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Two site comparison of a single PCR enrichment and nanopore sequencing workflow for complex clinically-relevant variants in seven genes associated with hereditary diseases

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Background

Variants associated with severe genetic disorders typically require multiple workflows beyond NGS for resolution in diagnostic and screening applications. Complex pathogenic variants involving short tandem repeats, copy number/structural variation (CNV/SV), and differentiating from genetically similar pseudogenes confound NGS. At two independent sites we evaluated the AmplideX® Nanopore Carrier Plus Kit (Asuragen, Austin, TX, USA) to genotype 7 challenging targets (*SMN1/2*, *FMR1*, *HBA1/2*, *HBB*, *GBA1*) using a combination of PCR enrichment, nanopore sequencing (Oxford Nanopore Technologies, Oxford, United Kingdom), and automated analysis software in a novel and unified workflow.

Methods

The study cohort included 98 previously genotyped residual clinical samples analyzed using orthogonal methods, including multiplex ligation-dependent probe amplification, reverse dot blot, long-range PCR followed by Sanger sequencing, targeted short-read next-generation sequencing, and fragment analysis. The cohort comprised 33 samples tested for *SMN1/SMN2*, 11 for *FMR1*, 40 for *HBA1/HBA2* and *HBB*, and 14 for *GBA1*. Samples were analyzed using the AmplideX® Nanopore Carrier Plus Kit (Asuragen), which enables the simultaneous detection of copy number variations and sequence variants through gene-specific PCR and long-read nanopore sequencing. Genomic DNA was amplified, barcoded, pooled, and sequenced using MinION™ R10.4.1 flow cells on a GridION™ device (Oxford Nanopore Technologies) at two independent sites. In total, 34 samples were analyzed at both laboratories and 64 at one laboratory. Data analysis and variant classification were performed using the AmplideX One Reporter software (Asuragen), which provides fully automated quality control and variant reporting. Results were compared across sites and validated against orthogonal methods. The workflow of the AmplideX Nanopore Carrier Plus Kit is illustrated in **Figure 1**.

Results

Overall Performance. Across all samples, 100% showed concordance with orthogonal results and across laboratories for all variant types.

SMN1/SMN2 analysis. A total of 33 samples underwent *SMN1* and *SMN2* genotyping: 10 samples were analyzed at both laboratories, while 23 were processed at a single site. The *SMN1* copy number (ranging from 0 to 3) and *SMN2* copy number (ranging from 0 to 4) were concordant with orthogonal data in all cases (**Figure 2**).

FMR1 analysis. Eleven *FMR1*-genotyped samples were analyzed: nine at both laboratories and two at a single site. All samples (11/11) showed categorical concordance with orthogonal data. **Figures 3A**, **3B**, and **3C** show representative genotypes, including one normal (23;30 CGG repeats) and two premutation cases (29;57/75, and 32;110/120 repeats). In all cases, AGG interruption patterns were clearly visible in the waterfall plots. Notably, one premutation case (57/75) lacked AGG interruptions on either allele, indicating an increased risk of repeat expansion.

HBA1/2 and HBB analysis. A total of 40 samples were analyzed—24 at both laboratories and 16 at a single site. Of these, 38 passed quality control based on sequencing depth. Among samples with available orthogonal data, 17 *HBA1/2* and 25 *HBB* genotypes were concordant. **Figure 4A** shows an example of a heterozygous anti-3.7 α-globin gene duplication, and **Figure 4B** shows a subject heterozygous for an anti-3.7 α-globin gene deletion.

GBA1 analysis. Fourteen *GBA1-genotyped* samples were analyzed—eight at both laboratories and six at a single site. The assay demonstrated 100% concordance with orthogonal methods for the detection of single nucleotide variants. All 14 samples were correctly genotyped, including nine distinct missense variants. Zygosity was reliably determined in all cases. One individual was accurately identified as compound heterozygous for two different missense variants, confirming the assay's precision for both variant detection and genotyping.

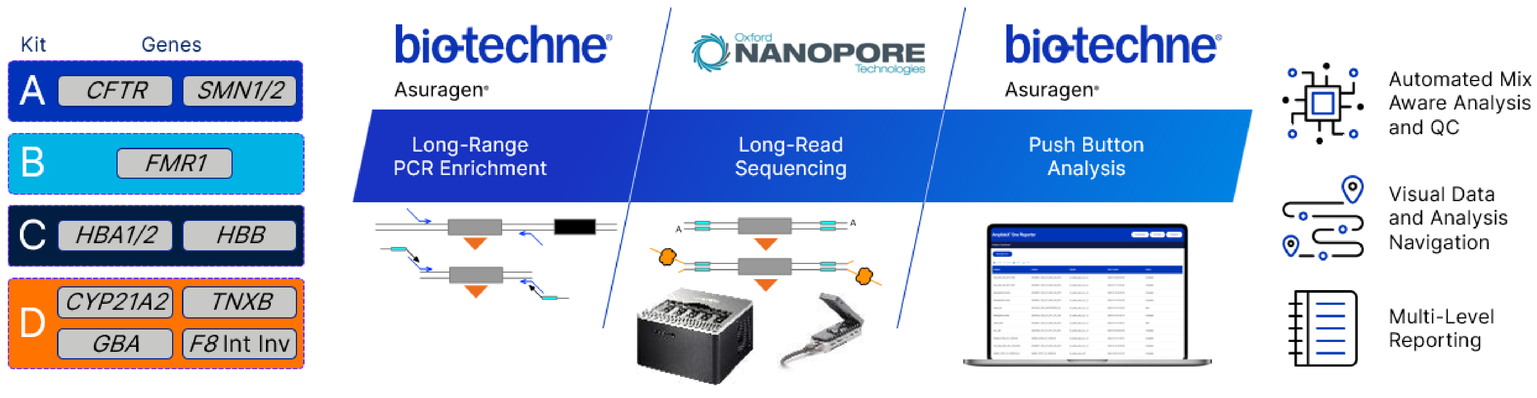

Figure 1. AmplideX Nanopore Carrier Plus Kit (Asuragen) Workflow. This kit enables simultaneous quantification of gene copy number and detection of sequence variants using long-read Nanopore sequencing (Oxford Nanopore Technologies). It combines targeted PCR amplification, barcoding, and nanopore-based sequencing to accurately analyze complex loci with high sequence homology—such as *SMN1* and *SMN2*, or *HBA1*, *HBA2* and *HBB*—*in* a single assay.

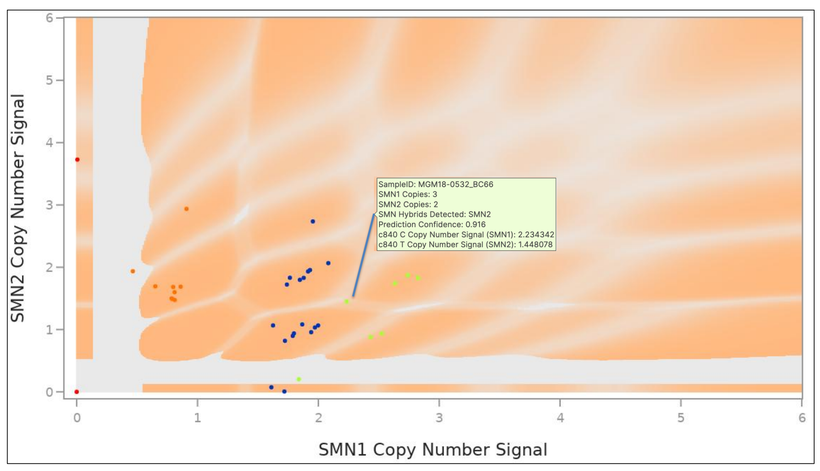

Figure 2. Representative plots from the analysis of *SMN1* (x-axis) and *SMN2* (y-axis) fold change values in clinical samples. Fold change values are normalized to two endogenous controls. Each dot represents one sample.

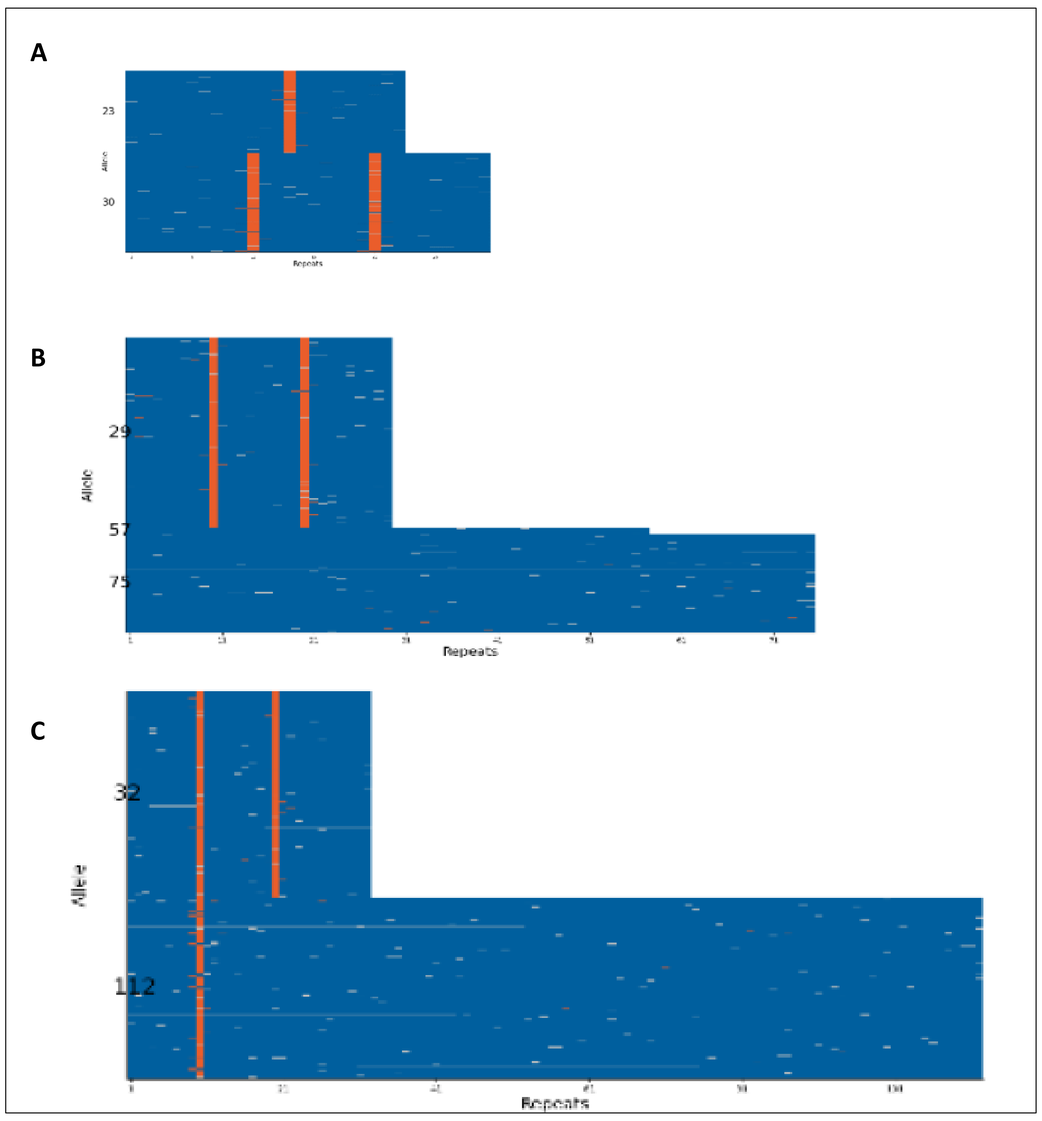

Figure 3. Waterfall plots showing representative *FMR1* genotypes. CGG repeats are shown in blue, and AGG interruptions are highlighted in orange. **Figure 3A** shows a normal female genotype with 23 and 30 CGG repeats. **Figure 3B** shows a heterozygous female with one normal allele (29 repeats) and a premutation allele (57/75 repeats). **Figure 3C** shows a heterozygous premutation genotype with 32 and 110/120 CGG repeats. AGG interruption patterns are clearly visible in all three plots and are useful for assessing the risk of repeat expansion.

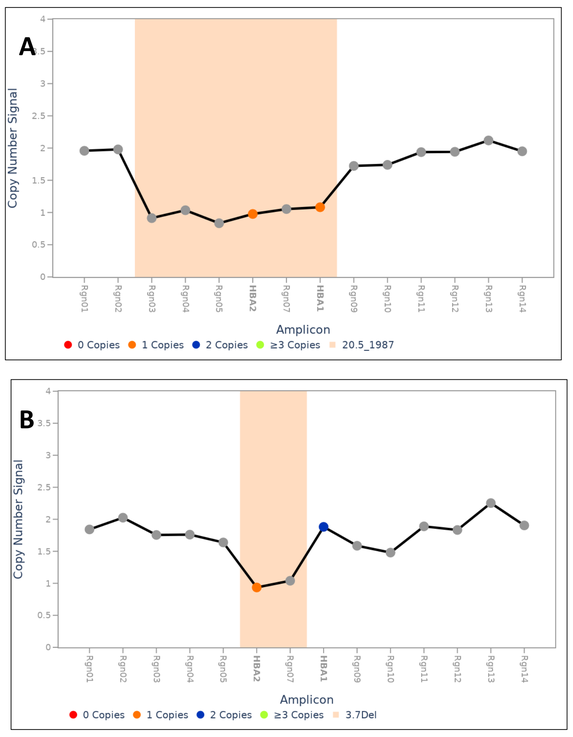

Figure 4. Analysis output from the AmplideX One Reporter software (Asuragen) showing (**A**) a subject compound heterozygous for the -20.5 deletion and single nucleotide variant c.2T>C in the *HBA* gene, and (**B**) a subject heterozygous for an anti-3.7 α-globin gene deletion. Blue dots represent two copies, indicating a normal copy number. Green dots indicate three or more copies, suggestive of a copy number variant consistent with duplication or amplification. Red dots represent zero copies, and orange dots indicate a single copy, consistent with a heterozygous deletion.

Conclusion

Our results highlight the reproducibility and accuracy of nanopore sequencing and dedicated bioinformatics software to consolidate workflows and automate both the analysis and interpretation of challenging variants relevant to hereditary genetic disease. These technological advances have the potential to simplify variant detection within molecular labs.

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