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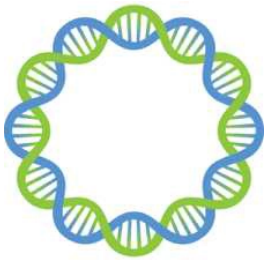
# Genomic Assembly of Plasmid DNA from Bacterial Cultures



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**Protocol status:** Working
**We use this protocol and it's working**

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## Abstract

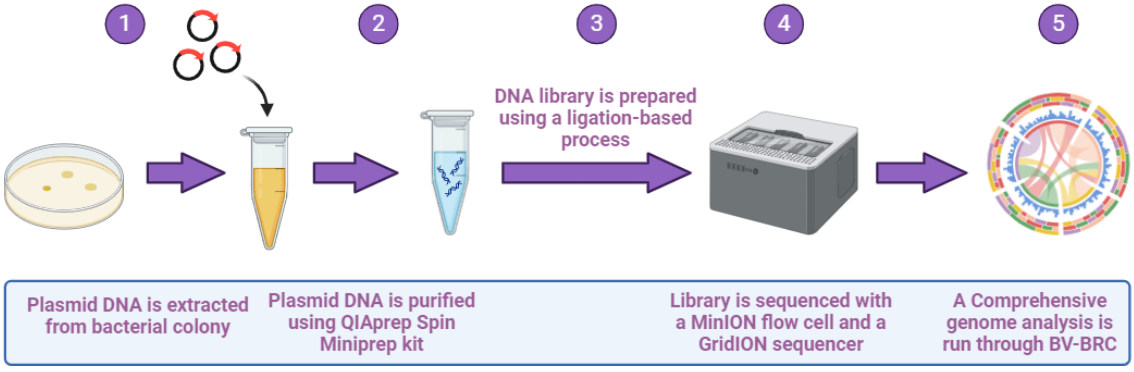
This protocol was developed for BIT 495: Special Topics in Biotechnology: Portable Genome Sequencing at North Carolina State University.

The purpose of this protocol is to consistently complete genomic assemblies of plasmid DNA from bacterial cultures. This is done by ensuring that the DNA is sequenced with long DNA reads, which helps the DNA preserve its integrity and decreases the chance of the sample having multiple contigs in the final assembly. This protocol has been optimized to produce high-quality reads from the most difficult-to-extract plasmid DNA.

This protocol is broken down into a few key steps:

1. A bacterial colony is grown, and then the plasmids are extracted using the method given below
2. The DNA is purified from the plasmids by using a slight variation of the QIAprep Spin Miniprep kit
3. The DNA library will be made using the Ligation Sequencing kit but with a custom protocol designed for plasmids
4. Load this sample into a MinION flow cell and use a MinION sequencer to extract the reads
5. The analysis of our samples will be completed using BV-BRC

*Note: For the last two steps of this process (the sequencing and analysis) there will be no strict procedures given, only brief descriptions.*


## Guidelines

When following this protocol, ensure that all steps are carried out under sterile conditions to avoid contamination of the plasmid DNA. Always use fresh reagents, and handle all materials with care to maintain the integrity of your samples. Pipette accurately and avoid excessive vortexing to prevent shearing of the DNA. Consistently monitor centrifugation speeds and times to ensure proper separation during plasmid extraction and purification. For DNA quantification, ensure calibration of equipment such as NanoDrop and Qubit Fluorometer. Follow manufacturer instructions when using commercial kits like the QIAprep Spin Miniprep kit and Ligation Sequencing Kit V14 to achieve optimal results. You may pause this process between any major section of this protocol.

## Materials

***For Section 1: Plasmid Extraction from Bacterial Colony***

- Desktop microcentrifuge
- Desktop vortexer
- Overnight culture of bacteria transformed with your plasmid
- **Resuspension buffer** [25 mM Tris-HCl (pH 8), 50 mM glucose, 10 mM EDTA]
- **Denaturing solution** [0.2 N NaOH, 1.0% SDS]
- **Renaturing solution** [120 mL 5M Potassium acetate, 23 mL glacial acetic acid, 57 mL of dH2O]

***For Section 2: DNA Purification From Plasmid***

- QIAprep Spin Miniprep kit
- Centrifuge
- Vortexer
- Heating Block
- Nutating Mixer
- NanoDropTM (Thermo Scientific, catalog number: ND2000C)
- Qubit Fluorometer
- Fume Hood
- Autoclave
- 1.5 mL Eppendorf tubes
- Various Pipette and Pipette tip sizes
- Absolute Ethanol
- **TE Buffer** [1 mL Tris-HCl (pH 8.0), .2 mL Na2EDTA (pH 8.0), 98.8 MiliQ water]
- **5 M NaCl** [Dissolve 29.2 g of NaCl in 80 mL of MilliQ water, Adjust to 100 mL using MilliQ water, Autoclave at 121 °C (15 psi) for 15 min]
- **20% SDS** [20g of SDS, dissolve into MiliQ water, Adjust to 100 mL using miliQ water, Autoclave at 121 °C (15 psi) for 15 min]
- **CIA** (Chloroform: isoamyl alcohol) [Add 2 mL of isoamyl alcohol in a 50 mL tube, Adjust to 50 mL by adding Chloroform, Mix well and cover with aluminum foil, Prepare in a fume hood]
- RNase (20 mg/mL)
- 70% Ethanol

***For Section 3.1: Library Preparation of DNA - DNA Repair and End Prep***

- Ligation Sequencing Kit V14
- 1 µg (or 100-200 fmol) gDNA
- DNA Control Sample
- (DCS)
- AMPure XP Beads (AXP)
- Qubit Fluorometer
- NEBNext® FFPE DNA Repair Mix (M6630) from the NEBNext® Companion Module v2 (NEB, E7672S or E7672L)
- NEBNext® FFPE DNA Repair Buffer v2 (E7363) from the NEBNext® Companion Module v2 (NEB, E7672S or E7672L)
- NEBNext® Ultra II End Prep Enzyme Mix (E7646) from the NEBNext® Companion Module v2 (NEB, E7672S or E7672L)

- Qubit dsDNA HS Assay Kit (Invitrogen, Q32851)
- Nuclease-free water (e.g. ThermoFisher, AM9937)
- Freshly prepared 80% ethanol in nuclease-free water
- Qubit™ Assay Tubes (Invitrogen, Q32856)
- 0.2 ml thin-walled PCR tubes
- 1.5 ml Eppendorf DNA LoBind tubes
- P1000 pipette and tips
- P100 pipette and tips
- P10 pipette and tips
- Microfuge
- Thermal cycler
- Hula mixer
- (gentle rotator mixer)
- Magnetic rack
- Ice bucket with ice

***For Section 3.2: Library Preparation of DNA - Adapter Ligation and Clean Up***

- Ligation Sequencing Kit V14
- Ligation Adapter (LA)
- Ligation Buffer (LNB)
- Long Fragment Buffer (LFB)
- Flow Cell Flush (FCF)
- Bovine Serum Albumin (BSA) at 50 mg/ml
- Flow Cell Tether (FCT)
- Sequencing Buffer (SB)
- Library Beads (LIB)
- AMPure XP Beads (AXP)
- Elution Buffer (EB)
- Salt-T4® DNA Ligase (NEB, M0467)
- 1.5 ml Eppendorf DNA LoBind tubes
- Qubit dsDNA HS Assay Kit (Invitrogen, Q32851)
- Qubit™ Assay Tubes (Invitrogen, Q32856)
- Magnetic rack
- Microfuge
- Vortex mixer
- P1000 pipette and tips
- P100 pipette and tips
- P20 pipette and tips
- P10 pipette and tips
- Qubit fluorometer (or equivalent for QC check)

***For Section 4: Nanopore Sequencing***

- MinION Flow Cell (R10.4.1)
- GridION Sequencing Device Mk1

***For Section 5: Sequencing Analysis***

- BV-BRC Website Software

## Safety warnings

Handle all chemicals, especially chloroform and ethanol, in a fume hood to avoid inhalation of fumes. Wear appropriate personal protective equipment (PPE) such as lab coats, gloves, and safety goggles at all times. Chloroform is toxic and should be handled with extreme caution—dispose of it in designated waste containers following hazardous waste protocols. Ensure that all centrifuge tubes are balanced before use to avoid damage to the equipment or sample loss. Use RNase with care, as it can degrade RNA unintentionally if mishandled. Finally, be cautious when handling electrical equipment such as the MinION sequencer and thermal cycler to prevent equipment damage or personal injury.

## 1. Plasmid Extraction from Bacterial Colony

1 To start, obtain an overnight culture of bacteria (of whatever type of bacteria)

2 Centrifuge the culture to pellet the bacteria

3 Remove all supernatant and resuspend the bacteria in the **resuspension buffer**

4 Add a **denaturing solution** to the resuspended bacteria
*This will lyse the bacteria to release their contents (including plasmid DNA)*

5 Add a **renaturing solution** to the denatured bacteria
*This causes proteins and genomic DNA to precipitate while leaving smaller plasmids in the solution*

6 Pellet the proteins and genomic DNA by centrifugation, and remove the plasmid-containing supernatant

## 2. DNA Purification From Plasmid

7 Add an equal volume of absolute ethanol into each tube and mix by inverting the tubes a few times

8 Centrifuge the tubes at 20,000× g (15,000 rpm) for 5 min

9 Decant the supernatant and carefully remove the liquid completely using a pipette

10 Add 300 µL of **TE buffer** (pH 8.0) (see recipe above), 150 µL of **5 M NaCl** (see recipe above), and 45 µL of **20% SDS** (see recipe above). Mix by inversion and incubate at 65 °C in a heating block for at least 10 min

11 Add an equal volume (500 µL) of **CIA** (see recipe above) and keep shaking on a Nutating Mixer for at least 5 min

12 Centrifuge at 20,000× g (15,000 rpm) for 7–10 min

13 Carefully transfer the upper phase (~400 µL) into new 1.5 mL Eppendorf tubes, add 16 µL (20 mg/mL) of RNase, mix well by inversion, and incubate at 37 °C in a heated block for at least 15 min. Add an equal volume of CIA and keep shaking on a Nutating Mixer for at least 5 min

14 Centrifuge at 20,000× g (15,000 rpm) for 7–10 min

15 Carefully transfer the upper phase (300 µL) into new 1.5 mL Eppendorf tubes, add an equal volume of absolute ethanol, and mix well by inversion

16 Centrifuge at 20,000× g (15,000 rpm) for 7–10 min

17 Carefully decant or pipette out the liquid phase and add 500 µL of 70% ethanol. Tap the tube to release the pellet

18 Centrifuge at 20,000× g (15,000 rpm) for 1–2 min and repeat step 11

19 Add 50 µL (if the pellet is large, add more volume) of **TE Buffer**. Completely dissolve the pellet by finger-tapping

20 Measure the quantity of the plasmid DNA using NanoDrop and using Qubit (this concentration will be helpful for the next steps

## 3.1 Library Preparation of DNA - DNA Repair and End Prep

21 Use the Qubit Fluorometer to find the concentration of plasmid DNA in your sample

22 Adjust the volume/concentration to get 1 ug of plasmid DNA
*This ensures that long reads will be obtained in this process*

23 Transfer 1 µg input DNA into a 1.5 ml Eppendorf DNA LoBind tube

24 Adjust the volume to 48 µl with nuclease-free water

25 Mix thoroughly by pipetting up and down

26 Spin Down with the microfuge

27 In a 0.2 ml thin-walled PCR tube, mix the following (pipette mix between each addition):
- 48 uL of the DNA sample
- 2 uL of NEBNext FFPE DNA Repair Buffer v2
- 2 uL of NEBNext FFPE DNA Repair Mix
- 3 uL of Ultra II End-prep Enzyme Mix

28 Mix the reaction and gently spin it down

29 Incubate with the Thermocycler at 20°C for 5 minutes and 65°C for 5 minutes

30 Transfer the DNA sample to a clean 1.5 ml Eppendorf DNA LoBind tube

31 Resuspend the AMPure XP Beads (AXP) by vortexing

32 Add 60 µl of resuspended AMPure XP Beads (AXP) to the end-prep reaction and mix by flicking the tube

33 Incubate on a Hula mixer for 10 minutes at room temperature

34 Prepare 500 µl of fresh 80% ethanol in nuclease-free water

35 Spin down the sample and pellet on a magnet until the supernatant is clear and colorless. Keep the tube on the magnet, and pipette off the supernatant

36 Keep the tube on the magnet and wash the beads with 200 µl of freshly prepared 80% ethanol without disturbing the pellet. Remove the ethanol using a pipette and discard

37 Repeat the previous step

38 Spin down and place the tube back on the magnet. Pipette off any residual ethanol. **Allow to dry for ~15 seconds, but do not dry the pellet to the point of cracking**

39 Remove the tube from the magnetic rack and resuspend the pellet in 61 µl nuclease-free water. Incubate for 2 minutes at room temperature

40 Pellet the beads on a magnet until the eluate is clear and colorless, for at least 1 minute

41 Remove and retain 61 µl of eluate into a clean 1.5 ml Eppendorf DNA LoBind tube

42 Quantity 1 uL of the eluted sample using a Qubit Fluorometer

## 3.2 Library Preparation of DNA - Adapter Ligation and Clean Up

43 Make note of the sample's DNA concentration

44 Thaw all reagents, individually spin them down, and store them on ice

45 In a 1.5 ml Eppendorf DNA LoBind tube, mix in the following order:
- 60 uL of the DNA sample
- 5 uL of the ligation adapter
- 25 uL of the ligation buffer
- 10 uL of the Salt-T4® DNA Ligase

46 Thoroughly mix the reaction by gently pipetting and briefly spinning down

47 Incubate the reaction for 10 minutes at room temperature

48 Resuspend the AMPure XP Beads (AXP) by vortexing

49 Add 40 µl of resuspended AMPure XP Beads (AXP) to the reaction and mix by flicking the tube

50 Incubate on a Hula mixer (rotator mixer) for 10 minutes at room temperature

51 Spin down the sample and pellet on a magnet. Keep the tube on the magnet, and pipette off the supernatant when clear and colorless

52 Wash the beads by adding 250 µl Long Fragment Buffer (LFB). Flick the beads to resuspend, spin down, then return the tube to the magnetic rack and allow the beads to pellet. Remove the supernatant using a pipette and discard

53 Repeat the previous step

54 Spin down and place the tube back on the magnet. Pipette off any residual supernatant. Allow to dry for **~15 seconds, but do not dry the pellet to the point of cracking**

55 Remove the tube from the magnetic rack and resuspend the pellet in a 15 µl Elution Buffer (EB). Spin down and incubate for **10 minutes at 37°C to improve the recovery of long fragments**

56 Pellet the beads on a magnet until the eluate is clear and colorless, for at least 3 minutes

57 Remove and retain 15 µl of eluate containing the DNA library in a clean 1.5 ml Eppendorf DNA LoBind tube

58 Measure the concentration of the sample using the Qubit Fluorometer

59 Prepare your final library as at least 300 ng of Plasmid DNA into 12 uL of elution buffer
*This ensures long reads of the plasmid DNA*

60 The priming mixture is made as follows in a 1.5 mL Eppendorf LoBind tube:
- 1170 uL of Flow Cell Flush
- 5 uL of Bovine Serum Albumin (BSA) at 50 mg/ml
- 30 uL of Flow Cell Tether

61 The Final Library mixture is made as follows in a 1.5 mL Eppendorf LoBind tube:
- 37.5 uL of Sequencing Buffer
- 25.5 uL of Library Beads (LIB) mixed immediately before use
- 12 uL of DNA library

## 4. Nanopore Sequencing

62 This section won't be in detail, but the library sample will be inserted into the MinION flow cell, which will hook up to the GridION sequencer
*Here is a video tutorial on priming and loading the MinION Flow Cell:*
*https://www.youtube.com/watch?v=IknVaEnuDz0*

## 5. Sequencing Analysis

63 This section will also not be in detail, but the results from the sequencing will be run into a comprehensive genome analysis on the BV-BRC website software

## Protocol references

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