Oxford Nanopore Technologies

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

RNA modifications add an important layer of regulation to the transcriptome, influencing RNA stability, splicing, localisation, and translation[1]. Accurately capturing transcriptomic diversity, including gene expression, isoforms, and RNA methylation, can help elucidate the molecular disease mechanisms. For example, RNA modifications, such as m^{6}A, impact RNA metabolism[2] and play significant roles in the initiation and progression of cancers[3,4], as well as several neurological disorders[5,6].

Legacy short-read RNA-seq does not preserve RNA modifications because RNA is reverse transcribed into cDNA before sequencing. Instead, antibody-based enrichment is required before short-read sequencing, such as MeRIP-seq for m^{6}A detection. However, these methods typically identify broad modification peaks that can cover multiple modification sites, are not fully quantitative, and suffer from low specificity and reproducibility.

Oxford Nanopore sequencing is the only available technology that allows you to directly read native RNA molecules for accurate detection of eight RNA modifications: m^{6}A, inosine, m^{5}C, pseU, 2′-OMe-A, 2′-OMe-C, 2′-OMe-G, and 2′-OMe-U. With direct RNA sequencing, you can also capture complete transcripts in single reads, supporting gene and isoform expression analysis without PCR bias for transcriptomic research. Additionally, you can achieve a more complete view of the epitranscriptome with modification stoichiometry analysis, co-modification detection within individual molecules, and isoform-level characterisation of RNA modifications.

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, you can enrich poly(A) transcripts from total RNA using the **NEBNext High Input Poly(A) mRNA Isolation Module**. For non-poly(A) transcripts, we recommend using **NEB *E. coli* Poly(A) Polymerase** reagent to add poly(A) tails for library preparation compatibility.

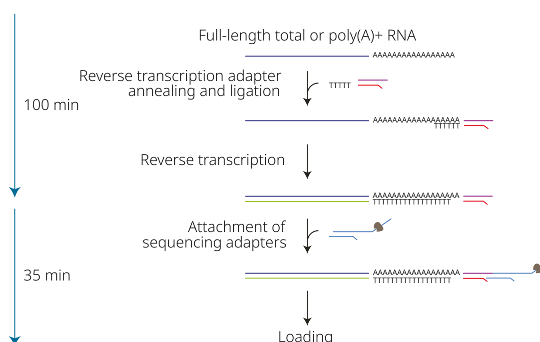
Before proceeding to library preparation, we recommend assessing RNA quality via a **Nanodrop** instrument and degradation using the **Agilent Bioanalyzer RNA Analysis Kit**.

Library preparation:
preparing native RNA samples

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

To sequence 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. With the **Direct RNA Barcoding Kit,** you can prepare 4–24 samples in one run, reducing cost and input requirements per library, while still maintaining the read length, output, and modification accuracy of the singleplex kit.

During library preparation, adapters are ligated onto the RNA strand before a second cDNA strand is synthesised via 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.

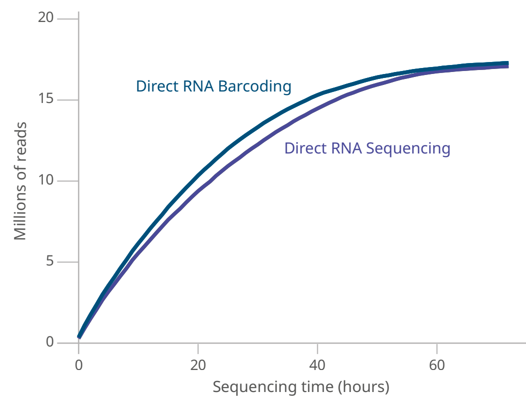


Sequencing:
generating high outputs of long native RNA reads

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

For high-output sequencing of long native RNA transcripts and m⁶A methylation detection, RNA-specific flow cells must be used. You can scale RNA sequencing to your requirements using the PromethION devices. The high-throughput **PromethION 24** sequencing device provides the capacity to run up to 24 high-output flow cells, while for lower throughput requirements, the compact **PromethION 2 Integrated** device allows sequencing on up to two independent flow cells. A single direct RNA sequencing run on a PromethION Flow Cell can generate 10–25 million reads.

For experiments with high sequencing output, we recommend basecalling using the high accuracy (HAC) mode in MinKNOW™, the device software. Poly(A) tail length estimation and modified RNA basecalling are now integrated in MinKNOW, enabling seamless RNA analysis.

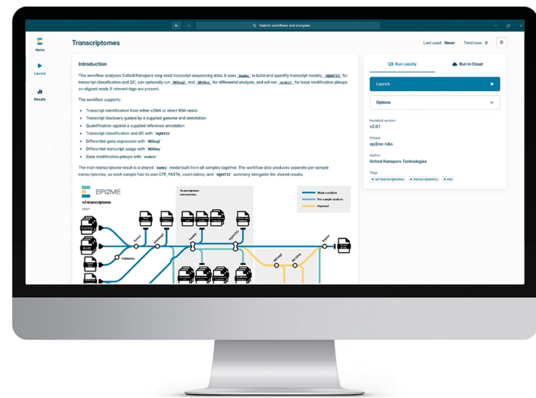


Analysis:
accurately detect RNA transcripts and methylation

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

The workflow **wf-transcriptomes**[7] 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. You can run them 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 prepares sequencing files for downstream RNA modification analysis, identifies RNA transcripts, assembles and annotates transcriptomes, detects gene fusions, and calculates differential gene expression and transcript usage.



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

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