A Comprehensive and Streamlined Approach to Deciphering Challenging ACMG Tier 3 Carrier Screening Genes

Poster Number: G038

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Summary

- Traditional sequencing methods often miss complex variants in key genes associated with common inherited disorders, requiring cumbersome workflows and yielding incomplete pathogenic variant detection.
- In response, we expanded the AmplideX® Nanopore Carrier Plus Kit† with a prototype‡ that addresses all challenging ACMG Tier 3 genes. The prototype adds *AFF2*, *FXN*, and *ARX* to the current panel interrogating *CFTR*, *SMN1/2*, *FMR1*, *HBA1/2*, *HBB*, *GBA*, *CYP21A2*, *TNXB*, and *F8* intron inversions with a streamlined end-to-end workflow.
- The prototype combines targeted PCR enrichment, nanopore sequencing, and companion analysis software to resolve complex pathogenic variants in a unified workflow for different variant types: short tandem repeats, copy number variation, structural variation, and paralog differentiation.
- Feasibility was demonstrated using a mixed sample set consisting of genomic DNA isolated from 34 cell-line and 204 whole blood samples, demonstrating >97% agreement across all variant categories and genes included in the assay.

Introduction

Precise identification of pathogenic variants associated with severe genetic disorders is fundamental for diagnostic accuracy and carrier screening. Although advances in next-generation sequencing (NGS) have simplified screening for most ACMG tier 3 genes[1], Guha et al.[2] identified 10 genes with complex pathogenic variants that still require multiple specialized workflows. Consequently, many laboratories need to maintain several unique assays, equipment, and expertise to detect variants in these genes, increasing costs and operational complexity.

The AmplideX Nanopore Carrier Plus Kit integrates short- and long-range PCR enrichment with nanopore sequencing in a modular, kit-based panel, enabling accurate genotyping of both NGS-accessible genes (*CFTR*, *HBB*) and challenging targets (*SMN1/2, FMR1, HBA1/2, GBA, CYP21A2, TNXB*, and *F8* intron inversions) within a unified workflow. Here, we report an expansion of the panel with a prototype addressing the remaining hard-to-decipher genes (*AFF2, ARX, FXN*) allowing labs to address all 113 Tier 3 genes with a sequencing solution (when combined with traditional NGS) regardless of variant complexity.

Materials and Methods

We evaluated a total of 238 isolated genomic DNA (gDNA) samples from 34 cell-line (CL) including 30 from Coriell Cell Repository (CCR) and 204 residual clinical whole blood (WB). Target regions of 46 samples (plus 2 controls) were enriched with all five PCR mixes and processed in a unified assay workflow. A cohort of 192 presumed normal samples were screened with 2 mixes: B (FMR1 and *AFF2*) and E (*FXN* and *ARX*) to assess performance of the new content. Nanopore sequencing was performed on MinION® flow cells (R10.4.1) with a Mk1D or GridION® (Oxford Nanopore Technologies®). Custom software automated analysis and reporting for the new content, and results were compared to data obtained from multiple orthogonal methods where available including PCR/CE, MLPA, Sanger sequencing, qPCR and long-range PCR.

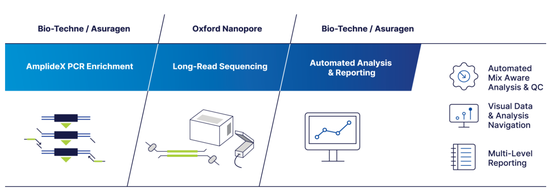
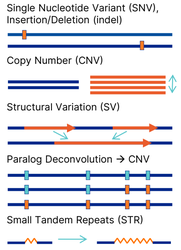


Figure 1. Prototype AmplideX Nanopore Carrier Plus Kit Panel Design. A modular panel design and workflow identifies pathogenic variants for 14 genes responsible for common inherited genetic disorders and/or hard-to-decipher genes. A total of 5 PCR mixes are used to amplify regions of interest across 14 genes (including all 10 challenging genes). Custom software identifies and reports multiple variant classes across genes. Unified prototype workflow involves PCR enrichment and sample barcoding, library preparation followed by nanopore sequencing, automated sequencing analysis and variant reporting.

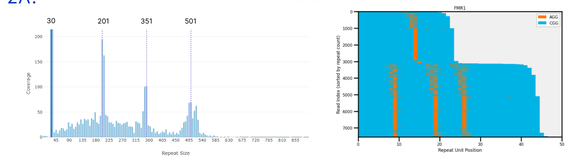
Results

Table 1. Percent agreement for 48-Sample sequencing across 5 Mixes. Data are shown for a set of 46 samples and 2 calibrators enriched with all 5 primer mixes and compared to variant truth. The panel includes 34 CL and 12 WB including 9 previously characterized for AFF2 repeat status. Two samples were flagged for low coverage and not included in the analysis. *Indicates a WB with discordant pseudogene copy number for *GBA/GBAP* (expected 2/1, called 2/2) of no clinical consequence. Only wildtype sequence was detected in the SNV hotspot for *AFF2* (c.1262+1G>C and c.1262+1G>T) or *ARX*. However, all samples showed high quality reads with >200X coverage.

Gene	Mix	% Agreement	Expected Variants
CFTR	A	100%	SNV (5), indel(1)
SMN1	A	100%	SNV (3), CNV (7)
SMN2	A	100%	CNV (23)
FMR1	B	100%	STR (4)
AFF2	B	100%	STR (9)
HBA1/2	C	100%	SV (6)
HBB	C	100%	STR (1), SV (1)
CYP21A2/A1P	D	100%	SNV (2)
F8	D	100%	SV (1)
GBA1/P	D	98%	CNV (1*), SV(1)
TNXB/A	D	100%	CNV (1), SV(1)
FXN	E	100%	STR (16)



FMR1 Repeats

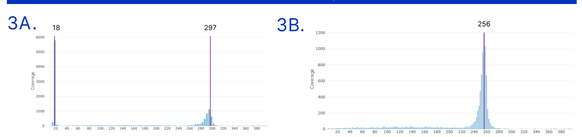


2C.

FMR1 Category		Expected NOR	INT	PRE	FM
Measured	NOR	39	0	0	0
	INT	0	1	0	0
	PRE	0	0	2	0
	FM	0	0	0	2

Figure 2. Signal processing visuals allow users to thoroughly investigate *FMR1* CGG repeats and AGG interrupts. A) Histogram of sequenced repeat lengths, which is used by the algorithm to determine the number of CGG repeats in each *FMR1* allele (purple line) and are identified in this sample at 30, 201, 351, and 501 repeats. B) Waterfall plot displays phased "AGG interrupts" (orange) within the 24 and 45 alleles, with 1 and 3 interrupts respectively, to allow for assessment of repeat expansion risk in *FMR1*. C) *FMR1* sample-level genotype agreement was 100% (44/44).

AFF2 (*FMR2*) Repeats



3C.

AFF2 Category		Expected NOR	INT	PRE	FM
Measured	NOR	35	0	0	0
	INT	0	1	0	0
	PRE	0	0	3	0
	FM	0	0	0	5

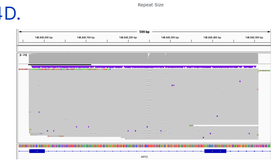
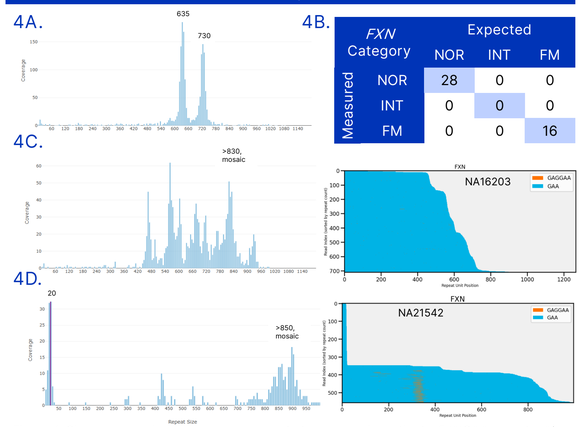


Figure 3. Signal processing visuals allow users to thoroughly investigate *AFF2 CCG* repeats. A) Histogram of sequenced CCG repeat lengths (18, 297) for female WB sample concordant with PCR/CE (18, >200). B) Sequenced CCG repeat length (256) for male WB sample concordant with Southern blot (286). C) *AFF2* sample-level genotype agreement was 100% (44/44). D) Alignment of a reads at the *AFF2* SNVs hot spot region for a wild type. None were detected across the panel.

FXN Repeats



FXN Category		Expected NOR	INT	FM
Measured	NOR	28	0	0
	INT	0	0	0
	FM	0	0	16

Figure 4. Signal processing visuals allow users to thoroughly investigate *FXN* GAA repeats. A) Histogram of sequenced GAA repeat lengths (635, 730) for NA16214 matches FM expansion reported by CCR (~600, ~700). B) Confusion matrix for categorical *FXN* genotype. Agreement was 100% (44/44 samples that passed QC). Samples in this cohort were between 200 and 1070 GAA repeats. C) Histogram and waterfall plot of NA16203 sized as >830 with mosaicism matches CCR (~670, ~830). No interrupts were detected. D) Histogram and waterfall plot of NA21542 sized as 20 | >850 with mosaicism. A GAGGAA interrupt was phased with the longer allele which may indicate delayed age of onset[3]. CCR reports this sample as clinically affected 20yo female donor with "Gly130Val on one allele, the other allele is as yet unstudied".

ARX Repeats

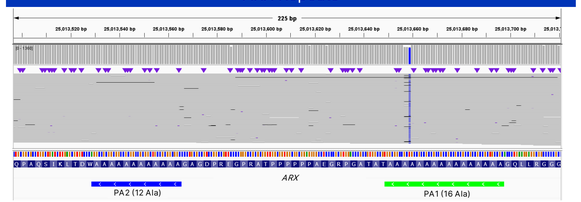


Figure 5. IGV plot showing *ARX* polyalanine regions 1 and 2 (225 bp) of the full sequenced gene. Although no PA1 or PA2 repeat variants were identified in the sample cohort, a benign SNV (c.336A>G; p.Ala112Ala) was identified within polyalanine region 1 in a presumed normal sample.

Repeat Distributions Across Genes in Presumed Normals

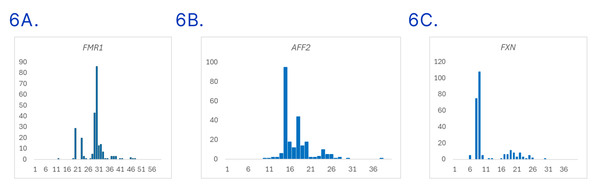


Figure 6. Repeat allele size distributions for *FMR1*, *AFF2*, and *FXN* in presumed normal samples. Histograms display repeat size on the x-axis and the number of alleles detected on the y-axis. A) *FMR1* CGG repeat distribution including 188 samples with alleles in the normal range (<45 CGG repeats) and 4 samples in the intermediate range (45-54 CGG repeats). B) *AFF2* CCG repeat distribution including 191 samples with alleles in the normal range (<30 CCG repeats) and 1 sample in the intermediate range (31-60 CCG repeats). C) *FXN* GAA repeat distribution including 192 samples with alleles in the normal range (5-33 GAA repeats).

Table 2. Carriers along with those with intermediate *FMR1* and *AFF2* expansions, were identified in 16/192 (8.3%) samples from a presumed normal cohort using the prototype assay. One donor sample (SID530 in blue) was a carrier for both *CFTR* and *SMN1*. Silent carrier haplotype associated variants (SC1, SC2) and the disease modifier variant (DM) are reported to further support interpretation of *SMN1* copies. No variants were detected for *FXN*, *ARX* or the *AFF2* hot spot SNV as expected given carrier frequency for these genes.

Sample ID	*FMR1*	*AFF2*	*CFTR* Allele 1	*CFTR* Allele 2	*SMN1* CNV	*SMN2* CNV	*SMN* DM	*SMN* SC1	*SMN* SC2	Carrier Status
SID481	31	15	F508del	wt	2	1	(-)	(-)	(-)	*CFTR*
SID489	30	15	R117H	wt	2	1	(-)	(-)	(-)	*CFTR*
SID530	30	15	F508del	wt	2	1	Pos	Pos	(-)	*CFTR, SMN1* SC
SID562	30	15	F508del	wt	2	2	(-)	(-)	(-)	*CFTR*
SID569	30	26	F508del	wt	2	2	(-)	(-)	(-)	*CFTR*
SID605	29	18	R1158X	wt	2	2	(-)	(-)	(-)	*CFTR*
SID637	30	15	F508del	wt	2	3	(-)	(-)	(-)	*CFTR*
SID535	39, 47	15, 15	wt	wt	2	2	Neg	Neg	Neg	*FMR1* Int
SID537	46	18	wt	wt	2	0	Neg	Neg	Neg	*FMR1* Int
SID542	47	16	wt	wt	2	0	Neg	Neg	Neg	*FMR1* Int
SID611	24, 46	15,18	wt	wt	3	2	Neg	Neg	Neg	*FMR1* Int
SID582	30	38	wt	wt	2	2	(-)	(-)	(-)	*AFF2* Int
SID461	20, 30	17,20	wt	wt	1	1	(-)	(-)	(-)	*SMN1*
SID546	29	15	wt	wt	1	2	(-)	(-)	(-)	*SMN1*
SID578	30,30	15,18	wt	wt	2	2	(-)	Pos	Pos	*SMN1* SC
SID589	30	18	wt	wt	2	2	(-)	Pos	Pos	*SMN1* SC

Conclusions

- The prototype assay streamlines workflows, shortens turnaround times, and delivers deeper insights into 14 ACMG Tier 3 genes—including 10 hard-to-decipher genes that previously required multiple ancillary methods across disparate platforms for accurate characterization.
- A cohort of 48 samples was successfully enriched with all 5 primer mixes and sequenced on a single MinION flow cell (R10.4.1). We expect further assay optimization will enable expanded sample multiplexing capacity.
- The prototype software automates genotyping and provides signal processing visuals of complex genotypes, allowing users to easily process and then review data to confirm results.
- The prototype assay produces precise and accurate results in a unified workflow that agreed with orthogonal methods for SNVs/INDELs (>99%), *SMN1/2* copy number (>99%), STR sizing and interrupt phasing in *FMR1*, *AFF2*, and *FXN* (>99%), *HBA1/2* deletions, *CYP21A2* genotype carrier status (100%), *GBA* carrier status (97%), and *F8* intron inversions (100%).

References

1. Gregg AR, et. al. ACMG Professional Practice and Guidelines Committee. Screening for autosomal recessive and X-linked conditions during pregnancy and preconception: a practice resource of the American College of Medical Genetics and Genomics (ACMG). Genet Med. 2021 Oct;23(10):1793-1806.
2. Guha S, et. al. ACMG Laboratory Quality Assurance Committee. Laboratory testing for preconception/prenatal carrier screening: A technical standard of the American College of Medical Genetics and Genomics (ACMG). Genet Med. 2024 Jul;26(7):101137.
3. Long A, et. al. Somatic instability of the expanded GAA repeats in Friedreich's ataxia. PLoS One. 2017 Dec;12(12):e0189990.

All authors have a financial relationship to disclose: Employment by Asuragen.

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‡This product is under development; performance characteristics and final product features to be determined.

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Cell lines were obtained from the NIGMS Human Genetic Cell Repository at the Coriell Institute for Medical Research