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Performance Evaluation of a PCR/Nanopore Assay for Carrier Screening for Cystic Fibrosis, Spinal Muscular Atrophy, and Fragile X Syndrome



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Cystic fibrosis, spinal muscular atrophy, and fragile X syndrome are among the most common inherited genetic disorders, making carrier screening essential for identifying at-risk couples. Traditional screening often involves multiple workflows and may miss rare variants. Comprehensive sequencing offers broader variant detection across diverse populations. This study validated a PCR/Nanopore-based assay for comprehensive assessment of *CFTR*, *SMN1/2*, and *FMR1*. Samples included anonymized DNA from: whole blood (archival clinical samples; $n = 53$), cell lines ($n = 19$), and residual College of American Pathology proficiency testing material ($n = 3$). Using the AmplideX Nanopore Carrier Plus reagents, gene-specific PCR was performed, followed by barcoding and sequencing on MinION flow cells. Data were analyzed using the AmplideX One Reporter software. Results for *SMN1/2* and *FMR1* showed 100% concordance with orthogonal methods (PCR/fragment analysis laboratory-developed tests) and 97% for *CFTR*. These results were reproducible between interrun and intrarun repeats. Here, it is shown that the PCR/Nanopore-based assay is accurate and reproducible for identifying pathogenic variants and poly-T size in *CFTR*; *SMN1/2* copy number and single-nucleotide polymorphism detection; and *FMR1* CGG repeat sizes and AGG interruptions. The assay was also able to detect mosaicism as low as 10% for *FMR1*. This single workflow enables accurate, reproducible screening for multiple disorders, with the ability to identify more *CFTR* variants than traditional genotyping panels.
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Carrier screening is genetic testing that aims to identify individuals or couples who are at risk of having a child affected by an autosomal recessive or X-linked genetic disorder. Historically, carrier screening recommendations for genetic disorders were partially guided by ethnicity; however, recent guidelines are moving toward a more inclusive, population-wide approach.[1] The American College of Medical Genetics and Genomics recommends carrier screening be offered to all individuals who are currently pregnant or plan to become pregnant.[1] Expanded carrier screening guidelines include 113 genes, selected for having a carrier frequency >1:200 and X-linked conditions.[1] Although expanded carrier screening is becoming more common, individual decisions should be based on personal factors, including family history,[2] and some patients may opt for targeted screening. Notably, cystic fibrosis (CF), spinal muscular atrophy (SMA), and fragile X syndrome (FXS) are among the most common genetic disorders for which individuals seek targeted carrier screening.[3]

CF is an autosomal recessive condition caused by pathogenic variants in the *CFTR* gene, of which >1000 variants have been identified (*https://cftr2.org/mutations_history*, last accessed September 24, 2025), with severity of disease being correlated to the variant's effect on the protein. Traditionally, carrier screening for CF has involved PCR-based panels that screen for a specific number of pathogenic variants. The American College of Medical Genetics and Genomics has recently updated its

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guidelines to include 100 *CFTR* variants in carrier screening panels.[4] However, panel-based screening methods are limited in their ability to detect the full spectrum of potential pathogenic variants. Comprehensive *CFTR* sequencing offers improved coverage across diverse populations, thereby reducing residual risk across groups in the case of a negative result.

SMA is an autosomal recessive condition most frequently caused by the deletion of exon 7 of the *SMN1* gene. Although 95% of cases are due to homozygous loss of exon 7, other pathogenic variants within *SMN1* can also cause disease.[5] The clinical severity of SMA is influenced, in part, by the number of copies of *SMN2* present in an individual. *SMN2* is a nearly identical gene that produces a small amount of functional survival of motor neuron (SMN) protein, such that an increased *SMN2* copy number can lessen the severity of SMA symptoms, whereas fewer copies of *SMN2* lead to the most severe forms of SMA. *SMN1* carrier status is most commonly assessed by measuring *SMN1* exon 7 copy number via PCR or multiplex ligation-dependent probe amplification. Because of the high degree of sequence similarity between *SMN1* and *SMN2*, whole gene sequencing for *SMN1* has proven challenging.

Notably, individuals may be silent carriers for *SMN1*, with two copies of the gene on a single chromosome and none on the homologous chromosome. Standard testing will not identify these individuals as being carriers, despite their potential to transmit a chromosome without a functional copy of *SMN1*. Certain single-nucleotide polymorphisms (SNPs) mark this haplotype and are associated with increased risk for being a silent carrier.[6,7]

FXS is an X-linked condition caused by a trinucleotide CGG repeat expansion in the 5′ untranslated region of the *FMR1* gene and is the most common cause of inherited intellectual disability. Individuals with approximately 55 to 200 CGG repeats are carriers of FXS, with this repeat number being considered a premutation that has the potential to expand to the full mutation (>200 repeats) in subsequent generations. The risk for expansion is also impacted by the presence of intervening AGG interruptions, which act to stabilize the region.[8] Although only a full mutation will cause FXS, premutations can also cause clinical manifestations, including primary ovarian insufficiency and a tremor/ataxia syndrome.[9–11] Additionally, individuals with mosaicism for multiple CGG repeat sizes in *FMR1* have been identified, which can lead to phenotypic variability.[12] Testing for *FMR1* repeat expansions was historically performed using Southern blot analysis; however, the current prevailing method is triplet repeat primed PCR. Sequencing of *FMR1* has been difficult because of the repetitive sequence, as well as the high GC content of the region.

The development of long-read sequencing technology has provided a way to sequence complex regions of the genome that are difficult with short-read sequencing because of repetitive sequences, structural variants, and regions with high GC content. Nanopore sequencing is a long-read sequencing technology that identifies DNA sequences by monitoring changes in ionic current as the molecule moves through a nanopore, with each nucleotide producing a distinct signal that can be decoded into its corresponding base.[13] Recently, long-read sequencing assays have been developed to be used for carrier screening for genetic disorders.[14–16] In this study, the performance characteristics of a laboratory-developed test were evaluated using the reagents in the AmplideX Nanopore Carrier Plus Kit (Asuragen, Austin, TX). The test uses PCR enrichment followed by long-read sequencing to detect pathogenic variants (including the American College of Medical Genetics and Genomics 100 variants) and poly-T status in *CFTR,* copy number calling and variant detection for *SMN1* and *SMN2,* and CGG repeat sizing with AGG interruption determination for *FMR1*. These validation studies include analytical accuracy, analytical precision/reproducibility, and method comparison using established comparative methods (orthogonal methods).

Materials and Methods

Samples

Samples included DNA obtained from: whole blood from anonymized residual clinical samples ($n = 53$), cell lines ($n = 19$), and College of American Pathology proficiency testing residual samples ($n = 3$) (Supplemental Table S1). This sample pool included 21 samples that were positive for at least one pathogenic *CFTR* variant, 23 samples with an *SMN1* exon 7 copy number of one or zero, 31 samples with an *SMN2* copy number of one or zero, and 17 samples that were positive for a premutation or full mutation in *FMR1*, with one of these having known mosaicism (NA20241/VCU70). Orthogonal testing results for each sample can be found in Supplemental Table S1.

Artificial mosaic *FMR1* samples were generated by diluting DNA from a female with a premutation with 30 and 80 repeats (NA20240/VCU11) or DNA from a female with a full mutation with 29 and >200 repeats (NA07537/VCU14) with normal female DNA with 29 and 30 repeats (NA20238/VCU71). Samples were prepared with 50%, 25%, 10%, 5%, and 1% of the premutation or full mutation allele.

Asuragen Carrier Plus Kit Reagents + Library Generation

Genomic DNA was diluted to 20 ng/μL, with 40 ng used per reaction. Gene-specific PCRs using mix A (*CFTR* and *SMN1/2*) and mix B (*FMR1*) were performed with the AmplideX Carrier Plus Kit reagents (catalog numbers A00627 and A00628; Asuragen). This was followed by purification with Agencourt AMPure XP beads (catalog number A63881; Beckman Coulter, Indianapolis, IN) and a sample-specific barcoding PCR. These products were quantified using Qubit dsDNA HS Assay reagents (Thermo

Fisher Scientific, Waltham, MA). Libraries for each mix were then pooled, to make an equimolar pool for each mix. A bead purification was then performed for each pooled, barcoded set of amplicons. The separate mix libraries were then combined for a final single library pool for library preparation. Sequencing libraries were prepared using the Oxford Nanopore Technologies (Oxford, UK) Ligation Sequencing Kit V14 reagents (catalog number SQK-LSK114). The pooled library was prepared for sequencing by end repair and A-tailing of the PCR products (New England Biolabs, Ipswich, MA), followed by bead purification. Adapters were ligated, bead cleanup was performed, and the final library was quantified by Qubit.

Sequencing

Libraries were sequenced on the MinION Mk1B (MIN-101B; Oxford Nanopore Technologies) with MinION R10.4.1 flow cells (FLO-MIN114) using MinKNOW software version 25.03.9. Flow cells were re-used when possible if meeting the minimum available number of pores. The flow cell was primed, the final library preparation was loaded onto the flow cell, and sequencing was initiated in the MinKNOW software. Once sequencing, base calling, and demultiplexing were complete, the data were analyzed with AmplideX One Reporter software version 1.0.11 with the AmplideX Nanopore Carrier Plus Analysis Module version 1.0.5 (Asuragen). The software detects all variants that differ from the reference sequence (GRCh38) within the designed amplicon regions and annotates variants based on a static ClinVar database (*http://www.ncbi.nlm.nih.gov/clinvar*, updated March 17, 2025).

Data Analysis

Sequencing results were compared with those obtained with PCR- and capillary electrophoresis (CE)–based orthogonal methods. These included using the AmplideX PCR/CE CFTR Kit (catalog number A00520) reagents for *CFTR,* AmplideX PCR/CE SMN1/2 Plus Kit (catalog number A00054) reagents for *SMN1/2*, and AmplideX PCR/CE FMR1 (catalog number 49402) reagents for *FMR1*, all from Asuragen.

Results

CFTR

Analytical accuracy was assessed by comparing results with the orthogonal method. Samples tested included 17 that were heterozygous for a pathogenic *CFTR* variant, 4 that were either homozygous ($n = 1$) or compound heterozygous ($n = 3$) for *CFTR* pathogenic variants, and 52 wild-type samples. These samples represented 14 unique *CFTR* pathogenic variants detected by the orthogonal method (Table 1 and Supplemental Figure S1).

Long-read sequencing with the AmplideX Carrier Plus kit reagents produced results that were 100% concordant with the orthogonal method for samples with two wild-type alleles ($n = 52$) or two pathogenic variants ($n = 4$). However, the concordance for heterozygous samples carrying a single pathogenic variant was 88% (Table 2).

This discrepancy arose from two patient samples reported by the orthogonal method to carry the c.1853T>C variant but actually carried the c.1972dup variant, which was correctly identified with the PCR/Nanopore assay (Supplemental Figure S1A). Importantly, the c.1972dup allele was not part of the variant panel used in the orthogonal method. Subsequent analysis revealed that the c.1972dup allele was linked to a 9-bp deletion (c.1977_1985del), which lies within the primer binding sites for the c.1853T>C allele for the orthogonal method, thus shortening the size of the expected wild-type PCR product size, causing it to be incorrectly called as the c.1853T>C allele (Supplemental Figure S1B).

In addition to evaluation of *CFTR* pathogenic variants, the accuracy of the poly-T status being called for each allele was assessed. The poly-T tract is a string of thymidine bases in intron 9 of *CFTR* that affects the splicing of exon 10. The 7T and 9T variants produce normal splicing, whereas 5T causes the skipping of exon 10, which leads to a reduction in functional *CFTR* protein.[17] Although previously thought to not have a clinical impact, more studies are showing that the presence of the 5T allele when inherited in *trans* with a pathogenic *CFTR* variant can manifest in a spectrum of clinical consequences, most commonly CF-related disorders.[18,19] Results showed 100% concordance of poly-T status for 5T, 7T, and 9T variants (Table 3).

SMN1/2

Results showed 100% concordance for all *SMN1* and *SMN2* copy numbers (Tables 4 and 5).

Table 1 *CFTR* Variants Detected

HGVS (cDNA)	HGVS (protein)	Legacy
c.1521_1523del	p.Phe508del	F508del
c.1519_1521del	p.Ile507del	I507del
c.1585-1G>A	N/A	1717-1G>A
c.1624G>T	p.Gly542X	G542X
c.1652G>A	p.Gly551Asp	G551D
c.2657+5G>A	N/A	2789+5G>A
c.2988+1G>A	N/A	3120+1G>A
c.3140-26A>G	N/A	3272-26A>G
c.350G>A	p.Arg117His	R117H
c.3528del	p.Lys1177SerfsX15	3659delC
c.3744del	p.Lys1250ArgfsX9	3876delA
c.3909C>G	p.Asn1303Lys	N1303K
c.4046G>A	p.Gly1349Asp	G1349D
c.1972dup*	p.Arg658LysfsX7	2104insA

*The c.1972dup was not able to be detected with the orthogonal method.

HGVS, Human Genome Variation Society.

Table 2 *CFTR* Genotyping Accuracy

	Orthogonal data			
PCR Nanopore	WT/WT	Mut/WT	Mut/Mut	Overall
WT/WT	52	0	0	52/52
Mut/WT	0	15*	0	15/17*
Mut/Mut	0	0	4	4/4

*The c.1972dup allele was not able to be detected by the orthogonal method; these two samples were called c.1853T>C by PCR/capillary electrophoresis. See Supplemental Figure S1.

Mut, mutant; WT, wild type.

This assay does not distinguish between copy numbers 3 and 4 but rather pools these into a single call of ≥3. Although detecting silent carrier status via phasing is not possible with this assay, the concordance for detecting the SNPs associated with silent carrier status was also tested (*SMN1* c.*3+80T>G and c.*211_*212del). Although orthogonal SNP data were not available for all samples, results showed that SNP detection was 100% concordant with previous results, with 11 c.*3+80T>G and 9 c.*211_*212del alleles being accurately detected (Table 6).

FMR1

Concordance for *FMR1* CGG repeat sizes was 100% within one repeat of the orthogonal method. Long-read sequencing accurately called 11 premutation and 6 full mutation alleles, both heterozygous carrier females and hemizygous males (Figure 1). This assay was also able to detect the number and location of AGG repeats within each allele (Supplemental Figure S2, A and B). Although the orthogonal method does provide information about the presence or absence of AGG interruptions, as indicated by gaps in the dragon tail on an electropherogram (Supplemental Figure S2, C and D), it is not able to consistently quantitate the interruptions or provide phasing. Therefore, for result comparison, presence or absence of AGG repeats was compared. For the samples for which orthogonal AGG interruption data were available, 100% concordance was found: there were 63 cases with AGG interruptions and 6 cases without (Table 7).

The test's ability to detect *FMR1* mosaicism was assessed by generating artificial mosaic samples, where female DNA with premutation and full mutation alleles was diluted with female DNA with normal repeat sizes. The PCR/Nanopore assay was able to detect the full mutation allele down to 5%. Interestingly, the premutation allele was detected at 1% but not 5%, perhaps because of poor sample amplification. Ultimately, these data suggest that the PCR/Nanopore assay can accurately detect *FMR1* mosaicism with confidence down to 10% (Figure 2A). The Coriell cell line NA20241 (Coriell Institute for Medical Research, Camden, NJ) (VCU70) was also included, which is reported to have 29 repeats in allele 1 and is considered to be mosaic for 93 to 110 repeats for allele 2, as a consensus could not be reached among nine laboratories testing the sample.[20] The PCR/Nanopore assay detected repeat sizes of 29 repeats for allele 1 and three premutation sizes (90, 113, and 117) for allele 2, suggesting that this sample does have mosaic premutation alleles (Figure 2B).

Table 3 *CFTR* Poly-T Accuracy

	Orthogonal data			
PCR Nanopore	9T	7T	5T	Overall
9T	27	0	0	27/27
7T	0	111	0	111/111
5T	0	0	8	8/8

Table 4 *SMN1* Copy Number Accuracy

	Orthogonal data				
PCR Nanopore	0	1	2	≥3	Overall
0	2	0	0	0	2/2
1	0	21	0	0	21/21
2	0	0	38	0	38/38
≥3	0	0	0	10	10/10

Interrun and Intrarun Precision

Sixteen samples were selected to test precision (Supplemental Table S1). These 16 samples were run with both mix A and mix B in three separate runs completed on different days by different users to assess interrun precision. Two of the 16 samples were also selected to test intrarun precision and were run in triplicate on two different days. Results obtained were highly reproducible between the interrun and intrarun repeats for all four genes tested.

Discussion

Carrier screening has become common for individuals and couples who want to know their risk of having a child with an autosomal recessive or X-linked condition. As the number of individuals who participate in carrier screening increases, so does the need to be able to detect rare variants in the genes being screened. Traditional carrier screening has involved targeted genotyping for the most commonly reported pathogenic variants in a given gene. Although this approach is effective for identifying carriers in certain

Table 5 *SMN2* Copy Number Accuracy

	Orthogonal data				
PCR Nanopore	0	1	2	≥3	Overall
0	7	0	0	0	7/7
1	0	24	0	0	24/24
2	0	0	31	0	31/31
≥3	0	0	0	9	9/9

Table 6 *SMN1* Silent Carrier SNP Detection

	Orthogonal data				
PCR Nanopore	c.*3+80T>G present	c.*3+80T>G absent	c.*211_212del present	c.*211_212del absent	Overall
c.*3+80T>G present	11	0	0	0	11/11
c.*3+80T>G absent	0	58	0	0	58/58
c.*211_212del present	0	0	9	0	9/9
c.*211_212del absent	0	0	0	41	41/41

SNP, single-nucleotide polymorphism.

populations, an inclusive, population-wide approach necessitates the ability to detect rare variants, which can be accomplished by either expanding the size of the targeted panel or sequencing the entire gene. Here, the performance characteristics of a long-read sequencing assay for the detection of pathogenic variants and poly-T status in *CFTR*, variants and exon 7 copy number in *SMN1* and *SMN2*, and repeat size in *FMR1* for carrier screening for CF, SMA, and FXS, respectively, were evaluated.

American College of Medical Genetics and Genomics carrier screening guidelines for *CFTR* were revised and expanded in 2023 to include 100 recommended variants.[4] Although this updated list captures variants for 95% of the total population carrier frequency for multiple ethnicities, full gene sequencing still offers significant advantages over targeted genotyping, particularly as the list of recommended pathogenic variants for testing continues to evolve. Full gene sequencing effectively future proofs this assay, minimizing the need for frequent updates in response to evolving clinical guidelines. Importantly, it also allows for the detection of rare variants that are not covered on traditional screening panels because of low population

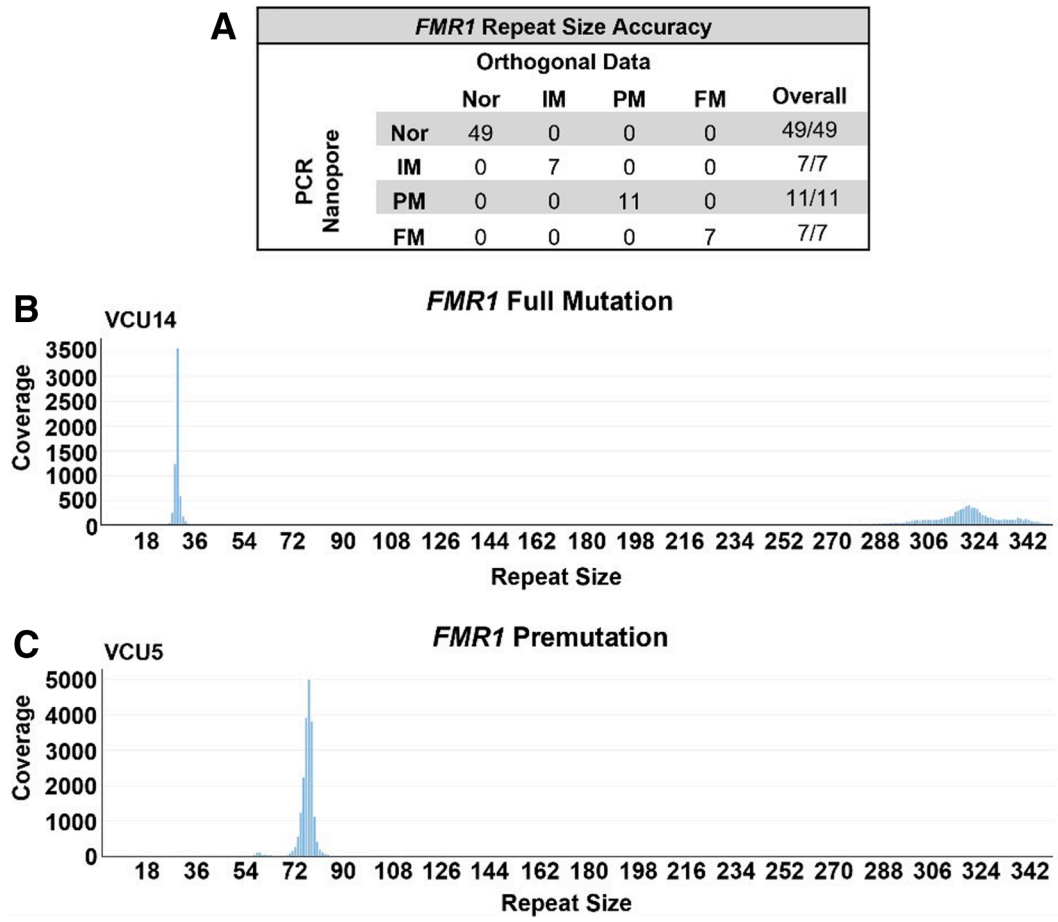


Figure 1 *FMR1* concordance results. **A:** Concordance results for *FMR1* repeat sizing. **B** and **C:** Repeat size plots generated by AmplideX One Reporter software version 1.0.11 with the AmplideX Nanopore Carrier Plus Analysis Module version 1.0.5 (Asuragen) for a female full mutation carrier (**B**) and a male hemizygous for a premutation (**C**). Normal (Nor): <45 repeats; intermediate (IM): 45 to 54 repeats; premutation (PM): 55 to 200 repeats; full mutation (FM): >200 repeats.

Table 7 Concordance Results for AGG Interruption Detection in *FMR1*

	Orthogonal data		
PCR Nanopore	Yes	No	Overall
Yes	63	0	63/63
No	0	6	6/6

frequency. In this study, two unrelated individuals who had previously been reported as carriers for the c.1853T>C variant were identified; however, both were, in fact, carriers for the c.1972dup allele, which was not included on the panel used in the orthogonal method. This discrepancy points to the strength of full exon sequencing over targeted/fixed content testing, as it can detect rare variants not included on the standard panel and overcomes a limitation of PCR/CE-based methods whereby benign DNA alterations can impact test results. Although both alleles are pathogenic, meaning the clinical consequences are the same, if the c.1972dup allele was not linked to the 9-bp deletion that resulted in the c.1853T>C allele being called by the orthogonal assay, neither of these patients would have been identified as carriers.

Comprehensive sequencing of *CFTR* is valuable not only for carrier screening, but also for diagnostic evaluation in individuals with suspected CF. In this study, the assay demonstrated reliable detection of pathogenic *CFTR* variants in both homozygous and compound heterozygous states, as well as poly-T and TG sizing. For carrier screening, poly-T status has primarily been reported in the context of the R117H variant.[21] However, emerging evidence indicates that a 5T variant occurring in *trans* with a pathogenic *CFTR* variant can result in a spectrum of clinical consequences, ranging from asymptomatic to classic CF.[18,19,22] These findings underline that identification and reporting of poly-T status can be beneficial for individuals undergoing a diagnostic CF workup. However, like other assays, poly-T tract phasing cannot be determined in this assay as the poly-T tract is on a different amplicon.

One advantage of long-read sequencing is the ability to sequence across complex regions of the genome that would be nearly impossible to capture with short-read next-generation sequencing because of its read length. Because of the sequence similarity between *SMN1* and *SMN2*, whole gene sequencing for these genes has proven difficult and, as such, carrier testing has historically been targeted to testing for *SMN1* exon 7 copy number. SMA is caused by homozygous deletion of exon 7 in *SMN1* in 95% of cases, but the remaining cases are caused by pathogenic variants in *SMN1*.[5] Long-read sequencing overcomes previous sequencing limitations and allows for complex regions of the genome, such as

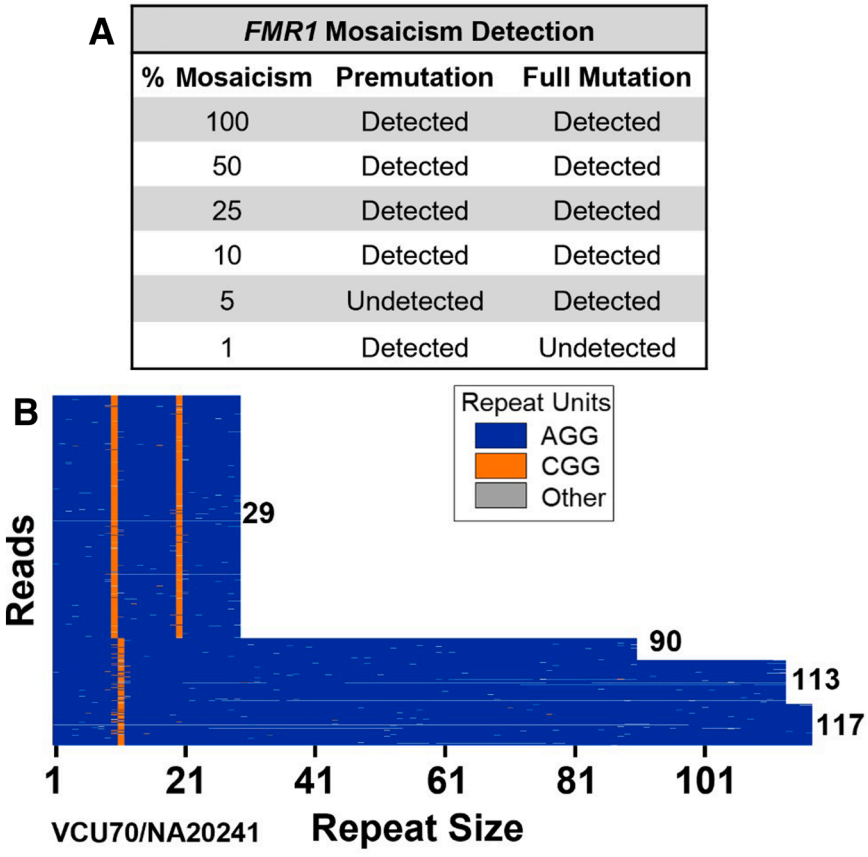


Figure 2 Mosaicism detection in *FMR1*. **A:** Detection of premutations and full mutations from 100% to 1% mosaicism. **B:** AGG repeat sizes and CGG interruptions for sample VCU70/Coriell cell line NA40421.

SMN1/2, to be sequenced and, therefore, allows for the detection of rare pathogenic variants that would remain undetected with traditional testing methods. Although no cases with rare pathogenic variants were available for inclusion in this study, the assay is designed to enable their detection. This assay was reliable for providing both *SMN1* and *SMN2* exon 7 copy number. As discussed above, with this method, phasing of exon 7 is not available, meaning that silent carriers, harboring two copies of *SMN1* in *cis*, are not able to be identified. However, the gene duplication SNPs can be assessed to inform the residual risk of silent carrier status. If the minor allele for the haplotype tagging SNP(s) for silent carrier are present, they will be reported (*SMN1* c.*3+80T>G and c.*211_*212del). Although the presence of these SNPs in an individual with two copies of *SMN1* exon 7 increases their residual risk of being an SMA carrier, their absence does not rule out carrier risk.[23] Thus, haplotype tagging SNP information can aid in the identification of silent carriers; however, phasing of *SMN1* copy number is a more reliable method for identifying these individuals, especially those who are negative for the SNPs. The assay also can detect *SMN2* disease modifier variants c.859G>C and c.835-44A>G; however, this was not assessed in the current study. In the present study, *SMN1* or *SMN2* copy numbers of 3 and above were grouped for analysis. This binning has limited implications for *SMN1*, as copy number 4 alleles are infrequently reported and, therefore, there are limited data on their impact on the adjusted risk of being a carrier.[24]

The trinucleotide expansion found in premutations and full mutations in *FMR1* has also previously proven difficult to sequence with limited read lengths. The PCR/Nanopore assay accurately called premutations and full mutations in hemizygous males and heterozygous females, with the additional advantage of being able to detect both the number and location of any AGG interruptions. The presence of AGG interruptions within an *FMR1* premutation have been shown to stabilize the allele and impact its expansion in subsequent generations.[8] In the PCR/CE method (orthogonal method), the AGG interruptions can be inferred visually in the electropherogram. However, for individuals with two X chromosomes, it is not always possible to unambiguously determine which allele harbors the interruptions. This limitation becomes particularly relevant for carriers of a premutation, as the AGG interruptions must be present on the premutation allele to impact its expansion; thus, the inability to phase the interruptions undermines the clinical utility of the result. In contrast, the PCR/Nanopore assay not only includes AGG interruption data in the software output but also provides location and phasing information, allowing assignment of interruptions to the correct allele. Furthermore, subtle differences in interruption positions between alleles, such as one occurring at the 11th repeat, and the other on the second X chromosome occurring at the 13th repeat, may have peaks that are indistinguishable by CE, but are able to be resolved with nanopore sequencing. Despite the more precise visualization of AGG interruptions, one limitation of the CGG calling algorithm in the current version of the AmplideX software is its inability to reliably distinguish between specimens that are homozygous and ones that are heterozygous with the two alleles only separated by one repeat. Although visual inspection of the output plot may allow for this distinction in some cases, the software does not consistently make these calls. Although this does not have clinical implications, it could impact the accuracy of the result. For full mutations, there is variability in the software output depending on read count and quality. The size may be reported as >200 CGG repeats or the specific number, if there is high confidence in the repeat sizing. This is in contrast to the CE method, which only reports >200, as opposed to a specific size.

There are reported cases of fragile X syndrome that exhibit mosaicism.[12] This study shows that mosaic premutation and full mutation alleles can be identified and at low levels of mosaicism that might be missed by other technologies. Coriell sample NA20241 is reported to carry 29 repeats on allele 1 and 93 to 110 repeats on allele 2 and is considered to be mosaic for a premutation allele.[20] Subsequent studies using long-read sequencing confirmed this suspected mosaicism. Sequencing using the PacBio platform from different institutions has shown this sample is mosaic for 90 and 120 repeat alleles[25] or 89 and 112 repeat alleles.[26] Meanwhile, an Oxford Nanopore Technologies–based assay indicated the premutation repeat sizes were 92 and 113.[27] These results showed bimodal distribution with mosaicism for two premutation allele sizes. In concordance with previous studies using sequencing methods,[25–27] this study proved robust for detecting a 29 repeat allele and mosaicism for three premutation alleles with 90, 113, and 117 repeats.

Conclusions

This study showed the validation of a robust, accurate, and reproducible assay using PCR/Nanopore sequencing technology. This assay can accurately detect carrier status across a variety of genes and allows for the detection of more pathogenic variants than traditional panel-based genotyping assays. It has the ability to pool samples and sequence multiple genes in a single workflow, at a cost that is comparable to running these assays individually.

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Supplemental Data

Supplemental material for this article can be found at *https://doi.org/10.1016/j.jmoldx.2026.03.003*.

References

1. Gregg AR, Aarabi M, Klugman S, Leach NT, Bashford MT, Goldwaser T, Chen E, Sparks TN, Reddi HV, Rajkovic A, Dungan JS: 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, 23: 1793−1806
2. American College of Obstetricians and Gynecologists Committee on Genetics: Committee opinion no. 690 summary: carrier screening in the age of genomic medicine. Obstet Gynecol 2017, 129:595−596
3. Ontario Health: Carrier screening programs for cystic fibrosis, fragile X syndrome, hemoglobinopathies and thalassemia, and spinal muscular atrophy: a health technology assessment. Ont Health Technol Assess Ser 2023, 23:1
4. Deignan JL, Gregg AR, Grody WW, Guo MH, Kearney H, Monaghan KG, Raraigh KS, Taylor J, Zepeda-Mendoza CJ, Ziats C: Updated recommendations for CFTR carrier screening: a position statement of the American College of Medical Genetics and Genomics (ACMG). Genet Med 2023, 25:100867
5. Keinath MC, Prior DE, Prior TW: Spinal muscular atrophy: mutations, testing, and clinical relevance. Appl Clin Genet 2021, 14:11−25
6. Alías L, Bernal S, Calucho M, Martínez E, March F, Gallano P, Fuentes-Prior P, Abuli A, Serra-Juhe C, Tizzano EF: Utility of two SMN1 variants to improve spinal muscular atrophy carrier diagnosis and genetic counselling. Eur J Hum Genet 2018, 26:1554−1557
7. Luo M, Liu L, Peter I, Zhu J, Scott SA, Zhao G, Eversley C, Kornreich R, Desnick RJ, Edelmann L: An Ashkenazi Jewish SMN1 haplotype specific to duplication alleles improves pan-ethnic carrier screening for spinal muscular atrophy. Genet Med 2014, 16:149−156
8. Yrigollen CM, Durbin-Johnson B, Gane L, Nelson DL, Hagerman R, Hagerman PJ, Tassone F: AGG interruptions within the maternal FMR1 gene reduce the risk of offspring with fragile X syndrome. Genet Med 2012, 14:729−736
9. Jacquemont S, Hagerman RJ, Leehey MA, Hall DA, Levine RA, Brunberg JA, Zhang L, Jardini T, Gane LW, Harris SW, Herman K, Grigsby J, Greco CM, Berry-Kravis E, Tassone F, Hagerman PJ: Penetrance of the fragile X-associated tremor/ataxia syndrome in a premutation carrier population. JAMA 2004, 291:460−469
10. Hagerman RJ, Leehey M, Heinrichs W, Tassone F, Wilson R, Hills J, Grigsby J, Gage B, Hagerman PJ: Intention tremor, parkinsonism, and generalized brain atrophy in male carriers of fragile X. Neurology 2001, 57:127−130
11. Allingham-Hawkins DJ, Babul-Hirji R, Chitayat D, Holden JJA, Yang KT, Lee C, et al: Fragile X premutation is a significant risk factor for premature ovarian failure: the International Collaborative POF in Fragile X Study—Preliminary data. Am J Med Genet 1999, 83:322
12. Nolin SL, Glicksman A, Houck GE, Brown WT, Dobkin CS: Mosaicism in fragile X affected males. Am J Med Genet 1994, 51:509−512
13. Jain M, Olsen HE, Paten B, Akeson M: The Oxford Nanopore MinION: delivery of nanopore sequencing to the genomics community. Genome Biol 2016, 17:239
14. Li S, Hua R, Han X, Xu Y, Li M, Gao L, Ma R, Meng W, Mao A, Wang J, Wang Y: Targeted long-read sequencing facilitates effective carrier screening for complex monogenic diseases including spinal muscular atrophy, [alpha]-/[beta]-thalassemia, 21-hydroxylase deficiency, and fragile-X syndrome. J Transl Med 2025, 23:307
15. Xia Z, Deng Q, Hu P, Gao C, Jiang Y, Zhou Y, Guo Q: Fragile X syndrome carrier screening using a nanopore sequencing assay. J Mol Diagn 2025, 27:645−656
16. Long J, Cui D, Yu C, Meng W: Evaluating the clinical efficacy of a long-read sequencing-based approach for carrier screening of spinal muscular atrophy. Hum Genomics 2024, 18:110
17. Chu CS, Trapnell BC, Curristin S, Cutting GR, Crystal RG: Genetic basis of variable exon 9 skipping in cystic fibrosis transmembrane conductance regulator mRNA. Nat Genet 1993, 3:151−156
18. Celestino-Soper PBS, Simpson E, Tumbleson Brink D, Lynnes TC, Dlouhy S, Vatta M, Yeley J, Brown C, Bai S: Intragenic CFTR duplication and 5T/12TG variant in a patient with non-classic cystic fibrosis. Sci Rep 2016, 6:38776
19. Tosco A, Castaldo A, Colombo C, Claut L, Carnovale V, Iacotucci P, Lucarelli M, Cimino G, Fabrizzi B, Caporelli N, Majo F, Ciciriello F, Padoan R, Poli P, Taccetti G, Centrone C, Casciaro R, Castellani C, Salvatore D, Colangelo C, Bonomi P, Castaldo G, Terlizzi V: Clinical outcomes of a large cohort of individuals with the F508del/5T;TG12 CFTR genotype. J Cystic Fibrosis 2022, 21:850−855
20. Wilson JA, Pratt VM, Phansalkar A, Muralidharan K, Highsmith WE, Beck JC, Bridgeman S, Courtney EM, Epp L, Ferreira-Gonzalez A, Hjelm NL, Holtegaard LM, Jama MA, Jakupciak JP, Johnson MA, Labrousse P, Lyon E, Prior TW, Richards CS, Richie KL, Roa BB, Rohlfs EM, Sellers T, Sherman SL, Siegrist KA, Silverman LM, Wiszniewska J, Kalman LV; The Fragile Xperts Working Group of the Association for Molecular Pathology Clinical Practice Committee: Consensus characterization of 16 FMR1 reference materials: a consortium study. J Mol Diagn 2008, 10:2−12
21. Moskowitz SM, Chmiel JF, Sternen DL, Cheng E, Gibson RL, Marshall SG, Cutting GR: Clinical practice and genetic counseling for cystic fibrosis and CFTR-Related disorders. Genet Med 2008, 10: 851−868
22. Nykamp K, Truty R, Riethmaier D, Wilkinson J, Bristow SL, Aguilar S, Neitzel D, Faulkner N, Aradhya S: Elucidating clinical phenotypic variability associated with the polyT tract and TG repeats in CFTR. Hum Mutat 2021, 42:1165
23. Milligan JN, Blasco-Pérez L, Costa-Roger M, Codina-Solà M, Tizzano EF: Recommendations for interpreting and reporting silent carrier and disease-modifying variants in SMA testing workflows. Genes (Basel) 2022, 13:1657
24. Hendrickson BC, Donohoe C, Akmaev VR, Sugarman EA, Labrousse P, Boguslavskiy L, Flynn K, Rohlfs EM, Walker A, Allitto B, Sears C, Scholl T: Differences in SMN1 allele frequencies among ethnic groups within North America. J Med Genet 2009, 46: 641−644
25. Tsai Y-C, Greenberg D, Powell J, Höijer I, Ameur A, Strahl M, Ellis E, Jonasson I, Pinto RM, Wheeler VC, Smith ML, Gyllensten U, Sebra R, Korlach J, Clark TA: Amplification-free, CRISPR-Cas9 targeted enrichment and SMRT sequencing of repeat-expansion disease causative genomic regions. BioRxiv 2017, [Preprint], https://doi.org/10.1101/203919
26. Liang Q, Liu Y, Liu Y, Duan R, Meng W, Zhan J, Xia J, Mao A, Liang D, Wu L: Comprehensive analysis of fragile X syndrome: full characterization of the FMR1 locus by long-read sequencing. Clin Chem 2022, 68:1529−1540
27. Grosso V, Marcolungo L, Maestri S, Alfano M, Lavezzari D, Iadarola B, Salviati A, Mariotti B, Botta A, D'Apice MR, Novelli G, Delledonne M, Rossato M: Characterization of FMR1 repeat expansion and intragenic variants by indirect sequence capture. Front Genet 2021, 12:743230