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# Title: Generating Whole Bacterial Genomes from Clinical Samples using A Target Enrichment Workflow

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# Author Questionnaire

1. **Microscopy**: Does your protocol require the use of a dissecting or stereomicroscope for performing a complex dissection, microinjection technique, or something similar? **No**

2. **Software:** Does the part of your protocol being filmed include step-by-step descriptions of software usage? **No**

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

   100m

**Current Protocol Length**
Number of Steps: 26
Number of Shots: 57

# Introduction

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

1.1. **Helena Seth-Smith:** Genomes can provide information on the ancestry, transmission, and capabilities of a pathogen. For unculturable bacteria, this information can only be obtained with target enrichment.
   1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

What are the current experimental challenges?

1.2. **Mariantonietta Letteri:** Sequencing bacterial pathogens directly from clinical samples is challenging because of overwhelming human DNA, diverse microbiota, and extremely low bacterial loads. In many cases, culture is not an option due to sampling methods and fastidious bacteria.
   1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

What significant findings have you established in your field?

1.3. **Karina Büttner:** While applying this technology to sexually transmitted bacteria, we discovered a novel *Chlamydia trachomatis* LGV lineage, called *ompA*-genotype L4, and confirmed global expansion of the dominant L2b lineage, delivering key insights for molecular diagnostics and epidemiological surveillance.
   1.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

What advantage does your protocol offer compared to other techniques?

1.4. **Very Bregy:** This probe-based enrichment protocol enables complete genome recovery in many cases, from DNA in diagnosed positive STI clinical samples. We found that this technique outperforms deep metagenomic sequencing, host DNA depletion, and nanopore adaptive sequencing.
   1.4.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

How will your findings advance research in your field?

1.5. **Helena Seth-Smith:** We are investigating the circulating strains of these pathogens across Europe and in Argentina. This has the potential to inform regarding transmission and antimicrobial stewardship.
   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.***

How do you think publishing with JoVE will enhance the visibility and impact of your research?

1.6. **PD Dr. Helena M.B. Seth-Smith, Co-Leiterin Microbial Genomics (NGS) / Leitung Bioinformatik, Research Group Leader of Enriching Microbiology Laboratory, Institute of Medical Microbiology, University of Zurich**

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

Can you share a specific success story or benefit you've experienced—or expect to experience—after using or publishing with JoVE? (This could include increased collaborations, citations, funding opportunities, streamlined lab procedures, reduced training time, cost savings in the lab, or improved lab productivity.)

1.7. **Dr. Karina Andrea Büttner, Postdoctoral Researcher, Institute of Medical Microbiology, University of Zurich**

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

# Protocol

## 2. DNA Fragmentation and Library Preparation

**Demonstrators:** Karina Büttner, Vera Bregy, Mariantonietta Letteri

2.1. To begin, measure the DNA concentration of the DNA extracts from clinical samples using a sensitive and accurate fluorescence-based method **[1]**. Dilute each DNA sample using nuclease-free water to 10 to 200 nanograms in a final volume of 17 microliters in each PCR tube strip **[2-TXT]**.

2.1.1. WIDE: Talent using a benchtop fluorometer to measure DNA concentration.

2.1.2. Talent pipetting nuclease-free water into PCR tube strips containing DNA to adjust the volume to 17 microliters. **TXT: Use the maximum input DNA within the recommended range for optimal sequencing**

2.2. Add 3 microliters of fragmentation master mix to each well containing the 17 microliters of diluted DNA **[1]**. Pipette up and down 20 times to mix thoroughly, then seal the PCR tube strip **[2].** Vortex the strip at high speed for 10 seconds **[3]** and spin briefly to collect the contents **[4]**.

2.2.1. Talent pipetting 3 microliters of fragmentation master mix into PCR tube wells.

2.2.2. Talent pipetting up and down 20 times to mix the reaction in each well and sealing the tube.

2.2.3. Talent vortexing the tube at high speed.

2.2.4. Talent placing the sealed strip in a microcentrifuge.

2.3. Then, place the PCR tube strip in the thermal cycler and start the pre-programmed protocol for fragmentation and incubation **[1]**.

2.3.1. Talent placing the sealed PCR tube strip into the thermal cycler and selecting the corresponding pre-loaded program.

2.4. Next, prepare the end repair and dA-tailing master mix as shown in the table **[1]**. Vortex and spin the tube as shown earlier and keep it on ice **[2]**.

2.4.1. LAB MEDIA: Table 5

2.4.2. Talent placing the 1.5mL tube on ice.

2.5. Then, pipette 20 microliters of the end repair and dA-tailing master mix into each well

containing 50 microliters of DNA fragments **[1]**. Seal the strip and vortex at high speed for 8 seconds **[2]**. After briefly spinning the strip, place it back into the thermal cycler **[3]**.

2.5.1. Talent pipetting 20 microliters of end repair/dA-tailing mix into each well with DNA.

2.5.2. Talent sealing and vortexing the PCR strip at high speed.

2.5.3. Talent placing the strip in the thermal cycler.

2.6. Once the thermal cycler program finishes, place the PCR tube strip on ice **[1]**.

2.6.1. Talent removing the strip from the thermal cycler and placing it on ice.

2.7. Next, add 25 microliters of room temperature ligation master mix, which was prepared 30 to 45 minutes in advance, to each well containing the DNA fragments **[1]**. Add 5 microliters of adaptor oligo mix for MBC-tagged libraries to each well **[2]**. Immediately place the PCR tube strip back into the thermal cycler and continue the program **[3]**.

2.7.1. Talent pipetting 25 microliters of ligation master mix into each sample well.

2.7.2. Talent pipetting 5 microliters of adaptor oligo mix into the same wells.

2.7.3. Talent quickly placing the sealed strip into the thermal cycler and starting the next protocol.

2.8. To begin DNA purification, retrieve the magnetic beads from 4 degrees Celsius and allow them to come to room temperature before use **[1]**, then add 80 microliters to each sample well **[2]**.

2.8.1. Talent retrieving magnetic beads from a 4 degrees Celsius storage and placing them on the benchtop.

2.8.2. Talent pipetting 80 microliters of magnetic bead suspension into each sample well.

2.9. Place the PCR tube strip on the magnetic stand and wash each well twice with 200 microliters of 70 percent ethanol **[1]**. Wait for 1 minute during each wash and carefully remove the ethanol without disturbing the magnetic beads **[2-TXT]**.

2.9.1. Talent placing the PCR tube strip into a magnetic stand and adding 200 microliters of 70 percent ethanol to each well. NOTE: This shot is filmed in two takes: the first showing the placement of the PCR tube strip into the magnetic stand, and the second showing the addition of 200 µL of 70% ethanol to each well.

2.9.2. Talent slowly aspirating ethanol after 1 minute, ensuring beads remain undisturbed. **TXT: Allow the beads to dry for up to 5 min in the unsealed PCR tube strip**

2.10. After mixing and incubating the beads with water for 5 minutes, carefully aspirate the cleared supernatant from each well **[1]** and transfer it into a corresponding well in a new PCR tube strip **[2]**. Place the new PCR tube strip on ice and discard the magnetic beads **[3-TXT]**.

2.10.1. Talent removing 34 microliters of supernatant from the PCR tube strip. NOTE: 2.10.1 with 2.10.2 are filmed in a single shot

2.10.2. Talent adding the aspirated liquid into a new PCR strip using a pipette.

2.10.3. Talent placing the new PCR tube strip with transferred DNA on ice**.**

2.11. Take the prepared pre-capture PCR reaction mix and add 11 microliters of it into each sample well containing the purified DNA library **[1]**. Add 5 microliters of the selected index primer pair to each reaction and seal the PCR tube strip **[2-TXT]**.

2.11.1. Talent pipetting 11 microliters of pre-capture PCR reaction mix into each well.

2.11.2. Talent adding 5 microliters of index primer mix to each corresponding sample and sealing the tube. **TXT: Vortex and spin the tube briefly**

2.12. Start the thermal cycler program for PCR amplification as specified in the table to perform the pre-capture PCR **[1]**. Only add the PCR tube strip once the thermal cycler has reached 98 degrees Celsius **[2].**

2.12.1. LAB MEDIA: Table 7

2.12.2. Talent place the PCR strip in the thermal cycler with the shot of the display showing the mentioned temperature.

2.13. After completing the pre-capture PCR, perform another bead-based clean-up to purify the library. Incubate the PCR product with magnetic beads **[1],** then place the PCR strip tube on a magnetic separation device and wait 5–10 minutes until the solution becomes clear **[2]**. While the strip remains on the magnetic stand, carefully remove and discard the clear supernatant from each well **[3-TXT]**.

2.13.1. Talent adding beads to the PCR products.

2.13.2. Talent placing the PCR tube strip into a magnetic separation stand and waiting for separation.

2.13.3. Talent slowly aspirating and discarding the cleared solution from each well

without disturbing the beads. **TXT: Wash 2x with EtOH**

2.14. Then, elute the DNA in 15 microliters of nuclease-free water and measure the DNA concentration of the purified DNA library **[1]**.

2.14.1. Talent at the benchtop fluorometer checking the DNA concentration.

## 3. Sample Hybridization/Capture

3.1. For sample hybridization, program the thermal cycler according to the protocol specified here **[1].** Start the program and immediately pause the run **[2]**.

3.1.1. LAB MEDIA: Table 9

3.1.2. Talent starts the program and pauses immediately.

3.2. Dilute 500 to 1000 nanograms of the prepared DNA libraries to a final volume of 12 microliters using nuclease-free water **[1].** After vortexing and spinning down the oligonucleotide blocker mix, add 5 microliters to each sample tube **[2]**. Seal the PCR tube strip and place it in the thermal cycler **[3]**.

3.2.1. Talent adding water to the prepared DNA libraries.

3.2.2. Talent pipetting 5 microliters of blocker mix into each sample well.

3.2.3. Talent loading it into the thermal cycler.

3.3. Pause the thermal cycler when it reaches Step 3 of the protocol **[1]**. Add 13 microliters of the prepared room temperature probe hybridization mix to each sample while keeping the strip inside the thermal cycler **[2]**. Pipette slowly up and down 8 to 10 times to mix **[3-TXT]**.

3.3.1. LAB MEDIA: Table 9 *Video editor: Please highlight step 3*

3.3.2. Talent pipetting 13 microliters of probe hybridization mix into each sample while it remains inside.

3.3.3. Talent slowly pipetting up and down 10 times in each well to ensure proper mixing. **TXT: Vortex and spin the sealed tubes**

3.4. Ensure no air bubbles are present and place the strip back into the thermal cycler **[1]**. Then, confirm that all tubes are sealed tightly **[2-TXT]**.

3.4.1. Talent inspecting for bubbles and placing the strip into the thermal cycler.

3.4.2. Talent pressing down on each tube lid to confirm tight seals. **TXT: Resume thermal cycling to hybridize library DNA with probes**

3.5. To capture the hybridized library, transfer the entire volume from each tube to the corresponding tubes containing 200 microliters of pre-washed streptavidin beads. ~~Incubate the PCR tube strip on a 96-well plate mixer at 1800 rpm for 30 minutes at room temperature~~ **[1-TXT]**. Collect the streptavidin beads before washing them with room-temperature wash buffer. **[2-TXT]**. NOTE: The VO is edited for the deleted shot

3.5.1. Talent transferring 30 microliters of hybridized DNA samples to PCR tube strip with pre-washed streptavidin beads. **TXT: Incubation: 1800rpm, RT, 30 min**

3.5.2. ~~Incubate the tube strip on a 96-well plate mixer at 1800 rpm for 30 minutes at room temperature~~ NOTE: Not filmed

3.5.3. Talent slowly aspirating room temperature wash buffer and discarding the supernatant from magnetic stand. **TXT**: **Remove and discard the supernatant.**

3.6. Once the PCR tube strip is removed from the magnetic separator, add 200 microliters of high-temperature wash buffer pre-warmed to 70 degrees Celsius **[1]** and resuspend the beads by pipetting up and down **[2]**. Seal the tube strip, vortex at high speed, and spin quickly as demonstrated earlier **[3]**.

3.6.1. Talent pipetting 200 microliters of 70 degrees Celsius wash buffer into each well.

3.6.2. Talent pipetting the beads up and down to ensure complete resuspension.

3.6.3. Talent sealing the tubes.

3.7. Place the sealed tube strip in the thermal cycler block at 70 degrees Celsius and incubate for 5 minutes **[1]**.

3.7.1. Talent placing the PCR tube strip into the thermal cycler block set at 70 degrees Celsius.

3.8. Transfer the PCR tube strip from the thermal cycler back to the magnetic separator at room temperature **[1]**. Wait for 1 minute until the solution becomes clear, then carefully remove and discard the supernatant **[2-TXT]**. Then, add 25 microliters of nuclease-free water to each tube containing the beads **[1]**.

3.8.1. Talent transferring the strip from the thermal cycler to the magnetic stand at room temperature.

3.8.2. Talent aspirating the clear supernatant and discarding it without disturbing the beads. **TXT: Repeat this washing procedure 6x**

3.8.3. Talent pipetting 25 microliters of nuclease-free water into each well of the strip tubes.

3.9. Program the thermal cycler as specified in the table shown here **[1]**.

3.9.1. LAB MEDIA: Table 12

3.10. Add 25 microliters of the prepared post-capture PCR reaction mix to each sample tube **[1]**. Mix the reaction until the suspension is uniform and securely seal the PCR tube strip without spinning it down **[2]**. Place the sealed PCR tube strip in the thermal cycler **[3-TXT]**.

3.10.1. Talent pipetting 25 microliters of post-capture polymerase chain reaction mix into each tube containing bead-bound DNA.

3.10.2. Talent mixing by pipetting thoroughly and sealing the strip tubes without centrifugation.

3.10.3. Talent placing the PCR tube strip into the thermal cycler. **TXT: Perform magnetic separation on PCR product**

3.11. After transferring the PCR product to a fresh tube, add 50 microliters of bead suspension to each sample tube and perform the DNA clean-up as shown earlier **[1].**

3.11.1. Talent pipetting 50 microliters of bead suspension into each sample tube.

3.12. Measure the DNA concentration of the final purified library DNA using a fluorescence-based quantification method **[1]**.

3.12.1. Talent using a benchtop fluorometer to measure the DNA concentration of the final library samples.

# Results

## 4. Results

4.1. Target enrichment resulted in significantly higher percentages of on-target reads, and therefore complete genomes, compared to direct sequencing across all pathogens tested, including *Chlamydia trachomatis (kluh-MID-ee-uh truh-KOH-muh-tis)* **[2]**, *Neisseria gonorrhoeae (nye-SEER-ee-uh gon-uh-REE-ee)* **[3]**, *Treponema pallidum (trep-oh-NEE-muh PAL-ih-dum)* **[4]**, and *Mycoplasma genitalium (MY-ko-plaz-muh jen-ih-TAY-lee-um)* **[5]**.

4.1.1. LAB MEDIA: Figure 1. *Video editor: Emphasize the upper-right cluster in the "CT" panel showing higher values in the "SureSelect" group*.

4.1.2. LAB MEDIA: Figure 1. *Video editor: Emphasize the "NG" panel, focusing on the "SureSelect" group where the values are visibly higher.*

4.1.3. LAB MEDIA: Figure 1. *Video editor: Highlight the "TP" panel, especially the "SureSelect" column showing raised box plot and dots.*

4.1.4. LAB MEDIA: Figure 1. *Video editor: Emphasize the "MG" panel's "SureSelect" column with values elevated compared to "Direct sequencing".*

4.2. Genome sequencing success was predominantly observed in samples with qPCR cycle threshold values below 30 for all four pathogens **[1]**, with a clear inverse correlation between Ct values and percentage of on-target reads for *Chlamydia trachomatis* **[2]**.

4.2.1. LAB MEDIA: Figure 2.

4.2.2. LAB MEDIA: Figure 2. *Video editor: Highlight the upper-left diagonal trend in the "CT" panel, showing high %OTR corresponding with lower Ct values*.

4.3. Using a hybridization temperature of 62.5 degrees Celsius compared to the previous 65 degrees Celsius improved genome coverage for *Mycoplasma genitalium* **[1]**, without reducing performance for the other target pathogens **[2]**.

4.3.1. LAB MEDIA: Figure 3. Video editor: *Highlight the red triangle symbols (representing M. genitalium) at 62.5°C*

4.3.2. LAB MEDIA: Figure 3. *Video editor: Highlight other microorganism symbols (circle, square, cross) at 62.5°C, showing consistently high coverage across types*.

4.4. Phylogenetic analysis of *Chlamydia trachomatis* genomes from Buenos Aires showed many samples clustering within the L2b clade **[1]**, and revealed a novel lineage proposed as ompA *(Omp-A)*-genotype L4, separated from all previous LGV genomes by approximately 600 single-nucleotide polymorphisms **[2]**.

4.4.1. LAB MEDIA: Figure 4. *Video editor: Highlight the cluster labeled "L2b" in the tree*.

4.4.2. LAB MEDIA: Figure 4. *Video editor: Emphasize the distinct lower-right clade labeled "L4",.*

**1. fluorometer**
**Pronunciation link:**
No confirmed link found *(not present in Merriam-Webster, OED, or HowToPronounce.com)*
**IPA:** /ˌflɔːrəˈmɪtər/
**Phonetic Spelling:** flaw-ruh-MIH-tər

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**2. nuclease**
**Pronunciation link:**
https://www.merriam-webster.com/dictionary/nuclease
**IPA:** /ˈnjuːkliˌeɪs/ or /ˈnuːkliˌeɪs/
**Phonetic Spelling:** NOO-klee-ays

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**3. ligation**
**Pronunciation link:**
https://en.wikipedia.org/wiki/Ligation_(molecular_biology)#Pronunciation
*(Note: Wikipedia doesn't always list pronunciation separately, but "ligation" follows standard phonetic patterns.)*
**IPA:** /laɪˈgeɪʃən/
**Phonetic Spelling:** ly-GAY-shun

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**4. fragmentation**
**Pronunciation link:**
No confirmed link found *(not listed in Merriam-Webster, OED, or HowToPronounce.com under this specific form; "fragment" is listed but we'll still provide.)*
**IPA:** /ˌfrægmənˈteɪʃən/
**Phonetic Spelling:** frag-MEN-TAY-shun

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**5. adaptor *(as in adaptor oligo mix)***
**Pronunciation link:**
No confirmed link found *(common alternate spelling of "adapter"; Merriam-Webster lists "adapter" but not "adaptor")*
**IPA:** /əˈdæptər/
**Phonetic Spelling:** uh-DAP-ter

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**6. streptavidin**
**Pronunciation link:**
No confirmed link found *(not in Merriam-Webster or OED typically; it's a biochemical term.)*

**IPA:** /strɛpˈtævədɪn/
**Phonetic Spelling:** strep-TAV-uh-din

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**7. hybridization**
**Pronunciation link:**
No confirmed link found *(standard technical term, but not always in general dictionaries)*
**IPA:** /haɪˌbrɪdɪˈzeɪʃən/
**Phonetic Spelling:** hy-brid-ih-ZAY-shun

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**8. Mycoplasma genitalium *(genus and species names)***
**Pronunciation link (genus):** No confirmed
**IPA (genus):** /ˌmaɪkoʊˈplæzmə/
**Phonetic Spelling (genus):** my-kuh-PLAZ-muh
**Pronunciation link (species):** No confirmed
**IPA (species):** /ˌdʒɛnɪˈteɪliəm/
**Phonetic Spelling (species):** jen-ih-TAY-lee-um