

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

Lebre A.S.^{1,2,3}, Yammine T.¹, Feyereisen L.^{1,2}, Moret H.³, Landais E.¹, Audrezet M.P.⁴, Girodon E.⁵, Raynal C.⁶, Metay C.⁷, Bonello N.⁸, Rouzier C.⁹, Chaussenot A.⁹, Pissard S.^{10,11}, Gueguen P.¹², Calmels N.¹³, Froissart R.¹⁴, Bekri S.^{15,16}, Bellanné-Chantelot C.¹⁷

¹) CHU Reims, Laboratoire de Génétique, Reims, France; ²) Université Reims Champagne-Ardenne (URCA), Reims, France; ³) Université Paris Cité, Institute of Psychiatry and Neuroscience of Paris (IPNP), INSERM U1266, Paris, France; ⁴) CHU Brest, Laboratoire de Génétique Moléculaire, Brest, France; ⁵) AP-HP Centre, Université de Paris, Hôpital Cochin, Service de Médecine Génomique des Maladies de Système et d'Organe, Paris, France; ⁶) CHU de Montpellier, Laboratoire de Génétique de Maladies Rares, Institut Universitaire de Recherche Clinique EA 7402, Montpellier, France; ⁷) AP-HP Sorbonne Université, Hôpital Pitié-Salpêtrière, Centre de Génétique Moléculaire et Chromosomique, UFR de Cardiogénétique et Myogénétique Moléculaire et Cellulaire, Paris, France; ⁸) APHM, Hôpital de la Timone, M2GM Laboratoire de Génétique Moléculaire, Marseille, France; ⁹) Service de Génétique Médicale, Centre de référence des maladies mitochondriales, CHU Nice, Université Côte d'Azur, CNRS, INSERM, IRCAN, Nice, France; ¹⁰) HGU Henri Mondor, Département de Génétique, Créteil, France; ¹¹) Université Paris-Est Créteil (UPEC), Créteil, France; ¹²) Service de Génétique, CHRU de Tours - Université de Tours, INSERM, Imaging Brain & Neuropsychiatry iBrain, Tours, France; ¹³) CHU de Strasbourg, Hôpital Civil, Laboratoire de diagnostic génétique, Strasbourg, France; ¹⁴) CHU de Lyon HCL, LBMS Service Biochimie et Biologie Moléculaire, Bron, France; ¹⁵) CHU Rouen Normandie, Laboratoire de Biochimie Métabolique, Rouen, France; ¹⁶) Université Rouen Normandie, UNIROUEN, INSERM U1245 Rouen, France; ¹⁷) AP-HP Sorbonne Université, Hôpital Pitié-Salpêtrière, Département de Génétique Médicale, Paris, France.

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 241 unique previously genotyped clinical samples provided by a network of eleven French clinical diagnostic laboratories.

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

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 phasing³.
- Beta test results:** (n=63) all sample level agreement for all samples that passed QC, including SVs and indels (figure 2A et B respectively), except for samples with very rare deep intronic variants (not included in the assay design).

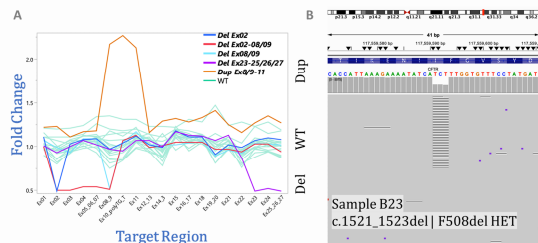
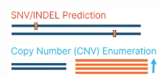


Figure 2. CFTR SV (A) and example indel (B) results.

SMN1 & SMN2



- ~71-95% gene deletions
- ~50% 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 QMPFS or MLPA), followed, if need be, by NGS sequencing combined with long-range PCR⁴.

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).

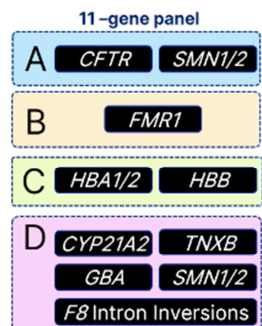
Methods

Deidentified and previously genotyped clinical genomic DNA samples isolated from whole blood (WB) donors were provided from collaborators across France (N=241). Target regions were enriched in 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.



Read length

~2kb

~10 kb

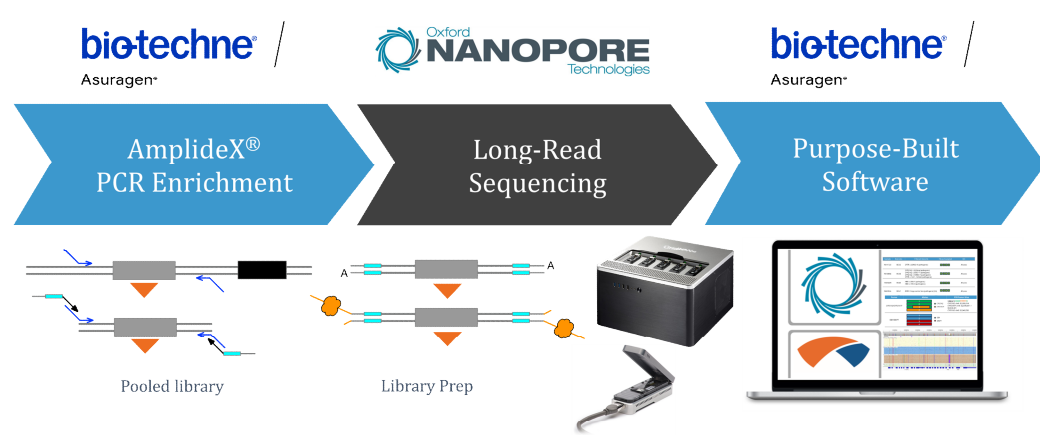


Figure 1. Single assay workflow. Wet lab steps are performed in 1.5 days, the sample run in 8-12 hours, and software analysis & processing in a couple of hours (8h maximum in our experience).

FMR1



- 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.

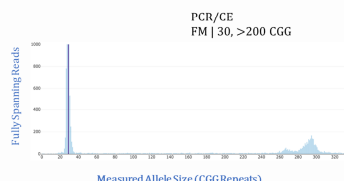


Figure 3. Example *FMR1* profile, showing a normal allele and a full mutation.

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 NGS⁶.
- Beta test results:** 100% sample level agreement for all samples that passed QC (n=11/12)

- Wish list for a v2 of the mix D: to include *F8* and *F9* full exonic sequences

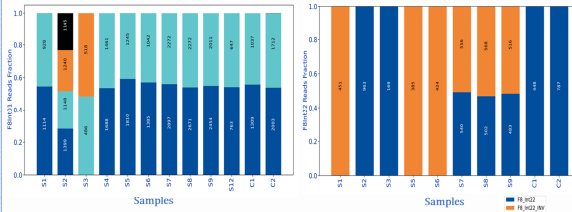
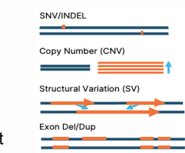


Figure 4. *F8* Variant Genotype. The algorithm identifies reads associated with the intronic H1 (intron 1 and 22) and H2 (intron 1 only) intergenic regions of the WT sample, or the H1 Fusion to H2 (intron 1) or H2/3 (intron 22) intergenic region indicating an inversion.

HBA1, HBA2 & HBB



- Current standard practice methods:** Gap PCR, followed by MLPA, Sanger Sequencing or NGS.
- Beta test results:** SNV and SV variant level classification was 100% for samples that passed QC (n=31/33).
- Wish list for a v2 of the Mix C: to include other *HB* genes

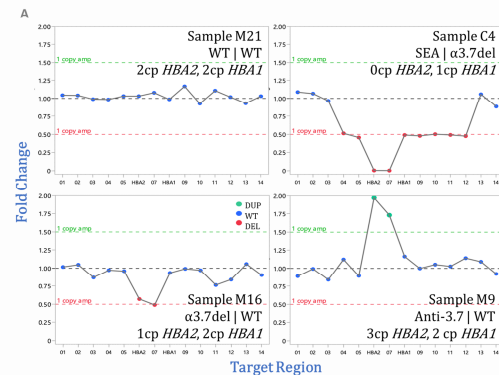
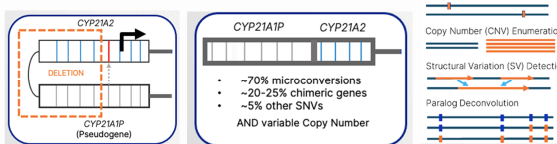


Figure 5. *HBA1/2* SV examples. A: A set of 14 "sentinel" amplicons target regions of the hemoglobin alpha cluster to differentiate known common breakpoints. Amplicon fold change patterns identify WT, compound HET SEA | 3.7del, HET α3.7del | WT, and anti-3.7 | WT. B: *HBB* SV accuracy. Two amplicons 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*.

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 phasing⁸.
- Beta test results:** 100% sample level agreement for all samples that passed QC (n=42/42), manual reporting was required for some samples.

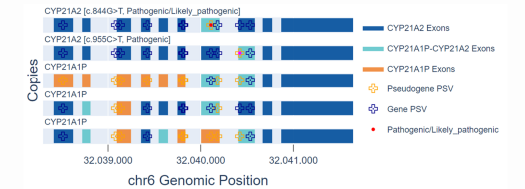
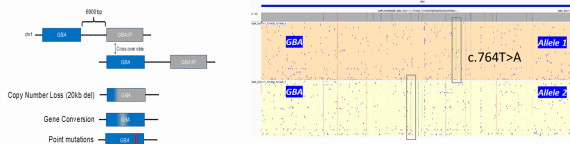


Figure 6. Example *CYP21A2* results, showing a composite heterozygous profile.

TNXB

- Current standard practice methods:** NGS panel and MLPA (P155)(EX1_31 and EX35) - High homology between *TNXB* EX32-44 and *TNXA* pseudogene - 2 probes of *CYP21A2* are included in MLPA kit (for CNV detection downstream of EX35)
- Beta test results:** 100% sample level agreement for all samples that passed QC (n=32/32), manual reporting was required for some samples.
- Wish list for a v2 of mix D: to include *TNXB* ex1-31 (ex32-44 are already included in mix D)

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.

Figure 7. Example *GBA* results, IGV view showing a composite heterozygous patient.

Conclusion

Data for 230 of 241 samples are presented here. Some *CFTR*, *CYP* and *TNXB* samples analyses are still in progress.

We have been satisfied by the ease of use, turnover-time, and performance of this Asuragen/ONT prototype assay.

We anticipate this assay will streamline testing conducted for screening as well as for diagnostic purposes.

For clinical use, we would recommend adjustments for future experiments, such as broadening primer design for *F8*, *TNXB*, and *HBB*.

Furthermore, genes with no underlying associated clinical phenotypes are currently grouped in the same reaction mix. Indeed, this assay was initially designed for carrier screening (which is not permitted in France for asymptomatic patients with no relevant family history), not clinical diagnosis.

Future versions could, we suggest, group "differential diagnosis" mixes.

Our wish list

- ✓ Access by individual mix A, B, C or D
- ✓ Accurate detection of all breakpoints
- ✓ Inclusion of our Variant Knowledge Bases (VKB)
- ✓ European IVDR certification

