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Title: Identifying the Binding Proteins of Small Ligands with the Differential Radial Capillary Action of Ligand Assay (DRaCALA)

Authors and Affiliations: Muriel Leandra Schicketanz[1], Paulina Długosz[1], and Yong Everett Zhang[1]

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Author Questionnaire

1. **Microscopy**: Does your protocol demonstrate the use of a dissecting or stereomicroscope for performing a complex dissection, microinjection technique, or similar? **NO**

2. **Software:** Does the part of your protocol being filmed demonstrate software usage? **YES**

3. **Interview statements:** Considering the Covid-19-imposed mask-wearing and social distancing recommendations, which interview statement filming option is the most appropriate for your group? **Please select one**.

   ☒ Interviewees wear masks until the videographer steps away (≥6 ft/2 m) and begins filming. The interviewee then removes the mask for line delivery only. When the shot is acquired, the interviewee puts the mask back on. Statements can be filmed outside if weather permits.

4. **Filming location:** Will the filming need to take place in multiple locations (greater than walking distance)? **NO**

Protocol Length
Number of Steps: 23
Number of Shots: 51 (4 SC)

Introduction

1. Introductory Interview Statements

REQUIRED:

1.1. **Muriel Leandra Schicketanz:** Small signaling molecules target various effector proteins to control bacterial virulence and human biology. However, these effector proteins are challenging to identify. **[1]**.

1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 2.2.1 for "effector proteins are challenging to identify"*

1.2. **Muriel Leandra Schicketanz:** As a systems biology tool, DRaCALA allows a feasible, rapid and highly sensitive identification of the unknown effector proteins by using an ORFeome library **[1]**.

1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 6.1.1 for "DRaCALA allows a feasible"*

OPTIONAL:

1.3. **Paulina Długosz:** DRaCALA could be used to study any small signaling molecule as long as it can be labelled with either radioactive isotopes or fluorescent dyes **[1]**.

1.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 5.1.2 for "labelled with either radioactive isotopes"*

Protocol

2. Preparation of Whole Cell Lysates

2.1. Start by inoculating *E. coli* into 1.5 milliliters of LB (L-B) supplemented with 25 micrograms/milliliter chloramphenicol in each well of 96-well deep well plates **[1-TXT]** for an overnight incubation at 30 degrees Celsius and 160 rotations per minute **[2]**.

2.1.1. WIDE: Talent adding bacteria to wells by using multichannel pipette from a 96x well microtiter plate. **TEXT: e.g., *E. coli* K-12 ASKA ORFeome**

2.1.2. Talent placing well plates in shaker incubator**.**

2.2. The next morning, treat the overnight cultures with 500 micromolar IPTG (I-P-T-G) for 6 hours at 30 degrees Celsius to induce protein expression **[1-TXT]**. At the end of the incubation, pellet the cells by centrifugation **[2-TXT]** and freeze the samples at minus 80 degrees Celsius **[3]**.

2.2.1. Talent adding IPTG to culture plates by using multichannel pipette from a plastic trove. **TEXT: IPTG: isopropyl beta-d-1-thiogalactopyranoside**

2.2.2. Talent placing plate in a centrifuge **TEXT: 10 min, 500 x *g*, RT**

2.2.3. Talent dumping supernatant and placing plate into freezer. NOTE: On take III (three): place plate in freezer

2.3. To initiate lysis, resuspend the pellets in 150 microliters of lysis buffer 1 per well for a 30-minute incubation at minus 80 degrees Celsius **[1-TXT]** before thawing the cells for 20 minutes at 37 degrees Celsius **[2-TXT]**. The lysate should then be stored at minus 80 degrees Celsius before use **[3].**

2.3.1. Talent adding buffer to wells by using multichannel pipettes and pipette up and down, and place plate in freezer -80°C. **TEXT: See text for all buffer and solution preparation details** *Videographer: This step is important!* NOTE: On take III: (three) place plate in freezer -80°C

2.3.2. Talent placing plate at 37 °C water bath. **TEXT: Repeat freeze/thaw x3**

2.3.3. Talent placing plate at -80oC.

3. Nickel-Nitrilotriacetic Acid (Ni-NTA) Affinity Purification of Relseq and GppA

3.1. To complete the lysis of cells with overexpressed proteins, resuspend the samples in 40 milliliters of ice-cold lysis buffer 2 **[1]** and sonicate the samples at a 60% amplitude at 2 seconds on-4 seconds off for 8 minutes **[2]**. Then clear the lysates by centrifugation **[3-TXT]**.

3.1.1. WIDE: Talent adding buffer to a Falcon tube and vortex to resuspend cells.

3.1.2. Talent sonicating cells in a beaker with ice.

3.1.3. Talent transferring cell to a spin tube and placing tubes in centrifuge. **TEXT: 40 min, 23,426 x *g*, 4 °C** NOTE: On take II (two), places tubes in centrifuge

3.2. While the samples are being centrifuged, add 500 microliters of homogenized nickel-NTA (N-T-A) resin to a standing polypropylene chromatography column **[1]**. After 15 minutes, wash the settled resin two times with 15 milliliters of ultrapure water and one time with 15 milliliters of lysis buffer 2 **[2]**.

3.2.1. Talent adding resin to column.

3.2.2. Talent adding water into the column.

3.3. At the end of the centrifugation, load the cleared lysate supernatant onto the column **[1]**. When all the lysate has flowed through the column, wash the column with 30 milliliters of washing buffer **[2]**.

3.3.1. Talent loading supernatant onto the column.

3.3.2. Talent adding buffer to column.

3.4. To elute the proteins, add 400-microliter volume of elution buffer to the column and repeat elution three times **[1]; another** 300-microliter volume of the elution buffer **to repeat elution three times**; and combine the eluted proteins in a final volume of 700 microliters **[2]**.

3.4.1. Talent adding buffer to column.

3.4.2. Talent combining the eluted volumes.

4. Gel Filtration

4.1. For gel filtration of the eluted sample, after washing a size exclusion column with 25 milliliters of freshly prepared gel filtration buffer **[1]**, load the entire volume of eluted protein onto the column by using a 500-microliter loop **[2],** run at 500 microliters per minute and collect two to three, 500-microliter volume fractions of the respective proteins **[3]**.

4.1.1. WIDE: Talent running gel filtration buffer to column.

4.1.2. Talent loading the sample on the column by using a syringe.

4.1.3. Protein fraction being collected

4.2. Then use individual spin columns to concentrate each protein **[1]** and use the Bradford assay kit to measure the protein concentrations according to standard protocols **[2]**.

4.2.1. Talent adding sample to spin column.

4.2.2. Talent opening Bradford assay or similar representative action.

5. Synthesis of ^{32}P-Labeled Guanosine Pentaphosphate (pppGpp) and Guanosine Tetraphosphate (ppGpp)

5.1. To synthesize the phosphorus-32 (thirty-two)-labeled pppGpp *(pronounce guanosine pentaphosphate)*, assemble a small-scale Rel-seq *(pronounce rell-seek)* reaction in a screw cap tube as outlined in the Table **[1]** and incubate the reaction in a thermomixer for 1 hour at 37 degrees Celsius and 5 minutes at 95 degrees Celsius **[2]** followed by 5 minutes on ice **[3]**.

5.1.1. LAB MEDIA: Table 1.

5.1.2. WIDE: Talent placing tube into thermomixer behind a perplex shield in a fumehood.

5.1.3. Talent placing tube on ice behind a perplex shield.

5.2. At the end of the incubations, spin down the precipitated protein by centrifugation **[1-TXT]** and transfer the synthesized phosphorus-32-pppGpp-containing supernatant to a new screw cap tube **[2]**.

5.2.1. Talent placing tubes in a centrifuge and closing the lid behind a perplex shield. **TEXT: 5 min, 15,700 x *g*, RT**

5.2.2. Talent transferring supernatant to a fresh screw cap tube by using filter tips.

5.3. For phosphorus-32- guanosine tetraphosphate synthesis, add 1 micromolar GppA *(pronounce G-P-P-A)* to half of the phosphorus-32-pppGpp product in a new screw cap tube **[1]** and incubate the reaction for 10 minutes at 37 degrees Celsius, 5 minutes at 95 degrees Celsius, and 5 minutes on ice **[2]**.

5.3.1. Talent adding GppA to tube.

5.3.2. Talent placing sample at 37 °C.

5.4. At the end of the incubation, spin down the precipitate by centrifugation **[1-TXT]** and transfer the phosphorus-32-ppGpp-containing supernatant to a new tube **[2]**. To analyze the isolated target proteins, run 1 microliter of each sample on a thin layer chromatography plate using 1.5 molar monopotassium phosphate as the mobile phase **[3]**.

5.4.1. Talent adding sample to centrifuge **TEXT: 5 min, 15,700 x *g*, RT**

5.4.2. Talent adding supernatant to a new screw cap tube

5.4.3. Talent adding sample to TLC plate. *Videographer: This step is important!*

5.5. After the analysis, place the dried plate in a transparent plastic folder **[1]** and expose the plate to a storage phosphor screen for 5 minutes **[2]** before visualizing and quantifying the data on a phosphorimager **[3]**.

5.5.1. Talent placing plate into folder.

5.5.2. Talent exposing TLC plate to phosphor screen.

5.5.3. Talent loading plate onto phosphorimager.

6. DRaCALA Screening of the Target Proteins of (p)ppGpp

6.1. For DraCALA (*pronounce drah-cah-lah*) screening of the target proteins, add 20 microliters of the thawed whole cell lysates to individual wells of a 96-well V-bottom microtiter plate **[1-TXT]** and add 2.5 units of *Serratia marcescens* endonuclease to each well **[2]**. After 15 minutes at 37 degrees Celsius, place the lysates on ice for 20 minutes **[3]**.

6.1.1. WIDE: Talent add the prestored whole cell lysates to a 96-well V-bottom microtiter plate by using multichannel pipette. **TEXT: After freeze-thaw lysis of cells**

6.1.2. Talent adding endonuclease to wells.

6.1.3. Talent placing plates on ice.

6.2. Next, mix equal volumes of phosphorus-32 pppGpp and -ppGpp **[1]** and add 1x lysis buffer 1 to the mixture to obtain a 4 nanomolar pppGpp solution **[2]**. Using a multichannel pipette and filtered pipette tips, mix 10 microliters of the pppGpp mixture with the cell lysate, for a 5-minute incubation at room temperature **[3]**.

6.2.1. Talent mix the pppGpp and ppGpp, behind a shield. *Videographer: This step is important!* NOTE: labelled 6.1.2. on the board

6.2.2. Talent adding lysis buffer to the mix.

6.2.3. Talent adding the (p)ppGpp mixture from a plastic trove to the cell lysate in microtiter plate. *Videographer: This step is important!*

6.3. At the end of the incubation, wash a 96x pin tool three times in a 0.01% solution of non-ionic detergent for 30 seconds **[1]** followed by 30 seconds of drying on a paper towel per wash **[2]** before placing the pin tool in the 96-well sample plate **[3]**.

6.3.1. Talent placing tool in detergent.

6.3.2. Talent placing tool onto paper towel.

6.3.3. Talent placing pin tool in sample plate. *Videographer: This step is important!*

6.4. After 30 seconds, lift the pin tool straight up and place it straight down on a nitrocellulose membrane for 30 seconds **[1]**.

6.4.1. Talent lifting pin tool and placing it on a nitrocellulose membrane. *Videographer: This step is important!*

6.5. After 5 minutes of drying, place the nitrocellulose membrane in a transparent plastic folder for storage phosphor screen exposure **[1]** and visualization by phosphorimaging as demonstrated **[2]**.

6.5.1. Talent placing membrane into folder.

6.5.2. Talent loading sample onto imager.

7. Quantification and Identification of Potential Target Proteins

7.1. To quantify and identify potential target proteins, in the analysis software associated with the phosphorimager, open the .gel file of the visualized plates **[1]**. To define the spots to be analyzed, use the **Array analysis** function to set up a 12-column x 8-row grid **[2]**.

7.1.1. WIDE: Talent opening file, with monitor visible in frame

7.1.2. SCREEN: 62331_screenshot_7.1.2.mp4. 0:00 – 0:26.

7.2. To circumscribe the outer edge of the whole spots, define big circles. Export the **Volume+Background** and **Area** of the defined big circles to a spreadsheet. To circumscribe the small inner dots, size down the defined circles **[1]**.

7.2.1. SCREEN: 62331_screenshot_7.2.1.mp4. 0:00 – 0.46 *Video Editor: please speed up a bit till 0:15.*

7.3. Export the **Volume+Background**, and **Area** of the defined small circles, and save all the data in the spreadsheet. Position circles to overlap with spots as necessary, resizing to slightly bigger than actual spots **[1]**.

7.3.1. SCREEN: 62331_screenshot_7.3.1.mp4. 0:00 – 0:30.

7.4. Use the equation to calculate the binding fractions in the spreadsheet **[1-TXT]** and plot the data **[2]**. Then identify the potential binding proteins in the wells that show high binding fractions compared to the majority of other wells **[3]**.

7.4.1. BLACK TEXT WHITE BACKGROUND: Binding fraction = $[S_1 – (S_2-S_1)/(A_2-A_1) \times A_1]/A_2$

7.4.2. Data being plotted.

7.4.3. SCREEN: 62331_screenshot_7.4.3.mp4. 0:00 – 0:06. *Video Editor: please emphasize high binding fractions when mentioned*

Results

8. Results: DRaCALA Plate Screening and Quantification

8.1. In this representative analysis **[1]**, a plate with relatively low background binding signals in most wells can be observed **[2]**.

8.1.1. LABMEDIA: Figure 3 A.

8.1.2. LABMEDIA: Figure 3 A. *Video Editor: Emphasize the binding fraction graph.*

8.2. The positive binding signal in well H3 **[1]** exhibited a binding fraction that was much higher than that observed for the other wells due to overexpression of the pppGpp binding protein Hpt (H-P-T) **[2-TXT]**.

8.2.1. LABMEDIA: Figure 3 A. *Video Editor: Emphasize on highlighted well in AKSA plate-50.*

8.2.2. LABMEDIA: Figure 3 A. *Video Editor: Emphasize on binding fraction graph for 'Hpt'.* **TEXT: Hpt: histidine phosphotransferase**

8.3. In the representative plates, several wells showed relatively higher background binding signals **[1]**, as indicated by the relatively strong inner dots observed in many wells **[2]** as well as the consistently high binding fractions observed after quantification **[3]**.

8.3.1. LABMEDIA: Figure 3 B, C.

8.3.2. LAB MEDIA: Figure 3 B, C. *Video Editor: Emphasize dark dots in center or well(s) in plate image.*

8.3.3. LAB MEDIA: Figure 3B, C. *Video Editor: Emphasize PrcC, NadR, HflX peaks in graph*

8.4. Notably, some true targets also gave variable false negative binding fractions. To clarify further, researchers used purified proteins to test the binding again **[1]**.

8.4.1. LABMEDIA: Figure 3 B, C. *Video Editor: Emphasize on red circles.*

Conclusion

9. Conclusion Interview Statements

9.1. **Muriel Leandra Schicketanz:** Once the target proteins are identified, one can purify them to homogeneity and confirm the interaction strength and specificity by using either DRaCALA, Isothermal Titration Calorimetry or other methods **[1]**.

9.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 7.4.3 for "the target proteins are identified"*

9.2. **Muriel Leandra Schicketanz:** Identifying the target proteins of small signaling molecules paves the way to an in-depth understanding of their roles in ranging from bacterial virulence to human biology **[1]**.

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