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Project Page Link: https://review.jove.com/files_upload.php?src=21372923

Title: Metagenomic Next-Generation Sequencing of Cerebrospinal Fluid for the Detection of Central Nervous System Pathogens

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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? **yes**
- 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: 27

Number of Shots: 53

Introduction

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

Authors: Please note that **the questions will not appear on screen**. Please answer in **stand-alone full sentence with sufficient context**

INTRODUCTION:

REQUIRED: Summarize your research focus and the main questions it aims to answer.

- 1.1. **Sumanth Reddy:** This is a research-based protocol that allows for metagenomic next-generation sequencing of cerebrospinal fluid samples for the detection of pathogens.
 - 1.1.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera.

~~Describe the research gap or limitation in existing methods and how this protocol addresses them.~~

- 1.2. **Sumanth Reddy:** Existing mNGS methods struggle with low RNA yield in CSF, affecting extraction and enzyme efficiency; this protocol optimizes workflows for low-biomass samples.
 - 1.2.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B roll: 3.4.1*

CONCLUSION:

~~Describe the most important consideration or challenge when performing this protocol.~~

- 1.3. **Jarod Morgenroth-Rebin:** A key challenge is precise pipetting due to low volumes, along with time-sensitive bead drying, where under- or overdrying can compromise results.
 - 1.3.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B roll: 3.7.2*

~~Are there any additional methods or analyses that can be performed following this procedure?~~

- 1.4. **Sumanth Reddy**: In addition to pathogen detection, host RNA transcripts obtained from this protocol can be further analyzed for various downstream applications.

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

~~How can future studies build on this work? Describe the questions or research directions that can be investigated next.~~

- 1.5. **Sumanth Reddy**: Future studies can compare emerging library preparation protocols for sparse samples to evaluate their performance in both pathogen detection and bulk transcriptomic analyses.

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.

Testimonial Questions (OPTIONAL):

Videographer: Please capture all testimonial shots in a wide-angle format with sufficient headspace, as the final videos will be rendered in a 1:1 aspect ratio. Testimonial statements will be presented live by the authors, sharing their spontaneous perspectives.

What is the key takeaway you hope viewers remember after watching your video article?

- 1.6. **Sumanth Reddy, MD Neuroimmunology Fellow, University of California San Francisco**, : (authors will present their testimonial statements live)

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

How does the video format of your article help you to share your work with the broader scientific community?

- 1.7. **Sumanth Reddy, MD Neuroimmunology Fellow, University of California San Francisco**, : (authors will present their testimonial statements live)

1.7.1. 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 Institutional Review Board (IRB) at the University of California, San Francisco

Protocol

***Videographer:** Please always record an audio slate while filming each shot mentioned in the shotlist.*

2. Extraction of Cerebrospinal Fluid (CSF) Nucleic Acid

Demonstrator: Jarod Morgenroth-Rebin

- 2.1. To begin, thaw the CSF (C-S-F) samples on ice [1]. Transfer 100 microliters to 1 milliliter of each sample to a 1.5-milliliter tube [2] and centrifuge at 16000 g for 10 minutes at 4 degrees Celsius [3].
 - 2.1.1. WIDE: Talent thawing the cerebrospinal fluid samples on ice.
 - 2.1.2. Talent transferring each sample to a 1.5 milliliter tube.
 - 2.1.3. Talent placing the tubes in the centrifuge.
- 2.2. Then, carefully pipette off the supernatant, leaving behind the pellet and 100 microliters of supernatant [1].
 - 2.2.1. Talent pipetting off the supernatant from the tube.
- 2.3. Add 100 microliters of DNA and RNA Shield to the remaining 100 microliters of supernatant and pellet and mix thoroughly [1].
 - 2.3.1. Talent adding DNA/RNA Shield to the tube and mixing it. VIDEO NOTE: Use Take 2
- 2.4. Then, add 600 microliters of DNA and RNA lysis buffer and mix by pipetting until the solution is homogeneous [1].
 - 2.4.1. Talent adding deoxyribonucleic acid/ribonucleic acid lysis buffer to the tube and mixing it.
- 2.5. Perform the DNA and RNA extraction protocol as per the manufacturer's instructions [1]. Perform all centrifugation steps for 1 minute at maximum speed, except the final DNA or RNA wash, which should be centrifuged for 3 minutes [2].
 - 2.5.1. Talent adding a reagent to the tube with the labelled vials of all the reagents in the frame.
 - 2.5.2. Talent places the tube in the centrifuge and setting the parameters.
- 2.6. To elute the sample, add 22 microliters of nuclease-free water directly onto the column matrix, then incubate for 3 minutes at room temperature [1].
 - 2.6.1. Talent adding nuclease-free water directly onto the column matrix and leaving it to incubate.
- 2.7. After elution, pipette the flow-through back onto the column matrix [1]. Centrifuge

again for 1 minute at maximum speed before transferring the sample to a new 1.5-milliliter storage tube [2]. NOTE: The VO is edited for the deleted step

2.7.1. Talent pipetting the flow-through back onto the column matrix.

2.7.2. Talent placing the column in the centrifuge again.

~~2.7.3. Talent transferring the sample to a new 1.5 milliliter storage tube.~~ NOTE: Not filmed

3. CSF RNA Library Preparation

3.1. Pipette the components required for RNA fragmentation and priming in a PCR tube [1]. Incubate the tube in a thermocycler with the heated lid set to 105 degrees Celsius [2].

3.1.1. TEXT ON A PLAIN BACKGROUND

RNA sample: 3.5 μ L

ERCC spike-in (1:2500): 0.5 μ L

First-strand reaction buffer (5x): 4 μ L

Random primers: 1 μ L

rRNA (1:100): 1 μ L

3.1.2. Talent placing the polymerase chain reaction tube into the thermocycler.
and

TEXT ON A PLAIN BACKGROUND

75 °C for 2 min

70 °C for 2 min

65 °C for 2 min

60 °C for 2 min

55 °C for 2 min

37 °C for 5 min

25 °C for 5 min *Video editor: Please use split screen to show both the shots simultaneously*

3.2. Pipette mix the fragmented and primed RNA with 8 microliters of nuclease-free water and 2 microliters of first-strand synthesis enzyme mix [1]. Incubate the tube in a thermocycler with the heated lid set to 105 degrees Celsius [2-TXT].

3.2.1. Talent pipetting and mixing the fragmented and primed ribonucleic acid with reagents.

- 3.2.2. Talent placing the tube into the thermocycler. **TXT: 25 °C for 10 min; 42 °C for 15 min; 70 °C for 15 min**
- 3.3. Then mix the first-strand synthesized DNA with 48 microliters of nuclease-free water, 8 microliters of second-strand synthesis reaction buffer, and 4 microliters of second-strand synthesis enzyme mix [1]. Place the tube in a thermocycler and incubate for 1 hour at 16 degrees Celsius with the heated lid turned off [2].
 - 3.3.1. Talent pipetting and mixing the first-strand synthesized deoxyribonucleic acid with reagents.
 - 3.3.2. Talent placing the tube into the thermocycler.
- 3.4. Next, perform magnetic bead purification using a 1.8 times ratio of solid-phase reversible immobilization magnetic beads [1]. After incubation, elute the sample into 53 microliters of nuclease-free water [2]. Transfer 50 microliters of the final supernatant containing purified double-stranded cDNA to a clean nuclease-free PCR tube [3].
 - 3.4.1. Talent adding solid-phase reversible immobilization magnetic beads to the sample.
 - 3.4.2. Talent adding nuclease-free water to elute the sample.
 - 3.4.3. Talent transferring the supernatant to a clean polymerase chain reaction tube.
- 3.5. If the sample was stored at minus 20 degrees Celsius overnight, thaw on ice before restarting [1]. Pipette mix 50 microliters of purified double-stranded cDNA with 7 microliters of end-preparation reaction buffer and 3 microliters of end-preparation enzyme mix [2].
 - 3.5.1. Talent thawing the sample on ice.
 - 3.5.2. Talent pipetting and mixing the purified double-stranded complementary deoxyribonucleic acid with reagents.
- 3.6. Place the samples in a thermocycler and incubate at 20 degrees Celsius for 30 minutes and 65 degrees Celsius for 30 minutes with the heated lid turned off [1].
 - 3.6.1. Talent placing the tubes into the thermocycler.
- 3.7. Next, perform the adapter ligation step on ice by first creating a 1 to 100 dilution of adapter in nuclease-free water [1]. Pipette the end-preparation reaction mixture into a tube containing 30 microliters of ligation master mix, 1 microliter of ligation enhancer, and 2.5 microliters of the diluted adapter [2-TXT].
 - 3.7.1. Talent preparing a diluted adapter solution on ice.
 - 3.7.2. Talent pipetting the reaction mixture, ligation master mix, ligation enhancer, and adapter sequentially into a tube.

3.8. Place the samples in a thermocycler and incubate at 20 degrees Celsius for 15 minutes with the heated lid turned off [1].

3.8.1. Talent placing the tubes into the thermocycler.

3.9. After that, proceed immediately to magnetic bead purification using a 0.9 times ratio of solid-phase reversible immobilization magnetic beads [1].

3.9.1. Talent adding solid-phase reversible immobilization magnetic beads to the sample.

3.10. Once the sample is incubated, elute into 17 microliters of nuclease-free water [1]. Transfer 15 microliters of the final supernatant to a clean nuclease-free PCR tube [2].

3.10.1. Talent adding nuclease-free water to elute the sample.

3.10.2. Talent transferring the supernatant to a clean polymerase chain reaction tube.

3.11. Now, pipette purified adapter-ligated cDNA into a tube with 3 microliters of USER (*User*) enzyme, 25 microliters of Q5 Master Mix, and 10 microliters of unique barcoded primers [1]. Place the tube in a thermocycler with the heated lid set to 105 degrees Celsius [2] and incubate at the presented conditions [3].

3.11.1. Talent pipetting and mixing the complementary deoxyribonucleic acid with USER enzyme, Q5 Master Mix, and barcoded primers.

3.11.2. Talent placing the tube in the thermocycler.

3.11.3. TEXT ON A PLAIN BACKGROUND

37 °C for 15 min

98 °C for 30 s

98 °C for 10 s and 65 °C for 75 s for 19 cycles

65 °C for 5 min.

3.12. Perform a final magnetic bead purification using a 0.8 times ratio of magnetic beads [1]. Then, elute the sample into 23 microliters of nuclease-free water [2]. Transfer 20 microliters of the final supernatant to a clean nuclease-free PCR tube [3].

3.12.1. Talent adding magnetic beads to the sample.

3.12.2. Talent adding nuclease-free water to elute the sample.

3.12.3. Talent transferring the supernatant to a clean polymerase chain reaction tube.

3.13. Quantify the concentration of the sample libraries using a DNA quantification kit and the corresponding fluorometer [1]. Verify that water controls have substantially lower or unquantifiable concentrations compared to the other

samples [2].

3.13.1. Talent places the sample in the flurometer.

3.13.2. Display fluorometer readings showing low or undetectable signal for water controls compared to sample libraries.

NOTE: 3.13.1. and 3.13.2 are combined in a single shot

3.14. Assess the sample library sizes by running the samples on an automated capillary electrophoresis machine [1-TXT].

3.14.1. Talent loading the samples into the automated capillary electrophoresis machine. **TXT: Perform CSF DNA library preparation following a similar workflow**

4. Data Analysis for Pathogen Detection

Demonstrator: Sumanth Reddy

4.1. Use the open-source web-based platform Chan Zuckerberg ID to perform metagenomic analysis for pathogen detection [1].

4.1.1. SCREEN: 70075_screenshot_1.mp4: 00:00-00:02

4.2. Log into Chan Zuckerberg ID and click **Upload** [1]. Select **Metagenomics** as the analysis type and choose the input FASTQ (*Fast-Q*) files from the sequencing run [2]. Enter the required sample information, including sample name, sample type CSF, and nucleotide type RNA or DNA [3].

4.2.1. SCREEN: 70075_screenshot_1.mp4: 00:03-00:09, 00:17-00:19

4.2.2. SCREEN: 70075_screenshot_1.mp4: 00:49-01:02

4.2.3. SCREEN: 70075_screenshot_2.mp4: 00:00-00:20

4.3. Once processing is complete, click the project folder containing the samples and navigate to the summary dashboard [1].

4.3.1. SCREEN: 70075_screenshot_3.mp4

4.4. Scroll down to examine the number of reads per sample [1]. Review the percentage of reads passing quality control filtering [2] and the duplicate compression ratio to assess for biased PCR overamplification [3].

4.4.1. SCREEN: 70075_screenshot_4.mp4: 00:03-00:05 *Video editor: Highlight the left hand side graph*

4.4.2. SCREEN: 70075_screenshot_4.mp4: 00:03-00:05 *Video editor: Highlight the right hand side graph*

4.4.3. SCREEN: 70075_screenshot_4.mp4: 00:09-00:11 *Video editor: Highlight the*

left-hand side graph

- 4.5. To determine any Foreign DNA contamination, within the project folder, select each water control sample and click **Background model** to generate the model [1]. During analysis, apply the model and use **Threshold filters** to set an NTZ ($N-T-Z$) score greater than 1 to remove likely contaminants. Then, analyse the data using appropriate statistical software [2].

4.5.1. SCREEN: 70075_screenshot_5.mp4

4.5.2. SCREEN: 70075_screenshot_6.mp4

- ~~4.6. Utilize predefined reads per million thresholds by comparing sample reads per million values to control averages [1]. Download taxon-specific reads per million data by selecting samples, clicking **Download**, and choosing **Combined Sample Taxon Results** [2]. Analyze the resulting comma-separated values file using statistical software such as R or Stata [3].~~ **NOTE: Not filmed**

~~4.6.1. SCREEN: Selecting samples and clicking Download followed by Combined Sample Taxon Results.~~

~~4.6.2. SCREEN: Displaying the downloaded comma-separated values file.~~

~~4.6.3. SCREEN: Opening and analyzing the file in statistical software.~~

Results

5. Results

5.1. The capillary electrophoresis and electropherogram data demonstrated a cDNA sample library that was over-fragmented and contained adapter dimers not suitable for sequencing [1].

5.1.1. LAB MEDIA: Figure 2A and B. *Video editor: Highlight the small peak region at low size values indicating adapter dimers*

5.2. The cDNA library without significant adapter dimer contamination was ready for pooling and sequencing [1].

5.2.1. LAB MEDIA: Figure 2C and D. *Video editor: Show the smooth main peak representing the cDNA library*

5.3. CSF mNGS detected diverse pathogens in Cohort A and B, with at least one pathogen in 73.5 percent of Cohort A samples [1] and 51.2 percent of Cohort B samples [2]

5.3.1. LAB MEDIA: Figure 3

5.3.2. LAB MEDIA: Figure 3

1. Cerebrospinal

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

IPA: /səˈriːbrəʊˈspɑɪ.nəl/

Phonetic Spelling: suh·ree·broh·spy·nuhl

2. Centrifuge

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

IPA: /ˈsen.trəˌfjuːdʒ/

Phonetic Spelling: sen·truh·fyooj

3. Supernatant

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

IPA: /ˌsuː.pəˈnei.tənt/

Phonetic Spelling: soo·per·nay·tuhnt

4. Pellet
Pronunciation link: <https://www.merriam-webster.com/dictionary/pellet>
IPA: /'pel.ɪt/
Phonetic Spelling: pel·it
5. Deoxyribonucleic
Pronunciation link: <https://www.merriam-webster.com/dictionary/deoxyribonucleic%20acid>
IPA: /diˌɑːk.siˌraɪ.boʊ.nuː'kliː.ɪk/
Phonetic Spelling: dee·ok·see·ry·boh·noo·klee·ik
6. Ribonucleic
Pronunciation link: <https://www.merriam-webster.com/dictionary/ribonucleic%20acid>
IPA: /ˌraɪ.boʊ.nuː'kliː.ɪk/
Phonetic Spelling: ry·boh·noo·klee·ik
7. Homogeneous
Pronunciation link: <https://www.merriam-webster.com/dictionary/homogeneous>
IPA: /ˌhoʊ.mə'dʒiː.ni.əs/
Phonetic Spelling: hoh·muh·jee·nee·uhs
8. Nuclease
Pronunciation link: <https://www.merriam-webster.com/dictionary/nuclease>
IPA: /'nuː.kli.eɪs/
Phonetic Spelling: noo·klee·ays
9. Elute
Pronunciation link: <https://www.merriam-webster.com/dictionary/elute>
IPA: /i'luːt/
Phonetic Spelling: ee·loot
10. Thermocycler
Pronunciation link: No confirmed link found
IPA: /'θɜː.moʊ.sai.klə/
Phonetic Spelling: ther·moh·sy·kler
11. Fragmentation
Pronunciation link: <https://www.merriam-webster.com/dictionary/fragmentation>
IPA: /ˌfræɡ.mən'teɪ.ʃən/
Phonetic Spelling: frag·muhn·tay·shuhn
12. Priming
Pronunciation link: <https://www.merriam-webster.com/dictionary/priming>
IPA: /'praɪ.mɪŋ/
Phonetic Spelling: pry·ming
13. Polymerase
Pronunciation link: <https://www.merriam-webster.com/dictionary/polymerase>
IPA: /pə'lim.ə.reɪs/
Phonetic Spelling: puh·lim·uh·rays
14. Electrophoresis
Pronunciation link: <https://www.merriam-webster.com/dictionary/electrophoresis>

- IPA: /iˌlek.troʊ.fəˈriː.sɪs/
Phonetic Spelling: ih·lek·troh·fuh·ree·suhs
15. Electropherogram
Pronunciation link: No confirmed link found
IPA: /iˌlek.troʊˈfer.əˌgræm/
Phonetic Spelling: ih·lek·troh·fer·uh·gram
16. Metagenomic
Pronunciation link: <https://www.merriam-webster.com/dictionary/metagenomics>
IPA: /ˌmet.ə.dʒəˈnoʊ.mɪk/
Phonetic Spelling: met·uh·juh·noh·mik
17. FASTQ
Pronunciation link: No confirmed link found
IPA: /ˈfæst.kjuː/
Phonetic Spelling: fast·kyoo
18. Pathogen
Pronunciation link: <https://www.merriam-webster.com/dictionary/pathogen>
IPA: /ˈpæθ.ə.dʒən/
Phonetic Spelling: path·uh·juhn