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BIT495 PGS Individual Project Protocol

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Portable Genome Sequen… Joslene Morgan

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Protocol status: In development
I developed this protocol for my portable genome sequencing course at NC State.

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Abstract

This protocol is designed for sequencing DNA from human organ tissue samples. The information obtained from sequencing can then be used for various clinical applications.

Materials

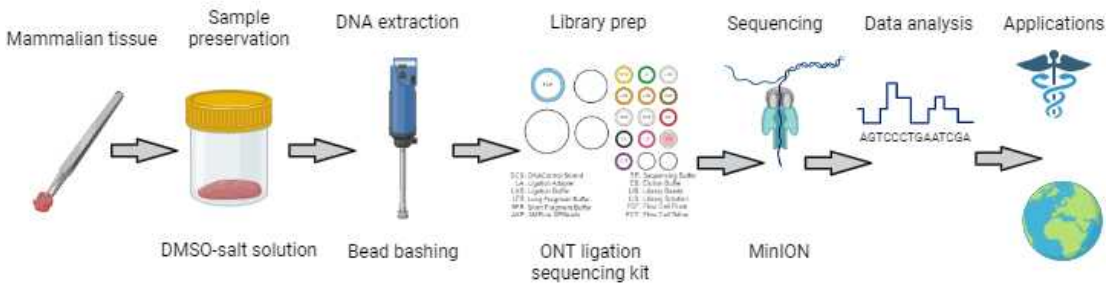
DNA extraction:
- ZYMO Quick-DNA Tissue/Insect Microprep Kit

Library prep:
- Oxford Nanopore Technologies Ligation Sequencing Kit v14

Sequencing:
- MinION flow cell

Overview

1



The figure above shows an overview of the protocol. First, a sample from human organ tissue is obtained. This sample must be preserved until the rest of the protocol is ready to be done. Then, the DNA is extracted and the library is prepared. The sample is then sequenced and analyzed. The data obtained can be used for various clinical applications such as rare disease diagnosis, oncology, infectious diseases and microbiota, and transplantation (4) (5).

Obtain sample from the field and preserve it (1)

2

Note

The human organ tissue sample must be preserved so no degradation or contamination occurs during the transportation process, especially if the patient is not nearby to a lab that can sequence it. This protocol describes preservation with a DMSO-salt solution that will protect the sample and even prevent further fragmentation during the later extraction process.

3 Cut organ tissue into 4-6 mm diameter pieces

4 Prepare DMSO-salt solution

4.1 20% DMSO

4.2 0.25 M sodium–EDTA

4.3 NaCl to saturation

4.4 pH 7.5

5 Add 3X volume of DMSO-salt solution to the tissue

6 When ready for next steps, rinse tissue with distilled water

DNA extraction (2)

7

Note

This is a bead-bashing based protcol specialized for mammalian tissue. 18 minutes of bead-bashing (total time for protocol, 7 min original+6 min extension) yields 0.48ug DNA per mg of tissue. It utilizes Zymo's BashingBead technology followed by column purification. https://www.zymoresearch.com/products/quick-dna-tissue-insect-microprep-kit?srsltid=AfmBOopAxZU1gFOF_Liu6lnGJDXkYGfq7Gi0T_peogRr2-3Sfzim3JtX

8 Zymo BashingBead Lysis Tubes

8.1 For every 2 mg of sample, add 550 µl BashingBead Buffer and 10 µl RNase A 20 mg/ml (invitrogen)

8.2 Vortex max speed 7 min

8.3 Centrifuge at 12,000 rcf for 1 min

8.4 Retrieve 200 µl supernatant for column purification

9 Column purification

9.1 Mix supernatant with 3X volume of dna binding buffer

9.2 Load samples in Zymo IC Spin columns

9.3 Centrifuge 10,000 rcf 30 sec

9.4 Wash with 500uL DNA wash buffer and centrifuge 10,000 rcf 30 sec

9.5 Wash with 200 uL DNA wash buffer and centrifuge 10,000 rcf 30 sec

9.6 Add 40uL zymo dna elution buffer and incubate at 37C for 1-3 min

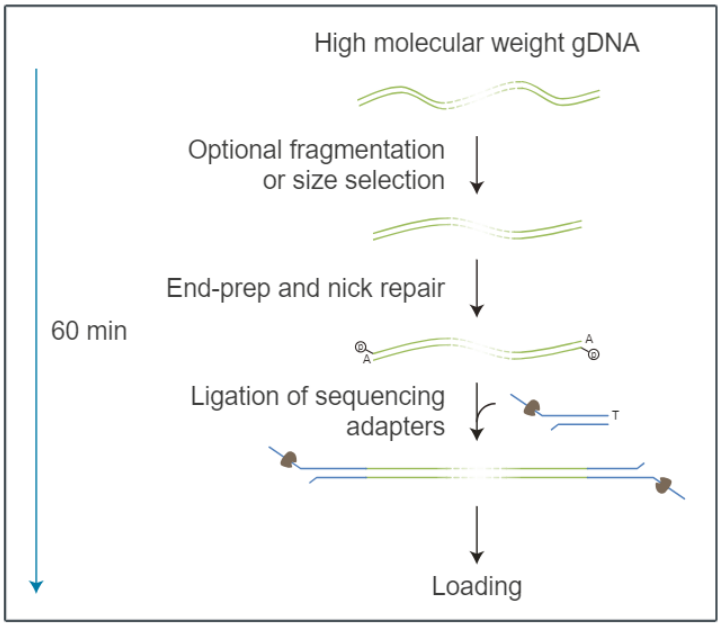
9.7 Centrifuge 10,000 rcf 30 sec

Library preparation (3)

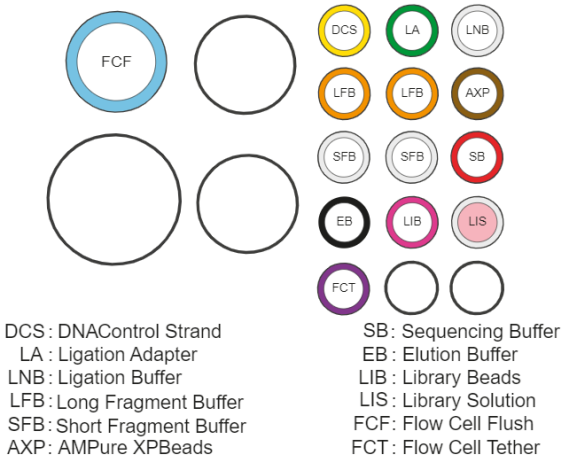
10

Note

This step describes library prep using Oxford Nanopore Technologies Ligation Sequencing Kit v14. This kit is good for long reads and helps repair any nicks in the DNA prior to sequencing. https://store.nanoporetech.com/us/ligation-sequencing-kit-v14.html



Ligation Sequencing Kit workflow (3)



Reagants included in Ligation Sequencing Kit (3)

11 DNA repair and end prep

11.1 Prep DNA in nuclease free water: 1 µg/100-200 fmol input DNA, adjust volume 47uL with nuclease free water

11.2 Mix the following:

Reagent	Volume
DNA from the previous step	47 µl
DNA CS (optional)	1 µl
NEBNext FFPE DNA Repair Buffer v2	7 µl
NEBNext FFPE DNA Repair Mix	2 µl
Ultra II End-prep Enzyme Mix	3 µl
Total	**60 µl**

(3)

11.3 Thoroughly mix the reaction by gently pipetting and briefly spinning down.

11.4 Using a thermal cycler, incubate at 20°C for 5 minutes and 65°C for 5 minutes.

11.5 Resuspend the AMPure XP Beads (AXP) by vortexing.

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

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

11.8 Incubate on a Hula mixer (rotator mixer) for 5 minutes at room temperature.

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

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

11.11 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.

11.12 Repeat the previous step.

11.13 Spin down and place the tube back on the magnet. Pipette off any residual ethanol. Allow to dry for ~30 seconds, but do not dry the pellet to the point of cracking.

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

11.15 Pellet the beads on a magnet until the eluate is clear and colourless, for at least 1 minute.

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

12 Adaptor ligation and cleanup

12.1 Mix the following:

Reagent	Volume
DNA sample from the previous step	60 µl
Ligation Adapter (LA)	5 µl
Ligation Buffer (LNB)	25 µl
Salt-T4® DNA Ligase	10 µl
Total	**100 µl**

(3)

12.2 Thoroughly mix the reaction by gently pipetting and briefly spinning down.

12.3 Incubate the reaction for 10 minutes at room temperature.

12.4 Resuspend the AMPure XP Beads (AXP) by vortexing.

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

12.6 Incubate on a Hula mixer (rotator mixer) for 5 minutes at room temperature.

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

12.8 Wash the beads by adding either 250 µl Long Fragment Buffer (LFB) or 250 µl Short Fragment Buffer (SFB). 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.

12.9 Repeat the previous step.

12.10 Spin down and place the tube back on the magnet. Pipette off any residual supernatant. Allow to dry for ~30 seconds, but do not dry the pellet to the point of cracking.

12.11 Remove the tube from the magnetic rack and resuspend the pellet in 15 µl Elution Buffer (EB). Spin down and incubate for 10 minutes at room temperature. For high molecular weight DNA, incubating at 37°C can improve the recovery of long fragments.

12.12 Pellet the beads on a magnet until the eluate is clear and colourless, for at least 1 minute.

12.13 Remove and retain 15 µl of eluate containing the DNA library into a clean 1.5 ml Eppendorf DNA LoBind tube.

Sequencing with MinION (3)

13

Note

Next, the library is loaded onto a MinION flow cell and sequenced. Sequencing with MinION gives a good data output betweent that of the flongle and PromethION. Reagants for these steps were obtained from the Ligation Sequencing Kit used in the previous step. https://store.nanoporetech.com/us/ligation-sequencing-kit-v14.html

14 Prep flow cell priming mix according to the table below:

Reagent	Volume per flow cell
Flow Cell Flush (FCF)	1,170 µl
Bovine Serum Albumin (BSA) at 50 mg/ml	5 µl
Flow Cell Tether (FCT)	30 µl
Total volume	**1,205 µl**

(3)

15 Draw 20-30uL from priming port to remove any air bubbles

16 Load 800uL of the priming mix into the priming port and wait 5 minutes

17 Load 200uL of priming mix into the priming port

18 Prep library for loading according to table below:

Reagent	Volume per flow cell
Sequencing Buffer (SB)	37.5 µl
Library Beads (LIB) mixed immediately before use, or Library Solution (LIS), if using	25.5 µl
DNA library	12 µl
Total	**75 µl**

(3)

19 Add 75uL of prepped sample to SpotON port dropwise, hovering above the port

Analysis with EPI2ME

20

Note

The output data from the sequencing run is then analyzed on EPI2ME. EPI2ME can now be run locally or on the cloud.

21 Concatenate the data and upload the resulting file to EPI2ME

22 Run workflow(s). Some options are listed in the substeps below, but you can browse EPI2ME and choose others based on your experimental goals.

22.1 wf-human-variation
Small variant calling, structural variant calling, CNV calling, STR expansion genotyping

22.2 wf-somatic-variation
Analyze variation between tumor and normal DNA. Identifies potential SNVs, structural variants, and modified sites

22.3 Various specialized workflows for certain infectious diseases and viruses

Protocol references

(1) Noncryogenic Preservation of Mammalian Tissues for DNA Extraction: An Assessment of Storage Methods
https://doi.org/10.1023/A:1014541222816
(2) A Rapid and Simple Bead-Bashing-Based Method for Genomic DNA Extraction from Mammalian Tissue
https://doi.org/10.2144/btn-2019-0172
(3) Ligation sequencing DNA V14
https://nanoporetech.com/document/genomic-dna-by-ligation-sqk-lsk114

Additional sources for information on clinical applications:
(4) The application of long-read sequencing in clinical settings
https://doi.org/10.1186/s40246-023-00522-3
(5) Clinical Versus Research Sequencing
https://doi.org/10.1101/cshperspect.a025809