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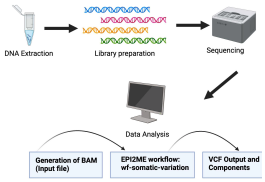
Oxford Nanopore Long-Read Sequencing for Identification of Somatic Variants in Tumor/Normal Pairs



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We use this protocol and it's working

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Disclaimer

This was created as part of a class assignment. It is based on published and well-documented protocols (referenced)

Abstract

Oxford Nanopore Technologies (ONT) offers a streamlined workflow for the identification of somatic variants in tumor samples. This workflow generates long-read and high-quality sequencing data that can be used for analysis with the EPI2ME wf-somatic-variation workflow. This workflow can be run through a user interface or the command line and minimizes hands-on analysis.

Image Attribution

Figure created with BioRender.com

Guidelines

All Centrifuge steps are done at room temperature (15-25°C)
Vortexing should be done by pulse-vortexing for 5 - 10 Seconds
Check kit specifications for other potentially hazardous material

Optional: Samples can be treated with RNAse A to digest RNA during the procedure (not supplied in listed DNA extraction kit)

To multiplex libraries, combine with the ONT Native Barcoding Kit

The recommended workflow for identifying somatic variants in tumor-normal sequencing data is the wf-somatic-variation through EPI2ME. This can be run through the GUI (recommended) or command line.

For other modified options including indels and methylation analysis refer to : https://github.com/epi2me-labs/wf-somatic-variation

Materials

- 1 ug (or 100-200 fmol) high molecular weight genomic DNA
- Or 100+ ng high molecular weight genomic DNA if performing DNA fragmentation
- Ligation Sequencing Kit V14 (SQK-LSK114)

Consumables Required:

- MinION and GridION Flow Cell
- Qubit dsDNA HS Assay Kit (Invitrogen, Q32851)
- NEBNext Companion Module for ONT Ligation Sequencing (NEB, E7180S or E7180L)

or

- NEBNext FFPE Repair Mix (NEB, M6630)
- NEBNext Ultra II End repair/dA-tailing Module (NEB, E7546)
- NEBNext Quick Ligation Module (NEB, E6056) (Optional)
- Bovine Serum Albumin (BSA) (50 mg/ml) (e.g Invitrogen™ UltraPure™ BSA 50 mg/ml, AM2616)
- Freshly prepared 80% ethanol in nuclease-free water
- Nuclease-free water (e.g. ThermoFisher, AM9937)
- 1.5 ml Eppendorf DNA LoBind tubes
- 0.2 ml thin-walled PCR tubes
- Qubit™ Assay Tubes (Invitrogen, Q32856)

Equipment Required

- MinION or GridION device
- MinION Flow Cell Light Shield
- Hula or other gentle rotator mixer
- Magnetic rack
- Microfuge
- Vortex Mixer
- Thermal Cycler
- P1000, P200, P100, P20, P10, P2 pipettes and tips
- Ice bucket
- Timer
- Fluorometer (Qubit or other for QC check)

Safety warnings

Buffers may contain hazardous chemicals. Check SDS before use.

Ethics statement

Not ethical concerns to disclose

Before start

Buffer ATL and Buffer AL may form precipitates upon storage. If necessary, warm to 56°C until the precipitates have fully dissolved. Buffer AW1 and Buffer AW2 are supplied as concentrates. Before using for the first time, add the appropriate amount of ethanol (96–100%) as indicated on the bottle to obtain a working solution. Preheat a thermomixer, shaking water bath or rocking platform to 56°C for use in step 2.

DNA Extraction (QIAGEN DNEasy Blood and Tissue Kit or alternative extraction method)

1 Add 180 ul Buffer ATL into a labeled 1.5 mL tube for each tissue specimen. Cut 25 mg of tissue into small pieces and place in the labeled tube.

2 Spin down the samples and add 20 ul Proteinase K. Mix by vortexing and incubate at 56°C until tissue is completely lysed. Vortex occasionally to check status.

2.1 (Optional): Add 4 ul RNase A (100 mg/ml), mix by vortexing, and incubate for 2 min at room temperature.

3 Vortex for 15 seconds. Add 200 ul Buffer AL to the sample and mix thoroughly by vortexing. Add 200 ul ethanol (96%-100%), and mix again by vortexing.

3.1 It is essential that the sample, Buffer AL, and ethanol are mixed immediately and thoroughly by vortexing or pipetting to yield a homogeneous solution. Buffer AL and ethanol can be premixed and added together in one step to save time when processing multiple samples. A white precipitate may form on addition of Buffer AL and ethanol. This precipitate does not interfere with the DNeasy procedure. Some tissue types (e.g., spleen, lung) may form a gelatinous lysate after addition of Buffer AL and ethanol. In this case, vigorously shaking or vortexing the preparation is recommended.

4 Pipet the mixture into the provided DNeasy Mini spin column in a 2 ml collection tube. Centrifuge at top speed for 1 minute. Discard the flow-through and collection tube.

5 Place the spin column in a new 2 ml collection tube, add 500 ul Buffer AW1 and centrifuge for 1 min at top speed. Discard flow-through and collection tube.

6 1. Place the spin column in a new 2 ml collection tube, add 500 ul Buffer AW2, and centrifuge for 1 minute at top speed. Discard flow-through but retain collection tube.

7 Add 500 ul of 70% (cold) ethanol. Centrifuge for 3 minutes at top speed to dry the DNeasy membrane. Discard flow through and collection tube

7.1 Remove the spin column carefully so it does not come into contact with the flow-through. This will result in the carry-over of ethanol.

8 Place the spin column in a new 2ml collection tube and add 200 ul Buffer AE directly onto the membrane. Let sit at room temperature for 1 minute and then centrifuge for 1 min at max speed to elute. Repeat the elution step to increase DNA yield.

9 Proceed to quantify the DNA.

Library Preparation (ONT Ligation Sequencing Kit): Note third-party consumables are required

10 1. Thaw the DNA Control Sample (DCS) at room temperature, spin down, mix by pipetting, and store on ice until ready to use

10.1 Recommended but 1 ul sample DNA or nuclease-free water can be used in place

11 Prepare the NEBNext FFPE DNA Repair Mix, and NEBNext Ultra II End Repair/ dA-tailing Module reagents according to manufacturer's recommendations. Store on Ice

11.1 a. Recommended to flix or invert the reagent tubes to mix
b. Do not vortex the FFPE DNA Repair Mix or Ultra II end Prep Enzyme Mix

12 Prepare the DNA in nuclease-free water:

13 1) Transfer 1 ug DNA into a 1.5 ml Eppendorf DNA LoBind tube

14 2) Adjust the volume to 47 ul with nuclease-free water

15 3) Mix by pipetting up and down gently or by flicking the tube

16 4) Spin down briefly in a microfuge

17 In a 0.2 ml thin-walled PCR tube, mix the following: (A = Reagent)(B = Volume ul)

DNA from Step 3	47
DNA CS (optional) or nuclease-free water	1
NEBNext FFPE DNA Repair Buffer	3.5
NEBNext FFPE DNA Repair Mix	2
Ultra II End-prep Reaction Buffer	3.5
Ultra II End-prep Enzyme Mix	3

Total	60 ul

18 Between additions of each reagent, pipette to mix 10-20 times (gently) ReagentVolume (ul)DNA from Step 347DNA CS (optional) or nuclease-free water1NEBNext FFPE DNA Repair Buffer3.5NEBNext FFPE DNA Repair Mix2Ultra II End-prep Reaction Buffer3.5Ultra II End-prep Enzyme Mix 3Total60 ul

19 Mix thoroughly by gently pipetting and spinning down

20 Using a thermal cycler incubate with the following conditions

20.1 20°C for 5 minutes

20.2 65°C for 5 minutes

21 Resuspend Ampure XP beads by vortexing

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

23 Add 60 ul of resuspended AmPure Beads to the end-prep reaction. Mix by flicking or inverting the tube

24 Incubate on hula or other rotator for 5 minutes at room temperature

25 Prepare 500 ul of fresh 80% ethanol in nuclease-free water

26 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

27 Keep the tube on the magnet and wash the beads with 200 ul of freshly prepared 80% ethanol without disturbing the pellet. Let sit for 30 seconds. Remove the ethanol using a pipette and discard.

28 Repeat the wash

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

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

31 Pellet the beads on a magnet until the eluate is clear and colorless (~ 1 min or more)

32 Remove and retain 61 ul of eluate into a clean 1.5 ml Eppendorf DNA LoBind tube

33 Use 1 ul of eluted sample using a fluoremeter

34 Spin down the Ligation Adapter (LA) and Quick T4 Ligase. Store on ice

35 Thaw Ligation Buffer (LNB) at room temperature, spin down and mix by pipetting. Store on ice immediately after thawing and mixing

35.1 Due to viscosity, vortexing this buffer is ineffective

36 Thaw the Elution Buffer (EB) at room temperature. Mix by vortexing. Spin down and store on ice

37 Thaw either the Long Fragment Buffer (LFB) or Short Fragment Buffer (SFB) at room temperature and mix by vortexing. Then spin down and place on ice

38 In a 1.5 ml Eppendorf DNA LoBind tube, mix in the following order:

38.1 Between addition of each reagent, pipette to mix (gently) 10 - 20 times

Reagent	Volume (ul)
DNA Sample from the previous step	60
Ligation Buffer (LNB)	25

NEBNext Quick T4 DNA Ligase	10
Ligation Adapter (LA)	5
Total	100 ul

Table adapted from the SQK-LSK114 Protocol

39 Mix thoroughly by gently pipetting and briefly spinning down.

40 Incubate the reaction for 10 minutes at room temperature

41 Resuspend the AMPure XP Beads (AXP) by vortexing

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

43 Incubate on a Hula mixer for 5 minutes at room temperature

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

45 Wash the beads by adding either 250 ul Long Fragment Buffer or 250 ul Short Fragment Buffer. Flick the beads to resuspend, spin down, return the tube to the magnet and allow the beads to pellet. Remove the supernatant and discard

46 Repeat

47 Spin down and place the tube back on the magnet. Pipette off any residual supernatant. Allow to dry for ~30 seconds

47.1 Do not dry the pellet to the point of cracking

48 Remove the tube from the magnet and resuspend the pellet in 15 ul Elution Buffer (EB). Spin down and incubate for 10 minutes at room temperature.

48.1 For high molecular weight DNA, incubating at 37°C can improve the recovery of long fragments

49 Pellet the beads on a magnet until the emulate is clear and colorless (~1 min or more)

50 Remove and retain 15 ul of eluate containing the DNa library into a clean 1.5 ml Eppendorf DNA LoBind tube. Dispose of the pelleted beads

51 Proceed to quantify 1 ul of eluted sample using a fluorometer

52 Depending on required output, prepare the final library to 35-50 fmol for high output of simplex data, or 10-20 fmol for duplex data, in 12 ul of Elution Buffer (EB).

52.1 If library yields are below input recommendations, load the entire library.

52.2 Recommended to use duplex data

52.3 Load 10 - 20 fmol of final library. Loading more than 20 fmol of DNA can reduce the rate of duplex read capture

53 Proceed to priming and loading of the flow cell

Data Analysis using EPI2ME

54 Following the sequencing run, BAM files (the required input for the workflow) can be generated from the POD5 or FAST5 files following the wf-basecalling workflow

54.1 Alternatively, BAM files can be generated from fastq files using the wf-alignment workflow

54.2 Both workflows generate aligned BAM files that are compatible with the wf-somatic-variation workflow.

54.3 If no matched normal sample is available, the tumor-only mode must be used.

55 QC of sequencing data and pre-processing

55.1 Computes depth of sequencing of the BAM files (mosdepth)

55.2 Computes read alignment statistics for each BAM (fastcat)

55.3 Other commands

55.4 --tumor_min_coverage :used to determine whether the input tumor BAMs have a depth greater than this value

55.5 --normal_min_coverage :used to determine whether the input normal BAMs have a depth greater than this value

55.6 Both steps can be skipped if the minimum coverage level is set to zero

56 Somatic Variant Calling (short variants) using ClairS

56.1 Version implemented by workflow is ClairS (v0.1.6) which is used to identify somatic variants in paired tumor-normal samples

56.2 Supported basecalling models can be found on https://github.com/epi2me-labs/wf-somatic-variation)

57 Clair3 is used in this workflow to call germline variants in both the tumor and normal sample

57.1 These are used to refine the somatic variant calling

57.2 Options for reducing computational demands

57.3 - - fast_mode: reduces accuracy of variant calling

57.4 - -normal_vcf: provide a pre-computed VCF file with germline calls for the normal sample

57.5 - - germline false: disables the germline calling process

58 Calling Somatic structural variants (SVs) with Nanomonsv

58.1 Workflow calls somatic structural variants with long-read sequencing data. It starts from paired tumor/normal samples and will:

58.2 Parse signatures of structural variants using nanomonsv parse

58.3 Call the somatic SVs with nanomonsv get

58.4 (Optional) Filters the SVs out in simple repeats with add_simple_repeat.py

58.5 (Optional) Annotate transposable and repetitive elements with nanomonsv insert_classify

Protocol references

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