Evaluation of an Amplification-Based Long-Read Nanopore Sequencing Assay for Simultaneous Detection of CGG Repeats and AGG Interruptions in *FMR1* Genotyping

Giulia Moschetti[1], Stela Filipovic-Sadic[1], Mia Mihailovic[1], Connor Parker[1], Theodore Markulin[1], Juliette Baker[1], Stephanie Borel[1], Jonathan Turner[1], Vivian Le[1], Bryan Killinger[1], Malia Deshotel[2], Scott Reading[2], Eric Fredrickson[2], Devin Close[2], Yuan Ji[2], Erica Andersen[2], Hunter Best[2], Jessica Wright[3], Mark Williams[3], Elizabeth Barrie[4], Kendall Martin[4], John Milligan[1], Brian Haynes[1], Bradley Hall[1]

[1] Asuragen, a Bio-Techne Brand, Austin, Texas, USA; [2] ARUP Laboratories, Salt Lake City, UT, USA; [3] Genomic Diagnostics, Heidelberg VIC, Australia; [4] VCU Health, Richmond, VA, USA

Summary

- Assessing CGG repeat size and AGG interruptions in the *FMR1* gene is essential for Fragile X carrier screening, particularly in premutation (PM) females, where AGG patterns influence intergenerational expansion risk.
- Traditional workflows often require separate assays to determine CGG repeat length and phase AGG interruptions.
- We verified and evaluated the AmplideX® Nanopore Carrier Plus Kit (Mix B)†, which uses PCR enrichment and long-read nanopore sequencing to deliver comprehensive *FMR1* genotyping in a single assay.
- Testing was performed on MinION™ and GridION™ platforms at four independent laboratories, with each site contributing its own samples, lab equipment, and personnel.

Introduction

Accurate resolution of genomic variants is essential for diagnostic and carrier screening applications. However, conventional next-generation sequencing (NGS) often falls short of fully characterizing complex variant types such as short tandem repeats (STRs), frequently necessitating multiple specialized workflows.[1,2] In the *FMR1* gene, carrier screening requires precise sizing of CGG repeats and phasing of AGG interruptions, which stabilize the repeat tract and reduce the risk of intergenerational expansion.[3–5]

To enable comprehensive characterization of the CGG repeat tract, we developed the AmplideX® Nanopore Carrier Plus Kit (Mix B) — an integrated assay-and-software workflow that combines PCR enrichment with nanopore sequencing to resolve *FMR1* CGG repeat length and in-phase AGG interruptions. The companion software automates CGG sizing, AGG localization and phasing, and QC review. Performance was evaluated across 4 sites.

Methods

A kitted, amplification-based nanopore sequencing assay was evaluated across four laboratories, each testing a subset of samples. Genomic DNA from whole blood and cell lines was used as input. In total, 292 samples (102 normal, 43 intermediate, 109 premutation, and 38 full mutation) were processed using AmplideX PCR enrichment and sequenced on Oxford Nanopore Technologies instruments (MinION and/or GridION). Libraries were barcoded, pooled, and sequenced on R10.4.1 flow cells. Data were analyzed with AmplideX® One Reporter† software (v1.0.4), and results were compared with orthogonal methods, including PCR/CE-based assays and Southern blot; orthogonal AGG interruption testing was performed using Xpansion Interpreter®‡.

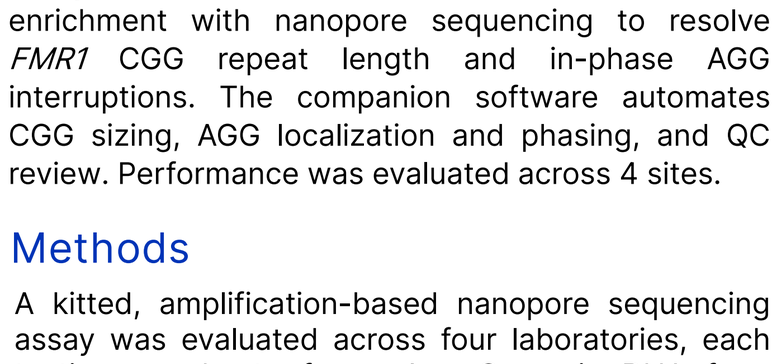

Figure 1. AmplideX Nanopore Carrier Plus Kit (Mix B) Workflow. Targeted PCR amplifies the *FMR1* CGG repeat region, producing amplicons that are barcoded, pooled, and sequenced on an ONT device. Automated analysis generates QC metrics, CGG repeat detection and sizing, AGG localization, and result visualizations, with the complete workflow finished within 3 days.

Results

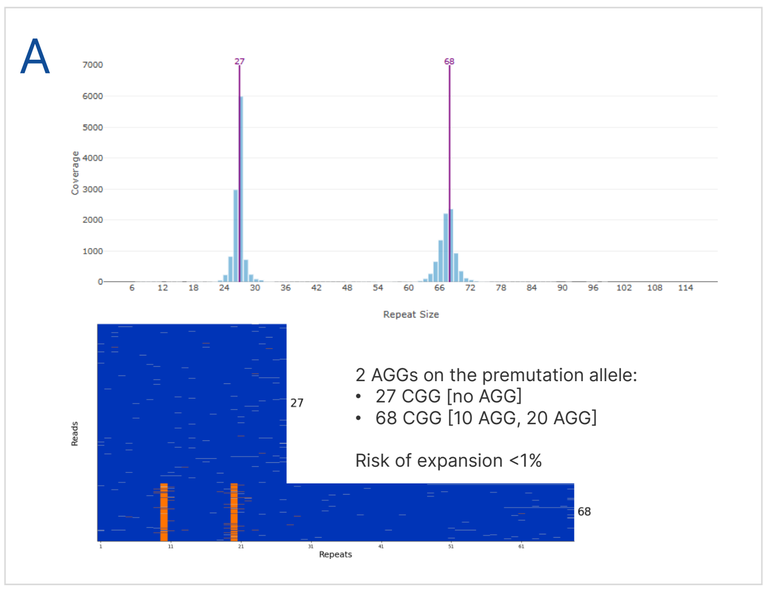

Figure 2. CGG/AGG Profiles and Expansion Risk in PM Samples. This amplification-based nanopore sequencing assay enables direct and accurate phasing of AGGs, improving the interpretation of expansion risk. For each sample, the top panel shows CGG histograms (allele sizes), and the bottom panel shows waterfall plots (CGG reads are shown in blue and AGG interruptions in orange). (A) PM allele [2×AGG]—low risk of expansion to a full mutation in the next generation. (B) PM allele [1×AGG]—higher risk than if two AGGs are present. (C) PM allele [no AGG]—high risk. (D) Mosaic with two PM alleles (73 CGG [2×AGG] and 105 CGG [2×AGG]); presence of a high-CGG allele may increase expansion risk compared to non-mosaic PM profiles. Expansion risk increases with CGG repeat length and decreases with the number of AGG interruptions[3-5].

Table 1. Categorical Genotype Agreement. A total of 292 genomic DNA samples (102 normal, 43 intermediate, 109 premutation, and 38 full mutation) were tested across four independent sites. Overall *FMR1* categorical genotype agreement was 99.3% (290/292). *Two discrepancies were due to low-level mosaicism, where PCR/CE identified low-frequency alleles that were observed in the nanopore data but not automatically called by its algorithm.

Measured \ Expected	Normal	Intermediate	Pre mutation	Full Mutation
Normal	102	0	0	0
Intermediate	0	43	*1	0
Premutation	0	0	107	0
Full Mutation	0	0	*1	38

Table 2. Multisite Accuracy. Accuracy was assessed at each site and summarized for all sites by allele detection and sample level categorical genotype. Thresholds to match alleles across methods were ±1 CGG for ≤70 repeats, ±3 CGG for 71–120 repeats, and ±5% for 121–200 repeats. Overall agreement was 96.7% for CGG allele detection (94.7–98.3% by site), 99.1% for AGG counts (93.2–100%), and 99.3% for categorical genotype (97.0–100%). Note: CGG repeats detected in only one of the comparator methods that corresponded to low-level size mosaicism confirmed by manual review of PCR/CE traces were excluded from calculation at Site 1; for Sites 2–4, all alleles were assessed since frequency per allele for mosaicism was unknown.

Variant	Site 1	Site 2	Site 3	Site 4	All 4 Sites
Genotype (Sample Number)	100% (96)	97.0% (33)	98.3% (59)	100% (104)	99.3% (292)
CGG Repeats (Allele Number)	98.3% (180)	94.7% (57)	95.8% (118)	96.3% (187)	96.7% (542)
AGG Interruptions (AGG Number)	99.6% (231)	NA	100.0% (144)	93.2% (44)	99.1% (419)

Conclusions

- The assay demonstrated 99.3% agreement for genotype classification and 96.7% concordance for CGG repeat detection, across 4 sites.
- AGG interruptions were detected and accurately phased in premutation alleles with 99.1% agreement, refining the assessment of expansion risk.
- Normal and premutation alleles were reliably sized up to 200 CGG repeats, while full mutations were either sized or correctly flagged as >200 CGG.
- Performance was consistent across four laboratories and on both MinION™ and GridION™ platforms, supporting assay robustness and reproducibility across diverse testing environments.
- This long-read nanopore sequencing assay provides a robust, scalable workflow that combines CGG repeat sizing with AGG phasing to enable comprehensive Fragile X insights without reflex testing.

1. Mantere T, Kersten S, Hoischen A. Long-Read Sequencing Emerging in Medical Genetics. Front Genet. 2019;10:426. Published 2019 May 7. doi:10.3389/fgene.2019.00426
2. Lincoln SE, Hambuch T, Zook JM, et al. One in seven pathogenic variants can be challenging to detect by NGS: an analysis of 450,000 patients with implications for clinical sensitivity and genetic test implementation. *Genet Med*. 2021;23(9):1673-1680. doi:10.1038/s41436-021-01187-w
3. Yrigollen CM, Durbin-Johnson B, Gane L, et al. AGG interruptions within the maternal *FMR1* gene reduce the risk of offspring with fragile X syndrome. Genet Med. 2012;14(8):729-736. doi:10.1038/gim.2012.34
4. Nolin SL, Glicksman A, Ersalesi N, et al. Fragile X full mutation expansions are inhibited by one or more AGG interruptions in premutation carriers. Genet Med. 2015;17(5):358-364. doi:10.1038/gim.2014.106
5. Latham GJ, Coppinger J, Hadd AG, Nolin SL. The role of AGG interruptions in fragile X repeat expansions: a twenty-year perspective. Front Genet. 2014;5:244. Published 2014 Jul 29. doi:10.3389/fgene.2014.0024

† *Research Use Only. Not for use in diagnostic procedures.*

‡ *Xpansion Interpreter® is a laboratory-developed test (LDT). Analytical and clinical performance have not been reviewed by the FDA.*

Oxford Nanopore Technologies products are not intended for use for health assessment or to diagnose, treat, mitigate, cure, or prevent any disease or condition.

Presented at the 6th International Conference on Fragile X Premutation Associated Conditions 2025.