BASE mRNA FACILITY

THE UNIVERSITY OF QUEENSLAND AUSTRALIA

The analysis of mRNA vaccines and therapies using RNA sequencing

USP Open Forum - Feb 29th 2024

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Disclaimer:

Tim Mercer and The University of Queensland have received research funding and travel support from Oxford Nanopore Technologies (United Kingdom). The views and opinions expressed herein are solely those of Tim Mercer, and do not represent The University of Queensland or Oxford Nanopore Technologies.

RNA Sequencing

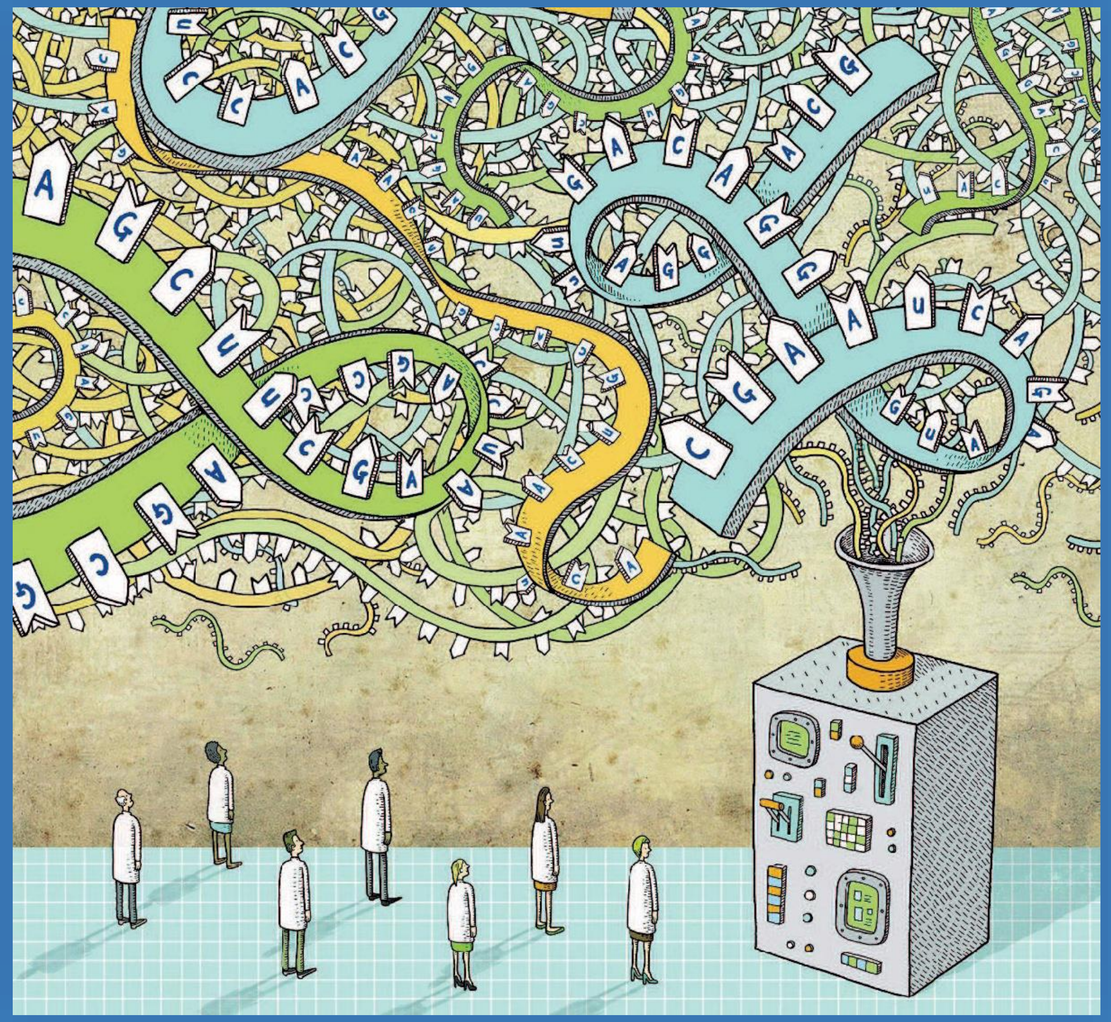

Image courtesy of Maxmillian Publishing

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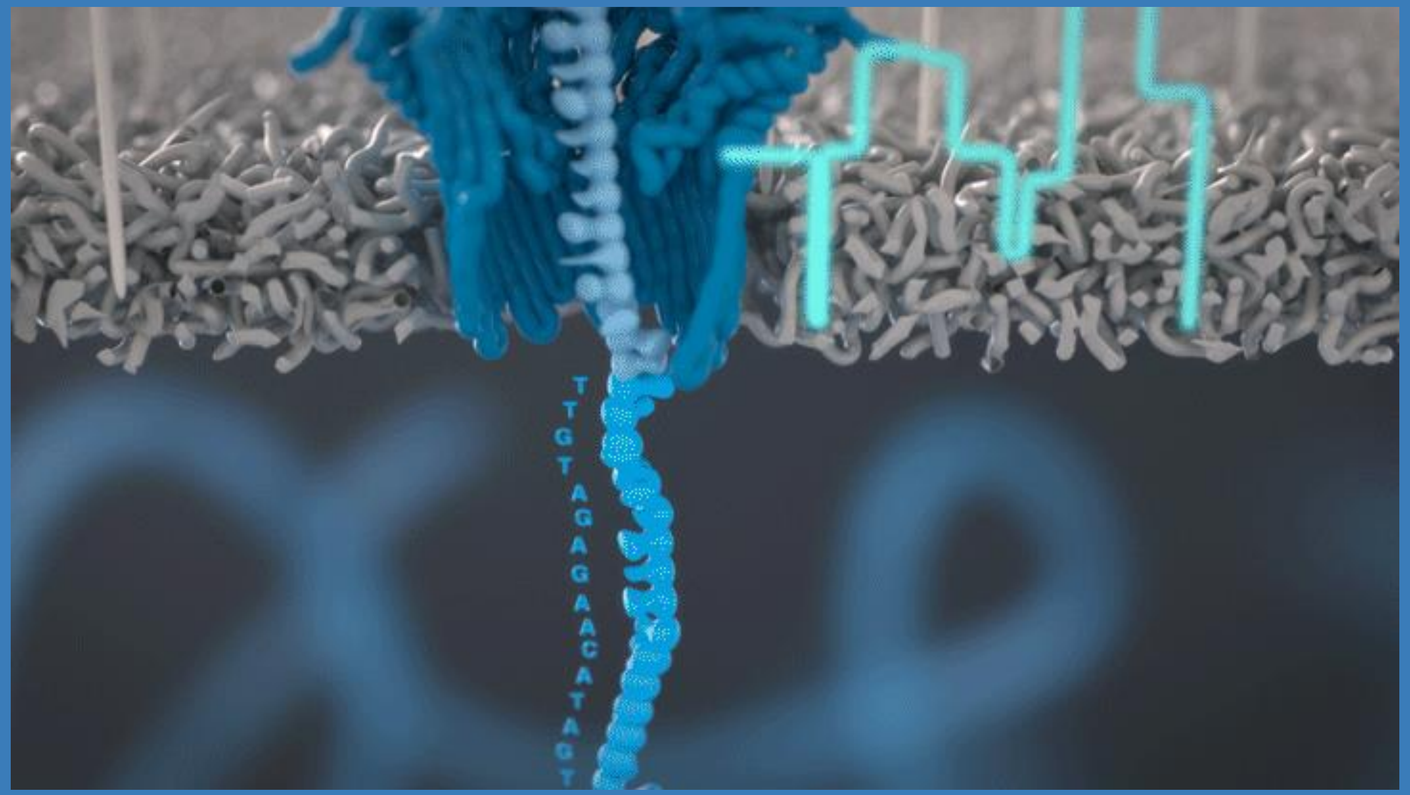
- RNA sequencing is a commonly-used sequencing technique to analyse the transcriptome.
- RNA sequencing is an ideal method to analyse mRNA vaccines and therapies.
- We use nanopore sequencing for quality control throughout the mRNA manufacture workflow.

Nanopore sequencing



- Nanopore sequencing determines the sequence of an DNA/RNA molecule as it traverses a protein nanopore embedded within a membrane.
- The DNA or RNA molecule impacts the ionic current and is base-called into a sequence.

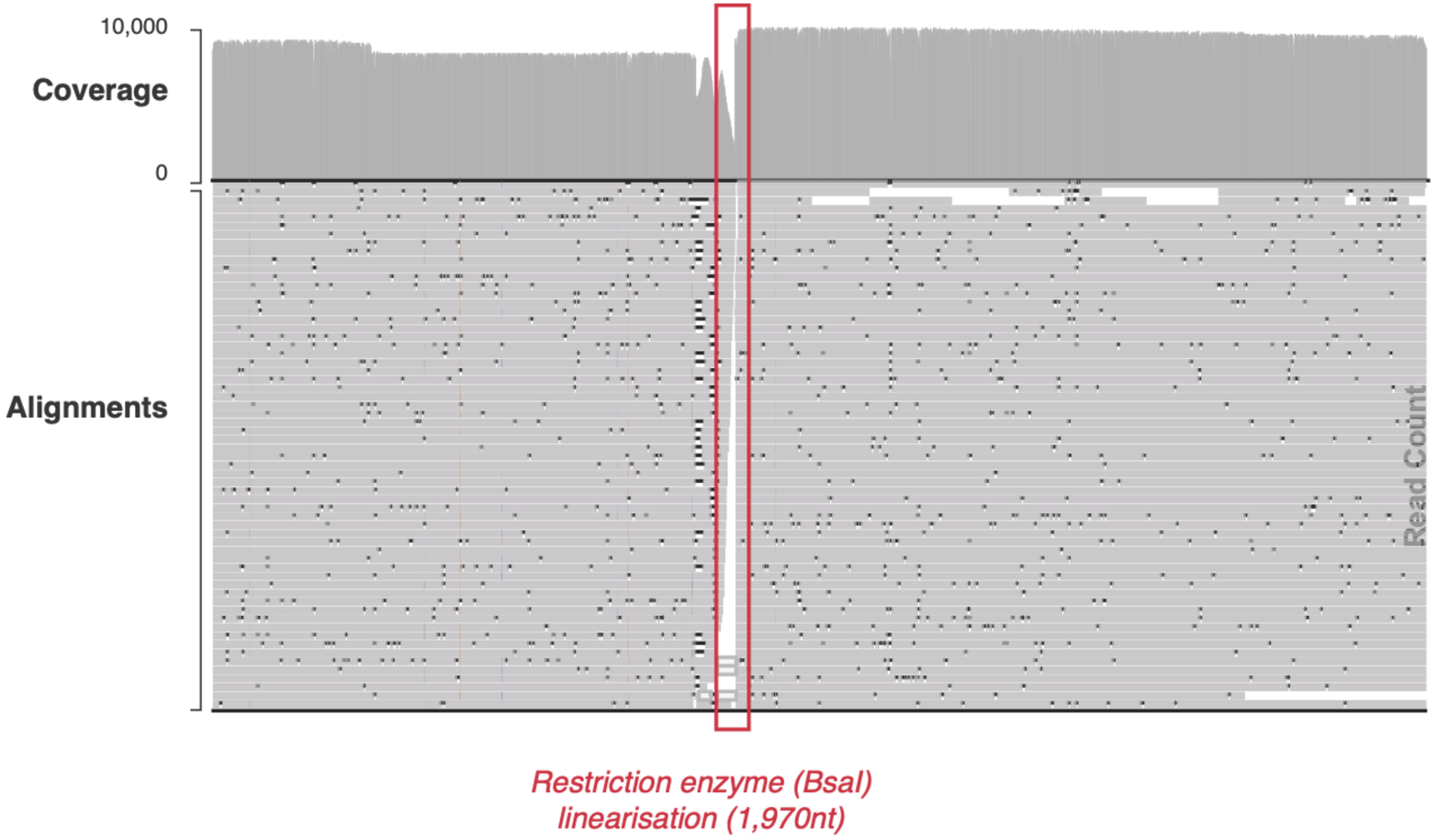
Image courtesy of Oxford Nanopore Technologies

GridION instrument

- GridION is a bench top instrument that can nanopore sequence with up to five flowcells.
- Oxford Nanopore sequencing is :
  - Full-length (longest DNA up to 4Mb)
  - Real-time (immediate results)
  - Direct (no amplification)
  - Single-molecule

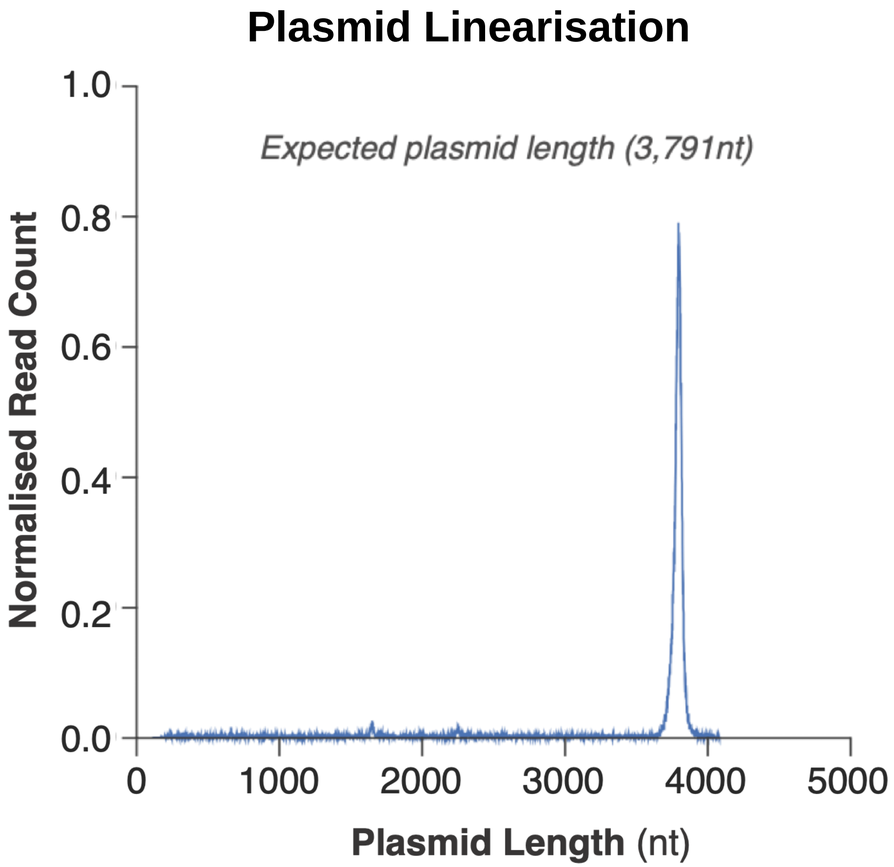
Plasmid DNA template

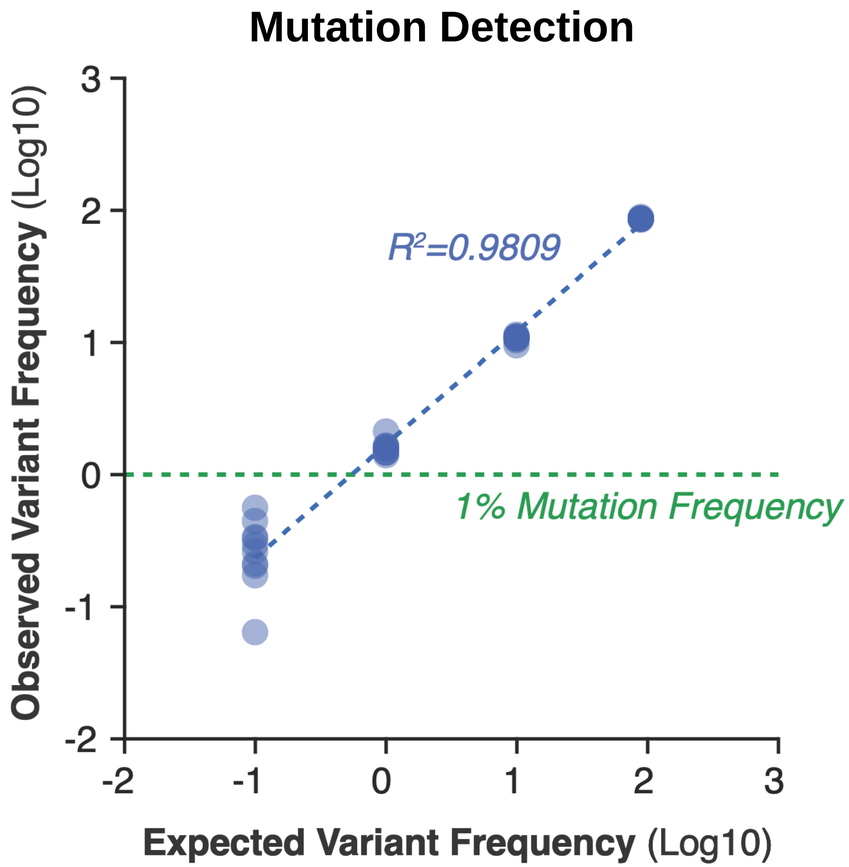
Sequencing data can be visualised through the Integrated Genome Viewer (igv.org)



- Sequencing linearised plasmid DNA template.
- Shows:
  - Errors, Deletions, Mismatches
  - Sub-Clonal errors
  - PolyA tail
  - DNA contamination
  - Linearisation

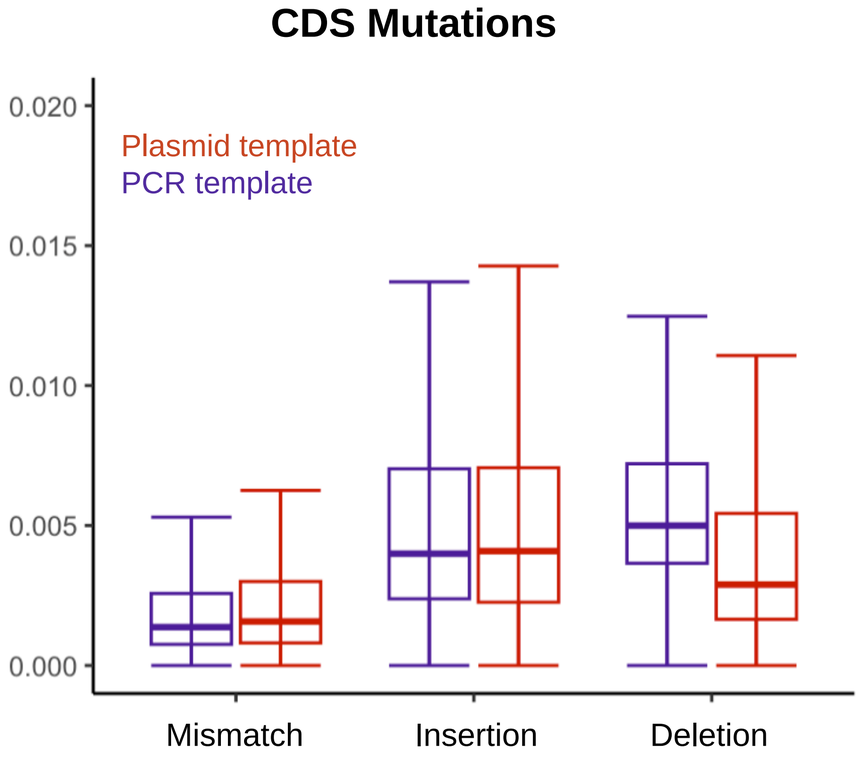
Linearisation & Errors

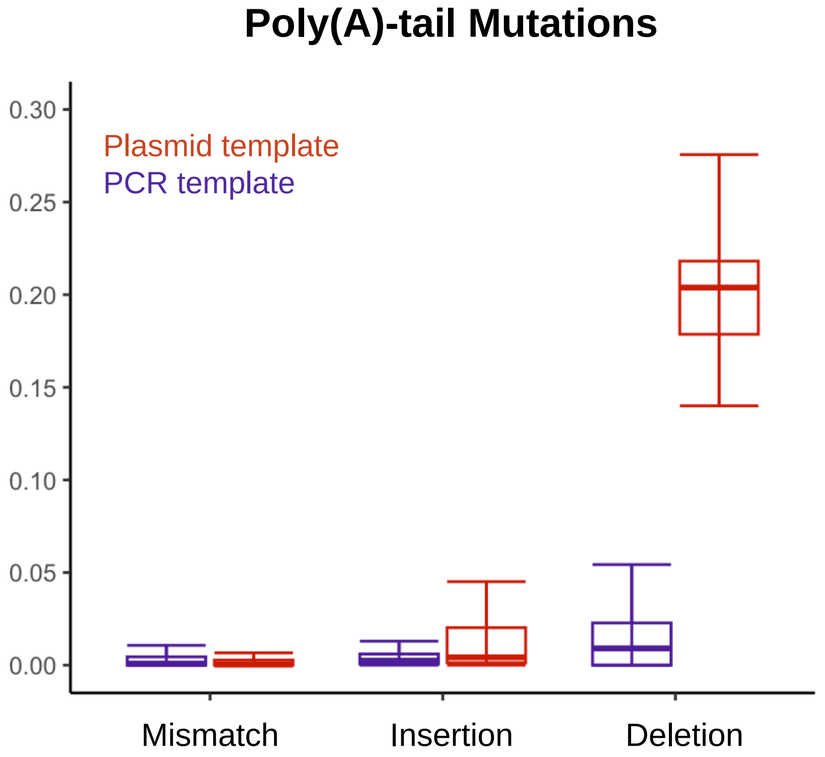




- **Plasmid Linearisation** - Full-length sequencing can determine start/end of linearised DNA.
- **Mutation Detection** - We sequenced staggered mixtures of plasmid DNA at varying allele frequency to show we can reliably detect mutations at >1% frequency.

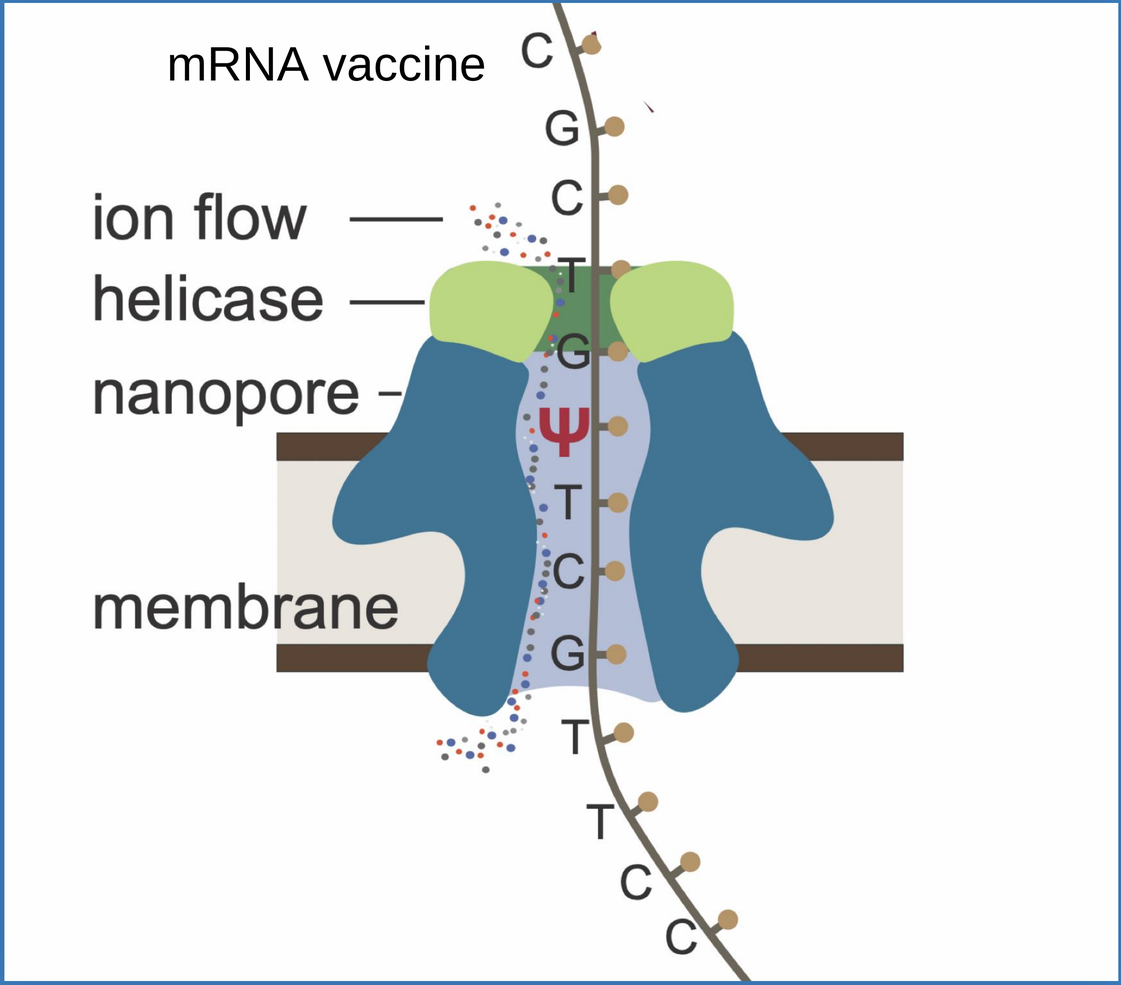
Synthetic DNA templates





- We used Oxford Nanopore sequencing to compare the mutation rate of plasmid DNA (red) and PCR-based (purple) DNA template.
- Within our limits of detection, we find mutation rate is similar between plasmid and PCR templates, but find routine deletion of poly(A) tails in plasmid DNA.

Direct RNA sequencing

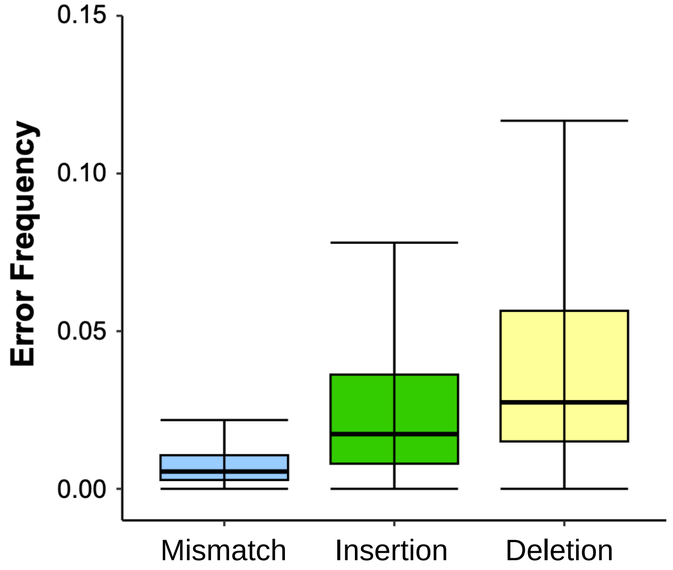


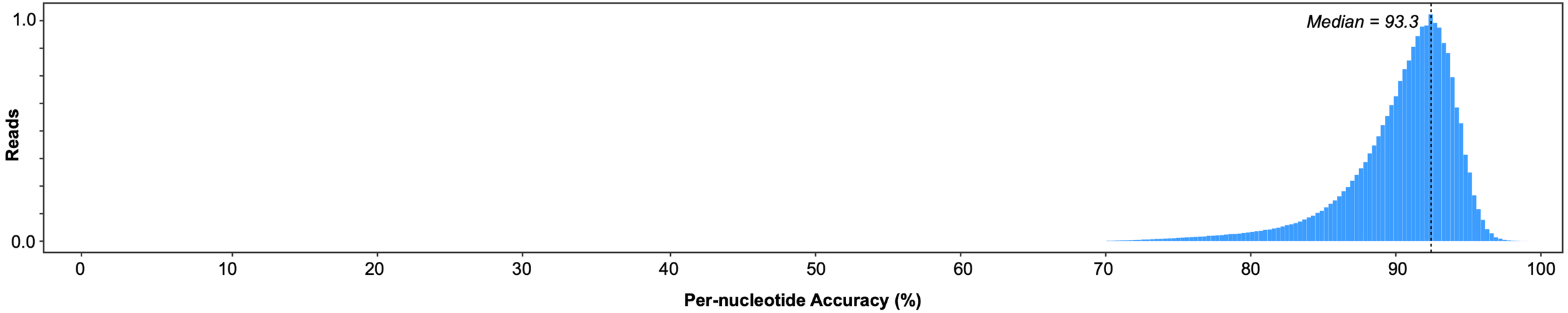
- Direct RNA sequencing of the mRNA vaccine molecule as it traverses the nanopore.
- Directly measures the quality features for millions of mRNA vaccine molecules.
- Full-length - does not require reverse transcription or amplification.
- Can detect modified nucleotides, such as N1-methyl-pseudouridine.
- Can measure polyA tail length
- Single-molecule sequencing - can measure functional vs non-functional mRNA molecules.

mage courtesy of Begik et. al., 2022

Accuracy

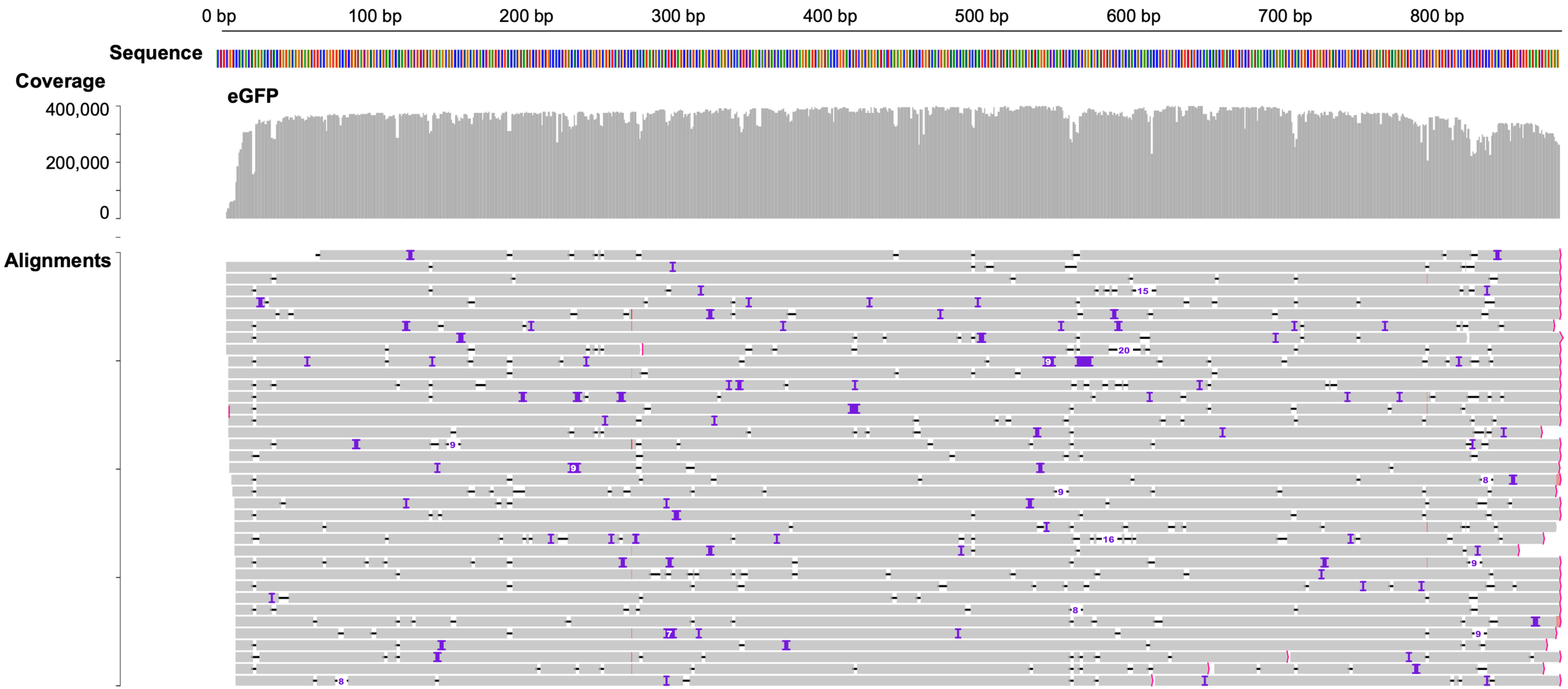
- Critical to confirm the sequence and identify of the mRNA vaccine.
- Base-calling can detect n1-methyl-pseudouridine or canonical uridine.
- Median accuracy 93.3%.
- Can confirm the consensus sequence, and also detect sub-clonal mutations.





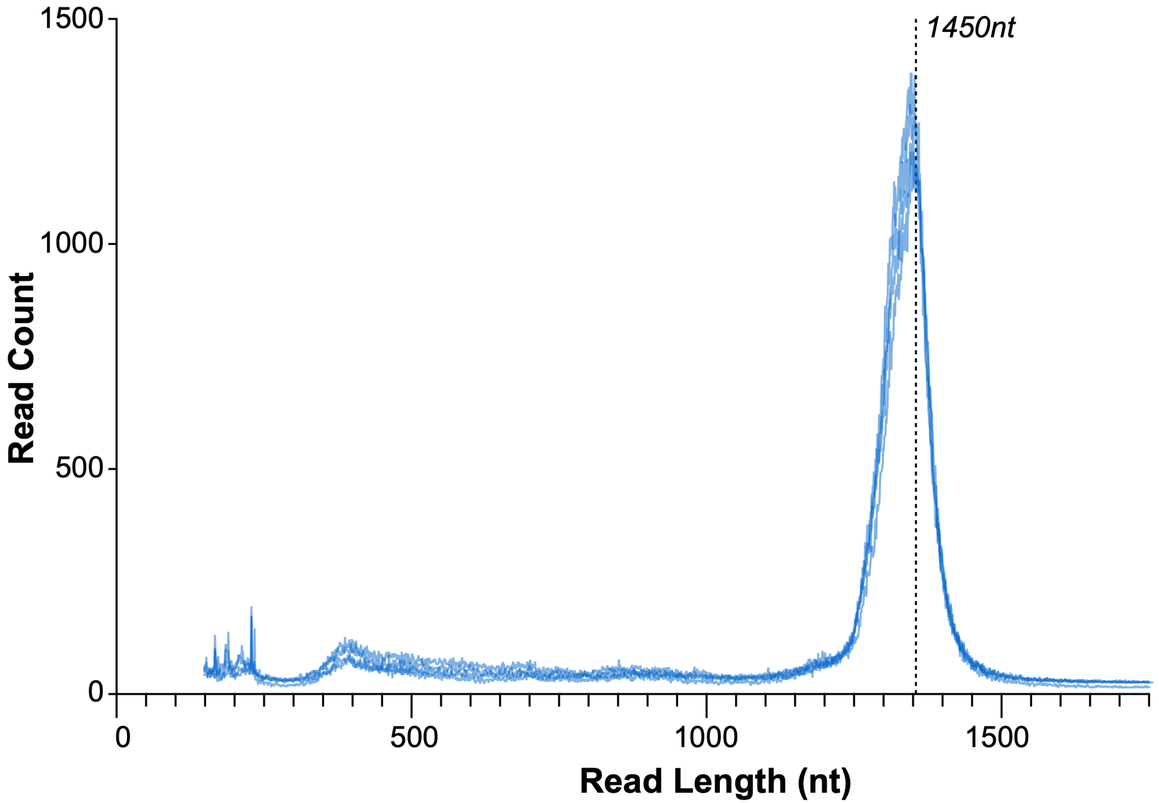
Sequencing mRNA vaccine

- Direct RNA sequencing of eGFP mRNA
- 3 hours 500,000 reads, 24 hours 2.5-4 million reads.

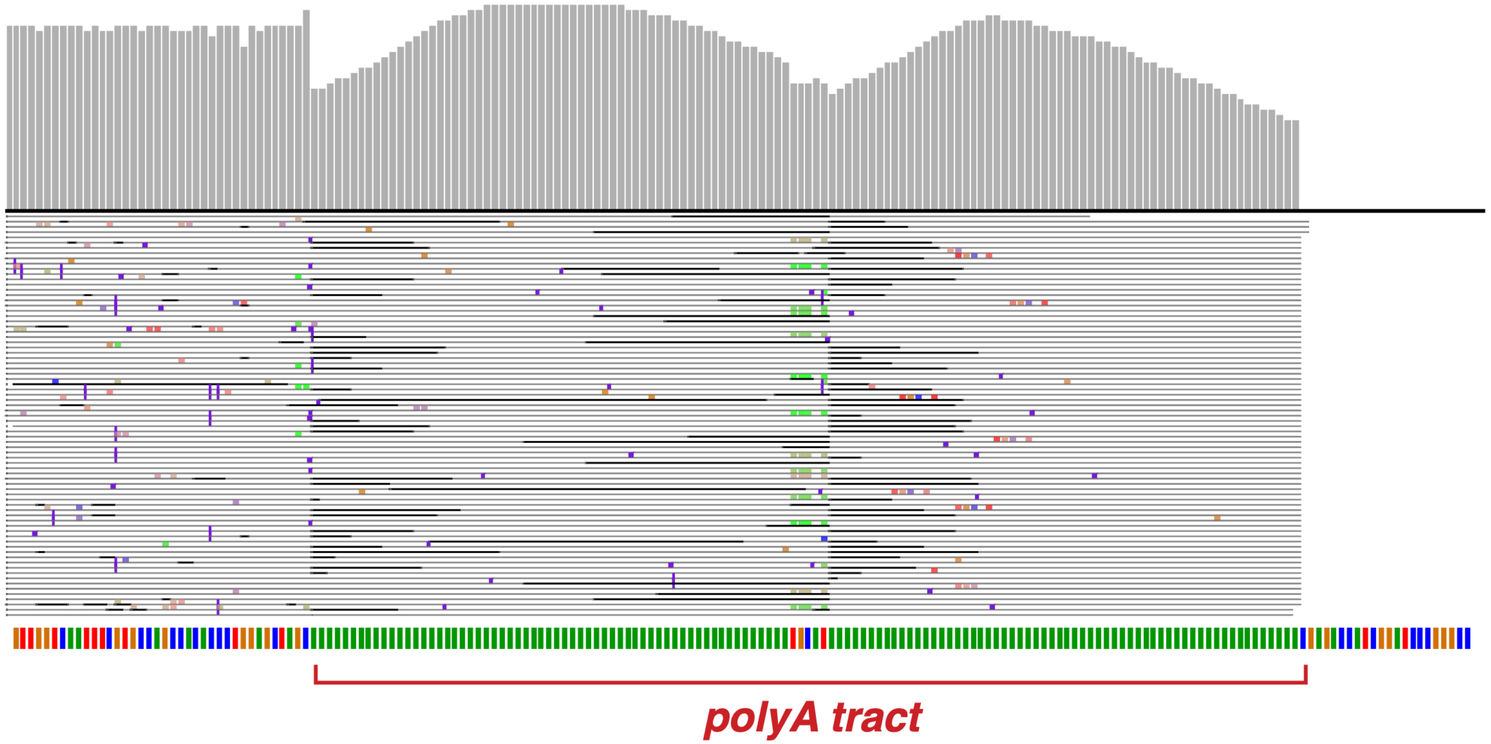


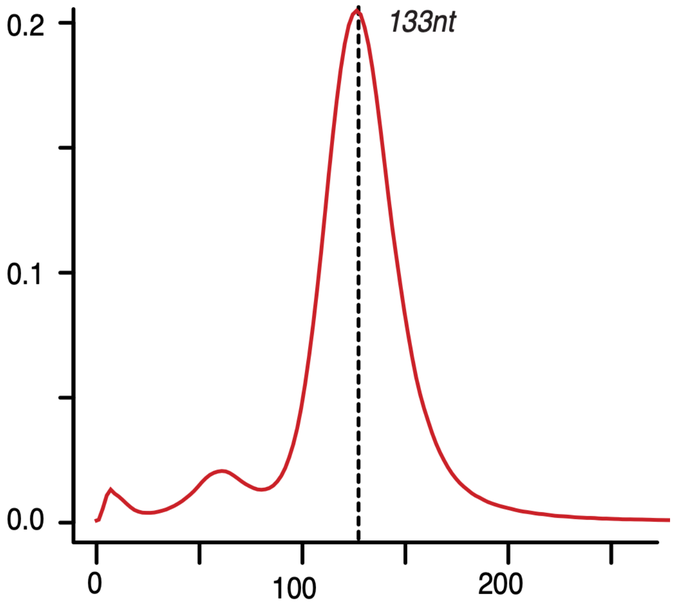
mRNA integrity

- mRNA can be truncated or fragmented during manufacture, storage and distribution - *no longer produce functional product, impacting performance and dosage.*
- Long-read sequencing (from start to end of mRNA molecule) can measure fragmented mRNAs.
- Not impacted by folding or secondary structures.
- Can investigate fragmented mRNA subpopulations.



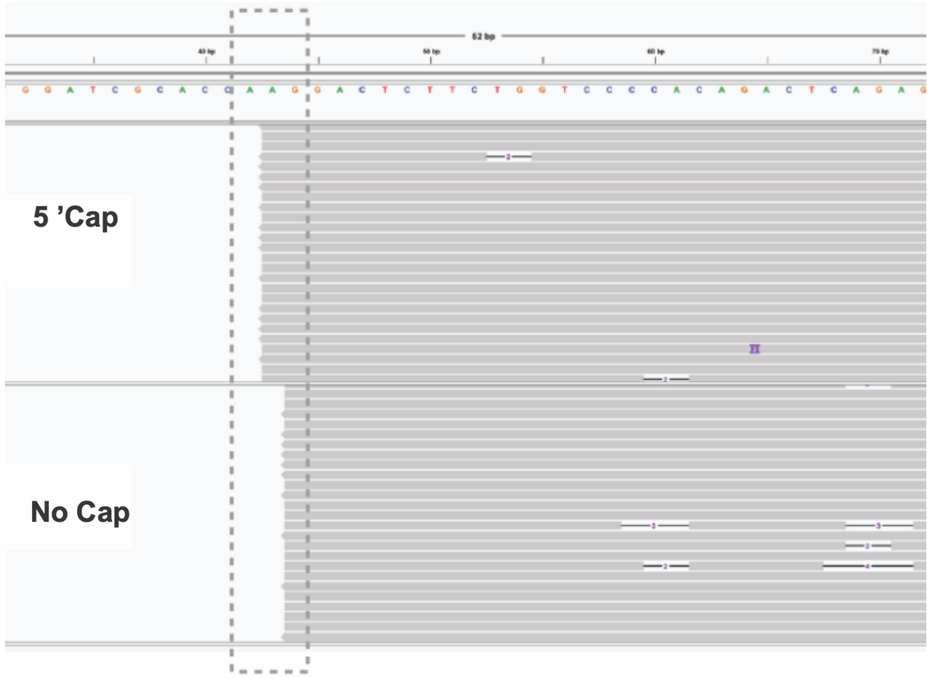
PolyA tail

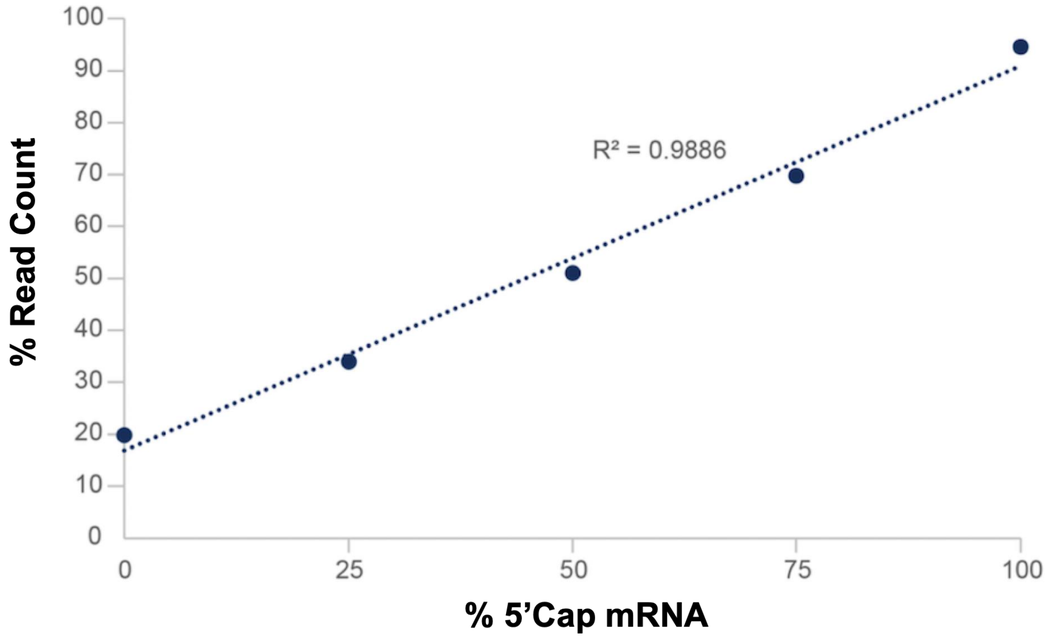




- Use *Dorado* to determine poly-A tail length from sequenced reads.
- Can resolve segmented, non-polyA and terminal modifications.

Co-transcriptional Capping

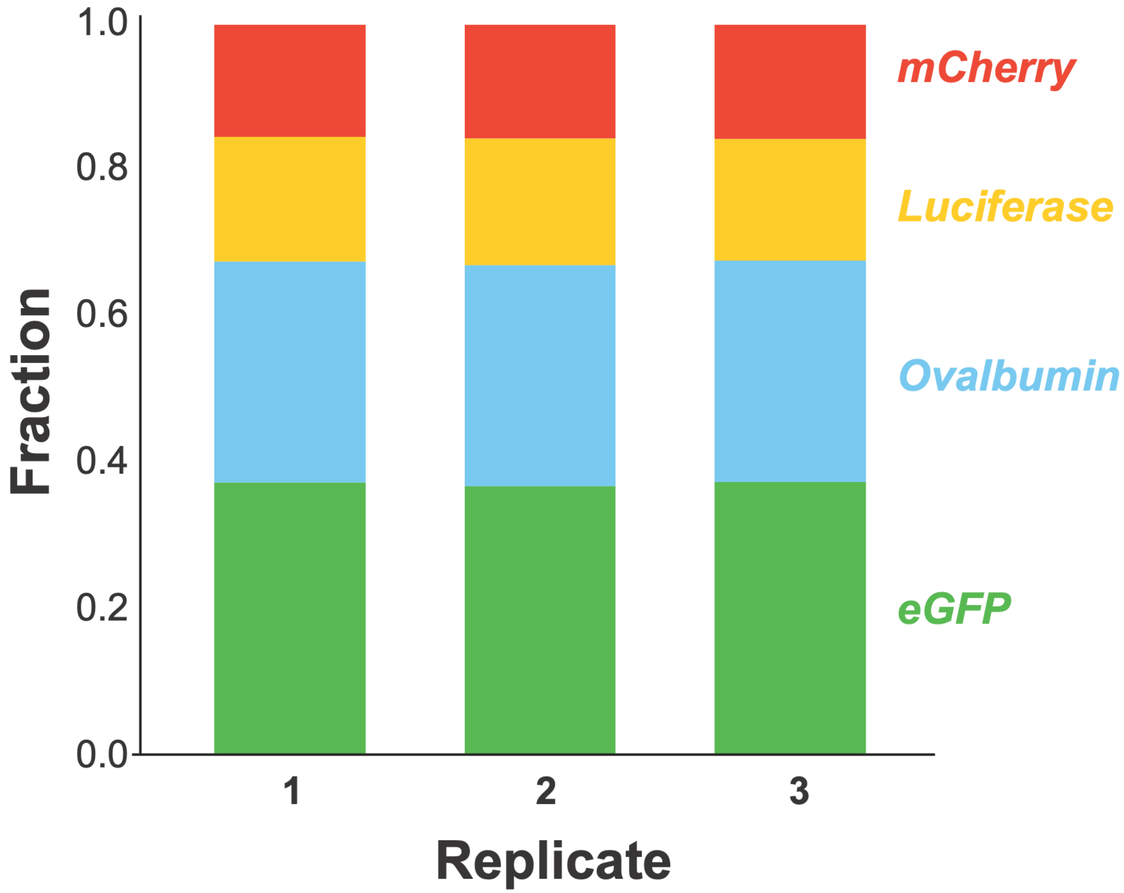




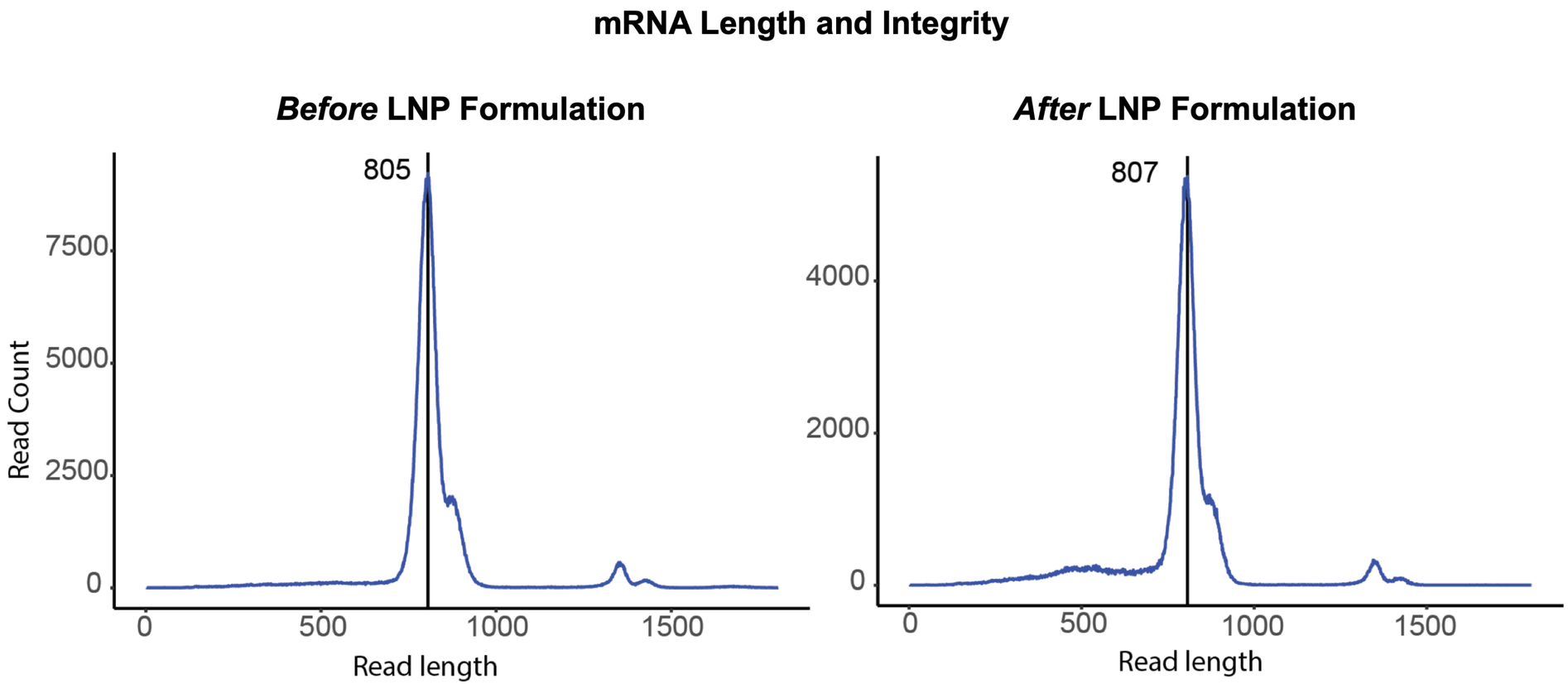
- Using cDNA sequence to measure the start nucleotide, we can determine whether an mRNA vaccine has incorporated a 5' Cap analog.
- (cDNA) Sequencing can quantitatively measure the presence of the 5' Cap Analog on each mRNA molecule.

Multivalent mRNA compositions

- Multiple mRNA vaccines can be combined into a single composition (e.g. COVID variants, influenza, combination respiratory viruses)
- Oxford Nanopore sequencing can measure the abundance of individual mRNAs within multivalent composition.
- Can further analyse quality features (errors etc.) of individual vaccines within composition.



Final drug product testing



- We have developed a protocol to extract mRNA from LNPs for sequencing with fidelity.
- Enables quality control measurement of mRNA in final formulated drug product.

Summary

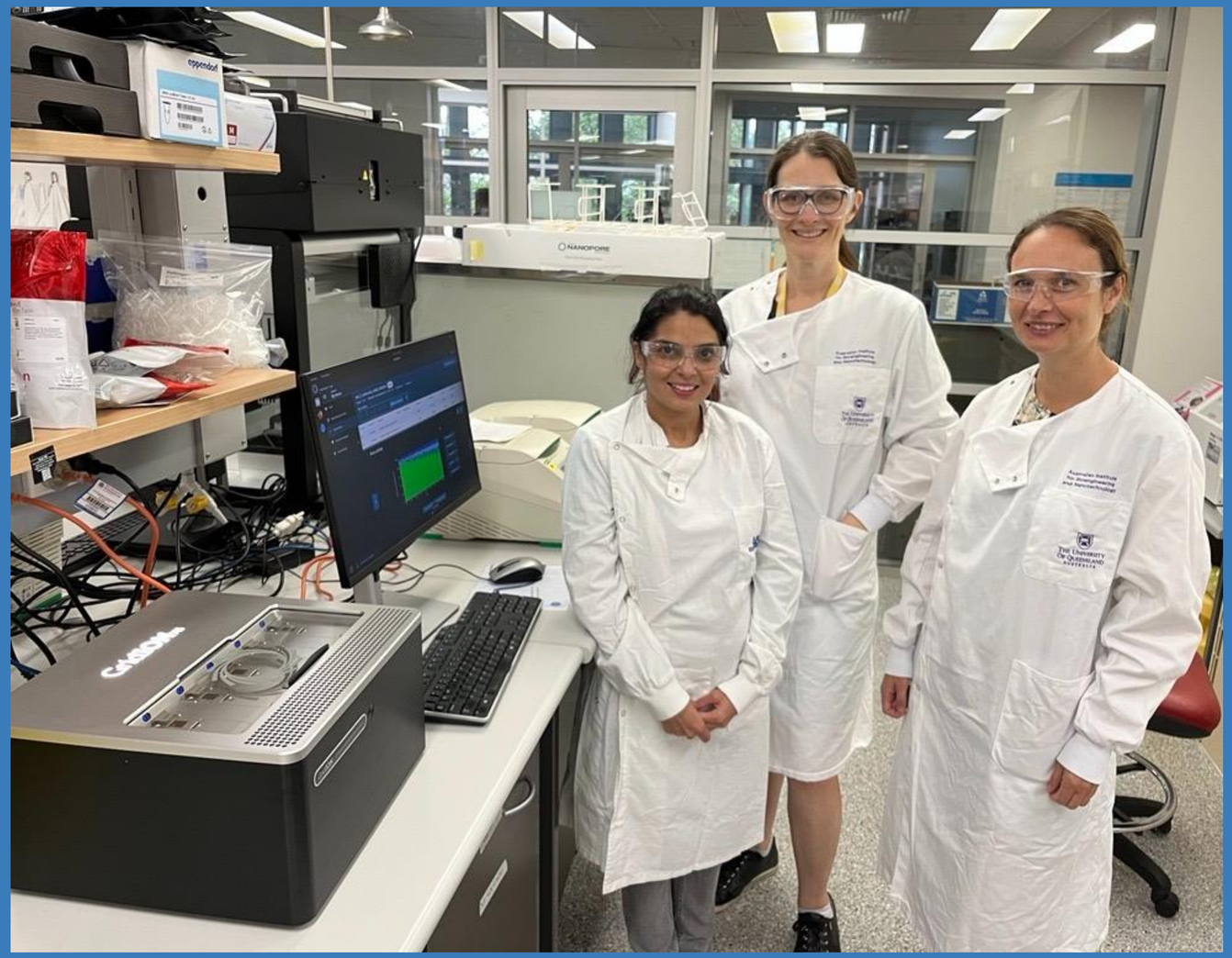
Quality	Attribute	Method
Identity	Sequence confirmation	Next generation sequencing (NGS)
		Sanger sequencing
		Reverse Transcriptase – PCR
Content	RNA content	RT-qPCR and RT–dPCR, Ultraviolet Spectroscopy
Integrity	Percentage of intact mRNA and fragment mRNA	Capillary gel electrophoresis
	5′ cap	IP–RP–HPLC
	3′ poly(A)	RP–HPLC
	mRNA Integrity	Gel electrophoresis
Purity	Product related impurities - dsRNA	Immunoblot
	Residual DNA template	qPCR
Safety	Endotoxin	USP <85>
	Bioburden	USP <61>, <62>, <1115>
	Sterility	USP <71>

- Nanopore sequencing provides real-time, full-length, single-molecule sequencing.
- DNA template sequencing can measure:
  - Sequence identity
  - Linearisation
  - Purity
- Direct RNA sequencing can measure:
  - Sequence identity
  - Fragmented mRNA
  - Modified nucleotides
  - 5’cap (via cDNA)
  - 3’ poly(A)

Functional mRNA molecules

- Nanopore sequencing can measure the multiple quality features of an individual mRNA molecule, from 5’cap to poly(A) tail.
- Can measure the fraction of *functional mRNA molecules* that will be proportional to *efficacy* and inverse to *dosage*.

Acknowledgements



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