Submission ID #: 61731
Scriptwriter Name: Anastasia Gomez
Project Page Link: https://www.jove.com/account/file-uploader?src=18828003

Title: MS2-Affinity Purification Coupled with RNA Sequencing in Gram-Positive Bacteria

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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? **No**

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**.

   ☒ Interview Statements are read by JoVE's voiceover talent.

4. **Filming location:** Will the filming need to take place in multiple locations? **No**

Current Protocol Length

Number of Steps: 13
Number of Shots: 38

Introduction

1. Introductory Statements

NOTE to VO Talent: Please record all introduction and conclusion statements.

1.1. MS2-Affinity Purification coupled with RNA Sequencing, or MAPS *(pronounce 'maps')*, has been developed to identify the whole targetome of any RNA of interest in bacteria. This technology makes it possible to identify any kind of sRNA partners, independently of the mechanism of regulation.

1.1.1. Suggested: Figure 1.

1.2. The characterization of sRNA -dependent regulatory networks will help understand better virulence factors production, biofilm formation and survival of the bacteria within the host cells, and ultimately fight against staphylococcal infections.

1.2.1. Suggested: Figure 2.

1.3. This protocol can be applied to any pathogenic or nonpathogenic Gram-positive bacteria.

Introduction of Demonstrator on Camera

1.4. Demonstrating the procedure will be Noémie Mercier, a PhD student from Dr. Pascale Romby's laboratory.

1.4.1. INTERVIEW: Author saying the above.

1.4.2. The named demonstrator(s) looks up from workbench or desk or microscope and acknowledges the camera.

Protocol

2. Bacteria Harvesting and Lysis

2.1. To begin, grow one colony of strains carrying either pCN51-P3-MS2-sRNA *(spell out 'P-C-N-51-P-3-M-S-2-S-R-N-A')* or pCN51-P3-MS2 *(spell out 'P-C-N-51-P-3-M-S-2')* plasmids in 3 milliliters of BHI medium supplemented with 10 micrograms per microliter erythromycin in duplicates **[1]**.

2.1.1. WIDE: Establishing shot of talent taking culture tubes out of the incubator.

2.2. Dilute each overnight culture in 50 milliliters of fresh BHI medium supplemented with erythromycin to reach an OD-600 of 0.05 **[1-TXT]**. Grow the cultures at 37 degrees Celsius with shaking at 180 rpm for 6 hours **[2]**.

2.2.1. Talent adding culture to the flask. **TEXT: 5:1 flask-to-medium ratio**

2.2.2. Talent putting the flask in the incubator and closing the lid.

2.3. Transfer each culture into a 50-milliliter centrifuge tube **[1]** and centrifuge the tubes at 2,900 x *g* for 15 minutes at 4 degrees Celsius **[2]**, then discard the supernatant **[3]**. Keep the pellets on ice and directly perform mechanical cell lysis or freeze and store them at -80 degrees Celsius **[4]**.

2.3.1. Talent transferring the culture into a 50mL tube.

2.3.2. Talent putting the tube in the centrifuge and closing the lid.

2.3.3. Talent discarding the supernatant.

2.3.4. Talent putting the pellet on ice.

2.4. To perform mechanical cell lysis, resuspend pellets in 5 milliliters of Buffer A **[1]** and transfer the resuspended cells to 15-milliliter centrifuge tubes with 3.5 grams of silica beads **[2-TXT]**. Insert the tubes in a mechanical cell lysis instrument and run a cycle of 40 seconds at 4 meters per second **[3]**.

2.4.1. Talent resuspending the pellet, with the Buffer A container in the shot.

2.4.2. Talent transferring the cells to a centrifuge tube with beads. **TEXT: 0.1 mm**

2.4.3. Talent putting the tubes in the mechanical lysis instrument and starting the cycle.

2.5. Centrifuge the tubes at 2,900 x *g* for 15 minutes **[1]**, then recover the supernatant **[2]** and keep it on ice. Make sure that the supernatant is clear, repeating the centrifugation if it is not **[3]**. *Videographer: This step is difficult!*

2.5.1. Talent putting the tubes in the centrifuge and closing the lid.

2.5.2. Talent recovering supernatant.

2.5.3. Talent putting the supernatant on ice.

3. MS2-affinity Purification

3.1. Remove the column tip **[2]**, then put a chromatography column in a column rack **[1]** and wash the column with ultrapure water **[3]**. Add 300 microliters of amylose resin **[added - 4]** and wash the column with 10 milliliters of Buffer A **[5]**. *Videographer: This step is important!*

3.1.1. Talent putting a chromatography column in a rack. NOTE: Move 3.1.2 before 3.1.1

3.1.2. Talent removing the column tip.

3.1.3. Talent washing the column with water.

Added shot: Talent shake the bottle containing the amylose resin

3.1.4. Talent adding amylose resin.

3.1.5. Talent washing the column with Buffer A, with the Buffer A container in the shot.

3.2. Dilute 1,200 picomole of MBP-MS2 protein in 6 milliliters of Buffer A **[1]** and load it into the column **[2]**. Wash the column with 10 milliliters of Buffer A **[3]**. *Videographer: This step is important!*

3.2.1. Talent diluting protein in Buffer A.

3.2.2. Talent loading the protein into the column.

3.2.3. Talent washing the column.

3.3. Next, perform MS2-affinity purification. Load the cell lysate into the column **[1]** and collect the flow-through fraction in a clean collection tube **[2]**. *Videographer: This step is important!*

3.3.1. Talent loading the cell lysate into the column.

3.3.2. Talent collecting the flow-through fraction.

3.4. Wash the column 3 times with 10 milliliters of Buffer A and collect the wash fraction **[1]**, then elute the column with 1 milliliter of Buffer E and collect the elution fraction in a 2-milliliter microtube **[2]**. Keep all collected fractions on ice until RNA extraction **[3]**. *Videographer: This step is important!*

3.4.1. Talent washing the column and collecting the wash fraction.

3.4.2. Talent eluting the column and collecting the elution fraction.

3.4.3. Talent putting the fractions on ice.

4. RNA Extraction of Collected Fractions (CE, FT, W and E)

4.1. Use 1 milliliter of each fraction for RNA extraction. Add 1 volume of phenol to the RNA and mix vigorously **[1-TXT]**, then centrifuge the mixture at 16,000 x *g* for 10 minutes at 20 degrees Celsius **[2]**. Transfer the upper phase to a clean 2-milliliter microtube **[3]**. Add 1 volume of chloroform-isoamyl alcohol and repeat the centrifugation **[4]**.

4.1.1. Talent adding phenol to the RNA and mixing, with the phenol container in the shot. **TEXT: CAUTION: Work under a fume hood!**

4.1.2. Talent putting the tubes in the centrifuge and closing the lid.

4.1.3. Talent transferring the upper phase to a new tube.

4.1.4. Talent adding chloroform/isoamyl alcohol to the tube and placing the tube in the centrifuge.

4.2. Then, transfer the upper phase to a new tube **[1]**. Add 2.5 volumes of 100% cold ethanol and 0.1 volume of 3 molar sodium acetate at pH 5.2 **[2]** and precipitate overnight at -20 degrees Celsius **[3]**.

4.2.1. Talent transferring the upper phase to a new tube.

4.2.2. Talent adding ethanol and sodium acetate to the tube, with the ethanol and sodium acetate containers in the shot.

4.2.3. Talent putting the tubes in the freezer and closing the door.

4.3. On the next day, centrifuge the tubes for 15 minutes at 16,000 x *g* and 4 degrees Celsius **[1]**. Slowly remove the ethanol with a pipette, taking care to not disturb the pellet **[2]**.

4.3.1. Talent putting the tubes in the centrifuge and closing the lid.

4.3.2. Talent removing the ethanol.

4.4. Add 500 microliters of 80% cold ethanol **[1]** and centrifuge for 5 minutes **[2]**. Discard the ethanol by pipetting it slowly **[3]**, then dry the pellet using a vacuum concentrator for 5 minutes on run mode **[4]**. Resuspend the pellet in an appropriate volume of ultrapure water **[5]**. *Videographer: This step is difficult!*

4.4.1. Talent adding 80% ethanol to the tube.

4.4.2. Talent putting the tube in the centrifuge and closing the lid.

4.4.3. Talent removing the ethanol.

4.4.4. Talent using the vacuum concentrator.

4.4.5. Talent resuspending the pellet.

Results

5. Results: Constructs Validation and MAPS Controls

5.1. This protocol was used to study the RsaC *(spell out 'R-S-A-C')* targetome in *S. aureus*. Several mRNAs interacting directly with RsaC were identified using MAPS technology, revealing its crucial role in oxidative stress and metal-related responses **[1]**.

5.1.1. LAB MEDIA: Figure 3 A.

5.2. To confirm the MS2-sRNA constructs and visualize their pattern of expression, cells were harvested after 2, 4, and 6 hours of growth in BHI medium. RNA was extracted and Northern blot analysis was performed using the RsaC-specific DIG probe **[1]**. The level of endogenous RsaC significantly increased after 6 hours of growth **[2]**.

5.2.1. LAB MEDIA: Figure 3 A.

5.2.2. LAB MEDIA: Figure 3 A. *Video Editor: Emphasize the WT lanes, 1 – 3.*

5.3. Importantly, the levels of MS2-RsaC$_{544}$ *(spell out 'M-S-2-R-S-A-C-544')* and MS2-RsaC$_{1,116}$ *(spell out 'M-S-2-R-S-A-C-1116')* were comparable to endogenous RsaC at 6 hours **[1]**.

5.3.1. LAB MEDIA: Figure 3 A. *Video Editor: Emphasize lanes 7 – 12.*

5.4. Affinity purification was performed and RNAs were extracted from the crude extract, flow-through, and elution fractions in the wild type strain expressing the MS2 tag alone and the mutant *rsaC* strain expressing the MS2-RsaC$_{544}$ construct **[1]**.

5.4.1. LAB MEDIA: Figure 3 B.

5.5. The 1,116-nucleotide long endogenous RsaC was enriched in the elution fraction but interacted non-specifically with the affinity column due to its length and complex secondary structure **[1]**.

5.5.1. LAD MEDIA: Figure 3 B. *Video Editor: Emphasize lanes 2 – 3*.

5.6. The MS2-RsaC$_{544}$ was highly enriched in the elution fraction, demonstrating that it was successfully retained by the MS2-MBP fusion protein **[1]**.

5.6.1. LAB MEDIA: Figure 3 B. *Video Editor: Emphasize lane 6.*

5.7. A bioinformatic analysis revealed putative mRNA targets according to the Fold-change between MS2-sRNA and MS2 control, indicating that *sodA* *(pronounce 'sod-A')* was the first putative target **[1]**.

5.7.1. LAB MEDIA: Figure 2.

5.8. A Northern blot analysis performed with a *sodA*-specific DIG probe after MS2-affinity purification shows that *sodA* was efficiently co-enriched with MS2-RsaC$_{544}$ compared to the MS2 control **[1]**.

5.8.1. LAB MEDIA: Figure 3 C.

Conclusion

6. Conclusion Interview Statements

6.1. When attempting this protocol, keep in mind that the addition of the MS2-tag can affect sRNA conformation, stability, or function. This should be carefully monitored.

6.1.1. *Suggested: 3.2.2, 3.3.1.*

6.2. MAPS provides a snapshot of sRNA-RNA targets pairings. Further experimental validation will unravel their function. This technique represents a powerful tool to build sRNA regulatory networks in any bacteria.

6.2.1. *Suggested: Figure 2.*