4.5.1. SCREEN: 71458_screening_9.mp4. 00:12–00:19
- 4.5.2. SCREEN: 71458_screening_9.mp4. 00:19–00:25
- 4.5.3. SCREEN: 71458_screening_10.mp4. 00:13–00:19
- 4.6. After the equipment stabilizes for 5 minutes, replace the collection vial to collect positive droplets [1]. Click **Stop** under the **Sorting** tab, then click **Yes** in the pop-up window to stop droplet sorting [2]. Gently remove the collection tube from the chip [3]. Place the tube and collection vial vertically to ensure that the droplets in the tube enter the collection vial [4].
- 4.6.1. Talent replacing the collection vial.
- 4.6.2. SCREEN: 71458_screening_11.mp4. 00:00–00:08
- 4.6.3. Talent removing the collection tube from the chip.
- 4.6.4. Talent placing the tube and collection vial vertically.
- 4.7. After droplet collection is completed, export the scatter plot data corresponding to the fluorescence signal values of all droplets and the sorted positive droplets to a spreadsheet for further analysis [1].
- 4.7.1. SCREEN: 71458_screening_12.mp4. 00:01–00:06 and
71458_screening_13.mp4, 00:00–00:06
- 4.8. Remove the chip, oil phase inlet vial, sample vial, and reagent vial from the stage [1]. Click on the **Close** icon, then select Yes in the pop-up window to close the software [2].
- 4.8.1. Talent removing the chip and vials from the stage.
- 4.8.2. SCREEN: 71458_screening_11.mp4. 00:13–00:26
- 4.9. Add 500 microliters of demulsifier to the droplet collection tube [1-TXT]. After 5 minutes, add 500 microliters of fresh screening medium for recovery, then perform culture validation of high-yielding strains [2].
- 4.9.1. Talent adding demulsifier to the droplet collection tube. **TXT: Demulsifier: 1H, 1H, 2H, 2H-perfluorooctanol**
- 4.9.2. Talent adding fresh screening medium to the droplet collection tube.

Results

5. Results

- 5.1. *In vitro* characterization of the lactic acid sensor showed that the fluorescence intensity increased with increasing concentrations from 100 micromolar to 10 millimolar [1].
 - 5.1.1. LAB MEDIA: Figure 5A. *Video editor: Please emphasize the rising curve at X-axis value 100 μ M to 10000 μ M, while simultaneously highlighting the highest bar in the inset image labelled L-lactate.*
- 5.2. The sensor's recovery rate measurement to validate the lactic acid detection showed a rate of approximately 109 percent in screening medium [1] and 98 percent in aqueous solution [2], with relative standard deviations remaining below 5 percent [3].
 - 5.2.1. LAB MEDIA: Figure 5C. *Video editor: Highlight the bar labelled 'Medium' along with the value 108.81 mentioned in the table above.*
 - 5.2.2. LAB MEDIA: Figure 5C. *Video editor: Highlight the bar labelled 'H2O' along with the value 98.39 mentioned in the table above.*
 - 5.2.3. LAB MEDIA: Figure 5C. *Video editor: Highlight the column STD% in the table along with the values under it.*
- 5.3. The sensor exhibited excellent linearity in the range of 0 to 0.6 grams per litre after 5 minutes of mixing 1 in 1 ratio with L-lactic acid standards in microplate assays [1]. During droplet-based screening validation on the microfluidic platform, the sensor exhibited a significant rightward shift in fluorescence signal with increasing L-lactic acid concentration [2].
 - 5.3.1. LAB MEDIA: Figure 5D. *Video editor: Highlight the red-color trend line and the legend corresponding to red dot labelled '5 min' simultaneously.*
 - 5.3.2. LAB MEDIA: Figure 5E. *Video editor: Except the control histogram on the extreme left and its corresponding legend, highlight each histogram peak with its corresponding legend one-by-one simultaneously.*
- 5.4. Dynamic monitoring of the growth status of the bacterial strains in pico-injected droplets showed that they entered the logarithmic phase at 6 hours of culture [1], and bacterial biomass in the droplets increased significantly at 10 hours [2].
 - 5.4.1. LAB MEDIA: Figure 6A. *Video editor: Highlight the droplet image labelled '6 h'.*
 - 5.4.2. LAB MEDIA: Figure 6A. *Video editor: Highlight the droplet image labelled '10 h'.*
- 5.5. Equal volume injection of sensor into droplets cultured for different durations showed the significantly enhanced fluorescence signal of the droplets after 10 hours of culture [1], indicating 10 hours as the optimal culture time for strains in droplets [2].

- 5.5.1. LAB MEDIA: Figure 6B. *Video editor: Highlight the red dotted circle in the fluorescence panel corresponding to '10 h' image.*
- 5.5.2. LAB MEDIA: Figure 6B. *Video editor: Highlight the red dotted circle in the bright field panel corresponding to '10 h' image.*

Pronunciation Guide:

1. **Bacillus coagulans**
Pronunciation link: <https://www.merriam-webster.com/dictionary/Bacillus>
IPA: /bəˈsɪləs/; *coagulans*: /koʊˈæɡjələns/
Phonetic Spelling: buh·SIL·uhs; koh·AG·yuh·luhnz
2. **Encapsulation**
Pronunciation link: <https://www.merriam-webster.com/dictionary/encapsulation>
IPA: /ɪnˌkæp.sjəˈleɪ.fən/
Phonetic Spelling: in·kap·syuh·LAY·shuhn
3. **Fluorinated**
Pronunciation link: <https://www.merriam-webster.com/dictionary/fluorinated>
IPA: /ˈflʊrəˌneɪtɪd/
Phonetic Spelling: FLOOR·uh·nay·tuhd
4. **Microfluidic**
Pronunciation link: <https://www.merriam-webster.com/dictionary/microfluidics>
IPA: /ˌmaɪkroʊfluˈɪdɪk/
Phonetic Spelling: MY·kroh·floo·ID·ik
5. **Automated**
Pronunciation link: <https://www.merriam-webster.com/dictionary/automated>
IPA: /ˈɔtəˌmeɪtɪd/
Phonetic Spelling: AW·tuh·may·tuhd
6. **Entrapping**
Pronunciation link: <https://www.merriam-webster.com/dictionary/entrap>
IPA: /ɪnˈtræpɪŋ/
Phonetic Spelling: en·TRAP·ing
7. **Calibration**
Pronunciation link: <https://www.merriam-webster.com/dictionary/calibration>
IPA: /ˌkæl.əˈbreɪ.fən/
Phonetic Spelling: kal·uh·BRAY·shuhn
8. **Incubate**
Pronunciation link: <https://www.merriam-webster.com/dictionary/incubate>
IPA: /ˈɪŋkjəˌbeɪt/
Phonetic Spelling: ING·kyuh·bayt
9. **Centrifuge**
Pronunciation link: <https://www.merriam-webster.com/dictionary/centrifuge>

IPA: /'sɛntrəˌfjuːdʒ/

Phonetic Spelling: SEN-truh-fyooj

10. **Laminar**

Pronunciation link: <https://www.merriam-webster.com/dictionary/laminar>

IPA: /'læmənər/

Phonetic Spelling: LAM-uh-ner

11. **Pico-injection**

Pronunciation link: No confirmed link found

IPA: /ˌpiːkɒʊ ɪnˈdʒɛkʃən/

Phonetic Spelling: PEE-koh in-JEK-shuhn

12. **Phosphate-buffered**

Pronunciation link: <https://www.merriam-webster.com/dictionary/phosphate>

IPA: /'fɑːsfɛrt/

Phonetic Spelling: FOS-fayt

13. **Saline**

Pronunciation link: <https://www.merriam-webster.com/dictionary/saline>

IPA: /'seɪlɪn/

Phonetic Spelling: SAY-line

14. **Electrode**

Pronunciation link: <https://www.merriam-webster.com/dictionary/electrode>

IPA: /ɪˈlɛkˌtroʊd/

Phonetic Spelling: ih-LEK-trohd

15. **Multimeter**

Pronunciation link: <https://www.merriam-webster.com/dictionary/multimeter>

IPA: /ˌmʌltiˈmiːtər/

Phonetic Spelling: MUL-tee-MEE-ter

16. **Continuity**

Pronunciation link: <https://www.merriam-webster.com/dictionary/continuity>

IPA: /ˌkɔːntəˈnuːəti/

Phonetic Spelling: kon-tuh-NOO-uh-tee

17. **Fluorescence**

Pronunciation link: <https://www.merriam-webster.com/dictionary/fluorescence>

IPA: /flʊˈresəns/

Phonetic Spelling: floor-ESS-uhns

18. **Hyperproducing**

Pronunciation link: No confirmed link found

IPA: /ˌhaɪpərprəˈduːsɪŋ/

Phonetic Spelling: HY-per-pruh-DOO-sing

19. **Nanometers**

Pronunciation link: <https://www.merriam-webster.com/dictionary/nanometer>

IPA: /ˈnænəˌmiːtər/

Phonetic Spelling: NAN-oh-mee-ter

20. Histogram

Pronunciation link: <https://www.merriam-webster.com/dictionary/histogram>

IPA: /'hɪstəˌgræm/

Phonetic Spelling: HIS-tuh·gram

21. Deflection

Pronunciation link: <https://www.merriam-webster.com/dictionary/deflection>

IPA: /dɪ'flekʃən/

Phonetic Spelling: dih·FLEK-shuhn

22. Demulsifier

Pronunciation link: No confirmed link found

IPA: /di:'mʌlsɪˌfaɪər/

Phonetic Spelling: dee·MUL-sih·fyer

23. Perfluorooctanol

Pronunciation link: No confirmed link found

IPA: /ˌpɜːfluˌɔːrɒʊˈɑːktəˌnɔːl/

Phonetic Spelling: per·floor·oh·ok-tuh·nawl