Beta evaluation of AmplideX® PCR enrichement technology with Nanopore long-read DNA sequencing of a panel of eleven challenging genes

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Introduction

- Short-read DNA sequencing is the main technical approach used in clinical diagnostics. However, for some challenging genes, additional non-NGS methods are required, such as PCR/CE, qPCR, MLPA or Sanger sequencing. This often entails increased labor and turnover times.
- In this beta-test, we evaluated a prototype assay based on the Asuragen AmplideX Nanopore Carrier Plus kit* , which targets the following challenging genes : *CFTR, FMR1, HBA1/2, HBB, SMN1/2, F8, GBA, CYP21A2* and *TNXB*. This assay relies upon PCR-enrichment, Oxford Nanopore Technology (ONT) sequencing, and machine learning models to enable multiplex detection of diverse variant classes including SNVs, INDELs, exon del/dups, SVs, gene CNVs and STRs in a single workflow.
- We evaluated performance on a set of 155 unique previously genotyped clinical samples provided by a network of French clinical diagnostic laboratories.

*NB: genetic carrier screening for asymptomatic patients with no relevant family history is not authorized in France.

Methods

Deidentified and previously genotyped clinical genomic DNA samples isolated from whole blood (WB) donors were provided from collaborators across France (N=155). Target regions were enriched in 1-4 PCR reactions, barcoded, pooled and sequenced on MinION flow cells (R10.4.1) with a Mk1B (ONT).

Data analysis and reporting were performed by collaborators at Asuragen as follows. Bespoke bioinformatics pipelines automated analysis of exon and whole gene gain or loss, gene-pseudogene fusions, and large structural variants. Clair3 and Sniffles2 were utilized for SNV/INDEL and SV identification[1,2].

Genotype agreement of the results obtained with this new assay to those determined with current French gold standard clinical diagnostic methods was then assessed.

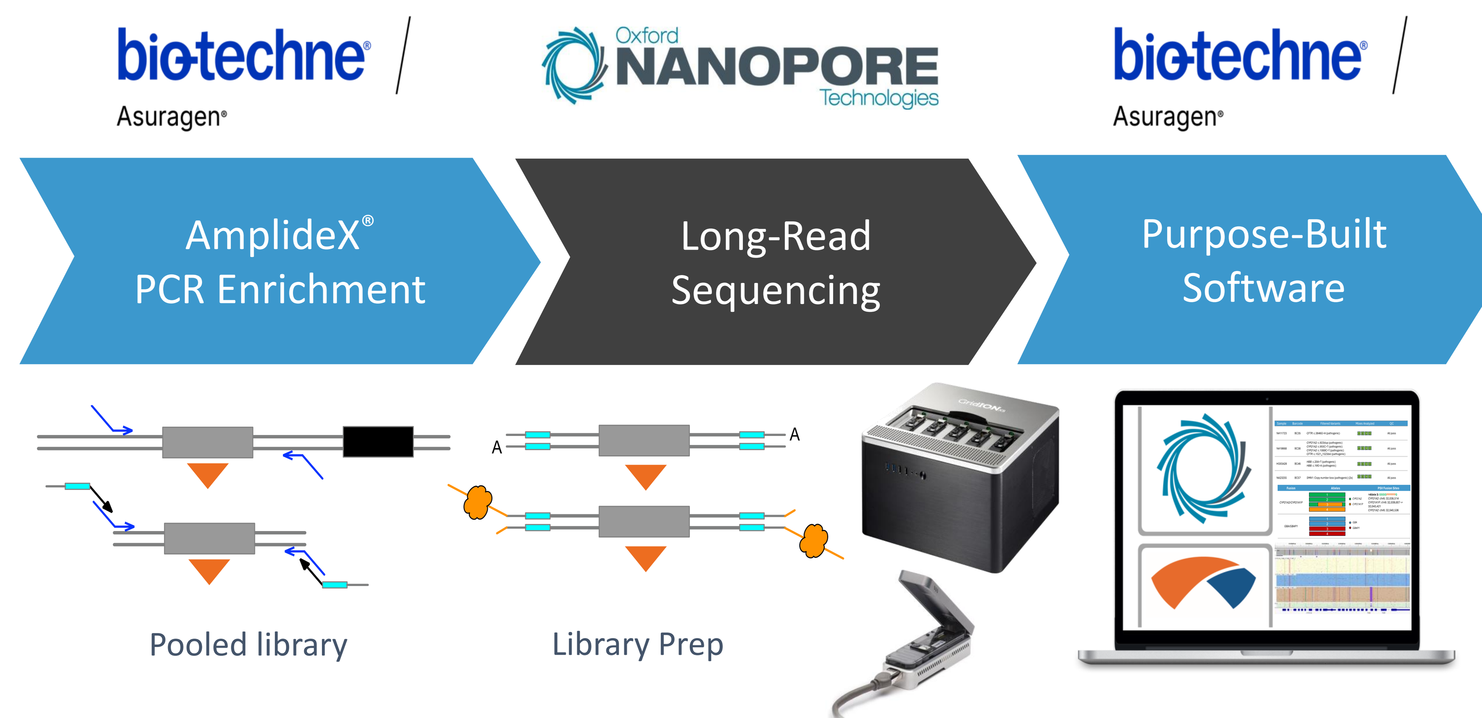
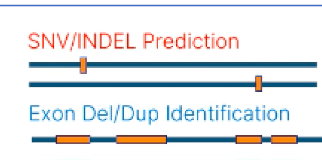


Figure 1. Single assay workstream. Wet lab steps are performed in 1.5 days, the sample run in 8-12 hours, and software analysis and processing in a couple of hours (8h maximum in our experience). There are 4 gene specific reaction mixes (mix A: *CFTR, SMN1/2*; mix B : *FMR1*; mix C : *HBA1/2, HBB*; mix D : *CYP21A2, TNXB, GBA, SMN1/2*, and *F8* intron inversions), which can be combined as needed.

Results

CFTR



- Current standard practice methods: ARMS-multiplex PCR for the detection of the most common pan-European pathogenic variants, Sanger or NGS sequencing for the identification of rare SNVs, parental segregation study for phasing[3].
- Beta test results: (n=24) 100% sample level agreement for all samples that passed QC, including SVs and indels (figure 2A et B respectively)

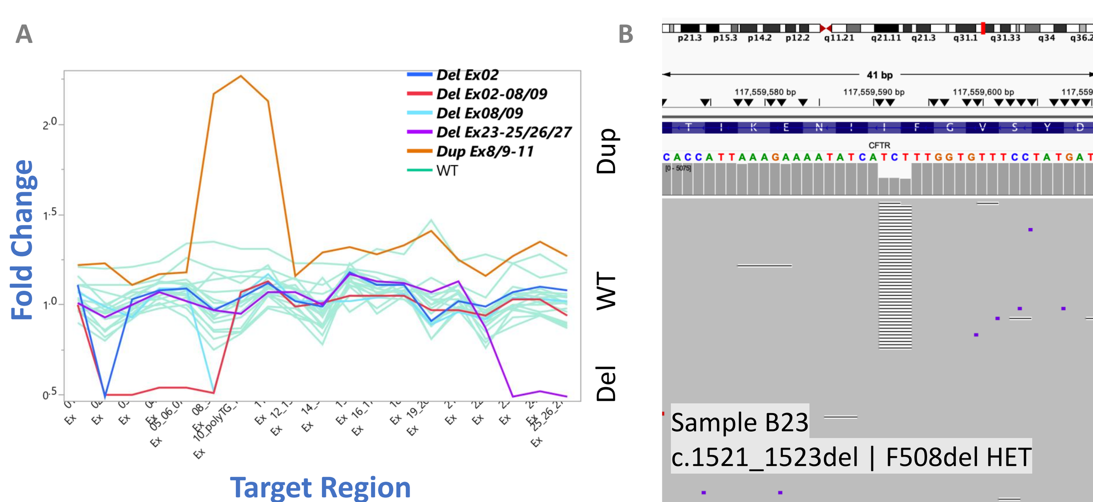


Figure 2. CFTR SV (A) and example indel (B) results.

F8 (introns 1 & 22)

- Current standard practice methods: breakpoint-specific multiplex PCR for the detection of intron 1 or 22 inversions, Sanger sequencing, complemented if needed by MLPA or NGS[6].
- Beta test results: 100% sample level agreement for all samples that passed QC (n=11/12)

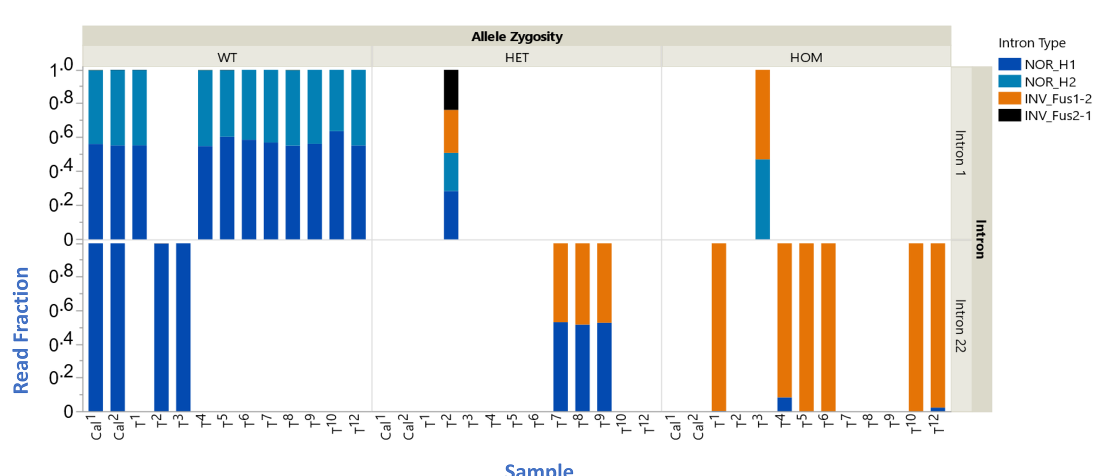
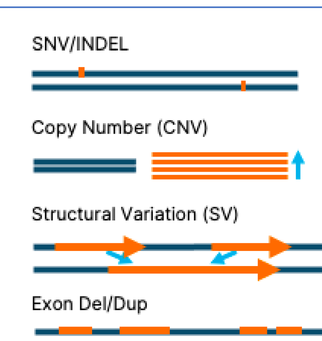


Figure 3. *F8* Variant Genotype. The algorithm identifies reads associated with the intronic H1 (intron 1 and 22) and H2 (intron 1 only) intergenic regions of the WT sample, or the H1 Fusion to H2 (intron 1) or H2/3 (intron 22) intergenic region indicating an inversion.

HBA1, HBA2 & *HBB*



- Current standard practice methods: Gap PCR, followed by MLPA the Sanger Sequencing or NGS.
- Beta test results: SNV and SV variant level classification was 100% for samples that passed QC (n=31/33).

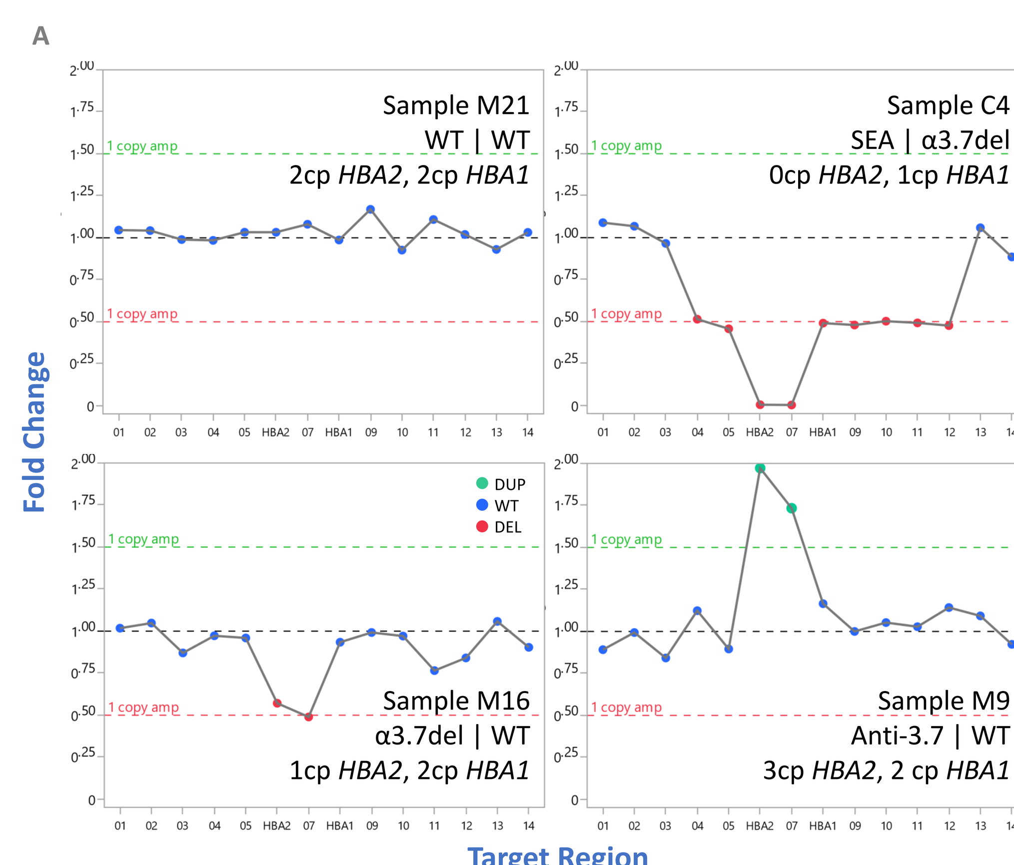
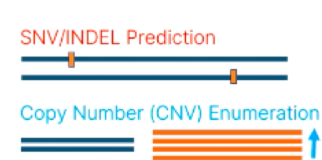


Figure 4. *HBA1/2* SV examples. A: A set of 14 "sentinel" amplicons target regions of the hemoglobin alpha cluster to differentiate known common breakpoints. Amplicon fold change patterns identify WT, compound HET SEA | 3.7del, HET α3.7del | WT, and anti-3.7 | WT. **B:** HBB SV accuracy. Two amplicons cover the *HBB* gene and differentiate two classes of SVs: Sicilian/HPFH-like (full-gene deletion) from Hb Leopore-like (only Exon 1-2 deletion). **C:** HBB SNV example, a compound HET *HBB* Hb S "sickle cell" SNV. Sample M16 has 0 functional allele copies of *HBB*, 1 copy of *HBA2* and 2 copies of *HBA1*.

SMN1 & *SMN2*



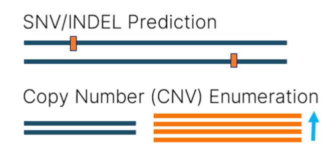
- ~71-95% gene deletions
- ~5% other SNVs
- ~5-30% [2+0] silent carriers

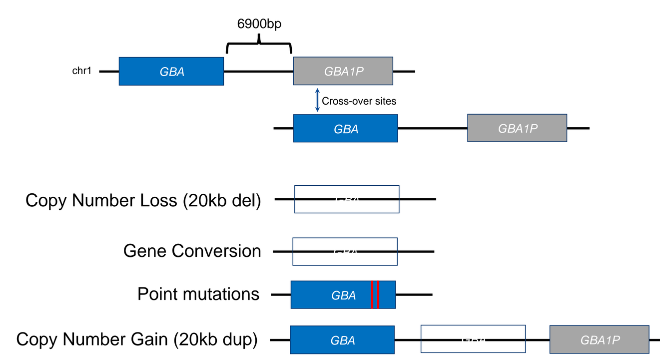
Variable copy number for both paralogs

Current standard practice methods: copy number for *SMN1* and *SMN2* is determined by qPCR (alternatively by QMPSF or MLPA), followed, if need be, by NGS sequencing combined with long-range PCR[4].

Beta test results: (n=13) 100% samples agreed with copy number reported in the comparator data for both *SMN1* and *SMN2*, including hybrids with an allele aligning to *SMN1* with yet c.840T from *SMN2* and a sample with silent carrier SNVs (c.*3+80T>G HET, and c.*211_*212del HET).

GBA





- Current standard practice methods: Sanger sequencing or NGS with a custom bioinformatic pipeline.
- Beta test results: 100% sample level agreement for samples that passed QC (n=10/12), troubleshooting will be required in future runs in order to increase read depth for this gene.

FMR1

Accurate Repeat Sizing

Repeat Interrupt Phasing

- Current standard practice methods: an Amplidex PCR-CE kit is used in order to detect all possible alleles (normal, intermediary zone, premutation, mutation, +/- cellular mosaicism)[5].
- Beta test results: (n=23) 3' UTR CGG repeat sizing agreement was 100% for alleles previously identified by PCR/CE.

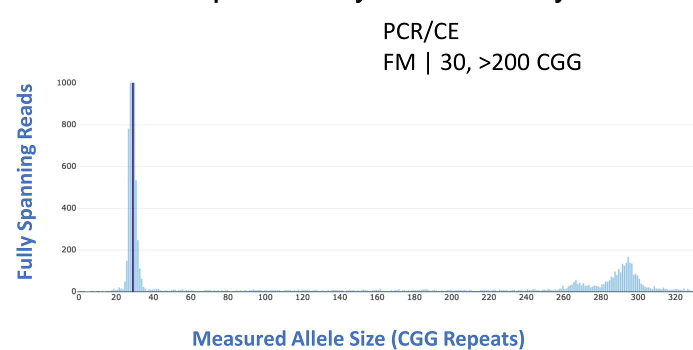
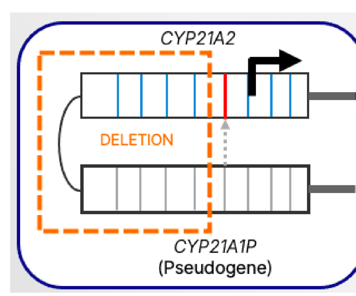
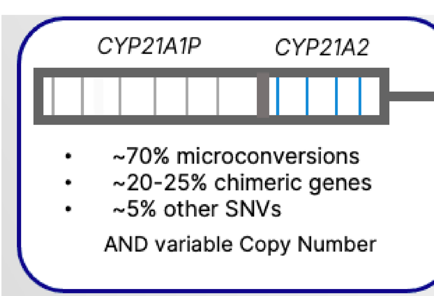
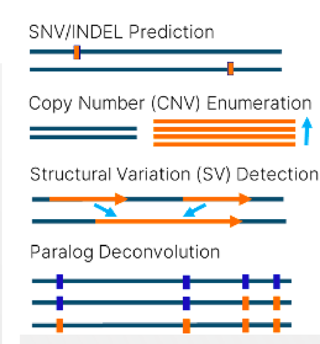


Figure 3. Example *FMR*1 profile, showing a normal allele and a full mutation.

CYP21A2







- Current standard practice methods: PCR with 2 couples of primers, followed by Sanger sequencing, in 2/3 cases a parental segregation study is required in order to determine phasing[8].
- Beta test results: 100% sample level agreement for all samples that passed QC (n=20/20), manual reporting was required for some samples.

sample	allele	Copy	haplotype	Category	Variants	Protein	Type
CYP-S-3	0	1	GPPGGGGGGG	Chimera			
CYP-S-3	1	1	GPPGPPPPPG	Pseudogene			
CYP-S-3	2	1	PPPPPPPGPPG	Pseudogene			
CYP-S-3	3	1	GAGGGGGGGPG	SNV	c.955C>T	Q318X	SW*
CYP-S-3	4	1	GAGGGGGPGGG	SNV	c.844G>T	V282L	NC*
CYP-S-6	0	1	GAGGGGGPGGG	SNV	c.844G>T	V282L	NC*
CYP-S-6	1	1	PPPPPPPPPGG	Pseudogene			
CYP-S-6	2	1	PPPPPPPPPPG	Pseudogene			
CYP-S-6	3	1	PPPPPPPGPPG	Pseudogene			

Figure 5. Example *CYP21A2* results, showing 2 composite heterozygous patients.

Conclusion

- **Data for 137 of 155 samples are presented here, 95% (130/137) passed QC. 100% agreed with expected sample-level genotypes.**
- Given this first experience with this prototype, we believe the single-platform and streamlined workflow has potential to reduce analysis turnaround time of these challenging genes compared to current methods used in standard French clinical diagnostic practice.
- Further adjustments will be required in future experiments, such as updates to the accompanying software, broadening of primer design (notably for *F8, TNXB* and *HBB*), or troubleshooting of read depth for some samples (i.e. *GBA*).
- Currently, genes with no underlying associated clinical phenotypes are grouped in the same reaction mix. Indeed, this assay was initially designed for carrier screening, not clinical diagnosis. Future versions could, we suggest, group "differential diagnosis" mixes.

GRANT DISCLAIMER:

Asuragen (PCR reagents, analysis software and laptop, library prep reagents, ESHG conference registration and accommodation costs for ASL)

Oxford Nanopore Technology Mk1B, Flow cells, library prep reagents).

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