Detecting isoforms and RNA modifications with PCR-free, direct RNA sequencing

Accurately capturing the range of RNA diversity — including gene expression, isoforms, and RNA methylation — can help elucidate the molecular mechanisms of disease and functional roles of RNA modifications. For example, RNA modifications, such as n^6-methyladenosine (m^6A), impact RNA metabolism[1] and play a significant role in initiation and progression of cancers[2,3], as well as neurological disorders, including Alzheimer's disease[4] and Huntington's disease[5].

Traditional short-read sequencing technology typically generates 50–100 bp reads, making it challenging to accurately assemble and quantify transcript isoforms and difficult to measure poly(A) tail length. Additionally, the reverse transcription and amplification steps used to prepare these libraries erase RNA modifications and can introduce PCR bias.

Oxford Nanopore sequencing is the only available technology that directly reads native RNA transcripts, enabling quantitation of gene and isoform expression without PCR bias. It also provides resolution of full-length isoforms[6] and accurately estimates poly(A) tail length. Furthermore, direct RNA nanopore sequencing provides direct detection of m^6A methylation[7] for the DRACH motif at single-base resolution without additional sample preparation.

Here we present a simple workflow for RNA modification analysis from a human blood research sample, using direct RNA sequencing on PromethION™.

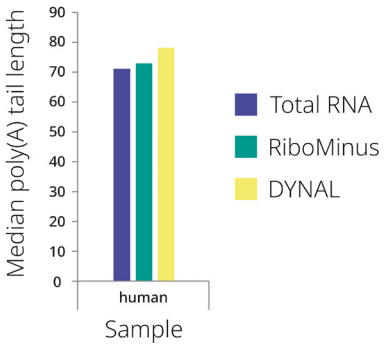
Extraction:
obtaining high-quality RNA

View extraction protocol recommendations for your sample type, plus guidance on RNA storage and contamination: nanoporetech.com/extraction-methods

To ensure high outputs of long reads from Oxford Nanopore sequencing, it is important to select an extraction method that preserves native RNA transcripts and minimises chemical contamination. For total RNA extraction from blood research samples, we recommend using the **QIAGEN PAXgene Blood RNA Kit**, followed by globin depletion using the **Invitrogen GLOBINclear-Human Kit**. If starting with human cell lines, we recommend using **Invitrogen TRIzol RNA Isolation Reagent**. When isolating RNA, we recommend working in an RNase-free environment to minimise degradation during extraction.

To maximise output, 3'-polyadenylated (poly(A)) transcripts can be enriched from total RNA using the **Invitrogen DYNAL Dynabeads mRNA Purification Kit**. For non-polyadenylated transcripts, we recommend the use of **NEB *E. coli* Poly(A) Polymerase** reagent to add poly(A) tails for library preparation compatibility.

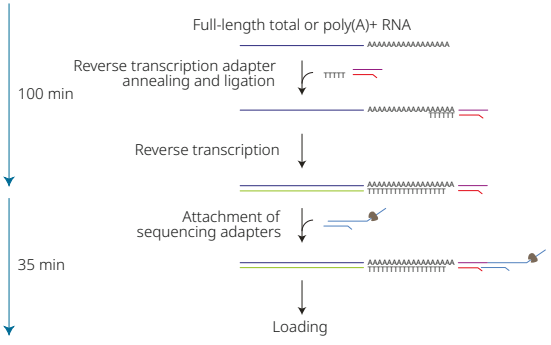
Prior to proceeding to library preparation, we recommend assessing RNA quality via a **Nanodrop** instrument and degradation using the **Agilent Bioanalyzer RNA Analysis Kit**.



Library preparation:
preparing native RNA samples

Learn more about Oxford Nanopore library preparation: nanoporetech.com/prepare

For direct sequencing of native RNA transcripts and modifications, prepare your libraries with the **Direct RNA Sequencing Kit**. This method does not require fragmentation or amplification, preserving long transcripts and RNA modifications. Starting from either total RNA or a poly(A)-enriched (poly(A)+) sample, adapters are ligated onto the RNA strand before a second complementary DNA (cDNA) strand is synthesised during reverse transcription. The cDNA strand is not sequenced but helps increase the sequencing output of native RNA molecules. Sequencing adapters are then attached to the RNA-cDNA hybrid.

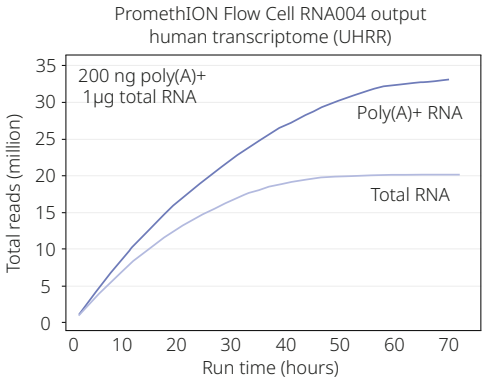


Sequencing:
generating high outputs of long native RNA reads

Find out more about PromethION sequencing devices:
nanoporetech.com/promethion

For high-output sequencing of long native RNA transcripts and m^{6}A methylation detection, RNA-specific flow cells must be used. RNA sequencing using nanopore technology can be scaled to your requirements with the PromethION family, which features the high-throughput PromethION 24 sequencing device, with the capacity to run up to 24 high-output flow cells. For lower throughput requirements, the compact PromethION 2 Solo and PromethION 2 Integrated devices allow sequencing on up to two independent flow cells. A single direct RNA PromethION Flow Cell can generate up to 30 million reads.

For the highest sequencing output and accuracy, we recommend using poly(A)+ samples as input and basecalling in high accuracy (HAC) mode.



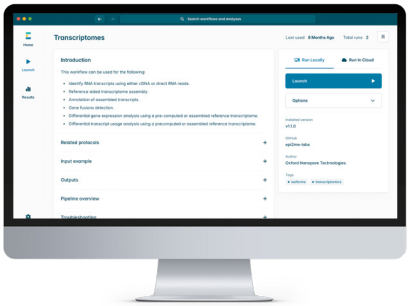
Analysis:
accurate RNA transcript detection and methylation calling

View the dedicated EPI2ME workflow:
nanoporetech.com/epi2me-wf-transcriptomes

The workflow **wf-transcriptomes**[8] is accessible through the **EPI2ME™** application and simplifies data analysis with an intuitive interface. **EPI2ME** workflows enable nanopore data analysis for all levels of expertise: the pre-configured analysis packages are free to access from an intuitive interface or the command line[8]. They can be run on local compute, via a cluster or cloud service, or on your nanopore sequencing device, such as GridION™ or PromethION.

The dedicated **wf-transcriptomes** pipeline identifies RNA transcripts, assembles, annotates transcriptomes, detects gene fusions, and identifies differential gene expression and transcript usage.

Direct RNA basecalling is enabled by Dorado, an optimised basecaller for nanopore sequencing, integrated into the device software, MinKNOW™. Poly(A) tail length estimation and m^{6}A modified RNA basecalling are currently available through the standalone version of Dorado[9] and will soon be integrated into MinKNOW, enabling seamless RNA analysis for immediate access to results.



View the direct RNA sequencing protocol:
nanoporetech.com/direct-rna-sequencing-protocol

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