

Submission ID #: 70645

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Title: Multi-Locus Sequencing for Strain Typing of *Toxoplasma gondii*

Authors and Affiliations:

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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**

~~*Videographer: Please record the computer screen for the shots labeled as SCREEN as back up*~~

3. Filming location: Will the filming need to take place in multiple locations? **Yes**

If **Yes**, how far apart are the locations? **15 metres**

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: 25

Number of Shots: 56 (10 SC)

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**

Answers will be slightly edited in accordance with journal guidelines.

INTRODUCTION:

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

- 1.1. **Paul Denny:** We focus on genetic variations in the food-borne parasite *Toxoplasma* to identify uncharacterized strains posing increased risk, specially during pregnancy.
 - 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. **Paul Denny:** Rapid genetic characterization of 'strains of interest' is key and this protocol outlines a rapid nanopore sequencing methodology.
 - 1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

CONCLUSION:

~~What does this protocol allow researchers to study or measure?~~

- 1.3. **Paul Denny:** This study has the potential to transform the detection and monitoring of foodborne protozoan parasites, particularly in low-resource settings.
 - 1.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

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

- 1.4. **Paul Denny:** Reference strains were utilized in this study. Expansion to field strains may require whole genome sequence data as well high-quality genomic DNA.

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

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

- 1.5. **Paul Denny:** This procedure could be expanded and multiplexed to include other food pathogens of interest, such as *Saracocystis*.
 - 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.

Videographer's NOTE: Only author Paul Denny's headshot was taken.

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.

- Testimonial statements will **not appear in the video** but may be featured in the journal's promotional materials.
- **Provide the full name and position** (e.g., Director of [Institute Name], Senior Researcher [University Name], etc.) of the author delivering the testimonial.
- Please **answer the testimonial question live during the shoot**, speaking naturally and in your own words in **complete sentences**.

What problem in your field of research does your JoVE article address?

1.6. **Paul Denny, Professor of Protozoan Biology and Director of Research (Biosciences)**

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

What value do you expect this video article to have for other researchers working in your field?

1.7. **Paul Denny, Professor of Protozoan Biology and Director of Research (Biosciences)**:
(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

Protocol

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

2. End Repair and dA-Tailing of *Toxoplasma gondii* Genomic DNA Amplicons

Demonstrator: Abeer Khadr

- 2.1. To begin, thaw the required reagents and the purified PCR amplicons generated from *Toxoplasma gondii* tachyzoite genomic DNA [1]. Pipette each DNA sample into 0.2 milliliter polymerase chain reaction tubes and adjust the volume to 11.5 microliters with nuclease-free water [2-TXT].
 - 2.1.1. Talent retrieving the reagents and DNA from refrigeration and placing the samples on ice box. **Videographer's NOTE: Shot 2.1 was incorrectly marked as 1.2**
 - 2.1.2. Talent pipetting each DNA sample into 0.2 milliliter polymerase chain reaction tubes and adding water **TXT: DNA: 200 fmol (~130 ng for a 1 kb fragment)**
- 2.2. Now, add 1 microliter of the diluted DNA control sample to each tube and mix by pipetting 10 to 20 times [1]. Prepare an end-prep master mix for each sample containing 1.75 microliters of Reaction Buffer and 0.75 microliters of Enzyme Mix, including 5% excess [2]. Add 2.5 microliters of the master mix to each sample and mix gently [3].
 - 2.2.1. Talent adding 1 microliter of diluted DNA control sample to each tube and mixing the contents by pipetting.
 - 2.2.2. Talent preparing the end-prep master mix for each sample with Reaction Buffer and Enzyme Mix.
 - 2.2.3. Talent adding 2.5 microliters of the master mix to each sample and mixing.
- 2.3. After a brief spin, incubate the samples in a thermal cycler at 20 degrees Celsius for 5 minutes, followed by 65 degrees Celsius for 5 minutes [1]. Purify the end-prepared DNA using DNA Cleanup magnetic beads and wash with ethanol to remove impurities [2]. After elution, measure the DNA concentration for downstream use [3].
 - 2.3.1. Talent placing the samples in the thermal cycler for incubation.
 - 2.3.2. Talent adding magnetic beads to a sample containing DNA. **Videographer's**

NOTE: Shot 2.3.2 had to be filmed in two parts

2.3.3. Talent loading 1 microliter of sample onto the spectrophotometer for measurement.

3. Native Barcoding and Pooling of End-Prepared DNA Samples

~~3.1. Assign individual native barcodes to samples numbered 1 to 24 [1].~~

~~3.1.1. Talent labeling and assigning individual native barcodes to corresponding samples.~~ **Videographer's NOTE:** Shot 3.1.1. was not filmed at the author's request, as said it was not required

3.2. Add 7.5 microliters of end-prepared DNA to each tube [1], followed by 2.5 microliters of the Native DNA Barcode and 10 microliters of Blunt TA Ligase Master Mix to assemble the barcoding reaction. Incubate at room temperature for 20 minutes [2-TXT]. **Authors:** We do not pronounce slash according to our journal guidelines

3.2.1. Talent adding 7.5 microliters of end-prepared DNA to a tube.

3.2.2. Talent adding 2.5 microliters of Native barcode and 10 microliters of Blunt/TA Ligase Master Mix to the tube. ~~TXT: Mix 10 – 20x; Incubate at RT (20 min)~~

3.3. After incubation, terminate the reaction with 4 microliters of EDTA and mix thoroughly by pipetting [1].

3.3.1. Talent adding EDTA and mixing by pipetting up and down.

3.4. Transfer the barcoded samples into a 1.5-milliliter low-DNA-binding microcentrifuge tube to pool them [1] and purify the DNA using magnetic beads [2 and 3].

3.4.1. Talent pooling barcoded samples into a 1.5 milliliter low DNA-binding microcentrifuge tube.

3.4.2. Talent adding magnetic beads to the sample.

3.4.3. TEXT ON 3.4.2's BACKGROUND:

- **Add magnetic beads:** 0.4x volume, mix and incubate for 10 min (RT)
- **Wash beads:** 2x, 700 µL Short Fragment Buffer (SFB)
- **Resuspension and Elution:** 35 µL nuclease-free water (37 °C, 10 min)

4. Adapter Ligation and Library Clean-Up of Barcoded Amplicon DNA

- 4.1. Add 30 microliters of purified pooled barcoded DNA to a tube [1] and mix it with 5 microliters each of Native Adapter and T4 DNA ligase, along with 10 microliters of 5x Ligation Buffer [2].
 - 4.1.1. Talent adding pooled barcoded DNA to a tube. **Videographer's NOTE: Shot 4.1.1 & 4.1.2 were filmed together**
 - 4.1.2. Talent adding the reaction mix to the barcoded DNA and mixing by pipetting.
- 4.2. After a brief spin, incubate the sample at room temperature for 20 minutes [1].
 - 4.2.1. Talent removing the tube from a centrifuge and placing it aside on a the work desk.
- 4.3. Next, add 20 microliters of resuspended magnetic beads to the reaction and mix by pipetting [1]. Incubate the sample on a rotator mixer at room temperature for 10 minutes [2].
 - 4.3.1. Talent adding 20 microliters of resuspended magnetic beads to the reaction and mixing the sample by pipetting.
 - 4.3.2. Talent placing the tube on a rotator mixer for incubation.
- 4.4. Spin down the sample [1] and place the tube on a magnetic rack to pellet the beads [2]. Pipette off the supernatant while keeping the tube on the magnet [3].
 - 4.4.1. Talent spinning down the sample.
 - 4.4.2. Talent placing the tube on a magnetic rack.
 - 4.4.3. Talent removing the supernatant with a pipette.
- 4.5. Next, add 125 microliters of Short Fragment Buffer to wash the beads [1]. Flick the tube to resuspend the beads [2]. Spin down briefly [3]. Place the tube on a magnetic rack and allow the beads to settle [4]. Remove the supernatant using a pipette and repeat the wash step with Short Fragment Buffer [5].
 - 4.5.1. Talent adding 125 microliters of Short Fragment Buffer to the tube.
 - 4.5.2. Talent flicking the tube to resuspend the beads.

- 4.5.3. Talent spinning down the tube briefly.
- 4.5.4. Talent placing the tube on a magnetic rack to pellet the beads.
- 4.5.5. Talent removing and discarding the supernatant.

- 4.6. After a brief spin [1], place the tube back on the magnetic rack [2] and pipette off any residual supernatant [3]. Remove the tube from the magnetic rack [4] and resuspend the pellet in 15 microliters of Elution Buffer [5].
 - 4.6.1. Talent spinning down the tube.
 - 4.6.2. Talent placing the tube on the magnetic rack.
 - 4.6.3. Talent removing residual supernatant with a pipette.
 - 4.6.4. Talent removing the tube from the magnetic rack.
 - 4.6.5. Talent resuspending the pellet in Elution Buffer.

- 4.7. Incubate the tube at 37 degrees Celsius for 10 minutes [1]. Agitate the tube every 2 minutes by gently flicking for 10 seconds to encourage DNA elution [2].
 - 4.7.1. Talent placing the tube for incubation in an incubator.
 - 4.7.2. Talent taking the tube out and flicking it and placing it back into the incubator.

- 4.8. Then, place the tube on a magnetic rack to let the beads settle until the eluate is clear and colorless for at least 1 minute [1]. Transfer 15 microliters of eluate containing the DNA library into a clean 1.5-milliliter low-DNA-binding microcentrifuge tube [2]. Measure the final library concentration using a spectrophotometer [3].
 - 4.8.1. Talent placing the tube on a magnetic rack and observing the tube for eluate.
 - 4.8.2. Talent transferring the eluate to a clean 1.5 milliliter low DNA-binding microcentrifuge tube.
 - 4.8.3. Talent loading the sample onto the spectrophotometer for quantification.

5. Flow Cell Preparation and Nanopore Sequencing

- 5.1. Inspect the flow cell and confirm at least 80 active pores [1].
 - 5.1.1. Talent inspecting the flow cell and checking pore availability on the device interface.

- 5.2. Add 117 microliters of flush solution to a microcentrifuge tube [1], followed by 3 microliters of tether solution to reach a total volume of 120 microliters [2]. Mix gently by pipetting to avoid introducing air bubbles [3]. Slowly load the entire 120 microliters of priming mixture into the flow cell priming port without introducing air bubbles [4].
 - 5.2.1. Talent adding flush solution to a microcentrifuge tube.
 - 5.2.2. Talent adding tether solution to the same tube. Videographer's NOTE: Shot 5.2.2. & 5.2.3. were filmed together
 - 5.2.3. Talent gently pipetting the mixture to mix.
 - 5.2.4. Talent slowly loading the priming mixture into the flow cell priming port.
- 5.3. Next, add 15 microliters of Sequencing Buffer to a tube [1]. Add 10 microliters of Library Beads along with 5 microliters of DNA library to prepare the sequencing mix [2]. Mix gently by pipetting up and down 3 to 5 times to avoid introducing bubbles [3].
 - 5.3.1. WIDE: Talent adding Sequencing Buffer to a tube.
 - 5.3.2. Talent adding Library Beads and DNA library to the tube.
 - 5.3.3. Talent gently pipetting the mixture up and down.
- 5.4. Slowly load a 30 to 45 microliter reaction volume of the sequencing mix into the flow cell sample port by dispensing dropwise [1]. Avoid introducing air bubbles and ensure the pipette tip does not touch the membrane [2]. Close the loading port securely after dispensing [3].
 - 5.4.1. Talent loading the sequencing mix into the flow cell sample port dropwise.
 - 5.4.2. Close-up of careful pipetting without touching the membrane.
 - 5.4.3. Talent closing the loading port securely.
- 5.5. Start sequencing after enabling real-time base calling and monitor the pore activity [1].
 - 5.5.1. SCREEN: (1)5.5.1a.mp4. and (2)5.5.1b.mp4 00:01-00:02
- 5.6. Continue sequencing until at least 500 times coverage per amplicon is achieved, with a recommended coverage of at least 1000 times for sufficient read depth and reliable analysis [1].
 - 5.6.1. SCREEN: (3)5.6.1.mp4. 00:57-01:05

5.7. Generate per-amplicon consensus sequences using a neural network–based consensus polishing algorithm with default parameters [1]. Export the consensus sequences in FASTA format for downstream analysis [2].

5.7.1. SCREEN: (5)5.8.2.mp4 00:10-00:20

5.7.2. SCREEN (5)5.8.2.mp4. 00:21-00:28

5.8. Perform a reference-based alignment and comparative sequence analysis using publicly available genomic databases [1]. Visualize the read alignments and polymorphic positions using a genome visualization software [2]. Next, perform high-accuracy base-calling using the platform-associated base-calling software with default high-accuracy settings to generate per-sample FASTQ files. Demultiplex the reads by barcode and retain the ones that pass the quality control filters [3].

5.8.1. SCREEN: (6)5.8.3.mp4. 00:05-00:09 and 00:22-00:27

5.8.2. SCREEN: (8)5.9.2.mp4. 00:25-00:28 and 00:45-00:48

5.8.3. SCREEN: (9)5.7.1&2.mp4 . 00:18-00:34

5.9. Finally, detect the variants using a consensus-based variant-calling algorithm [1]. Separate the single-nucleotide polymorphisms and the insertion or deletion events using variant selection tools [2].

5.9.1. SCREEN: (10)5.10.1.mp4 00:17-00:26.

5.9.2. SCREEN: (11)5.10.2b.mp4 00:10-00:18.

Results

6. Results

6.1. Genomic DNA of *Toxoplasma gondii* was successfully amplified targeting the SAG2 (*sag-2*) and SAG3 (*sag-3*) genetic markers [1].

6.1.1. LAB MEDIA: Figure 2. *Video editor: Show the full gel image and highlight labels "SAG2" and "SAG3"*

6.2. Agarose gel electrophoresis showed that the purified PCR products from RH and ME49 strains produced distinct bands corresponding to 639 base pairs for SAG2 [1] and 1158 base pairs for SAG3 [2].

6.2.1. LAB MEDIA: Figure 2. *Video editor: Highlight the bands labeled "639" under SAG2*

6.2.2. LAB MEDIA: Figure 2. *Video editor: Highlight the band labeled "1158" under SAG3*

6.3. High-throughput nanopore sequencing generated high-quality datasets across examined loci in both RH and ME49 strains [1].

6.3.1. LAB MEDIA: Table 1. *Video editor: Show the full table and highlight "Reads" column*

6.4. The ME49 strain showed good, relatively uniform sequencing coverage across the SAG2 locus with very few mismatches [1], whereas the RH strain showed multiple nucleotide substitutions across the same region [2].

6.4.1. LAB MEDIA: Figure 3. *Video editor: Show the left panel "SAG2_ME49" and highlight the even grey series of bars/lines in the segment labelled 2930 to 0*

6.4.2. LAB MEDIA: Figure 3. *Video editor: Show the right panel "SAG2_RH" and emphasize or draw boxes around the red, blue and green coloured lines between the series of grey lines/bars in the segment labelled 1977 to 0*

6.5. The ME49 strain showed limited sequence variation across the SAG3 locus with most reads matching the reference sequence [1], whereas the RH strain showed a markedly higher density of mismatches and clustered variants [2].

6.5.1. LAB MEDIA: Figure 4. *Video editor: Show the left panel "SAG3_ME49" and*

highlight the even grey series of bars/lines in the segment labelled 499 to 0

6.5.2. LAB MEDIA: Figure 4. *Video editor: Show the right panel "SAG3_RH" and emphasize the red blue and green coloured lines between the series of grey lines/bars in the segment labelled 3141 to 0*

6.6. At the SAG2 locus, only 1 single-nucleotide polymorphism was detected in ME49 [1], whereas 6 single-nucleotide polymorphisms were detected in RH [2].

6.6.1. LAB MEDIA: Table 2. *Video editor: Highlight the cell in the "SNPs" row for "ME49" under "SAG2," showing "1"*

6.6.2. LAB MEDIA: Table 2. *Video editor: Highlight the cell in the "SNPs" row for "RH" under "SAG2," showing "6"*

6.7. At the SAG3 locus, greater genetic variation was observed in RH, with 61 single-nucleotide polymorphisms and 14 deletions detected [1], while ME49 showed 34 single-nucleotide polymorphisms, 6 insertions, and 13 deletions [2].

6.7.1. LAB MEDIA: Table 2. *Video editor: Highlight the cells in "RH" column and under "SAG3," that show the SNPs row value of "61" and the Deletions row value of "14"*

6.7.2. LAB MEDIA: Table 2. *Video editor: Highlight the "ME49" columns under "SAG3," and emphasize the cells SNPs "34," Insertions "6," and Deletions "13"*

6.8. At the SAG3 locus, elevated transition-to-transversion ratios were observed in both ME49 [1] and RH strains [2], and the insertion-to-deletion ratios showed slightly more deletions than insertions in both strains [3].

6.8.1. LAB MEDIA: Table 2. *Video editor: Highlight the cell in the "Ts/Tv Ratio" row for "ME49" under "SAG3," showing "1.62"*

6.8.2. LAB MEDIA: Table 2. *Video editor: Highlight the cell in the "Ts/Tv Ratio" row for "RH" under "SAG3," showing "1.26"*

6.8.3. LAB MEDIA: Table 2. *Video editor: Highlight the cell in the "Insertion/Deletion Ratio" row for "ME49" under "SAG3," showing "0.46" and "Insertion/Deletion Ratio" row for "RH" under "SAG3," showing "0.57"*

1. Toxoplasma gondii

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

IPA: /ˈtɑːk.sʊʊˈplæz.mə ˈɡɑːn.di.əɪ/
Phonetic Spelling: tok·soh·plaz·muh gon·dee·eye

2. Amplicons

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

IPA: /ˈæm.plɪ.kɑːn/
Phonetic Spelling: am·pli·kon

3. Tachyzoite

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

IPA: /ˈtæk.ɪˈzoʊ.aɪt/
Phonetic Spelling: tak·uh·zoh·ite

4. Genomic

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

IPA: /dʒəˈnoʊ.mɪk/
Phonetic Spelling: juh·noh·mik

5. Polymerase

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

IPA: /pəˈlɪm.əˌreɪs/
Phonetic Spelling: puh·lim·uh·rays

6. Microliters

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

IPA: /ˈmaɪ.kroʊ.liː.tər/
Phonetic Spelling: my·kroh·lee·ter

7. Nuclease

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

IPA: /ˈnuː.kliː.əs/
Phonetic Spelling: noo·klee·ays

8. Spectrophotometer

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

IPA: /ˌspek.troʊ.foʊˈtɑː.mə.tər/

Phonetic Spelling: spek-troh-foh-tom-uh-ter

9. Barcoding

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

IPA: /ˈbɑːr.koʊdɪŋ/

Phonetic Spelling: bar-koh-ding

10. Ligase

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

IPA: /ˈlaɪ.geɪs/

Phonetic Spelling: ly-gays

11. EDTA

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

IPA: /ˌiː.di.tiˈeɪ/

Phonetic Spelling: ee-dee-tee-ay

12. Microcentrifuge

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

IPA: /ˌmaɪ.kroʊˈsen.trəˌfjuːdʒ/

Phonetic Spelling: my-kroh-sen-truh-fyooj

13. Nanopore

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

IPA: /ˈnæn.ɒp.ɔːr/

Phonetic Spelling: nan-oh-por

14. Sequencing

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

IPA: /ˈsiː.kwən.sɪŋ/

Phonetic Spelling: see-kwuhn-sing

15. Eluate

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

IPA: /'el.ju.ət/

Phonetic Spelling: el·yoo·ayt

16. Electrophoresis

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

IPA: /ɪˌlek.troʊ.fəˈriː.sɪs/

Phonetic Spelling: ih·lek·troh·fuh·ree·suhs

17. Agarose

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

IPA: /'æg.ə.roʊs/

Phonetic Spelling: ag·uh·rohs

18. Loci

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

IPA: /'loʊ.sai/

Phonetic Spelling: loh·sye

19. Polymorphisms

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

IPA: /ˌpɑː.li'mɔːr.fi.zəm/

Phonetic Spelling: pah·lee·mor·fi·zuhm

20. Transversion

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

IPA: /trænz'vɜː.zən/

Phonetic Spelling: tranz·vur·zhuhn

21. Insertion

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

IPA: /ɪnˈsɜːʃən/

Phonetic Spelling: in·sur·shuhn

22. Deletion

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

IPA: /dɪˈliːʃən/

Phonetic Spelling: dih·lee·shuhn

23. Consensus

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

IPA: /kənˈsɛn.səs/

Phonetic Spelling: kuhn·sen·suhs

24. Demultiplex

Pronunciation link:

<https://www.howtopronounce.com/demultiplex>

IPA: /ˌdiːˈmʌl.tɪ.pleks/

Phonetic Spelling: dee·mul·tuh·pleks