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Title: Phenol Red Thread-Based Sampling Procedure for Untargeted Tear Fluid Lipidomics in Biomarker Discovery

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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. **No**

Current Protocol Length

Number of Steps: 25

Number of Shots: 43

Introduction

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

INTRODUCTION:

~~What is the scope of your research? What questions are you trying to answer?~~

- 1.1. **Kenrick Chan:** We establish a more standardized, easy-to-operate Phenol Red Thread-based method to help achieve reproducible tear lipidomic profiles for future tear biomarker discovery.
 - 1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

~~What are the current experimental challenges?~~

- 1.2. **Hang Li:** The current challenge is that the Schirmer strips irritate, while capillaries show operator variability in tear collection. This hinders experimental reproducibility and inter-study comparisons.
 - 1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

CONCLUSION:

~~What research gap are you addressing with your protocol?~~

- 1.3. **Kenrick Chan:** This protocol addresses the absence of a standardized workflow for reproducible tear lipidomic analysis and can enhance inter-study comparability.
 - 1.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

~~What advantage does your protocol offer compared to other techniques?~~

- 1.4. **Hang Li:** This PRT-based method minimizes irritation, enables rapid tear collection, and reduces operator variability.
 - 1.4.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

~~What questions will future research focus on?~~

- 1.5. **Kenrick Kai-Yuen Chan:** Our future research will validate tear lipid biomarkers in large cohorts and integrate multi-omics for personalized diagnostics.
 - 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 Review Board (IRB) at the Hong Kong Polytechnic University

Protocol

2. Phenol Red Thread (PRT)-Based Collection of Human Tear Fluid

Demonstrators: Hang Li, Jimmy Cheung

- 2.1. To begin, wear gloves and disinfect the workstation thoroughly to prevent any contamination of the sample [1].
 - 2.1.1. WIDE: Talent wearing gloves and wiping down the entire workstation with disinfectant.
- 2.2. Hold the Phenol Red Thread by the end opposite the bent hook, making sure not to touch the bent hook region [1].
 - 2.2.1. Talent carefully gripping the PRT by its non-hooked end, avoiding contact with the bent hook side.
- 2.3. Ask the subject to direct their gaze towards the superior nasal direction [1].
 - 2.3.1. Talent gently instructing the subject to look towards the upper inside corner of their eye.
- 2.4. Then, pull down the subject's lower eyelid gently [1] and insert the Phenol Red Thread with the bent hook placed in the lower temporal palpebral conjunctiva near the outer corner of the eye [2].
 - 2.4.1. Talent gently pulling down the subject's lower eyelid.
 - 2.4.2. Talent precisely placing the PRT in the indicated conjunctival area near the outer canthus.
- 2.5. Now, ask the subject to close their eyes gently [1] and tilt their head slightly forward to prevent the Phenol Red Thread from contacting the skin on the face [2].
 - 2.5.1. Subject closing their eyes.
 - 2.5.2. Subject tilting their head forward.
- 2.6. Pull down the subject's lower eyelid again [1] and gently remove the Phenol Red Thread, avoiding contact with the sampled region [2].

2.6.1. Talent pulling down the eyelid.

2.6.2. Close-up of carefully extracting the PRT without touching the red-dyed segment.

2.7. Record the length of the sampled red region [1] and, using sterile forceps, transfer the PRT into a new sample tube [2].

2.7.1. Talent measuring the dyed region on the PRT.

2.7.2. Talent using sterile forceps to place the thread into a sample tube.

2.8. If the sampled length is less than 50 millimeters, ask the subject to rest for 1 to 3 minutes and then repeat the tear fluid collection using the same eye [1]. Place each newly sampled PRT into a separate sample tube [2].

2.8.1. Talent speaking to the subject and subject resting.

2.8.2. Talent placing a fresh tube with thread beside the first tube.

2.9. Now, label and cap sample tube properly [1].

2.9.1. Talent writing on sample tube labels and securely capping each one.

AUTHOR'S NOTE: Move shot 2.9.1 below shot 2.7.2

2.10. Using methanol and clean paper wipes, clean the gloves and forceps thoroughly to eliminate any tear residues or contaminants [1], then allow them to air dry completely [2-TXT].

2.10.1. Talent wiping the gloves and forceps with methanol-soaked paper.

2.10.2. Talent placing forceps on a clean surface and let dry. **TXT: Repeat the process for the subject's other eye**

2.11. Seal all sample tubes with parafilm [1] and immediately store them at minus 20 degrees Celsius for temporary storage or minus 80 degrees Celsius for long-term storage [2].

2.11.1. Talent sealing tubes with parafilm.

2.11.2. Talent placing the sealed tubes into designated freezer.

3. MTBE/Methanol Biphasic Separation for Lipid Extraction from Sampled PRT

Demonstrators: Kenrick Chan, Jimmy Cheung

- 3.1. Pre-wash the micro-scissors and forceps with methanol before processing the Phenol Red Threads [1].
 - 3.1.1. Talent rinsing a pair of micro-scissors and forceps thoroughly with methanol and drying by wiping paper.
- 3.2. Cut off the bent hook end, approximately the first 3 millimeters, from each Phenol Red Thread [1].
 - 3.2.1. Talent cutting off the bent hook segment of the PRT
- 3.3. Cut the sampled region up to the phenol red dye front into 2-millimeter pieces [1] and collect these segments with a clean 1.5-milliliter organic solvent-tolerant microcentrifuge tube [2]. Discard the bent hook end and unwanted region of PRT [3].
 - 3.3.1. Talent cutting the red-dyed region of the PRT into multiple 2-millimeter pieces. AUTHOR'S NOTE: This step was filmed together with shot 3.2.1
 - 3.3.2. Talent placing the cut pieces in a labeled organic solvent-tolerant microcentrifuge tube AUTHOR'S NOTE: This step was filmed together with shot 3.2.1. The tube is placed on the right side

Added shot: Talent discarding the bent hook end and unwanted region of PRT to waste bucket.
- 3.4. Place the samples on ice to maintain a low temperature before extraction [1].
 - 3.4.1. Talent placing microcentrifuge tubes containing samples onto an ice-filled container.
- 3.5. Now, add 232 microliters of ice-cold mass spectrometry grade methanol to each sample [1] and vortex for 15 seconds to mix thoroughly [2].
 - 3.5.1. Talent pipetting methanol into each microcentrifuge tube on ice.
 - 3.5.2. Talent vortexing the tubes one by one for 15 seconds.
- 3.6. Sonicate the samples in a pre-cooled ultrasonic cleanser for 15 minutes [1].
 - 3.6.1. Talent placing the tubes into the ultrasonic cleanser.
- 3.7. Then, add 774 microliters of HPLC (H-P-L-C) grade methyl tert-butyl ether and 194 microliters of deionized water to each sample [1]. Vortex again for 15 seconds to mix

the solvents thoroughly [2].

3.7.1. Talent pipetting MTBE and deionized water into each sample tube.

3.7.2. Talent vortexing the tubes for 15 seconds.

3.8. Sonicate the samples again in a pre-cooled ultrasonic cleanser for another 15 minutes [1].

3.8.1. Talent placing the tubes back into the ultrasonic cleanser and initiating the second sonication.

3.9. Post-sonication, incubate the samples in a thermomixer set to 4 degrees Celsius with shaking at 1,200 revolutions per minute for 8 hours [1].

3.9.1. Talent placing the tubes into the thermomixer.

3.10. Then, let the mixture sit at room temperature undisturbed for 10 minutes [1]. Centrifuge the mixtures at 10,000 g for 10 minutes at 4 degrees Celsius [2].

3.10.1. Talent removing the tubes from the thermomixer and placing them on the benchtop.

3.10.2. Talent loading the tubes into a refrigerated centrifuge.

3.11. After centrifugation, ensure the mixtures are visibly separated into two distinct liquid phases [1]. Carefully collect 700 microliters of the upper organic phase containing lipids and transfer it into a pre-chilled new 1.5-milliliter microcentrifuge tube [2]. Discard the lower aqueous phase, which contains residual methyl tert-butyl ether and methanol, as hazardous chemical waste [3].

3.11.1. Close-up of the centrifuged tubes to show the separation of the upper and lower phases.

3.11.2. Talent pipetting the upper clear layer into a new pre-chilled microcentrifuge tube labeled for lipid extracts.

3.11.3. Talent pouring the lower layer into a waste container clearly marked for chemical disposal.

4. Drying, Storage and Reconstitution of Extracted Tear Lipid for LC-MS/MS Analysis

4.1. Dry the lipid extracts using a refrigerated SpeedVac concentrator set at 4 degrees

Celsius for 4 hours [1]. Then, store the dried lipid extracts at minus 20 degrees Celsius for short-term storage or minus 80 degrees Celsius for long-term storage [2].

4.1.1. Talent placing the tubes with lipid extracts into a SpeedVac concentrator.

4.1.2. Talent transferring the dried tubes into appropriate freezers for storage.

- 4.2. Prepare the methanol-chloroform solvent mixture by mixing methanol with chloroform in a 1 to 1 volume ratio [1]. Reconstitute the dried lipid extracts using 50 microliters of the ice-cold methanol/chloroform solvent mixture [2]. Sonicate the reconstituted solution in a pre-cooled ultrasonic cleanser for 15 minutes [3].

Added shot: Talent pipetting and mixing methanol and chloroform to a new microcentrifuge tube to prepare the solvent mixture for reconstituting samples.

4.2.1. Talent pipetting the cold methanol/chloroform solvent mixture into each tube of dried lipid extracts.

4.2.2. Talent placing the tubes in the ultrasonic cleanser.

- 4.3. Now, centrifuge the reconstituted lipid extracts at 14,000 *g* and 4 degrees Celsius for 10 minutes [1]. Carefully transfer 40 microliters of the resulting supernatant into an autosampler glass vial equipped with a glass insert [2]. Tightly cap the vial with a non-slit septum open top cap to prepare it for liquid chromatography-tandem mass spectrometry analysis [3].

4.3.1. Talent placing the tubes into a high-speed refrigerated centrifuge.

4.3.2. Talent pipetting the clear upper supernatant into a labeled autosampler vial containing a glass insert.

4.3.3. Talent securing the vial with the appropriate cap and placing it into a rack designated for LC-MS/MS analysis.

Results

5. Results

NOTE: Please use updated Figure 5v2.

https://review.jove.com/files/ftp_upload/69474/69474fig5v2.jpg

5.1. Using stringent criteria, 773, 890, and 1,025 unique lipid species were identified in R1, R2, and R3 sample groups, respectively [1].

5.1.1. LAB MEDIA: Figure 5A. *Video editor: Highlight the bars labeled R1, R2, and R3 when the VO says 773, 890 and 1025 respectively*

5.2. Positive ion mode identified 1,302 lipid species, with 26.0% Grade A, 13.2% Grade B, and 60.8% Grade C identifications [1].

5.2.1. LAB MEDIA: Figure 5B. *Video editor: Highlight the pie chart showing positive ion mode.*

5.3. Negative ion mode identified 257 lipid species, with about 8.2% Grade A, 22.9% Grade B, and 68.9% Grade C identifications [1].

5.3.1. LAB MEDIA: Figure 5B. *Video editor: Highlight the pie chart showing negative ion mode.*

5.4. Despite storage-related variations, 394 unique lipid species were consistently identified across all three sample sets [1].

5.4.1. LAB MEDIA: Figure 6. *Video editor: Highlight the center intersection of the Venn diagram where all three circles overlap and show the number 394.*

5.5. Intra-subject coefficient of variation across three visits was 21.5% in positive mode [1] and 31.3% in negative mode [2].

5.5.1. LAB MEDIA: Figure 7A. *Video editor: Highlight the area of the chart representing CV for positive ion mode.*

5.5.2. LAB MEDIA: Figure 7B. *Video editor: Highlight the area of the chart representing CV for negative ion mode.*

Pronunciation Guide:

1. Phenol
Pronunciation link: <https://www.merriam-webster.com/dictionary/phenol>
IPA: /'fiːnɔːl/
Phonetic Spelling: fee·nawl
2. Lipidomics
Pronunciation link: <https://www.merriam-webster.com/dictionary/lipidomics>
IPA: /ˌlɪpɪ'dɑːmɪks/
Phonetic Spelling: lip·ih·daa·miks
3. Biomarker
Pronunciation link: <https://www.merriam-webster.com/dictionary/biomarker>
IPA: /'baɪoʊˌmɑːrkər/
Phonetic Spelling: bye·oh·maar·ker
4. Schirmer
Pronunciation link: <https://www.merriam-webster.com/dictionary/Schirmer>
IPA: /'ʃɪrmər/
Phonetic Spelling: shir·mer
5. Capillaries
Pronunciation link: <https://www.merriam-webster.com/dictionary/capillary>
IPA: /'kæpəˌleɪrɪz/
Phonetic Spelling: kap·uh·lair·eez
6. Reproducibility
Pronunciation link: <https://www.merriam-webster.com/dictionary/reproducibility>
IPA: /ˌrɪːprəˌduːsə'bɪləti/
Phonetic Spelling: ree·pruh·doo·suh·bil·uh·tee
7. Palpebral
Pronunciation link: <https://www.merriam-webster.com/dictionary/palpebral>
IPA: /'pælpɪbrəl/
Phonetic Spelling: pal·puh·bruhl
8. Conjunctiva
Pronunciation link: <https://www.merriam-webster.com/dictionary/conjunctiva>
IPA: /kənˌdʒʌŋk'taɪvə/
Phonetic Spelling: kuhn·juhngk·tie·vuh
9. Canthus
Pronunciation link: <https://www.merriam-webster.com/dictionary/canthus>
IPA: /'kænθəs/
Phonetic Spelling: kan·thus
10. Parafilm
Pronunciation link: <https://www.merriam-webster.com/dictionary/parafilm>
IPA: /'pærəˌfɪlm/
Phonetic Spelling: pair·uh·film

11. Methanol

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

IPA: /'meθəˌnɔːl/

Phonetic Spelling: meth·uh·nawl

12. Methyl tert-butyl ether

Pronunciation link: <https://www.merriam-webster.com/dictionary/methyl%20tert-butyl%20ether>

IPA: /'mɛθəlˌtɜːtˈbjuːtəl'iːθər/

Phonetic Spelling: meth·uhl tert·byoo·tuhl ee·ther

13. Chromatography

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

IPA: /ˌkroʊməˈtɑːɡrəfi/

Phonetic Spelling: kroh·muh·taa·gruh·fee

14. Thermomixer

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

IPA: /'θɜːmoʊˌmɪksər/

Phonetic Spelling: thur·moh·mik·ser

15. Centrifuge

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

IPA: /'sentrəˌfjuːdʒ/

Phonetic Spelling: sen·truh·fyooj

16. SpeedVac

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

IPA: /'spiːdˌvæk/

Phonetic Spelling: speed·vak

17. Chloroform

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

IPA: /'klɔːrəˌfɔːrm/

Phonetic Spelling: klor·uh·form

18. Autosampler

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

IPA: /'ɔːtoʊˌsæmplər/

Phonetic Spelling: aw·toh·sam·pler

19. Septum

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

IPA: /'septəm/

Phonetic Spelling: sep·tum

20. Tandem

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

IPA: /'tændəm/

Phonetic Spelling: tan·dum

21. Coefficient

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

IPA: /ˌkoʊtʃɪfənt/

Phonetic Spelling: koh·ih·fish·uhnt

22. Variation

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

IPA: /ˌvɛriˈeɪʃən/

Phonetic Spelling: vair·ee·ay·shuhn

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