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ROBIN: A unified nanopore-based sequencing assay integrating real-time, intraoperative methylome classification and next-day comprehensive molecular brain tumour profiling for ultra-rapid tumour diagnostics

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We use this protocol and it's working

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This protocol will require prior approval by the users' Institutional Review Board (IRB), or equivalent ethics committee, depending on the source of tissue used.

Abstract

Background

Advances in our technological capacity to interrogate brain tumour biology has led to the ever-increasing use of genomic sequencing in routine diagnostic decision making. Presently, brain tumours are routinely classified based on their epigenetic signatures, leading to a paradigm shift in diagnostic pathways. Such testing can be performed so rapidly using nanopore sequencing that results can be provided intraoperatively. This information greatly improves upon the fidelity of smear diagnosis and can help surgeons tailor their approach, balancing the risks of surgery with the likely benefit. Nevertheless, full integrated diagnosis may require subsequent additional assays to detect pathognomonic somatic mutations and structural variants, thereby delaying the time to final diagnosis.

Methods

Here, we present ROBIN, a tool based upon PromethION nanopore sequencing technology that can provide both real-time, intraoperative methylome classification and next-day comprehensive molecular profiling within a single assay. ROBIN uniquely integrates three methylation classifiers 1–3 to improve diagnostic performance in the intraoperative setting.

Findings

We demonstrate classifier performance on 50 prospective intraoperative cases, achieving a diagnostic turnaround time under 2 hours and generating robust tumour classifications within minutes of sequencing. Furthermore, ROBIN can detect single nucleotide variants (SNVs), copy number variants (CNVs) and structural variants (SVs) in real-time, and is able to inform a complete integrated diagnosis within 24 hours. Classifier performance demonstrated concordance with final integrated diagnosis in 90% of prospective cases.

Interpretation

Nanopore sequencing can greatly improve upon the turnaround times for standard of care diagnostic testing, including sequencing, and is furthermore able to reliably provide clinically actionable intraoperative tumour classification.

Guidelines

This protocol will require prior approval by the users' Institutional Review Board (IRB), or equivalent ethics committee, depending on the source of tissue used.

Materials

1. Qiagen QIAamp Fast DNA Tissue Kit (#51404)
2. Ultra-Long DNA Sequencing Kit V14 (SQK-ULK114)
3. AMPure XP SPRI Reagent (A63880)
4. PEGW buffer (10% PEG-8000/0.5M NaCl; filtered only)
5. 10 mM Tris-HCl pH 9.0
6. Qubit 1X dsDNA High Sensitivity (HS) Assay kit (Q33230 or Q33231)
7. Genomic DNA ScreenTape Analysis (5067-5365 and 5067-5366)
8. 1.5 ml Eppendorf DNA LoBind tubes (#0030108051)
9. 0.2 ml PCR tubes
10. PromethION flow cells R10.4.1
11. Flow cell wash kit (EXP-WSH004 - additional)

Before start

In order to minimise the turnaround time in the interoperative setting, prior to reciept of sample:

1. On ice, premix 9 µL **FDB buffer** with 3 µL **FRA** (from ONT SQK-ULK114).
2. Prepare 3 Qubit tubes with 199 µL 1x HS reagent, and store in a dark place.
3. Label all Eppendorfs required.
4. Thaw all required library reagents in fridge.
5. Prepare a DNA extraction master mix (from Qiagen QIAamp Fast DNA Tissue Kit) and pipette 265 µL into each of the 3 Tissue Disruption Tubes:

Reagents	**Volume (ul)**
AVE	600
VXL	120
DX	3
PK	60
RNAase A	12
Total	**265µl x 3**

Tissue Selection

1 Open tissue pot in Category 3 MSC and weigh total mass on petri dish.

2 Dissect out representative tumour tissue weighing 5-25 mg, then place in Tissue Disruption Tube.

Note

Reserve a portion of the adjacent tissue for intraoperative smear.

Note

Ensure lysis mix is added to Tissue Disruption Tubes prior to receipt of tissue.

3 Repeat steps 1-2 twice more, if tissue quantity allows.

Note

Ideally, three extractions from different representative parts of the tumour are performed.

4 Prepare a smear slide, using tissue adjacent to samples 1 to 3 arranged in a Left-to-Right orientation, labelled α, β, γ.

Note

Tissue labelled α, β, γ on slide to avoid confusion with routine laboratory nomenclature and sample labelling.

Note

During following DNA extraction steps, Neuropathologist to report smear and advise on most suitable sample to take forward for library preparation.

DNA Extraction from Brain Tissue

5 Homogenize tissue using vortexer at full speed for 00:05:00 .

> **Note**
>
> Proceed with step 4 regardless of whether there is residual tissue visible or not.

6 Incubate in a thermomixer at 1000 rpm, 56°C, 00:10:00 .

> **Note**
>
> If the lysate is homogenous after step 6, proceed directly with step 7. If there is still residual tissue left after step 6, repeat steps 5 and 6 a single time.

7 Add 265 µL **Buffer MVL** and mix by pipetting or vortexing.

8 Apply the mixture from step 7 to the QIAamp Mini spin column and centrifuge for 00:01:00 . Place the spin column into a new 2 ml collection tube.

> **Note**
>
> All centrifugation steps should be performed at **maximum speed (up to 20,000 x g)**. Unless otherwise stated, all centrifugation steps are performed for **30 seconds.**

9 Add 500 µL **Buffer AW1** to the spin column and centrifuge. Place the spin column into a new 2 ml collection tube.

10 Add 500 µL **Buffer AW2** to the spin column and centrifuge. Place the spin column into a new 2 ml collection tube.

11 Centrifuge for 00:01:30 . Place the spin column into a clean 1.5 ml microcentrifuge tube. 1m 30s

12 Add 100 µL **ATE** directly onto the spin column membrane, incubate at room temperature for 00:02:00 .

13 Centrifuge for 00:01:00 and store DNA On ice .

DNA shearing

14 Measure DNA concentration using 1X Qubit High Sensitivity (HS) reagents.

> **Note**
>
> As DNA concentration may reach >100 ng/ul, dilute the sample 10-fold when measuring with Qubit HS.

15 Take 2-10 µg DNA and dilute to 100 µL with [M] 10 millimolar (mM) **Tris-HCl pH 9.0** in a 1.5 ml Eppendorf LoBind tube. Aim for a DNA concentration of [M] 20-40 ng/µL

16 Shear DNA by slowly but steadily passing the sample 18 times through a 30G x 1/2 needle.

> **Note**
>
> Ensure not to aspirate air into the syringe.

17 Measure sheared DNA concentration again using Qubit HS.

Library Preparation

18 In a 1.5 ml Eppendorf LoBind tube, mix 600 ng DNA with the following components from the SQKULK114 (ONT) kit On ice :

	A	B
	Reagents	**Volume (ul)**
	DNA (~600ng)	x
	10mM Tris-HCl pH 9.0	73-x
	FDB buffer	15
	Total	**88.0**

	A	B

Note

Prior to experiment, prepare premixed Eppendorf with 9 µL **FDB** with 3 µL **FRA**.

19 Add the diluted **FRA** to the DNA mixture using a P200 low-retention filtered tip. Keep On ice at all times, stir the reaction with the pipette tip whilst expelling the diluted FRA to ensure an even distribution. Mix gently and thoroughly by pipetting.

Note

Total volume is 100 µL. Make sure sample is homogeneous and cold while mixing with FRA. The FRA enzyme is fast acting and we want to make sure it is distributed evenly through the DNA solution before reacting.

20 Transfer into a suitable 0.2 mL tube. In a thermocycler, incubate the sample as follows: 9m

30 °C for 00:05:00

80 °C for 00:02:00

4 °C for 00:01:00

Room temperature for 00:01:00

Note

Ensure that the thermocycler is able to start immediately when sample is ready (i.e. lid temperature has been reached and set to 90 °C if adjustable).

21 Add 1 µL **Rapid Adapter** (RA). Mix gently by pipetting, and spin down.

22 Incubate the reaction at Room temperature for 00:04:00 with rotation at 9-10 rpm. 4m

Note

Warm a PromethION flow cell at Room temperature in readiness for sequencing.

Library Clean-up

23 Add 100 µL of resuspended **AMPure XP Beads** to the reaction and mix by pipetting or flicking the tube.

Note

Ensure AMPure XP Beads are at Room temperature before use.

24 Incubate the reaction at Room temperature for 00:04:00 with rotation at 9-10 rpm. 4m

25 Spin down the sample and leave to pellet on a magnet rack for 00:03:00 . Pipette off the supernatant. 3m

26 Wash the beads by adding 250 µL **PEGW buffer** (10% PEG-8000/0.5M NaCl; filtered only). Flick the beads to resuspend, spin down, then return the tube to the magnetic rack and allow the beads to pellet for 00:03:00 . Pipette off the supernatant. 3m

27 Spin down and place the tube back on the magnetic rack. Pipette off any residual supernatant.

28 Remove the tube from the magnetic rack and resuspend the pellet in 50 µL of **10mM Tris-HCl pH 9.0**. Spin down.

29 Incubate at 37 °C for 00:05:00 .

Note

During incubation, mix flowcell priming reagents: 980 µL **Flow Cell Flush** (FCF) + 25 µL **Flush Tether UL** (FTU)

30 Prime the promethION flow cell with 500 µL of the priming mixture.

31 Pellet the beads on a magnet until the eluate is clear and colourless, for at least 00:01:00 .

32 Remove and retain the eluate containing the DNA library into a 1.5 ml Eppendorf DNA LoBind tube.

33 Quantify with Qubit HS.

Note

For troubleshooting, it may be necessary to quantify the DNA library using other methods, e.g. Agilent TapeStation genomic DNA and/or Nanodrop spectrophotometer, to validate library quantity and quality. Consider reserving 2ul of library for retrospective testing.

Library Loading and Sequencing

33.1 In a new 1.5 ml Eppendorf LoBind tube, prepare the library for loading as follows and mix well using a pipette:

Reagents	Volume
Sequencing Buffer (SBU)	100
Library Solution (LSU)	10
DNA library	45
10mM Tris-HCl pH 9.0	45

Total	**200**

Note

In order to save reagents, the total loading volume can be reduced to 150 µL as follows:

A	B
Reagents	**Volume**
Sequencing Buffer (SBU)	75
Library Solution (LSU)	7.5
DNA library	45
10mM Tris-HCl pH 9.0	37.5
Total	**150**

34 Prime the promethION flow cell with 500 µL of the priming mixture.

35 Load the library mix into the flow cell. Let tether for at least 00:05:00 prior to sequencing.

Note

It is recommended to protect the flow cell array from ambient light (covers supplied by ONT)