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Scriptwriter Name: Sri Lakshmi Priya

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Title: Optimized Yeast Surface Display Workflow for Directed Evolution of Proteases

Authors and Affiliations:

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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**

Videographer: Please film the shots labeled as SCREEN.

3. Filming location: Will the filming need to take place in multiple locations? **Yes**

If **Yes**, how far apart are the locations? Filming will take place in Dingal Lab space and another room that is a couple of feet away.

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: 33

Number of Shots: 56

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. **P. C. Dave P. Dingal**: Our research aims to reprogram protease-substrate specificity by evolving potyviral proteases to recognize new protein targets.
 - 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. **P. C. Dave P. Dingal**: This protocol can be applied to engineer proteases for synthetic biology, protein circuits, and future therapeutic applications.
 - 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. **Medel B. Lim Suan Jr.**: This procedure enables high-throughput identification of protease variants with desired substrate specificities.
 - 1.3.1. 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?~~

- 1.4. **Medel B. Lim Suan Jr.**: Additionally, following this procedure, sequenced variants can be characterized biochemically to determine catalytic efficiency and validated using fluorescence-based assays.
 - 1.4.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera.

~~How can future studies build on this work? Describe the questions or research directions that can be investigated next.~~

1.5. **Zuhair Quadri**: Future studies can engineer proteases for disease-relevant targets and incorporate them into programmable protein circuits and therapies.

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.

Ethics Title Card

This research has been approved by the Institutional Biosafety and Chemical Safety Committee at The University of Texas at Dallas

Protocol

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

2. Preparation of Reagents and Yeast Pre-culture for Electroporation

Demonstrator: Medel B. Lim Suan Jr.

- 2.1. To begin, prepare growth media, plates, electroporation buffer, and sterile water [1]. Store all prepared reagents at 4 degrees Celsius [2].
 - 2.1.1. WIDE: Talent arranging the growth media, plates, electroporation buffer, and sterile water on the workbench. *Videographer: Please obtain CU shot as well for this shot.*
 - 2.1.2. Talent placing the prepared reagents into 4 degrees Celsius storage.
- 2.2. Streak EBY100 (E-B-Y-One-Zero-Zero) yeast onto a Yeast Extract Peptone Dextrose or YPD (Y-P-D) agar plate from a glycerol stock [1] and grow the plate at 30 degrees Celsius for 2 days [2].
 - 2.2.1. Talent streaking EBY100 yeast onto a Yeast Extract Peptone Dextrose agar plate from a glycerol stock.
 - 2.2.2. Talent placing the inoculated plate into an incubator.
- 2.3. Prepare 125 milliliters of YPD in a 500-milliliter Erlenmeyer flask [1].
 - 2.3.1. Talent preparing 1x YPD in a 500-milliliter Erlenmeyer flask.
- 2.4. Transfer 25 milliliters of the prepared YPD to a 50-milliliter conical tube [1]. Using a micropipette tip, collect EBY100 cells from the previously prepared plate [2]. Transfer the yeast cells from the micropipette tip into the conical tube containing YPD [3].
 - 2.4.1. Talent transferring 25 milliliters of 1x Yeast Extract Peptone Dextrose in a 50-milliliter conical tube. NOTE: 2.4.1-2.4.3 are filmed in a single shot
 - 2.4.2. Talent collecting EBY100 cells from the previously prepared plate with a micropipette tip.
 - 2.4.3. Talent transferring the yeast cells from the micropipette tip into the conical tube containing 1x Yeast Extract Peptone Dextrose.
- 2.5. Then, vortex the conical tube to mix the yeast suspension thoroughly [1].
 - 2.5.1. Talent vortexing the conical tube. . NOTE: 2.5.1-2.6.2. are filmed in a single shot
- 2.6. To prepare a 150-milliliter pre-culture, transfer 25 milliliters of YPD containing the yeast cells into the 500-milliliter flask containing 125 milliliters of YPD [1]. Swirl the flask to

mix [2].

2.6.1. Talent transferring the yeast suspension into the 500 milliliter Erlenmeyer flask containing 125 milliliters of YPD.

2.6.2. Talent swirling the flask to mix.

2.7. Next, take two 1-centimeter path length microcuvettes and add 1 milliliter of YPD to one as the blank control and 1 milliliter of the pre-culture to the other [1]. Using a spectrophotometer, measure the optical density at 600 nanometers and achieve a value between 0.05 and 0.1 [2].

2.7.1. Talent filling a microcuvette with 1 milliliter of 1 × Yeast Extract Peptone Dextrose and a second microcuvette with 1 milliliter of the pre-culture.

2.7.2. SCREEN: Show the spectrophotometer display measuring the optical density at 600 nanometers and displaying a value between 0.05 and 0.1

Videographer: Please film the computer screen

2.8. Then, grow the 150-milliliter pre-culture overnight at 30 degrees Celsius with orbital shaking at 270 rpm [1].

2.8.1. Talent placing the flask containing the pre-culture into the shaking incubator.

3. Preparation of Yeast Cells for Electroporation

Demonstrator: Zuhair Quadri

3.1. After diluting the overnight pre-culture 1 to 20, using a spectrophotometer, measure the optical density at 600 nanometers [1].

3.1.1. Talent measuring the optical density at 600 nanometers of the 1:20 diluted overnight pre-culture with a spectrophotometer.

3.3. Calculate the volume of pre-culture required to inoculate a 100-milliliter subculture with an initial optical density at 600 nanometers of 3 to 4 [1]. NOTE: This is placed here as per author's request

3.3.1. Talent calculating the required pre-culture volume.

3.1. Dilute the overnight pre-culture to the desired initial optical density at 600 nanometers in 100 milliliters of 2x YPD in a 500-milliliter Erlenmeyer flask [1]. ~~Measure the optical density at 600 nanometers of the saturated overnight pre-culture [2].~~ NOTE: The VO is edited for the deleted shot

3.1.1. Talent diluting the overnight pre-culture in 100 milliliters of 2 × Yeast Extract Peptone Dextrose in a 500-milliliter Erlenmeyer flask.

~~3.1.2. Talent measuring the optical density at 600 nanometers of the saturated overnight pre-culture with a spectrophotometer.~~ **NOTE: Not filmed**

~~3.2. Calculate the volume of pre culture required to inoculate a 100-milliliter subculture with an initial optical density at 600 nanometers of 3 to 4 [1].~~ **NOTE: This step is moved before 3.2**

~~3.2.1. Talent calculating the required pre-culture volume.~~

3.3. Incubate the 100-milliliter subculture at 30 degrees Celsius with orbital shaking at 270 rpm for 4 hours [1].

3.3.1. Talent placing the 100-milliliter subculture into the shaking incubator.

3.4. Obtain the previously prepared ice-cold electroporation buffer and sterile water [1].

3.4.1. Talent arranging the ice-cold electroporation buffer and sterile water on the laboratory bench.

3.5. Prepare 100 milliliters of conditioning buffer and store it at room temperature between 20 and 23 degrees Celsius [1].

3.5.1. Talent preparing 100 milliliters of conditioning buffer.

3.6. Prepare 50 milliliters of outgrowth medium by mixing 25 milliliters of 2x YPD with 25 milliliters of 1 molar sorbitol and store it at room temperature between 20 and 23 degrees Celsius [1].

3.6.1. Talent mixing 25 milliliters of 2 × Yeast Extract Peptone Dextrose with 25 milliliters of 1 molar sorbitol.

4. Preparation of Electrocompetent EBY100 Cells

4.1. Split the 100-milliliter subculture into two 50-milliliter conical tubes [1]. Collect the cells by centrifugation at 1,500 g for 3 minutes at 4 degrees Celsius and decant the medium [2]. Keep the cells on ice until the conditioning step is complete [3].

4.1.1. Talent dividing the subculture into two 50-milliliter conical tubes.

4.1.2. Talent placing the tubes into the centrifuge.

4.1.3. Talent placing the tubes on ice.

4.2. Gently resuspend each cell pellet in 25 milliliters of ice-cold sterile water [1]. Centrifuge at 1,500 g for 3 minutes at 4 degrees Celsius [2]. Decant the medium and repeat the sterile water wash [3].

4.2.1. Talent resuspending each cell pellet with ice-cold sterile water.

4.2.2. Talent placing the tubes into the centrifuge.

4.2.3. Talent decanting the medium.

- 4.3. Gently resuspend each cell pellet in 25 milliliters of ice-cold electroporation buffer [1]. Centrifuge at 1,500 g for 3 minutes at 4 degrees Celsius and decant the medium [2].
 - 4.3.1. Talent gently resuspending each cell pellet with ice-cold electroporation buffer.
 - 4.3.2. Talent placing the tubes into the centrifuge.
- 4.4. Remove the cells from ice and gently resuspend each cell pellet in 10 milliliters of room temperature conditioning buffer [1]. Pool the cell suspensions into a single 125-milliliter Erlenmeyer flask [2] and incubate at 30 degrees Celsius with orbital shaking at 270 rpm for 30 minutes [3].
 - 4.4.1. Talent resuspending the cell pellet with conditioning buffer.
 - 4.4.2. Talent pooling the cell suspensions into a 125 milliliter Erlenmeyer flask.
 - 4.4.3. Talent placing the flask into the shaking incubator.
- 4.5. Transfer the cells into a 50-milliliter tube [1]. Centrifuge at 1,500 g for 3 minutes at 4 degrees Celsius and decant the medium [2].
 - 4.5.1. Talent transferring the cell suspension into a 50-milliliter tube.
 - 4.5.2. Talent placing the tube into the centrifuge.
- 4.6. Gently resuspend the cell pellet in 50 milliliters of ice-cold electroporation buffer [1]. Then, centrifuge again at 1,500 g for 3 minutes at 4 degrees Celsius and decant the medium [2].
 - 4.6.1. Talent gently resuspending the cell pellet with ice-cold electroporation buffer.
 - 4.6.2. Talent placing the tube into the centrifuge.
- 4.7. Then, resuspend the cell pellet to a final volume of 1 milliliter by adding approximately 100 to 200 microliters of electroporation buffer [1]. Keep the cells on ice until electroporation [2].
 - 4.7.1. Talent resuspending the cell pellet to a final volume of 1 milliliter with electroporation buffer. . NOTE: 4.7.1-4.7.2 are filmed in a single shot
 - 4.7.2. Talent placing the cell suspension on ice.

5. Electroporation and Recovery of Transformed Yeast Cells

- 5.1. Pre-chill 0.2-centimeter gap electroporation cuvettes on ice [1].
 - 5.1.1. Talent placing the electroporation cuvettes on ice.
- 5.2. Transfer 380 microliters of the resuspended cell pellet into a sterile 1.5-milliliter tube [1]. Add 4 micrograms of plasmid DNA or 4 micrograms of linearized plasmid vector and 12 micrograms of insert [2]. Gently mix by pipetting [3].
 - 5.2.1. Talent transferring the resuspended cell pellet into a sterile 1.5 milliliter tube.

- 5.2.2. Talent adding the DNA components to the tube. . **NOTE: 5.2.2-5.2.3 are filmed in a single shot**
- 5.2.3. Talent gently mixing the suspension by pipetting.
- 5.3. Transfer the cell suspension into the pre-chilled electroporation cuvette [1] and keep it on ice for 5 minutes [2].
- 5.3.1. Talent transferring the cell suspension into the pre-chilled electroporation cuvette. . **NOTE: 5.3.1-5.3.2 are filmed in a single shot**
- 5.3.2. Talent placing the cuvette on ice.
- 5.4. Wipe the outside of the cuvette with a lint-free laboratory wipe to remove residual moisture and ensure that no bubbles or cells remain above the metal plates in the cuvette [1].
- 5.4.1. Talent wiping the outside of the cuvette with a lint-free laboratory wipe.
- 5.5. Prepare 8 milliliters of outgrowth medium in a 50-milliliter conical tube [1]. Electroporate the cells [2].
- 5.5.1. Talent preparing 8 milliliters of outgrowth medium in a 50-milliliter conical tube.
- 5.5.2. TEXT ON A PLAIN BACKGROUND:
- Electroporation settings**
Voltage: 2.5 kV
Capacitance: 25 μ F
Resistance: 200 Ω
Time Constant: 2.8–4.5 ms
- 5.6. Immediately add approximately 200 microliters of outgrowth medium to the cuvette [1]. Transfer the electroporated cells into the 50-milliliter conical tube [2]. Add another 200 to 350 microliters of outgrowth medium until no visible liquid remains in the cuvette [3].
- 5.6.1. Talent adding outgrowth medium to the electroporation cuvette. **NOTE: 5.6.1-5.6.3 are filmed in a single shot**
- 5.6.2. Talent transferring the electroporated cells into the 50-milliliter conical tube.
- 5.6.3. Talent adding additional outgrowth medium to the cuvette.
- 5.7. Incubate the electroporated cells in 8 milliliters of outgrowth medium at 30 degrees Celsius without shaking for 1 hour, inverting the tube every 15 minutes to mix [1].
- 5.7.1. Talent placing the conical tube into the incubator.
- 5.8. Collect the cells by centrifugation at 1,500 g and 25 degrees Celsius for 3 minutes, then decant the supernatant [1].
- 5.8.1. Talent placing the conical tube into the centrifuge.

5.9. For plasmids containing the TRP1 (*T-R-P-one*) selection marker, resuspend the cells in 250 milliliters of Synthetic Dextrose Casamino Acids medium or SDCAA+ (*S-D-C-A-A-plus*) lacking tryptophan [1].

5.9.1. Talent resuspending the cells in 250 milliliters of SDCAA+ medium lacking tryptophan.

5.10. Incubate the culture overnight at 30 degrees Celsius with orbital shaking at 270 rpm [1].

5.10.1. Talent placing the culture into the shaking incubator.

5.11. After induction, fluorescent antibody staining, and fluorescence-activated cell sorting, analyze the data to evaluate the enrichment of functional variants and assess the performance of the gating strategy [1].

5.11.1. SCREEN: Display the fluorescence-activated cell sorting analysis showing the sorted populations.

Videographer: Please film the computer screen

Results

6. Results

- 6.1. Yeast cultures subcultured at initial optical density at 600 nanometres values ranging from 1 to 4 remained within the mid-log growth phase after 4 hours of incubation [1].
 - 6.1.1. LAB MEDIA: Figure 5A. *Video editor: Highlight the shaded area labeled 'final subculture OD600 range after 4-hr incubation'.*
- 6.2. Plasmid-only transformation efficiency was significantly higher at all initial optical density at 600 nanometres values above 1 [1], with the highest transformation efficiency observed at an initial optical density at 600 nanometres of 4 [2].
 - 6.2.1. LAB MEDIA: Figure 5B. *Video editor: Highlight the blue points from 1.5 to 4.0.*
 - 6.2.2. LAB MEDIA: Figure 5B. *Video editor: Highlight the blue point above the x-axis label '4.0'.*
- 6.3. Co-electroporation of a linearized vector and protease library insert showed significantly higher transformation efficiency at initial optical density at 600 nanometres values of 3.0 [1], 3.5 [2], and 4.0 [3], with the highest transformation efficiency observed at an initial optical density at 600 nanometres of 3.0 [4].
 - 6.3.1. LAB MEDIA: Figure 5B. *Video editor: Highlight the orange point above the x-axis label '3.0'.*
 - 6.3.2. LAB MEDIA: Figure 5B. *Video editor: Highlight the orange point above the x-axis label '3.5'.*
 - 6.3.3. LAB MEDIA: Figure 5B. *Video editor: Highlight the orange point above the x-axis label '4.0'.*
 - 6.3.4. LAB MEDIA: Figure 5B. *Video editor: Highlight the orange point above the x-axis label '3.0' at the highest position on the orange line.*
- 6.4. Co-electroporation of a linearized vector and protease library insert produced significantly higher transformation efficiencies than plasmid-only transformation across the tested initial optical density at 600 nanometres conditions [1], with a mean 121-fold increase in transformation efficiency [2].
 - 6.4.1. LAB MEDIA: Figure 5B. *Video editor: Highlight the orange line labelled 'vector + insert' and the blue line labelled 'plasmid'.*
 - 6.4.2. LAB MEDIA: Figure 5B. *Video editor: Zoom in on the bracket at the right side labelled '121x'.*
- 6.5. Applying a stringent gating strategy resulted in progressive enrichment of functional variants across successive rounds of sorting [1].

6.5.1. LAB MEDIA: Figure 6B.

6.6. Electroporated, induced, unstained yeast cells [1] and the corresponding anti-hemagglutinin fluorescein isothiocyanate or FITC (*F-I-T-C*) and anti-FLAG phycoerythrin or PE (*P-E*) fluorescence histograms were shown as negative controls [2].

6.6.1. LAB MEDIA: Figure 6C.

6.6.2. LAB MEDIA: Figure 6D.

1. Protease
Pronunciation link: <https://www.merriam-webster.com/dictionary/protease>
IPA: /'proʊ.ti.əs/
Phonetic Spelling: proh·tee·ays
2. Substrate
Pronunciation link: <https://www.merriam-webster.com/dictionary/substrate>
IPA: /'sʌb.streɪt/
Phonetic Spelling: sub·strayt
3. Potyviral
Pronunciation link: No confirmed link found
IPA: No verified IPA available
Phonetic Spelling: poh·tee·vy·ruhl
4. Synthetic biology
Pronunciation link: <https://www.merriam-webster.com/dictionary/synthetic>
IPA: /sɪn'θet.ɪk baɪ'ɑː.lə.dʒi/
Phonetic Spelling: sin·thet·ik bye·ol·uh·jee
5. Therapeutic
Pronunciation link: <https://www.merriam-webster.com/dictionary/therapeutic>
IPA: /,θer.ə'pjʊː.tɪk/
Phonetic Spelling: thair·uh·pyoo·tik
6. High-throughput
Pronunciation link: <https://www.merriam-webster.com/dictionary/high-throughput>
IPA: /,haɪ'θruː.pʊt/
Phonetic Spelling: hy·throo·put
7. Variants
Pronunciation link: <https://www.merriam-webster.com/dictionary/variant>
IPA: /'ver.i.ənt/
Phonetic Spelling: vair·ee·uhnt
8. Specificities
Pronunciation link: <https://www.merriam-webster.com/dictionary/specificity>
IPA: /,spes.ə'fis.ə.ti/
Phonetic Spelling: spess·uh·fis·uh·tee
9. Biochemically
Pronunciation link: <https://www.merriam-webster.com/dictionary/biochemical>
IPA: /,baɪ.oʊ'kem.i.kli/
Phonetic Spelling: bye·oh·kem·ik·lee
10. Catalytic
Pronunciation link: <https://www.merriam-webster.com/dictionary/catalytic>
IPA: /,kæt.ə'lɪ.tɪk/
Phonetic Spelling: kat·uh·lit·ik
11. Fluorescence
Pronunciation link: <https://www.merriam-webster.com/dictionary/fluorescence>
IPA: /flɔː'res.əns/
Phonetic Spelling: floor·ess·uhns

12. Electroporation
Pronunciation link: <https://www.merriam-webster.com/dictionary/electroporation>
IPA: /ɪˌlek.troʊ.pəˈreɪ.ʃən/
Phonetic Spelling: ih·lek·troh·puh·ray·shuhn
13. Reagents
Pronunciation link: <https://www.merriam-webster.com/dictionary/reagent>
IPA: /riˈeɪ.dʒənt/
Phonetic Spelling: ree·ay·juhnt
14. EBY100
Pronunciation link: No confirmed link found
IPA: No verified IPA available
Phonetic Spelling: ee·bee·why·one·zero·zero
15. Glycerol
Pronunciation link: <https://www.merriam-webster.com/dictionary/glycerol>
IPA: /ˈɡlɪs.əˌrɔːl/
Phonetic Spelling: glis·uh·rawl
16. Erlenmeyer
Pronunciation link: <https://www.merriam-webster.com/dictionary/Erlenmeyer%20flask>
IPA: /ˈɜː.lənˌmaɪ.ər/
Phonetic Spelling: er·len·my·er
17. Micropipette
Pronunciation link: <https://www.merriam-webster.com/dictionary/micropipette>
IPA: /ˌmaɪ.kroʊ.paɪˈpet/
Phonetic Spelling: my·kroh·pie·pet
18. Conical
Pronunciation link: <https://www.merriam-webster.com/dictionary/conical>
IPA: /ˈkɑː.nɪ.kəl/
Phonetic Spelling: kon·ih·kuhl
19. Biosafety
Pronunciation link: <https://www.merriam-webster.com/dictionary/biosafety>
IPA: /ˌbaɪ.oʊˈseɪf.ti/
Phonetic Spelling: bye·oh·sayf·tee
20. Programmable
Pronunciation link: <https://www.merriam-webster.com/dictionary/programmable>
IPA: /proʊˈɡræm.ə.bəl/
Phonetic Spelling: proh·gram·uh·buhl