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Title: Separation and Differential Characterization of Gut Microbial Extracellular Vesicles in Salt-Sensitive Rats Under High-Salt Diet Conditions

Authors and Affiliations:

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NOTE: The authors updated the email addresses for Luming Qi and Youli Tan.

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? **Yes**

   How far apart are the locations? **400 m**

Current Protocol Length
Number of Steps: 13
Number of Shots: 34

Introduction

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

REQUIRED:

1.1. **Lisha Wang:** This research explores the role of gut microbiota extracellular vesicles in salt-sensitive hypertensive rats on a high-salt diet, examining how high-salt diets alter these vesicles and influence hypertension and immune interactions.

1.1.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: LAB MEDIA: Figure 2A*.

What are the most recent developments in your field of research?

1.2. **Lisha Wang:** Recent advances in this field include understanding the role of gut microbiota-derived extracellular vesicles in hypertension, assessing the impact of high-salt diets on microbial diversity, and implementing plant-based interventions targeting vesicles for metabolic and immune regulation.

What are the current experimental challenges?

1.3. **Lisha Wang:** Current experimental challenges include isolating high-purity extracellular vesicles, distinguishing host-derived vesicles from microbial vesicles, minimizing sample loss, and standardizing protocols to ensure reproducibility across studies.

1.3.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 2.2.3.*

What advantage does your protocol offer compared to other techniques?

1.4. **Lisha Wang:** This density gradient centrifugation protocol provides higher-purity extracellular vesicles with improved integrity and reproducibility compared to traditional methods, enabling more reliable gut microbiota–extracellular vesicle research in hypertension studies.

1.4.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 4.6.1.*

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

Testimonial Questions:

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

1.5. **Lisha Wang:** Publishing with JoVE amplifies research impact through visual protocols that improve reproducibility and collaboration. The video format attracts interdisciplinary scientists, accelerating partnerships in gut microbiota–extracellular vesicle research. Greater visibility fosters innovation in hypertension studies, increases citations, and supports the standardization of methods for broader adoption.

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

Can you share a specific success story or benefit you've experienced—or expect to experience—after using or publishing with JoVE?

1.6. **Lisha Wang:** Publishing with JoVE will reduce laboratory costs by minimizing experimental errors through clear visual protocols. The standardized video format shortens training time for new team members and streamlines workflows, enhancing productivity while ensuring consistent, reproducible results in gut microbiota–extracellular vesicle isolation.

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

Ethics Title Card

This research has been approved by the Laboratory Animal Welfare and Ethics Committee of Chengdu University of Chinese Medicine

Protocol

2. Sample Preparation for the Extraction of Extracellular Vesicles

Demonstrator: Lisha Wang

2.1. To begin, transfer 5 grams of feces into a pre-weighed 50-milliliter centrifuge tube **[1]**. Add 50 milliliters of pre-heated 37 degrees Celsius endotoxin-free PBS to the tube **[2]** and rotate the tube for 30 minutes **[3]**. Cool the high-speed centrifuge to 4 degrees Celsius **[4]**.

2.1.1. WIDE: Talent using a spoon to transfer feces into a pre-weighed 50-milliliter centrifuge tube.

2.1.2. Talent adding 50 milliliters of pre-heated 37 degrees Celsius PBS into the centrifuge tube.

2.1.3. Talent placing the tube on a rotator.

2.1.4. Talent setting the centrifuge temperature to 4 degrees Celsius.

2.2. Next, place the samples symmetrically in the centrifuge **[1]** and spin at 8,000 *g* for 15 minutes **[2]**. After centrifugation, aspirate the supernatant and transfer it into a new sterile centrifuge tube **[3]**.

2.2.1. Talent loading samples symmetrically into a centrifuge.

2.2.2. Talent setting the centrifuge to 8,000 *g*.

2.2.3. Talent aspirating the supernatant and transferring it to a new sterile centrifuge tube.

2.3. After centrifuging again for 15 minutes, aspirate the supernatant carefully and use it for further analysis **[1]**.

2.3.1. Talent aspirating the supernatant and placing it aside.

3. Crude Extract Preparation

3.1. Place a sterile 0.22-micrometer filter unit on ice **[1]**. Transfer the supernatant onto the top of the filter **[2]** and turn on the vacuum pump **[3]** to collect the filtered samples **[4].**

3.1.1. Talent placing a sterile 0.22 micrometer filter unit on ice.

3.1.2. Talent transferring the supernatant onto the filter unit.

3.1.3. Talent turning on the vacuum pump to filter the sample.

3.1.4. A shot of the collected filtered samples.

3.2. Transfer the filtrate into a centrifugal filter with a 10-kilodalton cutoff and a 15-milliliter capacity **[1]**. Centrifuge at 4 degrees Celsius, 3,000 *g* for 30 minutes **[2]** to concentrate the sample to at least 1,400 microliters **[3-TXT]**. Immediately place the sample on ice **[4]**.

3.2.1. Talent transferring the filtrate to a centrifugal filter device.

3.2.2. Talent placing the filtrate obtained from the centrifugal filter device into a centrifuge.

3.2.3. A shot of the concentrated sample. **TXT: If needed, dilute the concentrated sample to 1400 µL with pre-cooled endotoxin-free PBS**

3.2.4. Talent placing the sample on ice.

4. Separation Procedure for Extracellular Vesicles

Videographer: Please make sure the labels on the reagents are clearly visible in the frame so that the shots don't look the same on camera.

4.1. Mix 1 volume of gradient buffer A with 5 volumes of density gradient medium to prepare the working solution **[1-TXT]**.

4.1.1. Talent mixing gradient buffer A and density gradient medium to prepare a working solution. **TXT: Density gradient medium: 60% iodixanol solution**

4.2. Prepare a 10% iodixanol solution by mixing 1 unit of working solution with 4 units of buffer B **[1]**. Similarly, mix 2 units of working solution with 3 units of buffer B for a 20% iodixanol solution and 4 units of working solution with 1 unit of buffer B for a 40% iodixanol solution **[2].**

4.2.1. Talent preparing a 10% iodixanol solution by mixing 1 unit of working solution with 4 units of buffer B.

4.2.2. A shot of the prepared 20% iodixanol solution and 40% iodixanol solution placed on ice. *Videographer: Please make sure the labels of the iodixanol solutions are clearly visible in the frame.*

4.3. Now, mix the concentrated sample prepared earlier with 7 milliliters of density gradient medium to make a 50% iodixanol solution **[1]**.

4.3.1. Talent mixing the concentrated sample with 7 milliliters of density gradient medium to prepare a 50 percent iodixanol solution.

4.4. To prepare a density gradient, add trypan blue solution to the 40% and 10% iodixanol solutions **[1]**. NOTE: The VO has been edited.

4.4.1. Talent adding trypan blue solution to 40 percent and 10 percent iodixanol solutions.

~~4.4.2. CU: A shot of the clear outline between the distinct layers.~~ NOTE: 4.4.2 has been removed.

Author's NOTE: Step 4.4.2 should be taken after step 4.5. It is the same operation as step 4.6.1. These two steps are repeated, and we missed the shooting of this step during the shooting process, but we will provide relevant pictures to explain this.

4.5. Transfer 8 milliliters of 50% iodixanol solution to the bottom of a thin-wall polypropylene centrifuge tube **[1]**. Tilt the tube to 70 degrees and transfer 8 milliliters of 40% iodixanol solution to the liquid surface **[2]**. Next, add 8 milliliters of 20% iodixanol solution **[3]**, followed by 7 milliliters of 10% iodixanol solution **[4]**, and 2 milliliters of endotoxin-free PBS **[5]**.

4.5.1. Talent transferring 8 milliliters of 50% iodixanol solution to the bottom of a thin-wall polypropylene centrifuge tube.

4.5.2. Talent tilting the tube and layering 8 milliliters of 40% iodixanol solution on top.

4.5.3. Talent adding 8 milliliters of 20% iodixanol solution to the tube.

4.5.4. Talent adding 7 milliliters of 10% iodixanol solution to the tube.

4.5.5. Talent adding 2 milliliters of endotoxin-free PBS to the tube.

4.6. Take the prepared density gradient tube **[1]** and place it into a pre-cooled ultracentrifuge **[2]**. Set the ultracentrifugation parameters to 100,000 *g*, 20 hours, and 10 degrees Celsius, and start the run **[3]**. NOTE: The VO has been edited.

4.6.1. A shot of the prepared density gradient tube. NOTE: Authors requested to use LAB MEDIA: 4.6.1-A-shot-of-the-prepared-density-gradient-tube.jpg for 4.6.1.

4.6.2. Talent placing the prepared density gradient tube in an ultracentrifuge.

4.6.3. Talent setting the ultracentrifuge parameters to 100,000 *g*, 20 hours, 10 degrees Celsius and starting the run. NOTE: The centrifugal force in step 4.6.3 has been changed to 100,000 *g* as the authors suggested.

4.7. For manual gradient density separation for the collection, the density gradient is about 34 milliliters of liquid. Two milliliters of solution are slowly extracted from the center of the liquid surface, and each 2 milliliter solution is a gradient, so the density gradient solution is divided into 17 gradients. Finally, using a pipette, transfer the density fractions into sterile sample tubes **[1]**. NOTE: The VO has been edited.

4.7.1. Talent slowly extracting 2 milliliters of gradient solution. NOTE: This shot was changed during the shoot. Author's NOTE: Step 4.7.1 should be to separate 2ml of liquid from the system, not to add.

~~4.7.2. A shot of the density gradient solution divided into 17 gradients.~~ Author's NOTE: steps 4.7.1, 4.7.2 and 4.8.1 should be the same step.

4.8. ~~Finally, using a pipette, transfer the density fractions into sterile sample tubes **[1]**~~ and immediately place them on ice **[2]**.

~~4.8.1. Talent using a pipette to transfer the density fractions into sterile tubes.~~ Author's NOTE: steps 4.7.1, 4.7.2, and 4.8.1 should be the same step. The VO of 4.8.1 has been added to 4.7.

4.8.2. Talent placing the tubes on ice.

Results

5. Results

5.1. Nanoparticle tracking analysis revealed the size distribution and concentration profiles of extracellular vesicles collected from different density gradient fractions **[1]**. The majority of extracellular vesicles across all fractions were concentrated within the 30 to 100 nanometer size range, following a typical distribution pattern **[2]**.

5.1.1. LAB MEDIA: Figure 2A.

5.1.2. LAB MEDIA: Figure 2A. *Video Editor: Highlight the black plots in all graphs.*

5.2. The concentration of extracellular vesicles reached its highest level in fraction 9, peaking at approximately 3.85×10^9 *(three point eight five times ten to the power of nine)* particles per milliliter **[1]**.

5.2.1. LAB MEDIA: Figure 2B. *Video Editor: Highlight the pink bar at 9.*

5.3. The protein content, measured using a bicinchoninic acid kit, was also highest in fraction 9 at 0.417 micrograms per microliter **[1]**.

5.3.1. LAB MEDIA: Figure 2C. *Video Editor: Highlight the pink bar at 9.*

5.4. Additionally, lipopolysaccharide expression levels, determined using an endotoxin detection kit, were significantly elevated in fractions 9 and 10 **[1]**.

5.4.1. LAB MEDIA: Figure 2D. *Video Editor: Highlight the bars at 9 and 10.*

5.5. Greater amounts of protein were also observed in fraction 9 during sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis **[1]**.

5.5.1. LAB MEDIA: Figure 2E.

5.6. Transmission electron microscopy or TEM *(T-E-M)* images revealed that extracellular vesicles possessed a circular membrane-like morphology **[1]**.

5.7.1. LAB MEDIA: Figure 2F.

Pronunciation Guides:

1. Iodixanol

- **Pronunciation Link:** https://www.howtopronounce.com/iodixanol
- **IPA:** /ˌaɪ.oʊˈdɪk.sə.nɔːl/
- **Phonetic Spelling:** eye-oh-DIK-suh-nawl

2. Trypan Blue

- **Pronunciation Link:** https://www.howtopronounce.com/trypan
- **IPA:** /ˈtraɪ.pæn bluː/
- **Phonetic Spelling:** TRY-pan bloo

3. Polypropylene

- **Pronunciation Link:** https://www.merriam-webster.com/dictionary/polypropylene
- **IPA:** /ˌpɒ.liˈproʊ.pəˌliːn/
- **Phonetic Spelling:** pah-lee-PROH-puh-leen

4. Ultracentrifuge

- **Pronunciation Link:** https://www.howtopronounce.com/ultracentrifuge
- **IPA:** /ˌʌl.trəˈsɛn.trəˌfjuːdʒ/
- **Phonetic Spelling:** UL-truh-SEN-truh-fyooj

5. Nanoparticle

- **Pronunciation Link:** https://www.howtopronounce.com/nanoparticle
- **IPA:** /ˈnæn.oʊˌpɑːr.tɪ.kəl/
- **Phonetic Spelling:** NAN-oh-par-ti-kuhl

6. Bicinchoninic Acid

- **Pronunciation Link:** No confirmed link found
- **IPA:** /ˌbaɪ.sɪn.kəˈnɪn.ɪk ˈæs.ɪd/
- **Phonetic Spelling:** BY-sin-ko-NIN-ik ASS-id

7. Lipopolysaccharide

- **Pronunciation Link:** https://www.howtopronounce.com/lipopolysaccharide
- **IPA:** /ˌlaɪ.poʊˌpɒl.iˈsæk.ə.raɪd/
- **Phonetic Spelling:** LIE-poh-pol-ee-SACK-uh-ride

8. Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis

- **Pronunciation Link:**
  No confirmed link found
- **IPA:** /ˈsoʊ.di.əm ˈdoʊ.dɛ.sɪl ˈsʌl.feɪt ˌpɒl.iˈæk.rɪ.lə.maɪd dʒɛl ɪˌlɛk.trə.fəˈri:.sɪs/
- **Phonetic Spelling:** SOH-dee-um DOH-deh-sil SUL-fate POL-ee-ACK-rih-luh-mide jel ee-LEK-troh-foh-REE-sis

9. Transmission Electron Microscopy

- **Pronunciation Link:**
  https://www.howtopronounce.com/transmission-electron-microscopy
- **IPA:** /trænsˈmɪʃ.ən ɪˈlɛk.trɒn maɪˈkrɒs.kə.pi/
- **Phonetic Spelling:** trans-MISH-un ee-LEK-tron my-KROSS-kuh-pee