Feasibility of PromethION® Platform Expansion with the AmplideX® Nanopore Carrier Plus Kit for High Throughput Applications

Poster Number: G023

Justin Janovsky, Connor Parker, Brian C. Haynes, John Milligan

Asuragen, a Bio-Techne Brand, Austin, TX

Summary

- The AmplideX Nanopore Carrier Plus Kit* was previously verified to detect challenging pathogenic variants in a panel that includes *CFTR*, *SMN1/2*, *FMR1*, *HBA1/2*, *HBB*, *GBA1*, *CYP21A2, TNXB*, and *F8* on the Oxford Nanopore Technologies (ONT) MinION® and GridION® sequencers.
- In order to meet demand for support on additional ONT devices, larger batch sizes, and faster turnaround times, we evaluated the feasibility of the Carrier Plus assay on the PromethION sequencer in addition to a dual barcoding strategy that increases the sample capacity of the assay.
- Here, we demonstrate equivalent performance of the AmplideX Nanopore Carrier Plus Kit on the ONT PromethION platform using the standard assay workflow.
- Additionally, we demonstrate equivalent performance on the PromethION platform of a dual barcoding strategy that increases the number of available barcodes beyond the 96 available in the standard assay to support high-throughput workflows.

Introduction

Long-read sequencing platforms represent a robust and powerful technology that overcomes many of the limitations of Sanger and short-read sequencing. In recent years, improvements in basecalling accuracy have made long-read sequencing an increasingly relevant technique in the clinical laboratory. The AmplideX Nanopore Carrier Plus Kit (Carrier Plus) was previously verified to detect challenging pathogenic variants in a panel that includes *CFTR*, *SMN1/2*, *FMR1*, *HBA1/2*, *HBB*, *GBA1*, *CYP21A2, TNXB*, and *F8* on the Oxford Nanopore Technologies (ONT) MinION and GridION sequencers. Here, we show equivalent performance of the kit on the PromethION platform using both the standard assay workflow as well as a dual barcoding approach that greatly expands the sample capacity of a single flow cell.

Materials and Methods

We tested 433 unique samples (139 cell line, 294 whole blood) to verify performance equivalence of the assay across platforms and across barcoding strategies. Using the AmplideX Nanopore Carrier Plus Kit (Asuragen, Inc.), target regions were enriched with 4 PCR reactions, barcoded, pooled, and sequenced in a single assay workflow (Figure 1). Sequencing was performed using R10.4.1 flow cells (FLO-PRO114M; FLO-MIN114) on PromethION 2 Solo, GridION, and MinION Mk1B devices (Oxford Nanopore Technologies).

Five Carrier Plus kits (2 of Kit A; 1 each of Kit B, C, and D), each consisting of 96 sample – or 94 samples and 2 calibrators for kits A and C – were prepared and sequenced. One set of amplicons were generated and all sequenced in each of three runs: 1) on a MinION/GridION using the standard assay workflow (the "comparator" run), 2) on a PromethION with single barcodes, and 3) on a PromethION with dual barcodes. Results from the PromethION runs were evaluated against the comparator run to evaluate the new sequencing platform and barcoding strategy.

The Carrier Plus protocol guide (00004909v2, 2025-04-30) was followed for sample preparation, gene-specific and barcoding PCR, pooling, and – for libraries sequenced on the MinION/GridION – library preparation and sequencing. For single-barcoded libraries sequenced on a PromethION, the ONT PromethION protocol for Ligation sequencing DNA V14 (SQK-LSK114) (VGDE_9161_v114_revAC_24Sep2025) was followed for library preparation and sequencing. Workflow differences for SQK-LSK114 on the PromethION were observed to be minimal compared to the MinION: the adapter ligation reaction is eluted in 25 µL rather than 15 µL, BSA is not required for the flow cell priming mix, and the library loading volume is scaled up to 200 µL (with component volumes scaled proportionally) to improve array coverage. For dual-barcoded libraries sequenced on a PromethION, the ONT PromethION protocol for Ligation sequencing DNA V14 – dual barcoding (SQK-NBD114.24 with EXP-PBC096) (VDBC_9184_v114_revO_03Oct2025) was followed for ligation of secondary barcodes (i.e., native barcodes), library preparation, and sequencing.

Processing and analysis of the sequencing data was performed with our AmplideX One Reporter software. Variant calls from the feasibility PromethION runs (single barcode and dual barcode) were compared to results generated by the standard assay workflow (single barcode library sequenced on a MinION/GridION device).

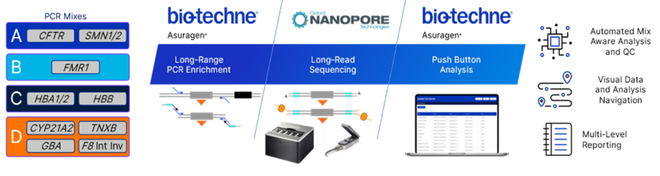

Figure 1. AmplideX Nanopore Carrier Plus Kit Panel Design and Workflow. Targeted PCR enriched panel-specific genetic regions in 11 genes responsible for common inherited genetic disorders. Kit workflow consisted of PCR enrichment, sequencing, and automated analysis software. Basecalled sequencing data was analyzed with Kit provided software to report multiple variant classes across genes.

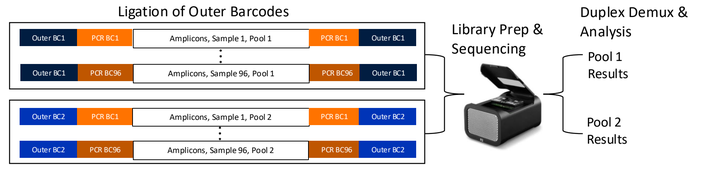

Figure 2. Modified Dual-barcoding Workflow to Enable Higher Sample Throughput. Standard workflow allows for using up to 96 barcodes in a single sequencing run. Ligation of the outer barcode to a pool of amplicons generated by the standard workflow allowed for combining multiple pools of samples in a single sequencing run. A first round of demultiplexing during sequencing separated the unique pools based on outer barcode, followed by a second round of demultiplexing to separate reads for each unique barcode in each pool, which were then analyzed with AmplideX One Reporter.

Results

Table 1. Genotype Agreement Relative to MinION-SingleBC Results. Across all genes assayed, >99% genotype call agreement was observed between the MinION and PromethION platforms. SNV/indel agreement was >99% across all genes. *SMN1/2* CNV (0 to ≥3) showed >97.5% agreement across both genes and 100% agreement for copy numbers <2. *FMR1* exhibited 99% agreement for repeat sizing and AGG interrupts. 100% agreement was observed for *CYP21A2/TNXB* and >97.5% for *GBA1*, which were differentiated from their pseudogenes, enabling resolution of fusions and SVs, as well as correct phasing of SNVs/indels. *F8* intron inversions showed 100% agreement. Rate of sequencing was observed to be on average 5.2 times faster on the PromethION compared to the MinION (with basecalling rate dependent upon available computational resources); all other sequencing metrics were observed to be similar between sequencing platforms. Dual barcoding results were nearly identical to single barcoding results with only slight differences in *SMN1, SMN2*, and *FMR1* genotype calls attributable to typical run-to-run variability observed due to random sampling during sequencing. Percentage of reads not assigned to a valid barcode after two rounds of demultiplexing was 8% greater than in standard workflow runs, suggesting a need to increase sequencing depth by approximately 10% when using this approach.

Kit	Gene	PromethION-SingleBC n*	PromethION-SingleBC Agreement	PromethION-DualBC n*	PromethION-DualBC Agreement
A	*CFTR*	182	99.5%	183	99.5%
A	*SMN1*	179	97.8%	176	96.6%
A	*SMN2*	183	98.9%	181	97.8%
B	*FMR1*	86	100%	91	97.8%
C	*HBA1/2*	91	100%	91	100%
C	*HBB*	91	100%	92	100%
D	*CYP21A2*	93	100%	93	100%
D	*F8*	89	100%	89	100%
D	*GBA1*	93	97.8%	93	97.8%
D	*SMN1*	90	100%	90	100%
D	*SMN2*	90	100%	90	100%
D	*TNXB*	93	100%	93	100%
Grand Total		**1,360**	**99.3%**	**1,364**	**98.8%**

*Number of samples with passing QC in both the experimental run and comparator experiment (MinION-SingleBC)

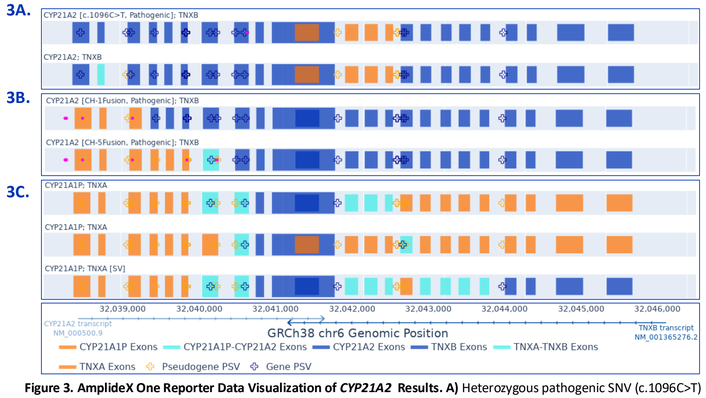

Figure 3. AmplideX One Reporter Data Visualization of *CYP21A2* Results. A) Heterozygous pathogenic SNV (c.1096C>T) in *CYP21A2*. **B)** Pathogenic *CYP21A2-CYP21A1P* fusions in both copies of *CYP21A2*. **C)** Homozygous loss of *CYP21A2*. Across sequencing platforms, *CYP21A2* genotypes exhibited 100% agreement.

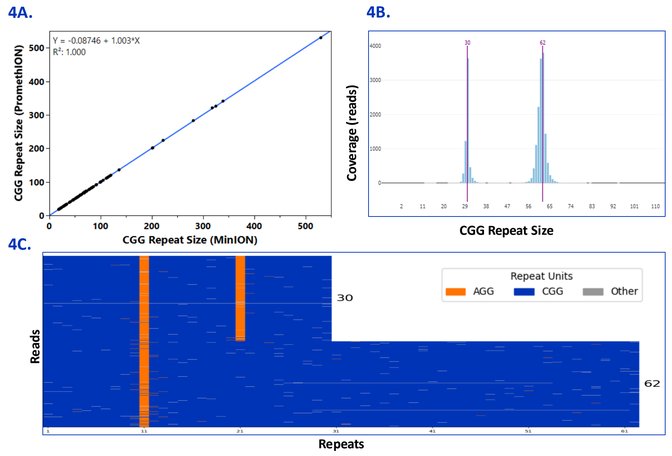

Figure 4. *FMR1* Results. A) *FMR1* CGG repeat size scatterplot by instrument (PromethION vs. MinION) demonstrated one-to-one correspondence in CGG repeat sizing, with the regression line (y = 1.003x – 0.08746, R^2=1) representing the line of identity. **B)** Example *FMR1* CGG size histogram plot displays read counts for each repeat size utilized to quantitate the CGG repeats in each allele (blue lines) for a sample with one normal allele (30 CGG) and one premutation allele (62 CGG). Agreement of CGG sizing was 99.4% across platforms. **C)** Example *FMR1* waterfall plot for same sample showing AGG interrupts (orange) at position 11 and 21 in the normal allele and position 11 in the premutation allele. AGG interrupt detection agreement was 99.4% across platforms.

5A.

MinION \ PromethION *SMN1* Copies	0	1	2	≥3
0	26	0	0	0
1	0	54	0	0
2	0	0	208	6
≥3	0	0	4	71

5B.

MinION \ PromethION *SMN2* Copies	0	1	2	≥3
0	42	0	0	0
1	0	82	0	0
2	0	0	178	7
≥3	0	0	0	60

Figure 5. *SMN1/2* Copy Number Agreement Tables between Sequencing Instruments. Overall agreement for **(A)** *SMN1* and **(B)** *SMN2* was 97% and 98%, respectively, and 100% agreement for all genotypes associated with SMA patients and carriers (*SMN1* copies of 0 and 1), demonstrating consistent copy number calling between the PromethION and MinION platforms.

Conclusions

- The described set of experiments demonstrate equivalent performance of the AmplideX Nanopore Carrier Plus Kit across the ONT MinION and PromethION long-read sequencing platforms.
- Across platforms and across all genes, overall genotype agreement was >99%. By gene, agreement was >99% for *CFTR*, 98% for *SMN1/2*, 99% for *FMR1*, 100% for *HBA1/2*, 100% for *HBB*, 100% for *CYP21A2*, 100% for *F8*, 98% for *GBA1*, and 100% for *TNXB*.
- Estimates of maximum throughput suggest a PromethION 2 Solo instrument can be used to assay hundreds of thousands of patient samples per year, and a single PromethION 24 sequencer is capable of testing >3 million samples per year (average of 380 samples per flow cell and 24 flow cells per day).
- We also demonstrate feasibility of a dual barcoding strategy to expand the multiplex capability of the Carrier Plus Kit. Utilizing this strategy, a total of 2,304 samples could be theoretically assayed with any combination of the four Carrier Plus kits (A, B, C, or D) on a single flow cell.

*For Research Use Only. Not for use in diagnostic procedures.