

Introduction

Abstract

In this project, we utilize a targeted sequencing strategy using in vitro Cas9 cleavage to investigate structural variations and haplotypes in the CYP2D6 gene, a critical determinant of tamoxifen metabolism in breast cancer patients. Variations in CYP2D6 influence the conversion of tamoxifen into its active form, endoxifen, thereby impacting therapeutic efficacy and patient outcomes for estrogen receptor-positive (ER+) breast cancer patients. Foundational to this effort is the optimization of adapter ligation efficiency, ensuring consistent and precise attachment of nanopore sequencing adapters to Cas9-cleaved DNA fragments. Nanopore sequencing adapters are specialized molecules that facilitate the attachment of DNA fragments to nanopore sequencing flow cells, enabling the generation of high-fidelity long-read sequencing data and real-time nucleotide analysis. Understanding ligation efficiency is essential to maximizing target molecule concentration when sequencing with Oxford Nanopore Technologies (ONT).

Background

Long-read sequencing technologies, such as ONT, offer a powerful approach for resolving full-length CYP2D6 haplotypes, enabling improved detection of clinically relevant variants. Quantifying the ligation efficiency metric enables fine-tuning of adapter-to-sample DNA ratios. Loading less than 75 fmol of target DNA can result in a low pore occupancy and can lead to inadequate reads, however, loading a larger amount of DNA can damage the pores in the technology (Oxford).

Figure 1: ONT Measurement of pore occupancy across varying library load. Suboptimal ligation efficiency can result in reduced pore occupancy (Oxford).

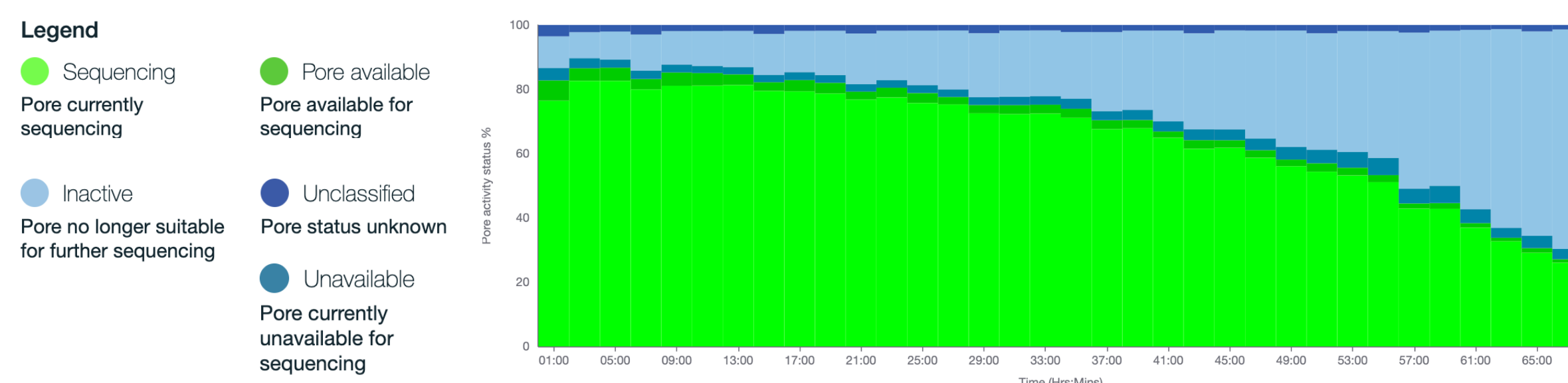


Figure 2: Example of Pore occupancy over a whole-genome sequencing run. Sequencing and available pores are maximized compared to what is typical for targeted sequencing.

Objective

This study focuses on optimizing adapter ligation efficiency to improve the accuracy and consistency of long-read sequencing. Establishing a standardized ligation protocol will improve not only pharmacogenomic applications but also broader structural variant analysis, advancing precision medicine strategies for genetic research and clinical diagnostics.

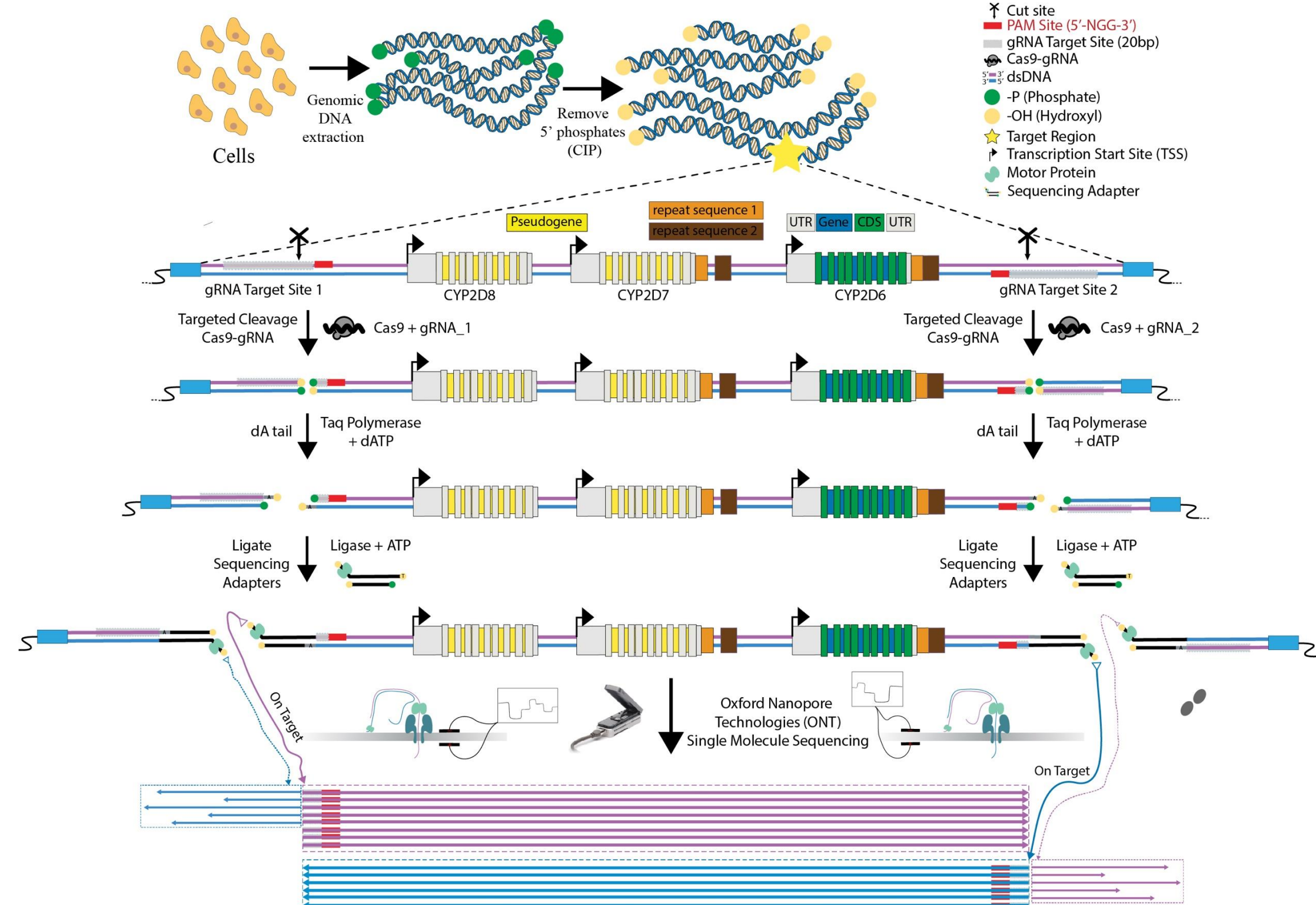


Figure 3: Targeted Sequencing Workflow. By leveraging Cas9-mediated enrichment, we will selectively isolate CYP2D6 fragments and prepare them for for sequencing.

Methods

A ligation assay was performed using 10 pmol of a 40 base pair synthetic target DNA oligonucleotide and 50 pmol of a 57 base pair dT-biotinylated adapter. The ligation reactions included varying concentrations of Hi-T4 DNA Ligase—specifically 0x, 1x, 2x, and 4x the standard ligase concentration. To monitor ligation kinetics, reactions were quenched at four time points: 0, 15, 30, and 60 minutes. Quenching was achieved by adding 30 mM EDTA to inhibit enzymatic activity. Following quenching, M-280 streptavidin-coated magnetic beads were used to isolate ligated DNA products. These beads were pelleted on a magnetic rack, allowing separation of the supernatant and retention of the ligation products. The final products were resolved using TapeStation D1000 Automated Electrophoresis and quantified through Qubit fluorescence analysis.

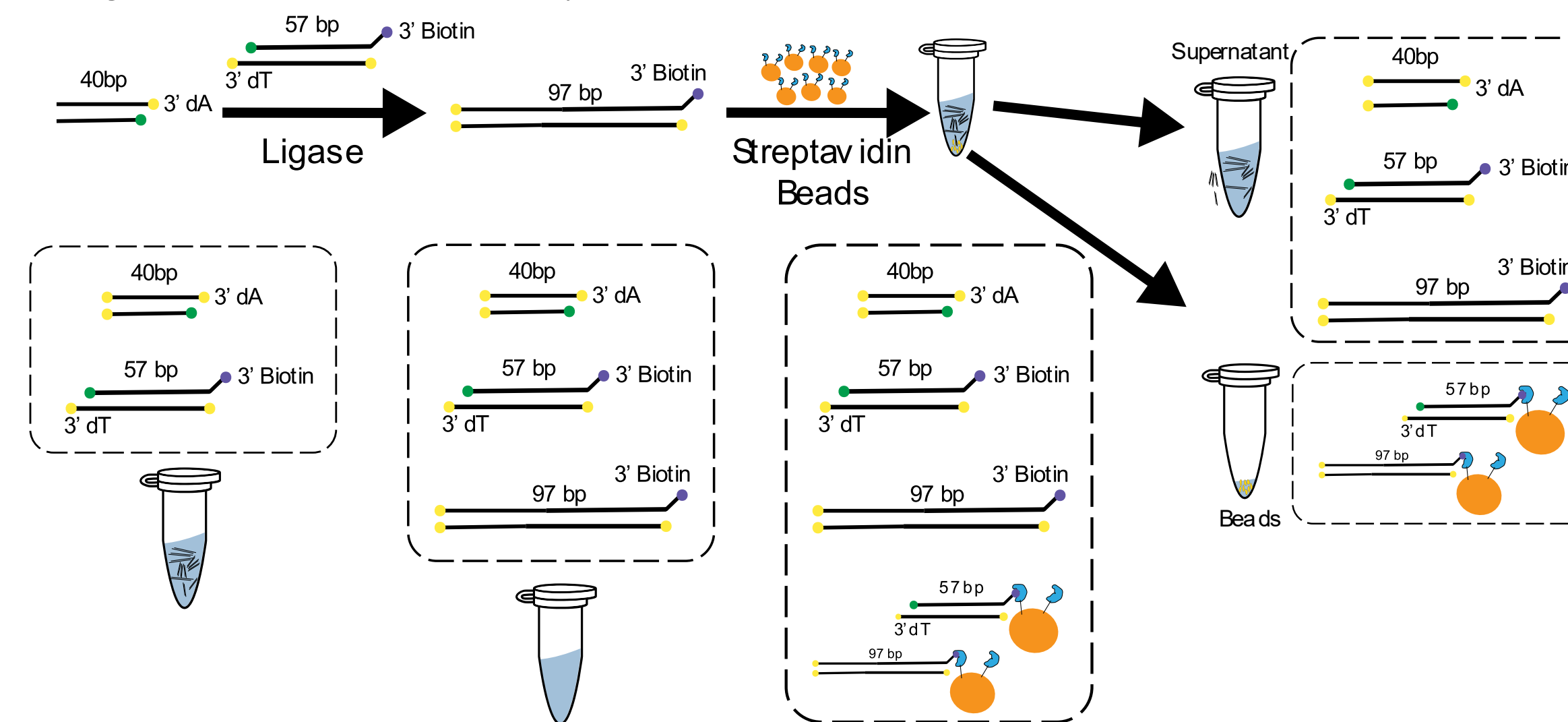


Figure 4: Adapter Ligation Procedure. Experimental workflow detailing use of M-280 streptavidin beads in ligation procedure.

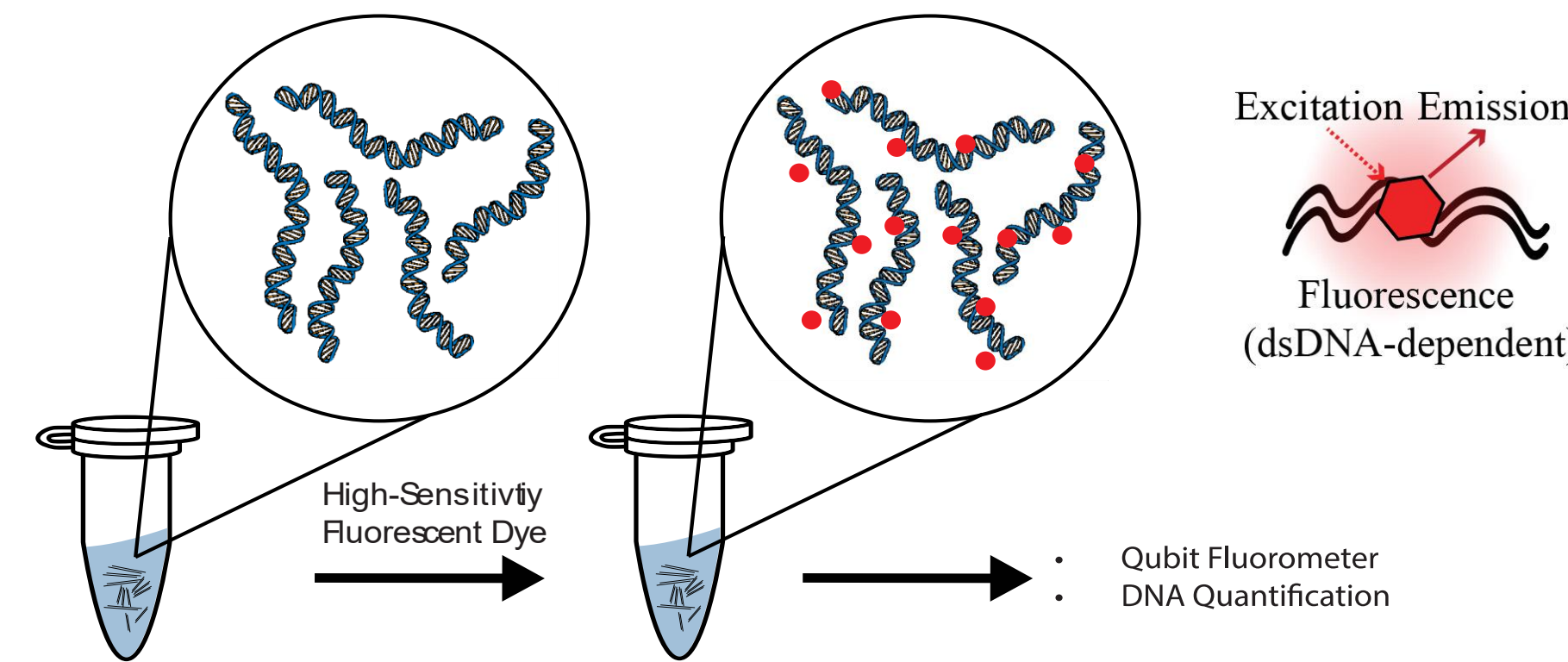


Figure 5: Qubit Fluorometers are used to quickly detect the concentration of dsDNA in a solution.

Results

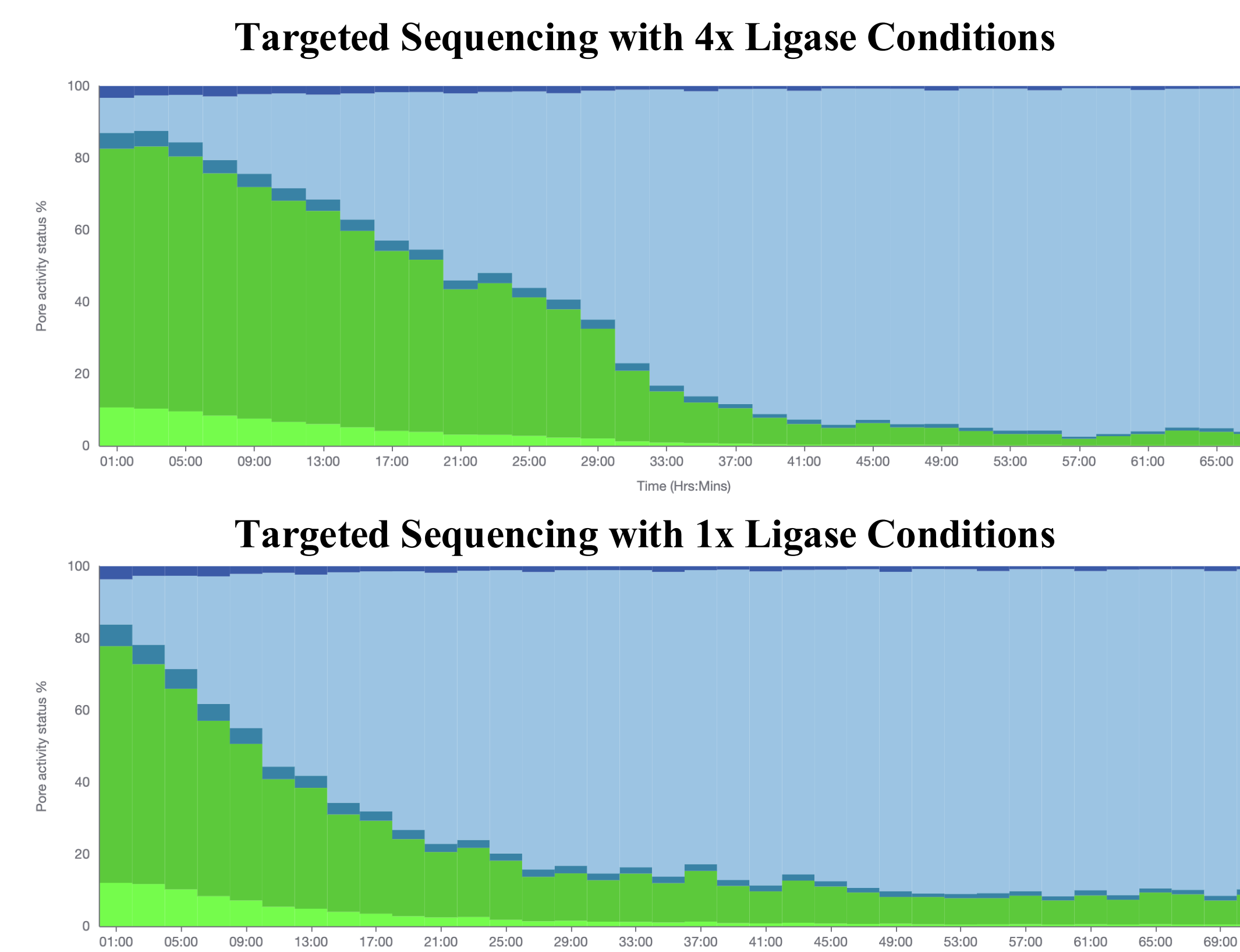


Figure 6: Graph of pore occupancy across two targeted sequencing experiments. 4x ligase (top) and 1x ligase (bottom). Observed correlation between ligase concentration and health of pores.

Results

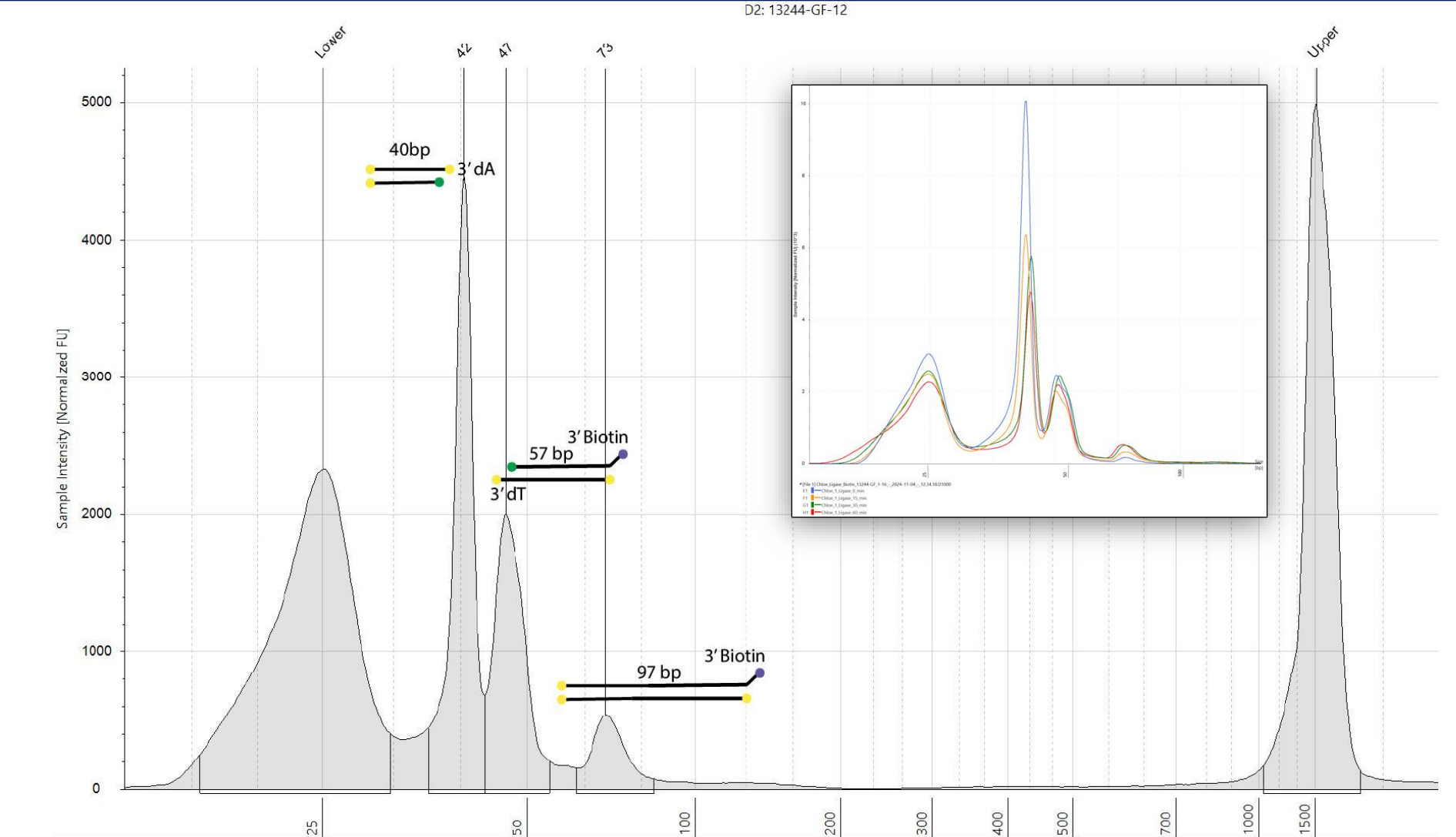


Figure 7: TapeStation D1000 analysis of ligation efficiency. Target depletion over time indicates successful ligation to adapters. Shown in the panel are results under 1x ligase conditions, demonstrating target depletion of the 40 bp species as ligation occurs.

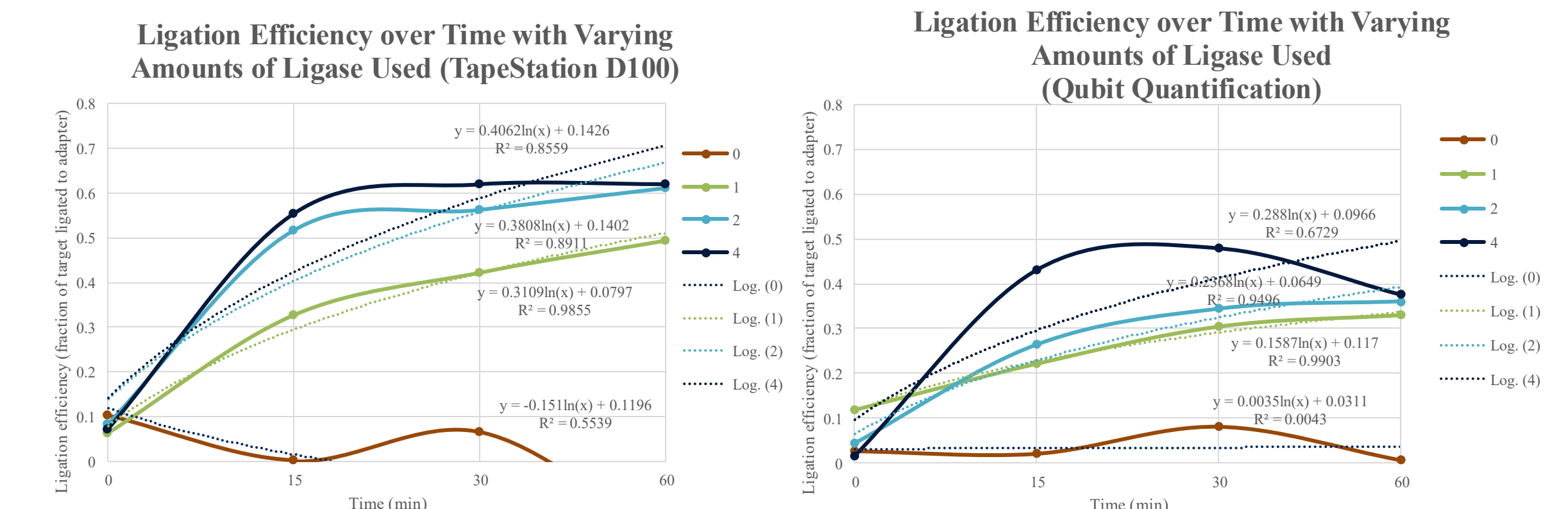


Figure 8: Ligation efficiency over time with varying amounts of ligase calculated using TapeStation D1000 data of the supernatant (Fig 4)

Figure 9: Ligation efficiency over time with varying amounts of ligase calculated using Qubit quantification of the target-depletion.

Ligation efficiency was assessed across multiple ligase concentrations. The no-ligase control (0x) exhibited minimal ligation, confirming the assay's specificity. A 1x ligase concentration resulted in moderate efficiency that increased with time. In contrast, 2x and 4x concentrations achieved higher efficiency at earlier time points, appearing to plateau between 30 and 60 minutes. These trends were consistently observed using both TapeStation and Qubit quantification methods.

Discussion & Conclusions

- This study establishes a quantitative approach to measuring Nanopore sequencing adapter ligation efficiency under different ligase concentrations and reaction times with two different methods.
- By using M-280 streptavidin beads to isolate biotinylated ligation products, the method allows direct quantification of unligated DNA in the supernatant using Qubit fluorescence, providing a measurement of ligation efficiency without sacrificing target material in a sequencing experiment.
- Accurate measures of ligation efficiency serve to reduce alignment errors and misclassification.
- Future work will apply bead-based quantification to detect ligation efficiency in order to predict and improve high-throughput sequencing workflows.

Acknowledgments

Supported by Phenomics Health Inc.. We thank the Advanced Genomics Core at the University of Michigan for running TapeStation Samples.

Oxford Nanopore Technologies. (2024, November 25). *Chemistry Technical Document (CHTD_500_v1_revAR_25Nov2024)*. <https://nanoporetech.com/document/chemistry-technical-document>.