

## Oxford Nanopore sequencing of native adeno-associated virus vectors for quality control

Adeno-associated virus (AAV) is a non-enveloped single-stranded DNA virus used in gene therapy. Accurate validation, contamination detection, and quality control (QC) of recombinant AAV (rAAV) vectors is crucial to ensure the correct rAAV genomes are packaged into capsids before therapeutic use, to confirm the safety and efficacy of the therapy. However, using legacy short-read sequencing technology for QC can present limitations: features such as inverted terminal repeats (ITRs) in AAV genomes are especially hard to map due to their high GC content, palindromic nature, and complex secondary structure. Moreover, short-read sequencing has limitations in accurately characterising genome truncations and heterogeneous vector populations, which can impact functionality and efficacy.

In contrast, long Oxford Nanopore reads can be used to sequence full-length, native rAAV genomes — both single-stranded and self-complementary AAV vectors — end to end, to aid their QC. This allows for ITRs to be fully characterised, enabling identification of any truncated rAAV genomes, contamination, or mutations. Transgenes and promoters of interest can also be identified to support validation of rAAV vectors.

Prior to rAAV sequencing, whole-plasmid sequencing should be performed for complete QC across the whole AAV production process. Further details can be found in our whole-plasmid sequencing workflow: [nanoporetech.com/resource-centre/plasmid-sequencing-workflow](https://nanoporetech.com/resource-centre/plasmid-sequencing-workflow).

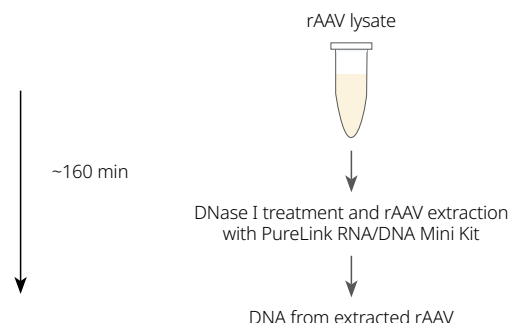
Here we present a workflow to sequence and characterise full-length, native rAAV vectors using MinION™ or GridION™ sequencing devices and the EPI2ME™ analysis platform.

**Extraction:**  
obtaining high molecular-weight DNA

Find extraction protocols and guidance on DNA storage and handling: [nanoporetech.com/documentation/prepare](https://nanoporetech.com/documentation/prepare)

For full-length sequencing of rAAV genomes in single reads, it is important to select an extraction method that preserves the ~4.7 kb DNA fragments. We recommend using the **PureLink Viral RNA/DNA Mini Kit**, which we have seen to produce more full-length rAAV genomes and ITR sequences compared with other methods, such as proteinase K and heat-based extractions. Prior to extraction, treat your samples with DNase I to remove any unencapsidated DNA contamination.

Next, you may self-anneal any remaining single strands of rAAV vector; however, this step is optional and we recommend omitting it to maximise the number of full-length ITR sequences. We also recommend quantifying your samples using the **Qubit ssDNA and dsDNA HS Assay Kits** and **Qubit fluorometer** before proceeding to library preparation.

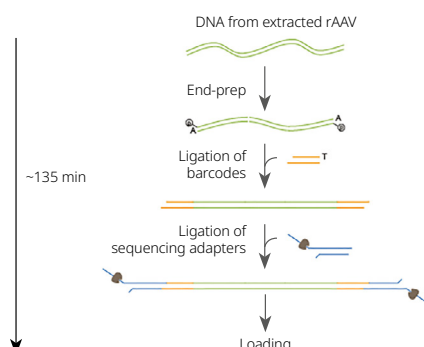


**Library preparation:**  
multiplexing samples

Learn more about Oxford Nanopore library preparation: [nanoporetech.com/prepare](https://nanoporetech.com/prepare)

To prepare your samples for sequencing in multiplex, use the Oxford Nanopore **Native Barcoding Kit 24**. This PCR-free library preparation method has been optimised for high sequencing output of native DNA.

Through multiplexed sequencing of samples on a single MinION Flow Cell, the cost per sample can be reduced; flow cells can also be washed and reused with the **Flow Cell Wash Kit**.



**Sequencing:**  
running until the necessary coverage is achieved

Find out more about Oxford Nanopore sequencing devices:  
[nanoporetech.com/sequence](https://nanoporetech.com/sequence)

We recommend sequencing up to six barcoded rAAV libraries on a MinION Flow Cell. This can be run on a portable **MinION** device for easily accessible, routine sequencing. For higher throughput needs, the **GridION** device can be used, enabling on-demand sequencing on up to five independent flow cells at a time.

For full-length rAAV sequencing, we recommend basecalling in high accuracy (HAC) mode using the **MinKNOW™** software. We recommend sequencing until you've achieved the necessary coverage for your experimental goals. To detect shorter reads that are often indicative of contamination, such as ITR-tetramers, minimum read length can be reduced in MinKNOW.

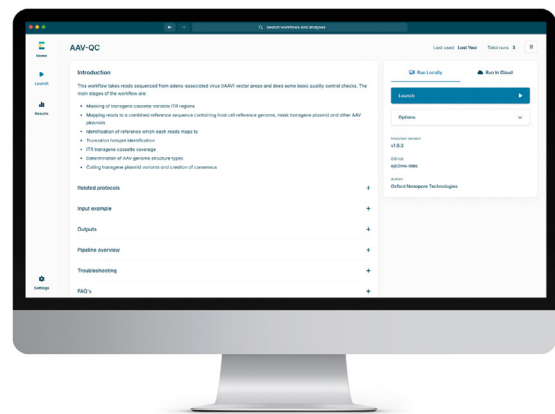


**Analysis:**  
using the EPI2ME wf-aav-qc workflow

View the dedicated EPI2ME workflow:  
[nanoporetech.com/epi2me-wf-aav-qc](https://nanoporetech.com/epi2me-wf-aav-qc)

QC of rAAV vectors is achieved using the **wf-aav-qc** workflow. The workflow — an EPI2ME solution — integrates several tools for the quick assessment of rAAV vectors, including **minimap2<sup>1</sup>** to map reads to a combined reference sequence containing host cell reference genome, the transgene plasmid, and other AAV plasmids, and **Medaka<sup>2</sup>** to call transgene plasmid variants and generate a consensus sequence.

**EPI2ME** is the user-friendly Oxford Nanopore data analysis platform, suitable for all levels of bioinformatics expertise. The report generated by this EPI2ME workflow includes contamination plots to show read sources distribution, a truncations plot, an ITR-ITR read coverage plot, and a genome type frequency plot.




Find out more at: [nanoporetech.com/biopharma](https://nanoporetech.com/biopharma)



View the end-to-end protocol: [nanoporetech.com/aav-sequencing-protocol](https://nanoporetech.com/aav-sequencing-protocol)



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#### References:

1. Li, H. Minimap2: pairwise alignment for nucleotide sequences. *Bioinformatics* 34(18):3094–3100 (2018). DOI: <https://doi.org/10.1093/bioinformatics/bty191>
2. GitHub. Medaka. Available at: <https://github.com/nanoporetech/medaka> [Accessed 05 June 2025]

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