Long read sequencing application of xGen™ Amplicon Technology as shown on Oxford Nanopore Technologies

Introduction

With global efforts intensifying to combat infectious diseases, there is an urgent need for advanced technologies that can accurately and efficiently decode rapidly evolving pathogen genomes. IDT's xGen Amplicon technology has a track record for comprehensive viral and bacterial sequencing [**Super amplicon technical note**]. An important benefit of xGen Amplicon technology is the formation of "super" amplicons (**Figure 1**). These are longer amplicons formed during the single tube workflow from distant forward and reverse primers. Super amplicons provide redundant coverage to mitigate the effect of primer dropout due to novel mutations. Combining the power of xGen Amplicon chemistry with Oxford Nanopore Technologies' (ONT) long read sequencing represents a way to open new fields of study and surveillance for infectious diseases.

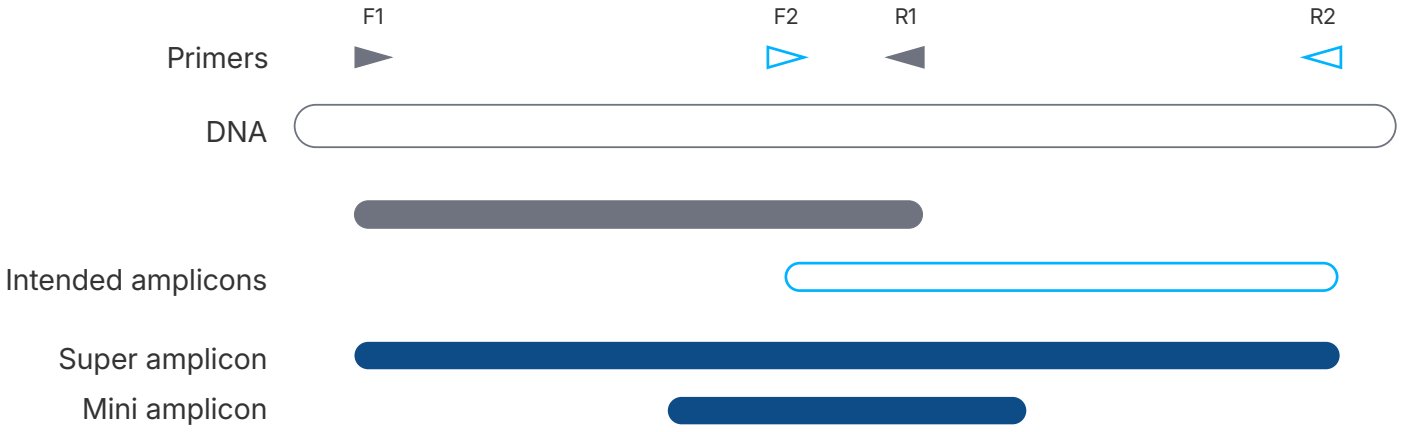

Figure 1. Creating super amplicons and mini amplicons. With two overlapping amplicons, the 4 primers involved can create 4 different aplicons: the two designed or intended amplicons, a super amplicon that spans both intended amplicons, and a mini amplicon. The super amplicon is created from F1 and R2 primers. The mini amplicon is created from F2 and R1 primers. While most PCR systems show a bias towards short products, xGen Amplicon's propriety chemistry favors the formation of intended and super amplicons over mini amplicons.

The xGen Amplicon technology can easily be adapted for ONT sequencing. Here we will show representative data for various custom xGen amplicon panels and describe the steps required for completing the xGen Amplicon for ONT workflow.

Results and discussion

To demonstrate that xGen Amplicon chemistry can generate long amplicons suitable for long-read sequencing, primers were designed to exon 11 of BRCA2 to create products of varying length: 1 kb, 1.5 kb, 2 kb, 3 kb, and 5 kb. Amplicons were generated using xGen Amplicon PCR1, followed by a bead cleanup and visualization on the BioAnalyzer (**Figure 2**). The expected product peak was observed for 1 kb, 1.5 kb, 2 kb and 3 kb amplicon designs, indicating that the xGen Amplicon chemistry can support amplicon creation up to approximately 3 kb. Results were compared against the xGen Amplicon BRCA1/BRCA2 Amplicon Panel, which contains amplicons ranging from 107–208 bp tiled along the coding exons of BRCA1 and BRCA2. Notably, evidence of super amplicon formation (~250–600 bp amplicons) was observed in the standard xGen Amplicon panel.

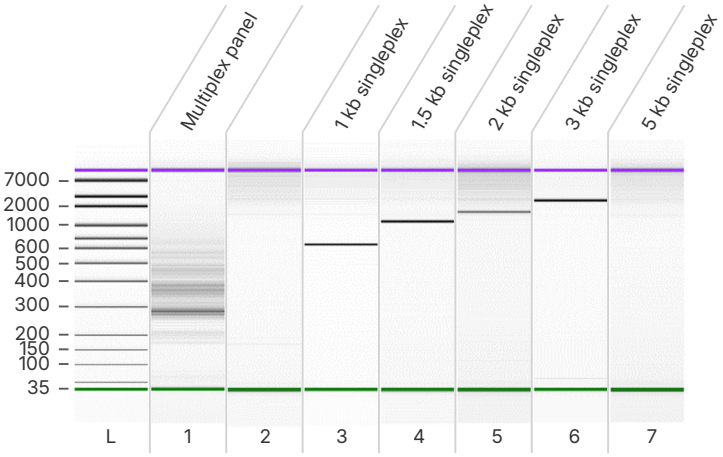

Figure 2. Amplicon size limits. Selected primers from the BRCA2_exon11 genome region to test varying length amplicons, specifically 1 kb, 1.5 kb, 2 kb, 3 kb, and 5 kb. Below is a BioAnalyzer HS trace of the PCR1 amplification products for the longer singleplex amplicons and the full BRCA1/2 multiplex product. In the bioanalyzer trace below it is clear that all the singleplex amplicons up to 3 kb were successfully formed by the xGen Amplicon chemistry.

The combination of xGen Amplicon's super amplicon chemistry with long-read sequencing leads to powerful new capabilities, especially when analyzing highly divergent sample types. Typical amplicon-based enrichment shows gaps in genome coverage as the input template deviates from the original design reference. Here we use the highly variable HIV genome to show that xGen Amplicon technology combined with long-read sequencing is especially robust against strain variability. Libraries were made from an HIV plasmid control sample that shared 93% identity to the HXB2 reference genome used for primer design. The same primer design was used to generate Illumina libraries via the xGen Amplicon Panels for viral genome sequencing protocol, or ONT libraries via the xGen Amplicon for ONT protocol. Final libraries were sequenced on the Illumina MiSeq using either 150PE or 250PE reads, or on the Oxford Nanopore MinION. Increasing the Illumina read length from 150 bp to 250 bp increased overall genomic coverage, while ONT sequencing achieved the highest genome coverage (**Figure 3**). This indicates that super amplicons created in the reaction often exceed the read length available to short-read technologies. Long-read sequencers can take full advantage of super-amplicon formation, leading to more comprehensive coverage of divergent strains.

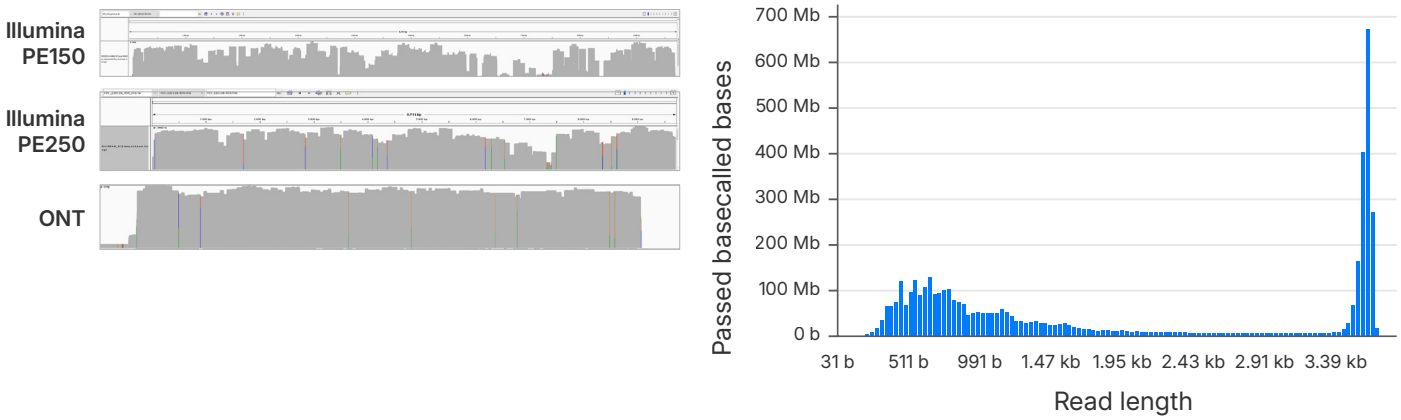

Figure 3. Super amplicons contribute to coverage. Left panel: ONT long-read sequencing enables complete coverage of super-amplicons, enhancing overall genome coverage in variable pathogen samples. Both Illumina and ONT data generated using the same primer design, which had amplicons tiled along the HIV reference genome with an average size of 147 bp. Right panel: size distribution of ONT reads shows super-amplicon formation >3 kb.

An important aspect of ONT sequencing is ease of use in the field to facilitate environmental studies. To demonstrate this, we paired the xGen 16S Amplicon Panel v2 primer design with the xGen Amplicon for ONT workflow to generate ONT libraries from *E.coli* DNA and mock microbial community control MSA-1003. MinION data showed full coverage for the *E.coli* 16S rRNA (rrSA) gene and included read lengths of ~1.5 kb, corresponding to the approximate length of the full 16S rRNA gene (**Figure 4**). Data from the MSA-1003 mixture reflected the expected representation of microbial genomes, indicating this workflow is capable of high-fidelity population representation for diverse samples.

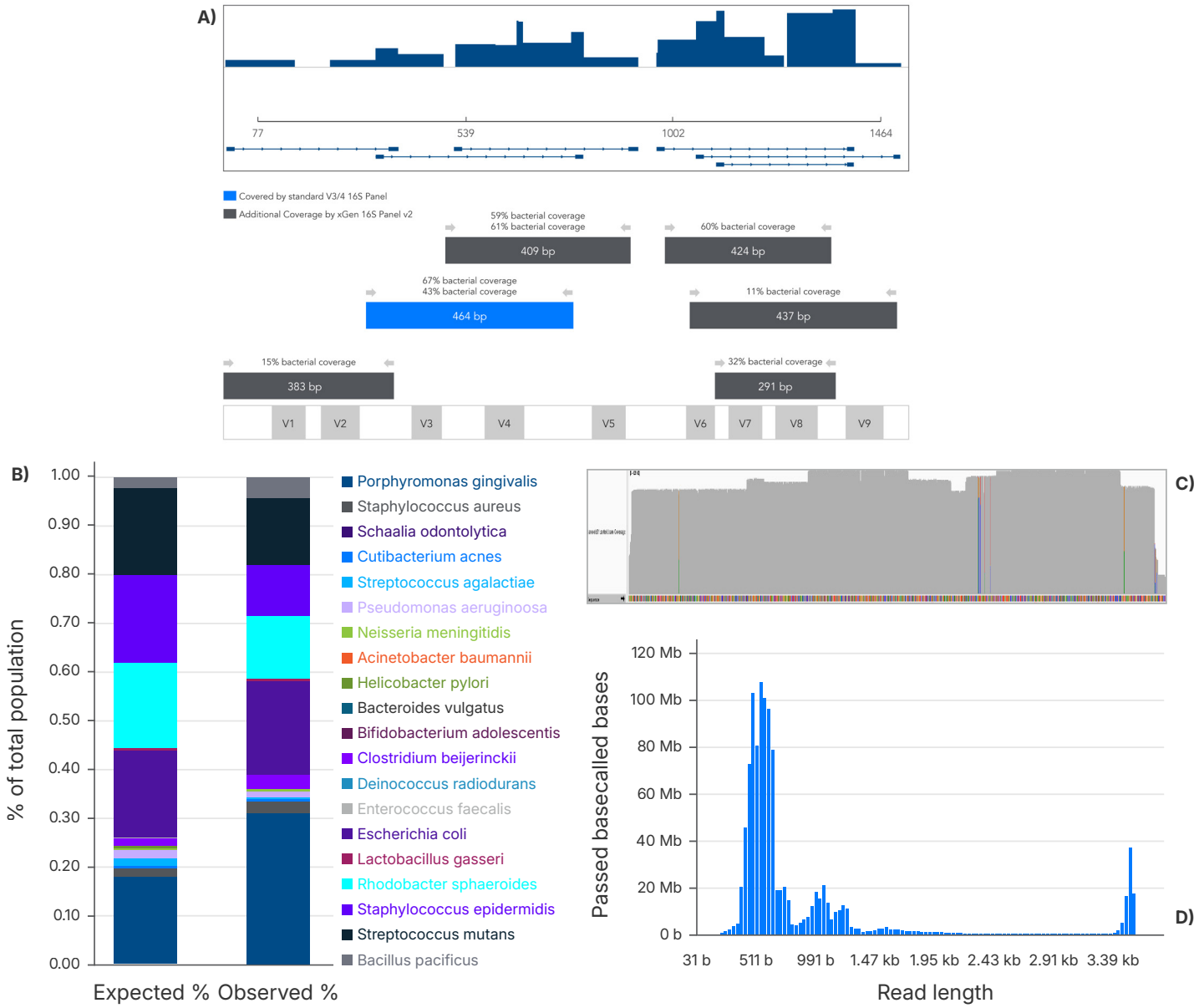

Figure 4: xGen Amplicon for ONT sequencing provides accurate identification of microbial samples. A) xGen 16S Amplicon Panel covers variable regions 1-9 in a single tube workflow B) xGen 16S Amplicon for ONT protocol showed expected genome representation for microbial control mixture MSA-1003 C) xGen 16S Amplicon for ONT protocol achieved full coverage for *E. coli* 16S rRNA gene. D) ONT read length distribution includes peak at ~1.5 kb, which corresponds to the full 16S rRNA gene (v1-v9).

Conclusions

We have shown that the xGen Amplicon can be adapted for ONT sequencing to facilitate both viral and metagenomic sequencing. Longer reads, corresponding to super amplicons created by the xGen Amplicon chemistry, were observed to increase genomic coverage in areas of high variability in a divergent genome (HIV) and no bias was observed in metagenomic sequencing. Combining xGen Amplicon with long read sequencing leverages the full potential of super amplicons and holds great value for infectious disease sequencing and surveillance.

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IDT offers custom xGen Amplicon designs for both the Illumina and Oxford Nanopore workflows. For more information, please contact **NGSDesign@idtdna.com**.

For more information, go to: **idtdna.com/ContactUs**

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