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# Quantitative proteomic profiling of murine ocular tissue and the extracellular environment

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**ABSTRACT:**

Mass spectrometry-based proteomics provides a robust and reliable method for detecting and quantifying changes in protein abundance among samples, including cells, tissues, organs, and supernatants. Physical damage or inflammation can compromise the ocular surface, permitting colonization by bacterial pathogens, commonly *Pseudomonas aeruginosa*, and the formation of biofilms. The interplay between *P. aeruginosa* and the immune system at the site of infection defines the host's ability to defend against bacterial invasion and promote clearance of infection. Profiling of the ocular tissue following infection describes the nature of the host innate immune response, and specifically, the presence and abundance of neutrophil-associated proteins to neutralize the bacterial biofilm. Moreover, detection of unique proteins produced by *P. aeruginosa* enable identification of the bacterial species and may serve as a diagnostic approach in a clinical setting. Given the emergence and prevalence of antimicrobial resistant bacterial strains, the ability to rapidly diagnose a bacterial infection, promoting quick and accurate treatment will reduce selective pressure towards resistance. Furthermore, the ability to define differences in the host immune response towards bacterial invasion enhances our understanding of innate immune system regulation at the ocular surface. Here, we describe murine ocular infection and sample collection, as well as outline protocols for protein extraction and mass spectrometry profiling from corneal tissue and extracellular environment (eye wash) samples.

**Basic protocol 1:** Murine model of ocular infection

**Basic protocol 2:** Murine model sample collection

**Basic protocol 3:** Protein extraction from eye wash



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**Basic protocol 4:** Protein extraction from corneal tissue

**Basic protocol 5:** Mass spectrometry-based proteomics and bioinformatics

**KEYWORDS:**

Mass spectrometry-based proteomics, bacterial keratitis, *Pseudomonas aeruginosa*, corneal tissue, eye wash, murine model, bioinformatics

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**INTRODUCTION:**

Mass spectrometry-based proteomics provides a reliable and robust method for profiling changes in protein abundance under a variety of conditions, including manipulated experimental conditions and during health and disease[1]. The information obtained from quantitative proteomic profiling can inform treatment strategies, patient stratification, molecular mechanisms underpinning phenotypic observations and details pertaining to signaling networks and protein-protein interactions critical to the evaluated criteria[2]. In addition, proteomic profiling can provide valuable insight into the interplay between host and pathogen during infection and generate large datasets outlining changes from these dual perspectives in a single experiment[3–5]. For example, proteomic profiling of the ocular surface during infection with *Pseudomonas aeruginosa*, a Gram-negative bacterial pathogen and the primary causative agent of bacterial keratitis, defines global and site-specific host responses to infection and uncovers potential biomarkers for prognostic and diagnostic purposes[6]. To evaluate the impact of bacterial infection of the ocular surface on the host, response both at the site of infection (i.e., cornea) and the adjacent uninfected eye, as well as define changes to the surface by analysing eye wash samples (representing a non-invasive strategy for examining



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the relationship between host and pathogen), we devised a step-by-step protocol (Fig. 1). These proteomics experiments provide the first *in vivo* identification of bacteria-specific responses to the host during bacterial biofilm formation of the eye and enhance our understanding of ocular health at the protein level.

Basic Protocol 1 describes the murine infection model for *P. aeruginosa* keratitis. Basic Protocol 2 outlines sample collection of eye wash and corneal tissue from the murine models. Basic Protocols 3 and 4 describe protein extraction and sample preparation from eye wash and corneal tissues, respectively, and Basic Protocol 5 demonstrates measurement and identification of the proteins by mass spectrometry-based proteomics. The protocols presented herein are optimized for protein extraction of corneal tissues and eye wash; however, the principles may be extended to other biological tissues and fluids. Overall, our strategy enables deep proteomic profiling of murine ocular tissues in health and disease and defines specific responses to infection from both the host and pathogen perspectives.

[*Copy Editor – insert Figure 1 here]

***BASIC PROTOCOL 1***

**MURINE MODEL OF OCULAR INFECTION**

**Introductory paragraph:**

The ocular surface, including the cornea and the surrounding mucosal sites and the extracellular environment, are highly susceptible to infection. During chronic infection, the formation of bacterial biofilms at the ocular surface promotes the recruitment and activation of neutrophils; however, neutrophils often fail to breach the bacterial biofilms, resulting in a limited host response[7,8]. We use a murine model of infection for bacterial keratitis to explore the



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relationship between host and pathogen during infection and tease apart components of defense response.

*Materials:*

Invasive *Pseudomonas aeruginosa* clinical isolate 6294 (the strain was isolated from a keratitis patient)

Pre-made Tryptic Soy Broth (TSB) Agar containing 5% sheep's blood plates (e.g., Becton Dickinson, cat. no. 221261)

Donor mice: C57BL/6N mice, 7- to 9-week old, male or female (e.g., Taconic Farms, Rensselaer, NY) (Note: C57BL/6N mice are susceptible to *P. aeruginosa* infection and therefore, recommended for the study; however, alternative mice strains (e.g., Swiss Webster) can be used, if preferred).

Ketamine HCL (e.g., Zoetis, cat. no. EA2489-564)

Xylazine (e.g., AnaSed, cat. no. Sc-362950Rx)

Static incubator (e.g., Thermo Fisher Scientific, cat. no. 151030515)

Cotton-tipped applicator (6 in. Fischer, cat. no. 50975)

Spectrophotometer (e.g., Spectronic Instruments, cat. no. 4001/4)

25G needle tip (e.g., Becton Dickinson, cat. no. 305122)

Pipette tip

Pipettes

*Solutions:*

Saline (0.9% NaCl)



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Intraperitoneal anesthetization solution (see Reagents and Solutions)

*Protocol steps:*

*Note:* Institutional review and approval is required for all animal protocols.

1. Plate *Pseudomonas aeruginosa* isolate 6294 in pre-made tryptic soy broth (TSB) agar with 5% sheep's blood plates and grow overnight at 37ºC.
2. Swab colonies with a cotton-tipped applicator from plate using a sterilized swab and inoculate saline solution to obtain optical density of 0.45 at 650 nm ($1x10^9$ cells/ml), measure with a spectrophotometer.
3. Dilute to $1x10^7$ cells/ml in saline solution.
4. Anesthetize mice with 200 µl intraperitoneal anesthetization solution injection per 20g body weight. Wait 10 min for anesthetization.

   *Typically, use 5 to 10 mice (both male and female mice should be used) per condition.*

5. Gently superficially scratch (3 x 5 mm) the cornea of mouse using a 25G needle by dragging the needle across the surface of the cornea.
6. Drop inoculum of $1x10^5$ colony forming units (CFU) of *P. aeruginosa* in 5 µl of saline solution onto the surface of the eye.

   *For control mice (i.e., uninfected), deliver 5 µl of saline solution. In addition, the counter lateral eye is the unscratched control. Preparation of eyes should alternate between right and left.*

7. Monitor recovery of mice from anesthesia.

   *Typically, animals recover within 1 h of challenge.*

8. Allow infection to proceed for designated amount of time (e.g., 24 h up to 5 days).



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*Infection in some strains (e.g., C57BL6/N) will result in perforation within 5 d post infectious challenge, depending on the dose and, if left untreated.*

9. *Euthanize mice upon signs of perforation or at the selected experimental time point*.

***BASIC PROTOCOL 2***

**MURINE MODEL SAMPLE COLLECTION**

**Introductory paragraph:**

To detect proteins released from the surface of the eye following non-invasive washing, we collect an eye wash sample following the murine model of infection for bacterial keratitis. In addition, to detect proteins at the ocular surface with altered changes in abundance following *P. aeruginosa* infection in a murine model, we collect the corneal tissue from the infected and adjacent eyes[9]. This approach allows us to detect and quantify proteins produced by the host in response to infection, including both general and site-specific defense responses. At the same time, we can measure bacterial proteins associated with biofilm formation at the ocular surface, representing potential biomarkers of infection.

*Materials:*

Donor mice: C57BL/6N mice, 7- to 9-week old, male or female (e.g., Taconic Farms, Rensselaer, NY) from Basic Protocol 1

Phosphate buffered saline (PBS), pH 7.4 (e.g., Boston BioProducts, cat. no. BM220)

Proteinase inhibitor cocktail tablet (e.g., Roche Diagnostics, cat. no. 11836170001)

Liquid nitrogen

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$CO_2$ euthanasia chamber (or comparable method for euthanasia, depending on specific animal facility conventions)

Pipette tips

Pipettes

1.5 ml microcentrifuge tubes, LoBind (e.g., Eppendorf, cat. no. 022431081)

Stereomicroscope (e.g., Motic, cat. no. 13017023)

Surgical blade

Surgical scissors

*Protocol steps:*

1. Sacrifice the mice from Basic Protocol 1 by placing them in $CO_2$ chamber and utilizing a secondary method for euthanasia (e.g., cervical dislocation).

   *Typically, samples are collected after 24 or 48 h of exposure to P. aeruginosa (as outlined in Basic Protocol 1) and used to proceed to Basic Protocol 2 immediately. The same time frames are used for the uninfected and counter lateral eyes.*
2. Wash eyes by pipetting up and down with 15 µl of phosphate buffered saline (PBS) containing 1 x proteinase inhibitor cocktail (PIC) tablet. Repeat wash step twice.

   *Pipette carefully as to not lose any liquid during washes and collection. Collect eye wash after each addition of 15 µl to the ocular surface.*
3. Collect eye wash in 1.5 ml microcentrifuge tube.
4. Flash freeze with liquid nitrogen. Store samples at -80ºC, until needed.



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5. Lift cornea with forceps. Generate a lateral cut at the limbus with the surgical blade to allow further lifting of the corneal flap. The blade should enter the anterior chamber.
6. Dissect the cornea out from ocular surface under stereomicroscope with surgical scissors. Make sure to cut out conjunctival and other non-corneal tissues.

   *Be careful to avoid contamination of the corneal samples with the iris, as the dark pigments may interfere with downstream protein quantification protocols.*
7. Collect infected and counter lateral (uninfected) cornea (as described in step 5) in 1.5 ml microcentrifuge tubes.
8. Flash freeze with liquid nitrogen. Store samples at -80ºC, until needed.

***BASIC PROTOCOL 3***

**PROTEIN EXTRACTION FROM EYE WASH**

**Introductory paragraph:**

To extract proteins from the eye wash samples, we perform an in-solution digest. This workflow is appropriate for many supernatant and/or extracellular environment analyses, as well as profiling of easily rupturable cells (e.g., neutrophils)[10]. The volume of reagents can be adjusted for compatibility with different sample volumes. Note: some supernatant samples with mass spectrometry-incompatible reagents (e.g., sodium dodecyl sulfate, high salt concentrations) may not



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process properly with this protocol and may require total proteome extraction as outlined in Basic Protocol 5.

*Materials*:

Eye wash samples from Basic Protocol 2 (approx. 45 µl)

8 M urea (e.g., Sigma-Aldrich, cat. no. U1250)

40 mM HEPES (e.g., Sigma-Aldrich, cat. no. H4034)

1 M dithiothreitol (DTT; e.g., Signal-Aldrich, cat. no. D0632)

0.55 M iodoacetamide (IAA; e.g., Sigma-Aldrich, cat.no. I6125)

Trypsin/Lys-C protease mixture, MS grade (e.g., Pierce, cat. no. A40007)

Stopping solution: 20% acetonitrile, MS grade (e.g., Pierce, cat. no. TS-51101) and 6% trifluoroacetic acid (e.g., Sigma-Aldrich, cat. no. 302031)

Ultrasonic bath, stored in 4°C cold room (e.g., Bransonic CPXH or comparable)

Pipettes

Vortex mixer (e.g., ThermoFisher Scientific, cat. no. 02-215-418)

Microcentrifuge (e.g., Eppendorf model 5430 or comparable)

1.5 ml microcentrifuge tubes, LoBind (e.g., Eppendorf, cat. no. 022431081)

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*Protocol steps:*

***Sample preparation***

1. In a 1.5 ml microcentrifuge tube, add 1/3 volume of pre-mixed 8 M urea/40 mM HEPES mixture to the eye wash samples (Basic Protocol 2).
2. Briefly vortex sample for 2 s.
3. Add 1 µl of 1 M dithiothreitol (DTT) per 100 µl sample to achieve a final concentration of 10 mM DTT and briefly vortex for 2 s.
4. Briefly vortex sample for 2 s.
5. Incubate the samples at room temperature for 30 min.

   *Alternatively, this step can be performed at 56ºC for 20 min.*
6. Add 1 µl of 0.55 M iodoacetamide (IAA) per 10 µL of sample to achieve a final concentration of 55 mM IAA.

   *To avoid degradation of IAA, perform this step in a dark environment.*
7. Briefly vortex sample for 2 s.
8. Incubate the samples at room temperature for 20 min in a dark environment.

   *Optional: a repeat of steps 3 - 5 can be performed to inactivate any remaining IAA after the incubation period.*

***Digestion***

9. Add 2:50 (enzyme volume:protein amount) of trypsin/Lys-C protease mixture.

   *Typically, from supernatant samples, we digest 50 to 100 µg of protein.*
10. Tap the tube gently to mix.



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11. Incubate samples (static) overnight at room temperature.

    *Alternatively, this step can be performed at 37°C, depending on manufacturer's instructions.*

12. Stop the digestion process by adding 1/10 volume of stopping solution.
13. To pellet any precipitate, centrifuge samples for 10 min at 10,000 x g.
14. Transfer supernatant to new 1.5 ml microcentrifuge tube. Discard any precipitate.
15. Proceed to Basic Protocol 6.

    *Alternatively, samples can be flash frozen and stored at -20°C for up to one week (although it is preferred to process samples immediately to avoid any peptide degradation).*

***BASIC PROTOCOL 4***

**PROTEIN EXTRACTION FROM CORNEAL TISSUE**

**Introductory paragraph:**

This protocol describes the extraction of murine corneal tissues to profile changes in protein abundance during health vs. disease. We have optimized the process for corneal tissues; however, the outlined method can also be applied to other ocular tissues and tested for other organs, as required.

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*Materials:*

Corneal tissue samples from Basic Protocol 3

10 X ice-cold Phosphate buffered saline (PBS), pH 7.4 (e.g., Boston BioProducts, cat. no. BM220)

100 mM Tris-HCl, pH 8.5 (e.g., Fisher Scientific, cat. no. BP152-1)

Protease inhibitor cocktail tablet (e.g., Roche, cat. no. 04693159001)

20% (w/v) sodium dodecyl sulfate (SDS; e.g., Sigma-Aldrich, cat. no. 75746)

1 M dithiothreitol (DTT; e.g., Signal-Aldrich, cat. no. D0632)

0.55 M iodoacetamide (IAA; e.g., Sigma-Aldrich, cat.no. I6125)

100 % ice cold acetone (stored at -20ºC)

80 % ice cold acetone (diluted with dd$H_2O$, stored at -20ºC)

8 M urea (e.g., Sigma-Aldrich, cat. no. U1250)

40 mM HEPES (e.g., Sigma-Aldrich, cat. no. H4034)

50 mM Ammonium bicarbonate (ABC; e.g., ThermoFisher Scientific, cat. no. A643-500)

Trypsin/Lys-C protease mixture, MS grade (e.g., Pierce, cat. no. A40007)

Stopping solution: 20% acetonitrile, MS grade (e.g., Pierce, cat. no. TS-51101) and 6% trifluoroacetic acid (e.g., Sigma-Aldrich, cat. no. 302031)

Pipettes

15 ml conical polypropylene centrifuge tubes (e.g., Corning, cat. no. C352196)

Vortex mixer (e.g., ThermoFisher Scientific, cat. no. 02-215-418)



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Probe sonicator (e.g., ThermoFisher Scientific, cat. no. 100-132-894 or comparable)

Thermal shaker (e.g., VWR, cat.no. NO89232-908 or comparable)

Microcentrifuge (e.g., Eppendorf model 5430 or comparable)

Ultrasonic bath, stored in 4°C cold room (e.g., Bransonic CPXH or comparable)

2 ml microcentrifuge tubes, LoBind (e.g., Eppendorf, cat. no. 022431102)

*Protocol steps:*

***Sample preparation***

1. Using the frozen murine corneal samples from Basic Protocol 3, wash the samples with 50 to 100 µl of ice-cold PBS by gently pipetting up and down.

   *Keep samples on ice during washing. Samples will thaw during first steps of Basic Protocol 4.*
2. Centrifuge the samples at 4°C briefly at 1,000 x g for 5 min to pellet sample and remove the supernatant by pipetting.
3. Repeat the washing step twice.
4. Remove the supernatant by pipetting and discard.
5. Transfer the corneal tissue to 15 ml conical polypropylene centrifuge tubes.
6. Resuspend the corneal tissue in 300 µl of ice-cold 100 mM Tris-HCl pH 8.5 with proteinase inhibitor cocktail (PIC) tablet.

   *Volume of Tris-HCl pH 8.5 added to the sample depends on samples size and can be adjusted, as needed; if adjusted, be sure to modify all subsequent volumes to maintain outlined ratios of reagents.*
7. Briefly vortex for 2 s to mix.



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8. Add 20% sodium dodecyl sulfate (SDS) to the sample to achieve a final concentration of 2% SDS.
9. Mechanically lyse the corneal samples using a probe sonicator while the samples are maintained in an ice bath (4 °C).

   *The power settings, cycle numbers, and on/off cycles may vary depending on instrument. We typically lyse samples for three cycles at 30% power, 30 s on/30 s off.*
10. Centrifuge the samples for 5 s at 1,000 x g to collect spray from the sides of the 15 ml conical polypropylene centrifuge tubes.

    *To avoid protein degradation, keep samples on ice throughout the protocol whenever possible.*
11. Transfer samples to clean 2 ml microcentrifuge tubes.

    *Samples should be homogenous. If any tissue pieces remain, repeat sonication and transfer all material to 2 ml microcentrifuge tube.*
12. Add 1 µl of 1 M dithiothreitol (DTT) per 100 µl sample to achieve a final concentration of 10 mM DTT.
13. Briefly vortex for 2 s to mix.
14. Place sample in a heating block with temperature set to 95°C. Shake at 800 rpm for 10 min.
15. Cool the samples on ice until they reach room temperature.
16. Add 1 µl of 0.55 M iodoacetamide (IAA) per 10 µl of sample to achieve a final concentration of 55 mM IAA.

    *To avoid degradation of IAA, perform this step in a dark environment.*
17. Incubate the samples at room temperature for 20 min in a dark environment.



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***Precipitation and re-solubilization***

18. Add ice-cold 100 % acetone to each sample to achieve a final concentration of 80% acetone.

    *Typically, following the protocol outlined above, we add 1,520 µl of 100% ice-cold acetone for a final volume of approx. 1.9 ml.*

19. Incubate the samples at -20°C overnight.
20. Centrifuge the samples at 10,000 x g at 4 °C for 10 min to pellet the precipitated protein.
21. Remove the supernatant.
22. Wash the samples twice with 500 to 1,000 µl of 80% ice-cold acetone.
23. Repeat steps 20 & 21 twice.
24. Air dry the pellet by incubating the samples at room temperature until the acetone has evaporated.

    *Acetone will evaporate in approx. 20 to 40 min.*

25. To resolubilize the precipitated protein, add 100 µl of 8 M urea/40 mM HEPES mixture to the samples and place in ultrasonic bath for 15 cycles of 30 s on/30 s off.

    *The volume of 8 M urea/40 mM HEPES can be increased depending on pellet size; ensure subsequent volumes are adjusted accordingly.*

***Protein quantification and digestion***

26. Quantify the concentration of protein in each sample (e.g., BSA tryptophan assay) as previously described[11].
27. Add 300 µl of 50 mM ammonium bicarbonate (ABC) to the samples to reach a final concentration of 2 M urea.



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*Volume of ABC compatible with 100 µl of 8 M urea/40 mM HEPES mixture; can be adjusted accordingly. A stock solution of ABC can be prepared ahead of time and stored at room temperature for up to four weeks.*

28. Add 2:50 (enzyme volume:protein amount) of trypsin/Lys-C protease mixture.

    *Typically, we digest 100 µg of protein. Remaining undigested samples can be flash frozen and stored at -20°C for two weeks or -80°C for longer.*

29. Tap the tube gently to mix.
30. Incubate the samples overnight at room temperature.
31. Stop the digestion process by adding 1/10 volume of stopping solution.
32. Centrifuge the samples for 5 min at 10,000 x g to pellet any precipitate
33. Transfer the supernatant to a new 2 ml microcentrifuge tube. Discard any precipitate.
34. Proceed to Basic Protocol 6.

    *Alternatively, samples can be flash frozen and stored at -20°C for up to one week (although it is preferred to process samples immediately to avoid any peptide degradation).*

***BASIC PROTOCOL 5***

**MASS SPECTROMETRY-BASED PROTEOMICS AND BIOINFORMATICS FROM EYE WASH AND CORNEAL TISSUE SAMPLES**

**Introductory paragraph:**

Mass spectrometry-based proteomics represents a powerful and unbiased platform for profiling changes in protein abundance within a variety of biological samples[1]. In this protocol, we outline our application of discovery-based proteomics for single-shot, label-free quantification



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tandem mass spectrometry. This approach enables exploration of differences in the host immune response and biofilm formation in a murine model of bacterial keratitis.

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*Materials*:

C18 resin (e.g., 3M Empore, cat. no. 3M2215)

Acetonitrile (ACN), MS grade (e.g., Pierce, cat. no. TS-51101)

Buffer B (see Reagents and Solutions)

Buffer A (see Reagents and Solutions )

Peptides from eye wash samples (see Basic Protocol 4)

Peptides from corneal tissue samples (see Basic Protocol 5)

Acetic acid (e.g., Thermo Fisher Scientific cat. no. A38-212)

C18 STAGE-tip

STAGE tipping centrifuge (e.g., Sonation, cat. no. STC-V2 or comparable)

0.2-ml PCR tubes (e.g., Thermo Fisher Scientific, cat. no. AB0620 or comparable)

Vacuum concentrator (e.g., Vacufuge, Eppendorf, cat. no. 07-748-15 or comparable)

Microvolume spectrophotometer (e.g., NanoDrop, ThermoFisher Scientific, cat. no. ND-2000 or comparable)

Nano-flow column, 15 cm Å~ 75 µm, PepMap C18, 2 µm (e.g., ThermoFisher Scientific, cat. no. ES803)

High performance liquid chromatography system (e.g., ThermoFisher Scientific, cat. no. LC140 or comparable)



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High-resolution mass spectrometer

Internet-connected computer running bioinformatics platforms (see Internet Resources)

*Protocol steps:*

*NOTE: All centrifugation steps should be performed at room temperature.*

***Peptide purification***

1. Prepare C18 STAGE-tip: wash tip with 100 µl of 100% acetonitrile and centrifuge for 1 min at 1,000 x g.

   *Typically, we use three layers of C18 resin to make a single STAGE-tip, which will capture between 15 and 45 µg of peptides. Alternatively, C18 spin columns may be used for peptide purification steps outlined below, according to manufacturer's protocol.*

2. Equilibrate C18 STAGE tip: wash tip with 50 µl Buffer B and centrifuge for 1 min at 1,000 x g.
3. Equilibrate C18 STAGE tip: wash tip with 200 µl Buffer A and centrifuge for 2 min at 1,000 x g.
4. Load C18 STAGE tip: add 15 to 45 µg of digested peptides onto C18 STAGE tip, and centrifuge 3 to 5 min at 1000 x g.

   *Depending on the sample composition, a higher speed or longer centrifugation time may be required to pass the sample through the C18 STAGE tip. We recommend a maximum speed of 3,500 x g. The remaining digested (non-purified) sample can be*



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*flash frozen in liquid nitrogen and stored at−20°C for up to 1 week or at −80°C for longer-term storage.*

5. Wash C18 STAGE tip: wash tip with 200 µl Buffer A, and centrifuge 3 to 5 min at 1000 x g.
6. Elute peptides from C18 STAGE tip: elute with 50 µl Buffer B, and centrifuge 2 min at 500 x g, into 0.2-ml PCR tubes.

   *For multiple samples, we recommend using 0.2-ml PCR strip tubes or a 96-well plate for elution.*
7. Evaporate Buffer B from the eluted sample using a vacuum concentrator at maximum speed for 30 to 40 min.

   *This step can be done at room temperature or 37°C for faster evaporation.*
8. Resuspend peptides in 10 µl Buffer A by pipetting up and down 3 to 5 times.

   *Samples can be loaded onto the mass spectrometer or stored at −20°C until needed.*
9. Measure peptide concentration on a microvolume spectrophotometer to determine the volume needed to inject approx. 1.5 µg peptides onto the MS column.

   *Samples typically range from 0.6 to 1 µg/µl at a wavelength of A280 nm, and sample type 1 Abs = 1 mg/ml. The amount of sample injected into the instrument varies depending on the reverse-phase column and instrument sensitivity, among other parameters, and should be optimized for each mass spectrometer.*

***Mass spectrometry and data analysis***

10. Separate peptides over a pre-determined gradient (e.g., 5% to 60% acetonitrile in 0.1% formic acid), and then wash out with up to 95% acetonitrile over a 10-min period using a nano-flow column (option: heat column to 50°C) with a flow rate of 250 - 300 nl/min on a high-performance liquid chromatography system.



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*For high-resolution mass spectrometry systems, we recommend a 60-min gradient for supernatant samples as the complexity is relatively low and a 2 to 3 h gradient for more complex samples (e.g., cellular proteomes). The gradient lengths can be adjusted based on the sample complexity and instrumentation, as needed.*

11. Operate the mass spectrometer in data-dependent acquisition mode with full scans (e.g., m/z 400 to 1600) acquired in the analyzer with a resolution of 60,000 to 120,000.

12. Process the data files using an appropriate bioinformatics platform (e.g., MaxQuant[12,13], PEAKS[14]).

    Filter protein identifications using a target-decoy approach at a false discovery rate of 1% with a minimum of two unique peptides for protein identification. Enable relative label-free quantification and match between runs, if desired. User and experiment-specific parameters can be set in consultation with available online resources (see Internet Resources).

13. Analyze the output file using an appropriate data analysis and visualization bioinformatics platforms (e.g., Perseus[15], PEAKS[14], R[16]).

    *User and experiment-specific parameters can be set in consultation with available online resources (see Internet Resources).*

14. It is encouraged to deposit mass spectrometry data, affiliated search and parameter files, and experimental metadata into the ProteomeXchange consortium (PRIDE).

    *The PRoteomics IDEntification (PRIDE) database is a centralized, standards-compliant, public data repository for proteomics data (see Internet Resources).*

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**REAGENTS AND SOLUTIONS:**

***Intraperitoneal anesthetization solution***

2 ml Ketamine (100 mg/ml)

400 µL xylazine (100 mg/ml)

27.6 ml saline

Prepare fresh

***Urea and HEPES buffer***

8 M Urea

40 mM HEPES

Prepared, aliquoted, and stored at -20ºC, until needed.

***Dithiothreitol***

1 M dithiothreitol

Prepared, aliquoted, and stored at -20ºC, until needed.

***Iodoacetamide***

0.55 M iodoacetamide

Prepared, aliquoted, and stored in the dark at -20ºC, until needed.



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***Trypsin/Lys-C enzyme mix***

Aliquots of trypsin/Lys-C protease mixture (0.5 µg/µl) can be prepared ahead of time by flash freezing and storing at -20°C. When adding enzyme to the samples, keep the protease aliquots on ice to prevent degradation of any unused enzyme. When finished with the aliquot, flash freeze the remaining volume and store at -20°C.

***Tris-HCl***

100 mM Tris-HCl pH 8.5

Proteinase inhibitor cocktail tablet

Prepared fresh in a 15 ml conical polypropylene centrifuge tube by adding one PIC tablet to 10 ml of ice-cold 100 mM Tris-HCl pH 8.5 and mixing with a vortex mixer until the mixture is homogenous.

***Sodium dodecyl sulphate solution (SDS)***

20% SDS solution

Prepared ahead of time and stored at room temperature for up to four weeks.

***Buffer A***

2% (v/v) acetonitrile



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0.1% (v/v) trifluoroacetic acid

0.1% (v/v) formic acid

MS-grade water

Store at room temperature for up to 1 month

***Buffer B***

80% (v/v) acetonitrile

0.1% (v/v) formic acid

MS-grade water

Store at room temperature for up to 1 month

**COMMENTARY**

**BACKGROUND INFORMATION:**

*Pseudomonas aeruginosa* is the most commonly isolated bacterium from contact lens-associated keratitis; it is a ubiquitous, Gram-negative, opportunistic pathogen with multiple mechanisms of antimicrobial resistance[17–19]. Pathogenesis is related to the production of a multitude of secreted and cell-associated virulence factors, as well as the formation of biofilms[20]. In *P. aeruginosa*, biofilms provide a protective extracellular polymeric substance (EPS) matrix consisting of polysaccharides (i.e., polysaccharide synthesis locus (Psl) and pellicle (Pel)), polymers (alginate), and extracellular DNA (eDNA). These biofilm components are important for cell attachment to surfaces,



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protection against phagocytic immune cells and oxidative stress, and it provides a first line of defense in premature biofilms against antibiotic penetration[21–26].

During acute and chronic infections, clearance of *P. aeruginosa* relies on multiple host cell types, including epithelial cells, as well as both resident and recruited phagocytic cells (e.g., lymphocytes, macrophages, and neutrophils). A hallmark of the inflammatory response to *P. aeruginosa* infection is the recruitment of neutrophils, which phagocytose and kill bacteria using highly-effective antimicrobial molecules[7]. These include reactive oxygen species for peroxidation of proteins and lipids, α-defensins for pore formation and membrane permeabilization of pathogens, and neutrophil extracellular traps to contain infection[8,27,28]. Despite the success of neutrophils to clear bacterial infections prior to biofilm formation, they often fail to breach bacterial biofilms due to limited accessibility or resistance of biofilms to degradative enzymes, resulting in a limited host response and persistent or re-occurring infections[28].

To explore the relationship between the host and pathogen during bacterial keratitis and define the presence of bacterial biofilms at the ocular surface, we use bottom-up mass spectrometry-based proteomics[6]. Using a clinical isolate of *P. aeruginosa* (strain 6294) in a murine model of ocular infection, we detect neutrophil marker proteins within the extracellular environment (eye wash), indicating neutrophil recruitment to the site of infection. We also identify the first potential diagnostic markers of *P. aeruginosa*-associated keratitis and we define proteins, categories, and networks critical to the host response to infection. Moreover, we provide the first identification of bacteria-specific proteins in response to the host during bacterial biofilm formation of the eye. Overall, our work provides comprehensive profiling of the host-pathogen interface and uncovers differences between general and site-specific host responses to infection. In this

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Protocol, we outline the techniques and methods used to comprehensively profile bacterial keratitis from both the host and pathogen perspectives by quantitative proteomics.

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**CRITICAL PARAMETERS:**

For murine models of infection, it is critical to follow all animal care and handling protocols and receive appropriate training prior to conducting the outlined experiments. Critical parameters for this work include defining adequate bacterial cell counts and infection times to establish *P. aeruginosa* infection at the ocular surface. We define these parameters based on the *P. aeruginosa* clinical isolate 6294 used for this study; however, these may differ for different strains or bacterial species. Moreover, we confirm the formation of bacterial biofilms by intravital microscopy and polysaccharide component staining methods[27].

For mass spectrometry-based analyses, nitrile gloves and laboratory coats should be worn, along with cleaning of the working area with 70% ethanol, to minimize the potential for contamination from dust, hair, etc., which can interfere with protein identifications. In general, the protein extraction protocols outlined above are optimized for eye wash and corneal tissue; however, protein identification rates can vary among samples, users, experiments, instruments, and bioinformatic processing. To improve protein identification rates, alternative approaches to single-shot mass spectrometry can be performed, including fractionation (e.g., size exclusion chromatography, high reversed-phase pH) to reduce sample complexity in a single run. We also describe the use of label-free quantification for measurement of relative protein abundance in the samples; however, other labeling methods (e.g., metabolic and chemical) can also be used to promote multiplexing of sample measurements on the mass spectrometer[29,30].



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**TROUBLESHOOTING:**

Table 1 presents common problems encountered with preparing proteomic samples, along with possible causes and recommended approaches to avoid or overcome these problems.

| Problem | Possible Cause | Solution |
|---|---|---|
| Poor bacterial biofilm development | Not enough time for biofilm to establish | Allow infection to proceed for 24 to 72 h to ensure adequate biofilm formation |
| Protein degradation | Improper sample handling | Key samples on ice whenever possible during processing |
| Poor digestion of proteins into peptides | Concentration of urea is too high for trypsin/LysC digestion<br><br>Lys-C and trypsin proteases are not working optimally | Ensure urea is 2 to 2.5 M during trypsin/Lys-C digestion; check pH during digestion (pH 8 is optimal)<br>Store enzyme mix at −20°C<br>Use fresh proteases stored according to manufacturer's instructions. Keep proteases on ice at all times during use and discard any remaining protease after thawing. |
| Poor peptide yield | In-house STAGE tips were | Ensure recommended centrifugation |

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| | | |
|---|---|---|
| | packed too loosely or too tightly | speeds and times are accurate for Buffer A. If tip is packed too loosely, Buffer A will pass very quickly; if packed too tightly, higher speeds and time may be required to wash the tip. |
| | STAGE tip is not sufficiently activated or has run dry during loading | Ensure C18 resin never runs dry during activation, loading, or washing |
| Lower than expected number of identified proteins | Complex sample eluting many peptides at the same time | Increase mass spectrometry gradient length (e.g., 90 min or 2 hr) to reduce sample complexity at a given elution time and to increase protein identifications<br>Use bioinformatics tools to ensure that peptides are not modified/undigested in an undesired fashion |

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**UNDERSTANDING RESULTS AND STATISTICAL ANALYSIS:**

The mass spectrometry analyses were performed on four biological replicates to achieve the results previously presented[27]. The methods described in this Protocol highlight the results of protein extraction and purification from both the extracellular environment (eye wash) and corneal tissue during health and disease. With the outlined protocols, we typically identify between 50 to 300 proteins from the eye wash samples and >2,500 proteins for the corneal tissue. These numbers include identification of bacterial proteins, as applicable; the number of proteins identified tends to vary depending on sample quantity and quality, the experimental conditions, mass spectrometer instrumentation, and bioinformatics platforms available. For our analyses, we filter for proteins present in >50% of replicates (e.g., protein must be detected in at least three of four replicates in at least one group) for further statistical testing and data interpretation. The statistical tests that we routinely perform include two-tailed Student's *t*-tests with multiple hypothesis testing correction using the Benjamini-Hochberg method and a false-discovery rate = 0.05[31]. These tests and parameters should be tailored, as appropriate, for each data set.

**TIME CONSIDERATIONS:**

Basic Protocol 1 uses 7- to 9-week old donor mice and an overnight culture of *P. aeruginosa* clinical isolate to initiate infection. Infection then proceeds for 24 to 48 h before sample collection. Basic Protocol 2 involves euthanasia of mice and collection of eye wash and corneal tissues. Depending on the number of samples required for Basic Protocols 1 & 2, these steps will take approx. 3 to 5 d. Basic Protocol 3 – the extraction and digestion of eye wash samples takes approx. 48 h to complete, whereas Basic Protocol 4 – the extraction and digestion of corneal tissues samples takes approx. 72 to complete. Note: Basic Protocols 3 & 4 can be performed simultaneously. Basic



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Protocol 5, which include peptide purification and mass spectrometry measurements will take approx. 24 to 48 h, depending on the number of samples measured and the gradient length selected. Finally, bioinformatics analyses take approx. two to five days depending on number of samples, computing power, and user experience. In total, the experiments outlined in this Protocol will require nine to 15 days.

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**CONFLICT OF INTEREST STATEMENT**:

J.Y., J.L., M.G. & J.G.-M. declare no conflicts of interest; J.R.K. is an employee of Bioinformatics Solutions Inc.

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**INTERNET RESOURCES:**

https://www.maxquant.org/

MaxQuant is a freely available, quantitative proteomics software package designed for analyzing large mass spectrometric data sets. Web site includes links to regularly updated wiki documentation and to MaxQuant Summer School, which provides hands-on training in computational analysis of proteomics data.

http://www.coxdocs.org/doku.php?id=maxquant:andromeda



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Documentation for the Andromeda search engine, which is based on probabilistic scoring (comparable to the commercially availableMascot) and is integrated with MaxQuant.

https://www.bioinfor.com//

PEAKS software is a comprehensive, vendor-neutral, proteomics tool to provide systematic identification and quantification of peptides/proteins in a complex protein mixture using tandem mass spectrometry. PEAKS workflows enable peptide and protein identification (e.g., *de novo* sequencing, database and spectral library searching), post-translational modification and mutation characterization, and quantification.

https://www.r-project.org

R is a free software environment for statistical computing and graphics. It compiles and runs on a wide variety of UNIX platforms, Windows, and MacOS.

https://maxquant.net/perseus/

Perseus software helps biological and biomedical researchers interpret protein quantification, interaction, and post-translational modification data. It offers a user-friendly, interactive workflow environment with complete documentation of computational methods used for publication. Web site also includes a link to regularly updated wiki documentation.

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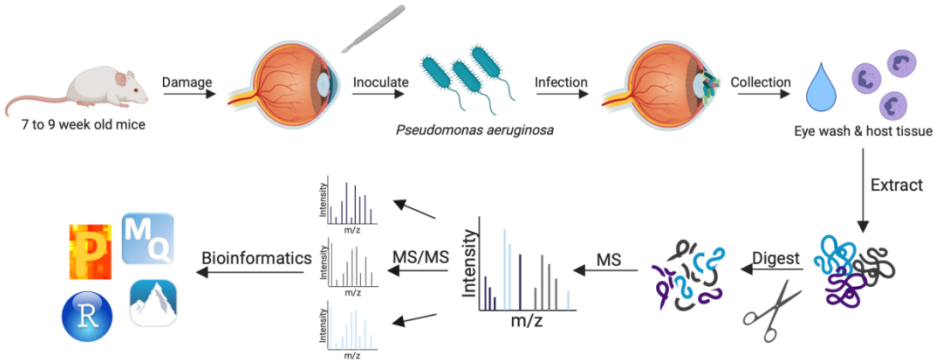
https://www.ebi.ac.uk/pride/archive/

The Proteomics IDEntification (PRIDE) database is a centralized, standards-compliant, public repository for proteomics data, including protein and peptide identifications, post-translational modifications, and supporting spectral evidence.

**Figure 1: Overview of mass spectrometry-based proteomics workflow for eye wash and corneal tissues.** Murine models of infection are used to initiate *P. aeruginosa* infection of the ocular surface. Corneal tissue and eye wash samples from infected, counter-lateral, and uninfected mice are collected. Protein extraction, digestion, and peptide purification is performed as described in the Protocol, followed by liquid chromatography and tandem mass spectrometry, as well as examples of data processing tools for analysis and visualization.



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