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Title: In-Vitro Reconstitution of Bacterial Ubiquitination and VCP/p97-Mediated Elimination

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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. 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. **Yes**

Current Protocol Length

Number of Steps: 16

Number of Shots: 43

Introduction

INTRODUCTION:

- 1.1. **Anirban Banerjee**: Our work reveals how VCP/p97 acts as a cytosolic defender against bacteria, unlocking new directions for future therapeutics and bioengineering strategies.

1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 2.3.1*

What technologies are currently used to advance research in your field?

- 1.2. **Anirban Banerjee**: In addition to routine biochemistry, super-resolution microscopy, Cryo-EM, and Cryo-ET reveal how host effectors organize to destroy the pathogen.

1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 2.5.1*

CONCLUSION:

What research gap are you addressing with your protocol?

- 1.3. **Anirban Banerjee**: Our protocol isolates VCP/p97's role using in-vitro reconstitution system, avoiding the complex effect of multiple factors in bacterial clearance.

1.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 3.3.1*

How will your findings advance research in your field?

- 1.4. **Anirban Banerjee**: Our findings could allow exploration to study how different ubiquitin ligases modify various pathogens and investigate effector mechanisms to clear the threat.

1.4.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 4.2.1*

What questions will future research focus on?

- 1.5. **Anirban Banerjee**: Our upcoming research aims to investigate how VCP/p97 is regulated and explore ways to manipulate it precisely to enhance bacterial clearance.
- 1.5.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 4.3.1*

Testimonial Question:

How do you think publishing with JoVE will enhance the visibility and impact of your research?

- 1.6. **Anirban Banerjee, Professor at the Dept of Biosciences and Bioengineering, Indian Institute of Technology Bombay**: (authors will present their testimonial statements live)

Can you share a specific success story or benefit you've experienced—or expect to experience—after using or publishing with JoVE? (This could include increased collaborations, citations, funding opportunities, streamlined lab procedures, reduced training time, cost savings in the lab, or improved lab productivity.)

- 1.7. **Anirban Banerjee, Professor at the Dept of Biosciences and Bioengineering, Indian Institute of Technology Bombay**: (authors will present their testimonial statements live)

Protocol

2. Setting Bacterial Culture for Ubiquitination Assay

Demonstrator: Udit Kumar Das

- 2.1. To begin, grow the *Streptococcus pneumoniae* R6 strain, Serotype II (2), overnight at 37 degrees Celsius with 5 percent carbon dioxide in Todd Hewitt yeast medium [1-TXT].
 - 2.1.1. WIDE: Talent placing the bacterial culture in an incubator set to 37 degrees Celsius with 5 percent carbon dioxide. **TXT: Todd Hewitt Yeast medium: Todd Hewitt broth powder + yeast extract powder**
- 2.2. Inoculate 1 part of the overnight culture into 10 parts of fresh Todd Hewitt yeast medium [1] and incubate at 37 degrees Celsius with 5 percent carbon dioxide until the optical density at 600 nanometers reaches 0.4 [2]. Then, mix 900 microliters of the *Streptococcus pneumoniae* culture with 600 microliters of 50 percent glycerol [3] and store the glycerol stock at minus 80 degrees Celsius [4].
 - 2.2.1. Talent pipetting overnight culture into fresh medium.
 - 2.2.2. Talent placing the culture in the incubator.
 - 2.2.3. Talent pipetting 900 microliters of culture and 600 microliters of glycerol into a labeled cryovial.
 - 2.2.4. Talent placing the cryovial into a minus 80 degrees Celsius freezer.
- 2.3. Then, thaw a fresh stock of *Streptococcus pneumoniae* from minus 80 degrees Celsius [1] and completely inoculate it into 5 milliliters of Todd Hewitt yeast medium [2-TXT].
 - 2.3.1. Talent removing cryovial from the minus 80 degrees Celsius freezer and thawing it.
 - 2.3.2. Talent inoculating the thawed culture into 5 milliliters of Todd Hewitt yeast medium. **TXT: Incubate until OD₆₀₀ reaches 0.4**

3. In-Vitro Ubiquitination Assay from Mammalian Lysate

Demonstrator: Sumit Rakshit

- 3.1. Take approximately 10^7 *Streptococcus pneumoniae* cells [1]. To wash the cells thoroughly, resuspend the bacteria in the reaction buffer [2] and centrifuge at 21,000 g for 5 minutes at room temperature [3-TXT].
 - 3.1.1. Talent transferring *Streptococcus pneumoniae* culture into a centrifuge tube.

- 3.1.2. Talent adding reaction buffer to the cells and mixing them.
- 3.1.3. Talent centrifuging the washed bacterial suspension at 21,000 g for 5 minutes.
TXT: Repeat centrifugation 1x; Wash 3x

3.2. Resuspend the pellet with A549 (*A-Five-Forty-Nine*) mammalian cell lysate [1]. Then, add purified ubiquitin and 1 millimolar adenosine triphosphate [2] and incubate the mixture for 1 to 2 hours at 27 degrees Celsius [3].

- 3.2.1. Talent resuspending the bacterial pellet in A549 mammalian cell lysate.
- 3.2.2. Talent adding purified ubiquitin and adenosine triphosphate to the mixture.
- 3.2.3. Talent placing the mixture into an incubator set at 27 degrees Celsius.

3.3. Then, wash the bacteria once with reaction buffer containing 1 molar urea, followed by two additional washes with only the reaction buffer [1]. Fix the bacteria with 1 percent paraformaldehyde [3]. After fixation, incubate the cells with PBS containing 1 milligram per milliliter glycine for 15 minutes [4], then wash twice with PBS [5].

- 3.3.1. Talent adding the bacteria with reaction buffer containing 1 molar urea.
- 3.3.2. Talent adding 1 percent paraformaldehyde.
- 3.3.3. Talent placing the sample aside.
- 3.3.4. Talent adding the cells with PBS.

4. In-Vitro Ubiquitination Assay and Visualization of Ubiquitinated Bacteria

4.1. Prepare a reaction mixture by combining ubiquitin with other required components in the reaction buffer [1-TXT]. Adjust the total volume of the reaction mixture to 100 to 150 microliters [2].

- 4.1.1. Talent pipetting the components sequentially into a microcentrifuge tube with all the labelled reagents in the frame. **TXT: Reaction components: Ubiquitin (350 μ M); UBE1 (400–800 nM); Ube2C (3 - 5 μ M); Rbx1-Skp1-Cul1-Fbxw7 complex (500 nM); 2 mM ATP; Reaction buffer**
- 4.1.2. Talent adjusting the final volume of the reaction mixture using reaction buffer.

4.2. Resuspend the bacteria in the ubiquitin enzyme-containing reaction mixture [1] and incubate the suspension for 2 hours at 27 degrees Celsius [2]. After washing the bacteria three times with reaction buffer containing urea, fix them with 1 percent paraformaldehyde [3]. After fixation, incubate the cells with PBS containing 1 milligram per milliliter glycine for 15 minutes [4-TXT].

- 4.2.1. Talent resuspending bacteria in the prepared reaction mixture.
- 4.2.2. Talent placing the tube into an incubator set at 27 degrees Celsius. Same as 4.3.1
- 4.2.3. Talent adding 1% PFA to the bacteria.
- 4.2.4. Talent keeping the sample added with PBS aside for incubation. **TXT: Wash the sample 2x with PBS**

- 4.3. Then, incubate the sample in 3 percent BSA [0] for 1 hour at 27 degrees Celsius [1] and wash the sample twice with PBS [2].
 - 4.3.0. Added shot: Add with 3% BSA to the fixed sample.
 - 4.3.1. Talent placing the fixed sample at 27 degrees Celsius.
 - 4.3.2. Talent removing the BSA solution.

- 4.4. Resuspend the ubiquitinated bacteria in a primary antibody solution prepared at higher concentrations than typically used in immunofluorescence assays [1-TXT]. Then, incubate for 1 hour at 27 degrees Celsius [2].
 - 4.4.1. Talent resuspending the sample in a high-concentration primary antibody solution. **TXT: Stain with bacteria Ab + Anti-ubiquitin (1:100) in PBS + 1% BSA**
 - 4.4.2. Talent placing the sample with the antibody mix at 27 degrees Celsius for 1 hour.

- 4.5. After washing the bacteria twice with PBS, resuspend them in the corresponding secondary antibody conjugated with Alexa Fluor 488 and Alexa Fluor 555 [1-TXT].
 - 4.5.1. Talent resuspending secondary antibody conjugates Alexa Fluor 488 and 555 to the bacteria. **TXT: Incubation: 27 °C; 1h** NOTE: The shot description is slightly edited

- 4.6. Now, drop-cast approximately 10 to 20 microliters of the labeled bacterial sample onto a glass slide [1] and mix in 5 to 10 microliters of mounting medium, with or without DAPI [2]. Place a coverslip over the sample and use tissue paper to blot any excess liquid [3-TXT].
 - 4.6.1. Talent placing a drop of the bacterial sample onto a slide.
 - 4.6.2. Talent mixing in the mounting medium containing or lacking DAPI.
 - 4.6.3. Talent placing a coverslip and blotting excess fluid with tissue paper. **TXT: Observe the sample under a confocal microscope on the same day**

5. In-Vitro Bacterial Killing Assay

Demonstrator: Udit Kumar Das

- 5.1. Combine 6 micromolar VCP/p97 (*V-C-P-P-ninety-seven*) with 1 micromolar NPLOC4 (*N-P-L-O-C-Four*) and 1 micromolar UFD1 (*U-F-D-One*) [1]. Adjust the volume of the reaction mixture to approximately 90 microliters using reaction buffer [2] and incubate on ice for 2 hours [3].
 - 5.1.1. Talent pipetting VCP/p97, NPLOC4, and UFD1 into a reaction tube.
 - 5.1.2. Talent adding reaction buffer to make up the volume.
 - 5.1.3. Talent placing the tube on ice for incubation.
- 5.2. Next, add 1 to 2 millimolar adenosine triphosphate to the reaction mixture containing p97-UFD1-NPLOC4 [1], then combine this with 10^7 ubiquitinated *Streptococcus pneumoniae* cells [2] and incubate for 2 hours at 37 degrees Celsius [3].
 - 5.2.1. Talent pipetting adenosine triphosphate into the pre-incubated reaction mixture.
 - 5.2.2. Talent adding the ubiquitinated bacteria to the reaction mixture.
 - 5.2.3. Talent placing the tube in a 37 degrees Celsius incubator.
- 5.3. Immediately after treatment, take a 10-microliter aliquot from the reaction mixture [1] and perform a serial dilution [2]. Spot 10 to 20 microliters of each dilution onto Brain Heart Infusion agar plates [3-TXT].
 - 5.3.1. Talent pipetting a 10 microliter sample from the reaction tube.
 - 5.3.2. Talent performing serial dilutions into separate labeled tubes.
 - 5.3.3. Talent spotting each dilution onto Brain Heart Infusion agar plates. **TXT: Use 10 μ L aliquot after 2 h incubation**
- 5.4. Assess colony-forming units between the zero-hour and 2-hour time points [1]. Calculate bacterial viability percentage by dividing the 2-hour CFU count by the zero-hour CFU count and multiplying by 100 [2].
 - 5.4.1. Show CFU plate for both 0 hour and 2 hour samples. **Videographer's NOTE: This was shot in 2 parts.**
 - 5.4.2. Talent working at the computer table to perform calculations. *Videographer: Please film the computer screen*

Results

6. Results

6.1. Significant ubiquitination of *Streptococcus pneumoniae* was observed when treated with either mammalian cell lysate [1] or a reconstituted E1–E2–E3 enzyme system [2].

6.1.1. LAB MEDIA: Figure 2A. *Video editor: Highlight the merged panel in the “Ub + Cell lysate” row showing strong magenta (ubiquitin) signal overlapping with cyan (SPN).*

6.1.2. LAB MEDIA: Figure 2A. *Video editor: Highlight the merged panel in the “Ub + SCF^{FBXW7}” row showing strong magenta signal overlapping with cyan SPN.*

6.2. Ubiquitination signal was absent in the control [1] and markedly diminished when ubiquitinated *Streptococcus pneumoniae* was exposed to OTUB1 (*O-T-U-B-One*), confirming K48-linked polyubiquitin modification [2].

6.2.1. LAB MEDIA: Figure 2A. *Video editor: Highlight the “No Ub” row’s Ub panel image.*

6.2.2. LAB MEDIA: Figure 2A. *Video editor: Highlight the “Ub + OTUB1” row’s Ub panel image .*

6.3. A line profile analysis showed full overlap between ubiquitin and bacterial signals, confirming their spatial colocalization [1]. Three-dimensional reconstruction confirmed the spatial overlap of ubiquitin and *Streptococcus pneumoniae* signals [2].

6.3.1. LAB MEDIA: Figure 2B. *Video editor: Show the line plot graph on the far right.*

6.3.2. LAB MEDIA: Figure 2B. *Video editor: Highlight the “3D reconstruction” panel.*

6.4. Wild-type p97 (*P-Ninety-Seven*), when combined with its cofactors UFD1 (*U-F-D-One*) and NPLOC4, reduced bacterial viability to 10 to 20% after 2 hours, regardless of whether ubiquitination was performed with cell lysate [1] or with purified enzyme system [2].

6.4.1. LAB MEDIA: Figure 3B. *Video editor: Highlight the red line group labeled “p97^{Active} + Ub-SPN” showing a steep decline in viability from 0 h to 2 h.*

6.4.2. LAB MEDIA: Figure 3C. *Video editor: Highlight the red line group labeled “p97^{Active} + Ub-SPN” showing significant drop in bacterial viability over 2 hours.*

6.5. No reduction in bacterial viability was observed when ubiquitination was omitted [1],

when p97 was heat-inactivated [2], or when using the catalytically inactive p97^{E578Q} (*P-ninety-seven-E-five-seven-eight-Q*) mutant, confirming that both ubiquitination and active p97 are essential for bacterial killing [3].

6.5.1. LAB MEDIA: Figure 3B and C. *Video editor: Highlight the lines labeled “p97^{ACTIVE} + Non-Ub-SPN” which remain flat between 0 h and 2 h in B.*

6.5.2. LAB MEDIA: Figure 3B and C. *Video editor: Highlight the lines labeled “p97^{Denature} + Ub-SPN” which remain flat between 0 h and 2 h in B and C.*

6.5.3. LAB MEDIA: Figure 3B and C. *Video editor: Highlight the Green lines labeled “p97^{E578Q} + Ub-SPN” showing no decrease in viability in B.*

1. Streptococcus pneumoniae
Pronunciation link: <https://www.merriam-webster.com/dictionary/Streptococcus%20pneumoniae>
IPA: /ˌstrɛp.təˈkɑːkəs nuːˈmoʊ.ni.iː/
Phonetic Spelling: strep·tuh·kah·kuhs noo·moh·nee·ee
2. Serotype
Pronunciation link: <https://www.merriam-webster.com/dictionary/serotype>
IPA: /ˈsɪr.oʊ.taɪp/
Phonetic Spelling: sir·oh·type
3. Carbon dioxide
Pronunciation link: <https://www.merriam-webster.com/dictionary/carbon%20dioxide>
IPA: /ˌkɑːr.bən daɪˈɑːk.səɪd/
Phonetic Spelling: kar·buhn dye·ok·side
4. Inoculate
Pronunciation link: <https://www.merriam-webster.com/dictionary/inoculate>
IPA: /ɪˈnɑː.kjə.leɪt/
Phonetic Spelling: ih·nah·kyuh·layt
5. Optical density
Pronunciation link: <https://www.merriam-webster.com/dictionary/optical%20density>
IPA: /ˈɑːp.tɪ.kəl ˈdɛn.sə.ti/
Phonetic Spelling: op·ti·kuhl den·suh·tee
6. Microliters
Pronunciation link: <https://www.merriam-webster.com/dictionary/microliter>
IPA: /ˈmaɪ.kroʊ.liː.tər/
Phonetic Spelling: my·kroh·lee·ter
7. Glycerol
Pronunciation link: <https://www.merriam-webster.com/dictionary/glycerol>
IPA: /ˈɡlɪs.ə.ɑːl/
Phonetic Spelling: gli·sir·awl
8. Cryovial
Pronunciation link: <https://www.merriam-webster.com/dictionary/cryovial>

- IPA: /'kraɪ.ʊʊ.vai.əl/
Phonetic Spelling: kry·oh·vy·uhl
9. Ubiquitination
Pronunciation link: <https://www.merriam-webster.com/dictionary/ubiquitination>
IPA: /juːˌbɪk.wɪ.təˈneɪ.ʃən/
Phonetic Spelling: yoo·bik·wuh·tay·shuhn
10. Centrifuge
Pronunciation link: <https://www.merriam-webster.com/dictionary/centrifuge>
IPA: /'sen.trɪˌfjuːdʒ/
Phonetic Spelling: sen·trih·fyooj
11. Lysate
Pronunciation link: <https://www.merriam-webster.com/dictionary/lysate>
IPA: /'laɪ.seɪt/
Phonetic Spelling: ly·sayt
12. Adenosine triphosphate
Pronunciation link: <https://www.merriam-webster.com/dictionary/adenosine%20triphosphate>
IPA: /əˈden.əˌsiːn traɪˈfɑːs.fert/
Phonetic Spelling: uh·den·uh·seen try·fahs·fayt
13. Paraformaldehyde
Pronunciation link: <https://www.merriam-webster.com/dictionary/paraformaldehyde>
IPA: /ˌpær.əˌfɔːrˈmæl.dəˌhaɪd/
Phonetic Spelling: pair·uh·for·mal·duh·hyde
14. Immunofluorescence
Pronunciation link: <https://www.merriam-webster.com/dictionary/immunofluorescence>
IPA: /ɪˌmjuː.nouˌflʊrˈes.əns/
Phonetic Spelling: ih·myoo·noh·floor·ess·uhns
15. Alexa Fluor
Pronunciation link: <https://www.howtopronounce.com/alexa-fluor>
IPA: /əˈlɛk.səˈflʊr/
Phonetic Spelling: uh·lek·suh floor
16. Confocal
Pronunciation link: <https://www.merriam-webster.com/dictionary/confocal>
IPA: /kɑːnˈfoʊ.kəl/
Phonetic Spelling: kon·foh·kuhl
17. VCP
Pronunciation link: <https://www.howtopronounce.com/vcp>
IPA: /ˌviː.siˈpiː/
Phonetic Spelling: vee·see·pee
18. NPLOC4
Pronunciation link: No confirmed link found
IPA: /ˌɛn.piː.loʊˈsiː.fɔːr/
Phonetic Spelling: en·pee·loh·see·for
19. UFD1
Pronunciation link: No confirmed link found

IPA: /ˌjuː.ɛf.diːˈwʌn/

Phonetic Spelling: yoo·ef·dee·wuhn

20. Serial dilution

Pronunciation link: <https://www.merriam-webster.com/dictionary/serial%20dilution>

IPA: /ˈsɪr.i.əl daɪˈluː.ʃən/

Phonetic Spelling: seer·ee·uhl dye·loo·shuhn

21. Colocalization

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

IPA: /ˌkoʊ.loʊ.kæl.əˈzeɪ.ʃən/

Phonetic Spelling: koh·loh·kal·uh·zay·shuhn

22. Polyubiquitin

Pronunciation link: <https://www.howtopronounce.com/polyubiquitin>

IPA: /ˌpɑː.li.juːˈbɪk.wɪ.tɪn/

Phonetic Spelling: pah·lee·yoo·bik·wih·tin

23. Viability

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

IPA: /ˌvaɪ.əˈbɪl.ə.ti/

Phonetic Spelling: vy·uh·bil·uh·tee