Native molecule whole genome sequencing of short fragment cfDNA and FFPE DNA

Adrian Kense, David T. Mulder, Melissa K. McConechy
Imagia Canexia Health, Vancouver, BC, Canada
Contact: akense@canexiahealth.com www.canexiahealth.com

INTRODUCTION

The Oxford Nanopore Technologies (ONT) sequencing platform is a promising tool in cancer diagnostics because it enables sequencing of DNA molecules of any length. Furthermore, nanopore sequencing can interrogate epigenetic methylation status without any template manipulation, making it valuable for rapid disease classification and sample stratification.

Nanopore technology has been largely optimized for long-read sequencing. However, useful templates like cell-free DNA (cfDNA) and DNA derived from formalin-fixed paraffin embedded tissue (FFPE DNA) are highly fragmented.

STUDY OBJECTIVES

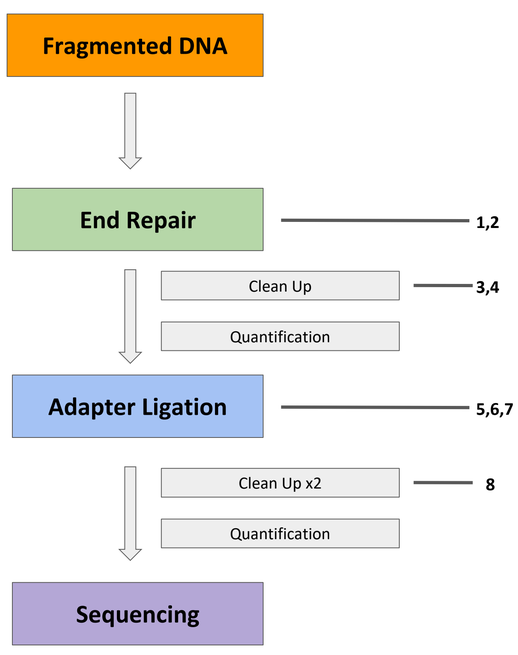
We sought to optimize the library preparation of both cfDNA and FFPE DNA templates to determine the feasibility of using short fragments for native molecule whole genome sequencing on the ONT platform.

This work was performed as research to assess future clinical applications. **Oxford Nanopore Technologies products are not intended for use for health assessment or to diagnose, treat, mitigate, cure, or prevent any disease or condition.**

METHODS

Native libraries were prepared using a modified version of the ONT SQK-LSK110 ligation protocol using the reagents provided in the kit and additional recommended reagents from New England Biolabs (end repair buffer, enzyme mix, and ligase) (**Figure 1**).

Figure 1. Library preparation workflow and modifications.



Modifications
1. Changed volumes of end repair reagents.
2. Increased end repair reaction time.
3. Increased bead-to-sample ratio during purification.
4. Reduced elution volume.
5. Reduced ligation volume.
6. Increased ligation time.
7. Added temperature cycling during ligation.
8. Added an additional post ligation purification.

Protocol modifications were first developed using genomic DNA templates from cell lines sheared by sonication (Covaris M220 Focused Ultra-Sonicator) to produce a 140-170 bp fragment length distribution similar to that of cfDNA.

Libraries were prepared using cfDNA extracted from normal or cancer plasma samples (Promega Maxwell RSC ccfDNA Plasma), as well as FFPE DNA extracted from FFPE sections (Qiagen QIAmp DNA FFPE Advanced UNG). Cancer samples had ethics approval for research use.

Adapter-ligated libraries were quantified by fluorescence (Qubit dsDNA High Sensitivity), and visualized by gel electrophoresis (Agilent Bioanalyzer High Sensitivity DNA).

Overall, four sheared cell line DNA libraries, 22 native cfDNA libraries, and four FFPE DNA libraries were sequenced in single-plex on MinION r9 flow cells (**Table 1**). Flow cells were run on either the MinION Mk1b or GridION, with the short-read setting (> 20 bp) selected in MinKNOW. Run times were between 30 - 72 hours.

RESULTS

Protocol modifications increased the ratio of fully adapter-ligated library molecules over semi-adapter-ligated and non-adapter-ligated molecules, and greatly reduced the amount of excess free adapter in the final sequencing libraries (**Figure 2a - 2c**). Yield was increased from 1 M to 40 M reads in sequencing runs with similar loading masses (**Figure 2d**).

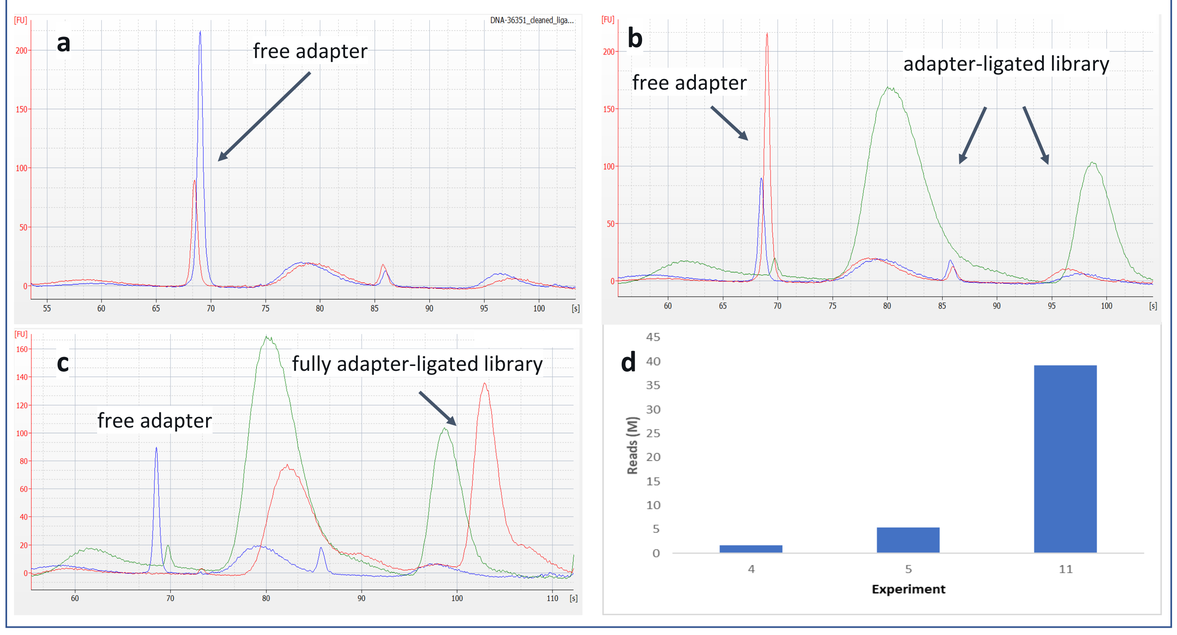
Using the improved protocol, sequencing of both sheared cell line DNA and native cfDNA produced high read yields, while sequencing of native FFPE DNA gave low yields (**Table 1**).

Table 1. Sequencing run metrics.

Metric	Cell line DNA	cfDNA	FFPE DNA
Number of library preps and runs	4	22	4
Conversion rate (template to adapted product) (%)	20	39	49
Average mass loaded onto flow cell (ng)	21	30	143
Average molar mass loaded onto flow cell (fmol)	175	216	345
Average N50	183	219	517
Average read count (M)	62.38	38.11	0.63
Average output (Gb)	10.09	9.25	0.32

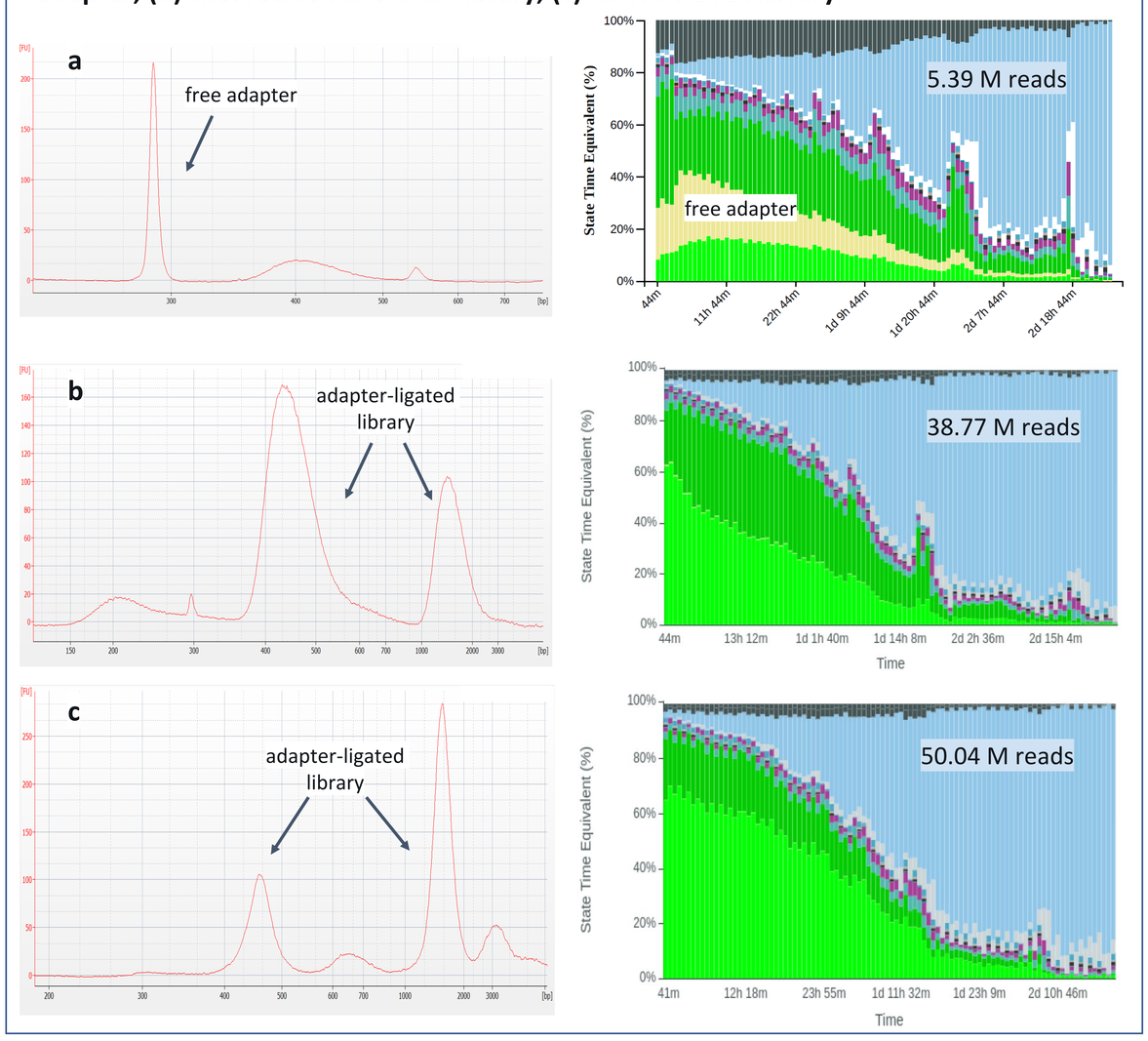
RESULTS

Figure 2. Improvements in adapter ligation efficiency. (a) Decrease in free adapter, (b) increase in adapter-ligated library yield, (c) increase in fully adapter-ligated library, (d) increase in read yield using similar loading masses.



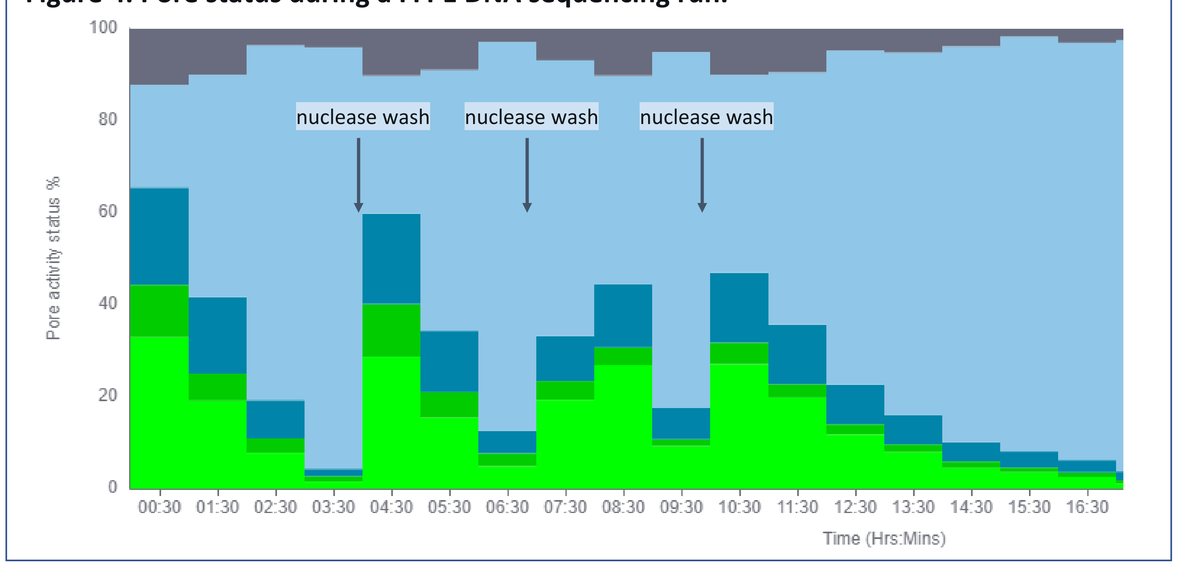
Limiting free adapter in the final library and increasing the amount of fully adapter-ligated molecules greatly improves pore occupancy and sequencing performance (**Figure 3**).

Figure 3. Improvements in sequencing performance. (a) Library with excess free adapter, (b) sheared cell line DNA library, (c) native cfDNA library.



Native FFPE DNA libraries cause significant and immediate pore clogging on r9 flow cells, but some data can be generated with repeated nuclease washes (**Figure 4**).

Figure 4. Pore status during a FFPE DNA sequencing run.



CONCLUSIONS

Optimizing the ONT SQK-LSK110 ligation protocol for use with fragmented templates improved sequencing performance. Modifications made to both the end repair and adapter ligation reactions decreased the amount of free adapter and increased the ratio of fully adapter-ligated molecules over semi-adapter-ligated and non-adapter-ligated molecules in the final libraries.

Native cfDNA was successfully sequenced with high read yields.

Despite optimizations, challenges remain with sequencing native FFPE DNA libraries.