

Submission ID #: 70202

Scriptwriter Name: Pallavi Sharma

Project Page Link: <https://review.jove.com/account/file-uploader?src=21411658>

Title: Long-Read Plasmid Sequencing and Assembly using Nanopore Sequencing-Based Workflows

Authors and Affiliations:

Gerardo Cortés-Cortés*, Caison Warner*, Juliet Ellenbach, Samara Katz, Jay Kim, and Manel Camps

Department of Microbiology and Environmental Toxicology, University of California, Santa Cruz

*These authors contributed equally.

Corresponding Authors:

Manel Camps (mcamps@ucsc.edu)

Email Addresses for All Authors:

Gerardo Cortés-Cortés	(gccortes@ucsc.edu)
Caison Warner	(cpwarner@ucsc.edu)
Juliet Ellenbach	(jcstephe@ucsc.edu)
Samara Katz	(Saakatz@ucsc.edu)
Jay Kim	(jaykim@soe.ucsc.edu)
Manel Camps	(mcamps@ucsc.edu)

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. **No**

Current Protocol Length

Number of Steps: 35

Number of Shots: 58

Introduction

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

INTRODUCTION:

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

- 1.1. **Caison Warner:** We investigate the replication, maintenance, and dissemination of self-replicating circular DNA elements, known as plasmids, which necessitates the determination of their sequences.

- 1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera *Suggested B roll: 2.3.1*

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

- 1.2. **Caison Warner:** This protocol addresses coverage and accuracy issues associated with long-read sequences by enriching plasmid DNA and optimising assembly methods.

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

~~Describe the research contexts or systems in which this protocol can be applied.~~

- 1.3. **Caison Warner:** Our protocol is designed to sequence plasmids in situations where the chromosomal sequence is either already known or irrelevant.

- 1.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera *Suggested B roll: 4.6.2*

CONCLUSION:

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

- 1.4. **Caison Warner:** The most important consideration is whether the chromosomal sequence is of interest; if so, standard whole-genome sequencing protocols are better.

- 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. **Caison Warner:** Our protocol's increased coverage opens the door to the study of heteroplasmy, i.e. plasmid sequence variation within a given population.
 - 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.

Protocol

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

2. Plasmid DNA Extraction from *Escherichia coli*

Demonstrator: Juliet Ellenbach

- 2.1. To begin, streak the samples onto LB agar plates supplemented with the appropriate antibiotic to select for the respective plasmid-borne resistance marker [1]. Incubate the plates at 37 degrees Celsius for 24 hours [2].
 - 2.1.1. Talent streaking samples onto Luria-Bertani agar plates supplemented with antibiotics.
 - 2.1.2. Talent placing the plates in the incubator.
- 2.2. The next day, inoculate 3 to 5 millilitres of LB medium supplemented with the appropriate antibiotic with a single isolated colony [1]. Incubate the culture with shaking at 37 degrees Celsius for approximately 8 hours, or until the optical density at 600 nanometers reaches 1 to 2 [2].
 - 2.2.1. Talent inoculating 3 to 5 milliliters of Luria-Bertani medium with a single isolated colony.
 - 2.2.2. Talent placing the culture into a shaking incubator.
- 2.3. Then, inoculate 400 millilitres of LB medium supplemented with the appropriate antibiotic with 400 microliters of the starter culture and incubate as demonstrated earlier [1].
 - 2.3.1. Talent inoculating 400 milliliters of Luria-Bertani medium with 400 microliters of starter culture.
- 2.4. Then, transfer the culture into centrifuge bottles appropriate for the culture volume [1]. Centrifuge at 4200 *g* for 10 minutes [2] and resuspend the pellet using the reagent volumes recommended by the manufacturer [3-TXT].
 - 2.4.1. Talent transferring the culture into centrifuge bottles appropriate for the culture volume.
 - 2.4.2. Talent placing the centrifuge bottles into the centrifuge.
 - 2.4.3. Talent resuspending the pellet with the recommended reagent volume. **TXT:**
$$\text{Vol. [mL]} = \text{Culture Volume [mL]} \times \left(\frac{\text{OD}_{600}}{50} \right)$$
- 2.5. Following cell lysis and neutralisation, centrifuge the lysate at 4200 *g* for 30 minutes [1]. Transfer the supernatant to a new tube and centrifuge it again [2-TXT].

- 2.5.1. Talent placing the lysate into the centrifuge.
- 2.5.2. Talent transferring the supernatant to a new tube.
- 2.6. Perform equilibration, washing, and DNA elution according to the manufacturer's instructions [1].
 - 2.6.1. Talent at the working bench with the components required for washing in the frame.
- 2.7. Then, resuspend the precipitated DNA in 100 microliters of nuclease-free water to maximize concentration for Nanopore library preparation [1]. Next, prepare the assay tubes as per the presented table [2].
 - 2.7.1. Talent adding nuclease-free water to the DNA pellet and resuspending.
 - 2.7.2. LAB MEDIA: Table 1
- ~~2.8. Vortex each tube for a few seconds and incubate the tubes at room temperature for 2 minutes [1].~~ NOTE: This step is not filmed
 - ~~2.8.1. Talent vortexing assay tubes and placing the tubes on the bench for incubation~~
- 2.9. After calibrating the fluorometer, insert the sample tubes into the fluorometer [1] and record the readings according to the instrument manual [2].
 - 2.9.1. Talent inserting sample tubes into the fluorometer.
 - 2.9.2. Talent recording readings from the fluorometer display.

3. Library Preparation for Plasmid Sequencing

- 3.1. Program the thermal cycler to 30 degrees Celsius for 2 minutes, followed by 80 degrees Celsius for 2 minutes [1].
 - 3.1.1. Show the thermal cycler interface while entering 30 degrees Celsius for 2 minutes and 80 degrees Celsius for 2 minutes.
- 3.2. Spin and mix the thawed kit components as per the presented table [1].
 - 3.2.1. LAB MEDIA: Table 2
- 3.3. Incubate the PCR tubes prepared for barcoding using the programmed thermal cycler [1]. Then, place the tubes on ice to cool [2].
 - 3.3.1. Talent placing PCR tubes into the thermal cycler.
AND
LAB MEDIA: Table 3 *Video editor: Use split screen and show this information*
 - 3.3.2. Talent placing tubes on ice.
- 3.4. Pool the barcoded samples in a clean 1.5-millilitre Eppendorf tube, ensuring the total volume does not exceed 1000 microliters [1].

- 3.4.1. Talent transferring barcoded samples into a 1.5 millilitre Eppendorf tube.
- 3.5. Once the beads are vortexed, add an equal volume of beads to the pooled barcoded samples, mix by flicking the tube followed by a 10-minute incubation [1]. ~~incubate the tubes at room temperature for 10 minutes on a Hula mixer [2].~~ NOTE: The VO for the deleted shot I added concisely in the previous shot
- 3.5.1. Talent adding beads to the pooled sample and flicking the tube to mix.
- ~~3.5.2. WIDE: Talent placing tubes onto a Hula mixer for incubation at room temperature.~~ NOTE: Not filmed
- 3.6. Then, wash the beads with 1 millilitre of 80 percent ethanol while on the magnet [1]. Remove the ethanol and repeat once [2].
- 3.6.1. Talent adding 1 milliliter of 80 percent ethanol to the beads on the magnet.
- 3.6.2. Talent removing ethanol from the tube while on the magnet.
- 3.7. Remove the tube from the magnet [1], resuspend the beads in elution buffer using the appropriate volume based on the number of barcodes in use and incubate for 10 minutes at room temperature [2].
- 3.7.1. Talent removing the tube from the magnetic rack.
- 3.7.2. Talent resuspending beads in elution buffer and keeping it aside on the bench.
- 3.8. Quantify the DNA concentration using 1 microliter of the eluted sample on the fluorometer to confirm successful library preparation [1].
- 3.8.1. Talent loading 1 microliter of sample into the fluorometer for measurement.
- 3.9. For adapter ligation, transfer 11 microliters of the DNA library into a new tube and label it [1].
- 3.9.1. Talent transferring 11 microliters of DNA library into a new tube and labeling the tube.
- 3.10. Then, mix the presented components in a fresh tube to dilute the thawed Rapid Adapter [1].
- 3.10.1. LAB MEDIA: Table 5
- 3.11. Next, add 1 microliter of diluted Rapid Adapter to the 11 microliters of DNA library and flick the tube to mix the contents [1]. Spin down the tube briefly to ensure the complete volume collects at the bottom, and incubate the tube on the bench for 5 minutes [2]
- 3.11.1. Talent adding diluted Rapid Adapter to the DNA library tube and flicking the tube.
- 3.11.2. Talent removes the tube from the centrifuge and keeps it on the bench.

4. Priming and Loading of the Flow Cell

Demonstrator: Caison Warner

- 4.1. Prepare the flow cell priming mix in a fresh DNA tube and mix by inversion and pipetting [1].
 - 4.1.1. Talent adding reagents into a fresh DNA tube
AND
LAB MEDIA: Table 6 *Video editor: Use split screen and show this information*
- 4.2. Lift the lid of the device [1] and insert the flow cell into it while applying gentle pressure to ensure proper placement [2].
 - 4.2.1. Talent lifting the lid of the device.
 - 4.2.2. Talent inserting the flow cell into the device.
- 4.3. After checking for air bubbles within or adjacent to the priming port, draw back 20 to 30 microliters of fluid to remove bubbles [1].
 - 4.3.1. Talent withdrawing 20 to 30 microliters of fluid from the priming port.
- 4.4. Then, draw up 800 microliters of priming mix and dispense it into the priming port while avoiding air bubbles, and let the device sit for 5 minutes [1].
 - 4.4.1. Talent drawing 800 microliters of priming mix into a pipette and dispensing the priming mix into the priming port.
- 4.5. Mix the DNA library with the appropriate volume of library beads in a new tube as presented here [1].
 - 4.5.1. LAB MEDIA: Table 7
- 4.6. Load 200 microliters of priming mix into the flow cell priming port [1]. Adjust the sample port cover to reveal the port [2]. Dispense 75 microliters of the DNA library with beads into the sample port one drop at a time, allowing each drop to absorb before adding the next [3]. NOTE: The VO is edited for the inverted shots
 - 4.6.2 Talent loading 200 microliters of priming mix into the priming port. NOTE: 4.6.1 and 4.6.2 are inverted
 - 4.6.1. Talent adjusting the sample port cover to open the port.
 - 4.6.2. Talent carefully dispensing the DNA library with beads into the sample port dropwise.
- 4.7. Then, reapply the sample port cover and close the priming port [1].
 - 4.7.1. Talent placing the sample port cover back onto the device and closing the priming port.
- 4.8. Once the device lid is closed, begin the sequencing run [1]. Set the run time limit based on the number of plasmids being multiplexed, with a default of 48 hours and a

maximum of 72 hours [2].

4.8.1. SCREEN: SCREEN_4_8_1.mp4; 00:06-00:09

4.8.2. SCREEN: SCREEN_4_8_2.mp4; 00:20-00:35

4.9. Select the super-accurate basecalling mode and the appropriate rapid-barcode kit option [1].

4.9.1. SCREEN: SCREEN_4.9.1_AND_4_10_1_AND_4_10_2.mp4: 00:07-00:25

4.10. Then, select the raw FAST5 (*Fast-Five*) or POD5 (*POD-Five*) data [1] and start basecalling [1].

4.10.1. SCREEN: SCREEN_4.9.1_AND_4_10_1_AND_4_10_2.mp4: 00:32-00:36

4.10.2. SCREEN: SCREEN_4.9.1_AND_4_10_1_AND_4_10_2.mp4: 00:38-00:40

4.11. To generate a filtered FASTQ file for downstream assembly, trim long reads using Filtlong to remove approximately 5 to 10 percent of the lowest-quality reads while retaining approximately 90 percent of the total yield [1].

4.11.1. SCREEN: 4_11_1_AND_4_11_2.mp4

4.12. Subsample reads using Autocycler according to recommended settings [1]. Assemble each read subset using Raven, Flye, Canu, miniasm, Myloasm, NECAT, Plasmsembler, and other selected assemblers [2]. ~~Record contig lengths and circularisation status for each assembly. Prioritise assemblies reporting circularisation for plasmid reconstruction [3].~~

4.12.1. SCREEN: 4_12_1.mp4; 00:00-00:05, 00:36-00:40

4.12.2. SCREEN: 4_12_1.mp4: 00:43-01:00

4.12.3. ~~SCREEN: Show reviewing contig lengths and circularization status in assembly outputs.~~ NOTE: Not filmed

4.13. Visualize each assembly graph file using Bandage. Evaluate contigs for circularity, size consistency across assemblers, and evidence of chimeric joints [1]. Remove linear contigs lacking support across multiple assemblers and retain circular contigs with consistent size estimates [2]. Remove minor unsupported contigs before consensus generation when major plasmids are circularized. Proceed to consensus generation after manual curation [3].

4.13.1. SCREEN: 4_13_1_REUPLOAD.mp4_fixed: 00:06-00:30.

4.13.2. SCREEN: 4_13_2_REUPLOAD.mp4_fixed: 00:00-00:38

4.13.3. SCREEN: 4_13_3_REUPLOAD.mp4_fixed

4.14. Generate the consensus sequence using Autocycler with the curated assemblies [1]. Execute the remaining Autocycler commands to produce the final consensus plasmid sequence [2].

4.14.1. SCREEN: 4_14_1_AND_4_14_2.mp4: 00:08-00:24.

4.14.2. SCREEN: 4_14_1_AND_4_14_2.mp4: 00:32-00:45

4.15. At last, polish the final assembly using trimmed long reads with Medaka to improve consensus accuracy [1].

4.15.1. SCREEN: 4_15_1.mp4: 00:00-00:20.

Results

5. Results

5.1. All conjugation assays produced transconjugants, confirming the presence of a conjugative plasmid in the donors [1]. The sizes of assembled plasmid sequences were congruent with estimates based on pulse-field gel electrophoresis mobility [2].

5.1.1. LAB MEDIA: Table 8. *Video editor: Show the table listing donor strains and plasmids to indicate the presence of conjugative plasmids across samples*

5.1.2. LAB MEDIA: Table 8. *Video editor: Highlight the column "Estimated PFGE Size" and "Assembly Length"*

5.2. Nanopore sequencing produced sufficient read output and quality for all samples analyzed [1]. Chromosomal contamination rates ranged between 72.1 percent and 28.0 percent with an average of 46.9 percent [2].

5.2.1. LAB MEDIA: Table 9.

5.2.2. LAB MEDIA: Table 10. *Video editor: Highlight the column "% Chromosomal Reads"*

5.3. Pulse-field gel electrophoresis analysis of strain pUPEC271 (*plasmid-U-PEC-Two-Seven-One*) revealed a band corresponding to a plasmid of approximately 145 kilobase pairs [1]. DNA from one of the transconjugants showed a sequence identical to the 145 kilobases [2].

5.3.1. LAB MEDIA: Figure 3A and B. *Video editor: Highlight the band near 145 kilobases in the donor lane in 3B*

5.3.2. LAB MEDIA: Figure 3A and B. *Video editor: Highlight the corresponding band in the transconjugant lane in 3B*

5.4. Pulse-field gel electrophoresis analysis of strain Blood_10_0913 (*Blood-Ten-Zero-Nine-One-Three*) identified two plasmids of approximately 70 and 110 kilobase pairs [1].

5.4.1. LAB MEDIA: Figure 4A. *Video editor: Highlight the two bands near 70 kilobases and 110 kilobases indicated by arrows*

5.5. Nanopore sequencing confirmed two plasmid assemblies of 71,182 base pairs [1] and 111,226 base pairs [2].

5.5.1. LAB MEDIA: Figure 4B. *Video editor: Zoom into the circular map labeled "pBlood_10_0913_1 71,182 bp"*

5.5.2. LAB MEDIA: Figure 4B. *Video editor: Zoom into the circular map labeled "pBlood_10_0913_2 111,226 bp"*

1. Luria-Bertani
Pronunciation link: <https://www.merriam-webster.com/dictionary/Luria-Bertani>
IPA: /'lʊr.i.ə bər'tɑː.ni/
Phonetic Spelling: LOOR·ee·uh ber·TAA·nee
2. Plasmid
Pronunciation link: <https://www.merriam-webster.com/dictionary/plasmid>
IPA: /'plæz.mɪd/
Phonetic Spelling: PLAZ·mid
3. Antibiotic
Pronunciation link: <https://www.merriam-webster.com/dictionary/antibiotic>
IPA: /,æn.ti.baɪ'ɑː.tɪk/
Phonetic Spelling: an·tee·by·AA·tik
4. Incubate
Pronunciation link: <https://www.merriam-webster.com/dictionary/incubate>
IPA: /'ɪŋ.kjə,bert/
Phonetic Spelling: ING·kyuh·bayt
5. Inoculate
Pronunciation link: <https://www.merriam-webster.com/dictionary/inoculate>
IPA: /ɪ'nɑː.kjə,leɪt/
Phonetic Spelling: ih·NAA·kyuh·layt
6. Centrifuge
Pronunciation link: <https://www.merriam-webster.com/dictionary/centrifuge>
IPA: /'sen.trə,fjuː.dʒ/
Phonetic Spelling: SEN·truh·fyooj
7. Supernatant
Pronunciation link: <https://www.merriam-webster.com/dictionary/supernatant>
IPA: /,suː.pər'nei.tənt/
Phonetic Spelling: soo·per·NAY·tuhnt
8. Resuspension
Pronunciation link: <https://www.merriam-webster.com/dictionary/resuspension>
IPA: /,riː.sə'spen.fən/
Phonetic Spelling: ree·suh·SPEN·shuhn
9. Neutralization
Pronunciation link: <https://www.merriam-webster.com/dictionary/neutralization>
IPA: /,nuː.trə.lə'zeɪ.fən/
Phonetic Spelling: noo·truh·luh·ZAY·shuhn
10. Equilibration
Pronunciation link: <https://www.merriam-webster.com/dictionary/equilibration>
IPA: /ɪ,kwiː.lə'breɪ.fən/
Phonetic Spelling: ih·KWEE·luh·BRAY·shuhn
11. Elution
Pronunciation link: <https://www.merriam-webster.com/dictionary/elution>

- IPA: /i'luː.ʃən/
Phonetic Spelling: ee·LOO·shuhn
12. Conjugation
Pronunciation link: <https://www.merriam-webster.com/dictionary/conjugation>
IPA: /ˌkɑːn.dʒə'geɪ.ʃən/
Phonetic Spelling: kaan·juh·GAY·shuhn
13. Nuclease
Pronunciation link: <https://www.merriam-webster.com/dictionary/nuclease>
IPA: /'nuː.kliˌeɪs/
Phonetic Spelling: NOO·klee·ays
14. Kilobase
Pronunciation link: <https://www.merriam-webster.com/dictionary/kilobase>
IPA: /'kɪl.əˌbeɪs/
Phonetic Spelling: KIL·uh·bays
15. Circularization
Pronunciation link: <https://www.merriam-webster.com/dictionary/circularization>
IPA: /ˌsɜː.kjə.lə.ə'zeɪ.ʃən/
Phonetic Spelling: sur·kyuh·lur·uh·ZAY·shuhn
16. Basecalling
Pronunciation link: No confirmed link found
IPA: /'beɪs.kɑː.lɪŋ/
Phonetic Spelling: bays·kaw·ling
17. Reconstruction
Pronunciation link: <https://www.merriam-webster.com/dictionary/reconstruction>
IPA: /ˌriː.kən'strʌk.ʃən/
Phonetic Spelling: ree·kuhn·STRUK·shuhn
18. Contamination
Pronunciation link: <https://www.merriam-webster.com/dictionary/contamination>
IPA: /kənˌtæm.ə'neɪ.ʃən/
Phonetic Spelling: kuhn·tam·uh·NAY·shuhn
19. Ligation
Pronunciation link: <https://www.merriam-webster.com/dictionary/ligation>
IPA: /laɪ'geɪ.ʃən/
Phonetic Spelling: ly·GAY·shuhn
20. Incubation
Pronunciation link: <https://www.merriam-webster.com/dictionary/incubation>
IPA: /ˌɪŋ.kjə'beɪ.ʃən/
Phonetic Spelling: ING·kyuh·BAY·shuhn