EVALUATION OF AUTOMATIC CLASS III DESIGNATION FOR
AmplideX® Fragile X Dx & Carrier Screen Kit
DECISION SUMMARY

A. DEN Number:

DEN190023

B. Purpose for Submission:

De Novo request for evaluation of automatic class III designation for the AmplideX® Fragile X Dx & Carrier Screen Kit

C. Measurands:

Trinucleotide (CGG) repeats in the FMR1 gene

D. Type of Test:

Polymerase chain reaction (PCR)-based nucleic acid amplification followed by size resolution with capillary electrophoresis

E. Applicant:

Asuragen, Inc.

F. Proprietary and Established Names:

AmplideX® Fragile X Dx & Carrier Screen Kit

G. Regulatory Information:

1. Regulation section:

   21 CFR 866.5970

2. Classification:

   Class II

3. Product code(s):

   OYV

4. Panel:

   Immunology (82)

H. Indications for use:

1. Indications for use:
   The AmplideX® Fragile X Dx & Carrier Screen Kit is an in vitro diagnostic device that uses polymerase chain reaction (PCR) and capillary electrophoresis to detect and identify the number of cytosine-guanine-guanine (CGG) repeats in the fragile X mental retardation-1 (FMR1) gene using genomic DNA isolated from peripheral whole blood specimens. It is solely intended as an aid in the post-natal diagnosis of fragile X syndrome, and fragile X-associated disorders [i.e., fragile X-associated tremor/ataxia syndrome (FXTAS) or fragile X-associated primary ovarian insufficiency (FXPOI)], and for carrier testing in adults of reproductive age. Assay results are solely intended to be interpreted by healthcare professionals who are board certified in molecular genetics and to be used in conjunction with other clinical and diagnostic findings, consistent with professional standards of practice. Reflex testing, clinical genetic evaluation, and genetic counseling should be offered as appropriate. The test is for use on the 3500Dx Series Genetic Analyzer.

   This test is not indicated for use for fetal diagnostic testing, newborn screening or for stand-alone diagnostic purposes.

2. Special conditions for use statement(s):

   For prescription use

   For *in vitro* diagnostic use

3. Special instrument requirements:

   Applied Biosystems® 3500 Dx Series Genetic Analyzer (k191030) using the AmplideX Fragile X Reporter Software v 1.0.

I. Device Description:

The AmplideX Fragile X Dx & Carrier Screen Kit (hereafter referred to as the AmplideX Kit) includes reagents sufficient for 100 reactions and are for use on the Applied Biosystems® 3500 Dx Series Genetic Analyzer (8 and 24 capillary) with AmplideX reporter software. A description of the reagents and the storage conditions are shown in Table 1 below.

Table 1. Components of the AmplideX Kit

Item	Storage Temperature
GC-Rich Amp Buffer	-15 to -30°C
GC-Rich Polymerase Mix	-15 to -30°C
ROX 1000 Size Ladder	-15 to -30°C
Diluent	-15 to -30°C
Fragile X Positive Control	-15 to -30°C
Fragile X Primer Mix	-15 to -30°C

Additional reagents required but not provided with the kit:

- Reagents for isolation and purification of genomic DNA (gDNA)
- POP-7 ™ Polymer
- Hi-Di™ Formamide
- DS-30 Matrix Standard Kit (Dye Set D)

J. Standard/Guidance Document Referenced (if applicable):

Guidance for Industry and FDA Staff: Guidance for the Content of Premarket Submissions for Software Contained in Medical Devices; May 11, 2005

General Principles of Software Validation; Final Guidance for Industry and FDA Staff; January 11, 2002

Guidance for Industry - Cybersecurity for Networked Medical Devices Containing Off-the- Shelf (OTS) Software; January 14, 2005

Guidance for Industry, FDA Reviewers and Compliance on Off-the-Shelf Software Use in Medical Devices; September 9, 1999

CLSI Standard EP05-A3, Evaluation of Precision Performance of Quantitative Measurement Methods; Approved Guideline – Second Edition.

CLSI Standard EP07-A2, Interference Testing in Clinical Chemistry, Approved Guideline – Second Edition.

CLSI Standard EP17-A2, Protocols for the Determination of Limits of Detection and Limits of Quantification, Approved Guideline – Second Edition.

CLSI Standard EP25-A, Evaluation of Stability on In Vitro Diagnostic Reagents, Approved Guideline.

K. Test Principle:

Fragile X syndrome (FXS) and Fragile X associated disorders are inherited genetic conditions characterized by expanded cytosine-guanine-guanine (CGG) nucleotide repeats in the 5' untranslated region of the *FMR1* gene. Expansion of the repeats is associated with hypermethylation and inactivation of gene expression and subsequent loss of protein expression. This expansion can lead to a variety of consequences depending on the length of the CGG expansion. The AmplideX Fragile X Dx & Carrier Screen Kit quantifies the number of CGG repeats in the alleles of a given sample of purified gDNA using polymerase chain reaction (PCR) followed by size resolution with capillary electrophoresis (CE).

Human gDNA is isolated from EDTA-treated whole human blood (fresh or stored at 2 to 8 °C for up to 14 days) using a DNA extraction kit capable of gDNA purity $A_{260/280}$ range of 1.6–2.0. A total DNA input of 20-80ng is needed to run the reaction. Purified gDNA is first added to a PCR reaction well containing a Master Mix with the GC-Rich Amp Buffer, GC-Rich Polymerase Mix, Fragile X Primer Mix, and either a specimen or control (Fragile X Diluent or Fragile X

Positive Control) in a total of 13 µL. After thermal cycling, unpurified PCR products are directly mixed with Hi-Di™ Formamide and ROX 1000 Size Ladder. Following denaturation, amplicons are resolved on an Applied Biosystems® 3500 Dx Genetic Analyzer running POP-7™ polymer and analyzed using the AmplideX Fragile X Reporter.

Calibration

The ROX 1000 Size Ladder included in the kit is added to each sample to allow for proper fragment sizing analysis by the AmplideX® Fragile X Reporter. Spectral calibration of the 3500 Dx instrument with DS-30 Matrix Standard Kit is required in order to properly detect and analyze samples containing ROX dye and is performed prior to the assay run.

Quality Control

The AmplideX Fragile X Reporter performs a series of QC checks on the batch controls No Template control (NTC) and Positive Control (PC) as well as on the individual samples. The software assesses three general modalities of failure: Ladder Error (LD), Low Signal (LS), and Contamination (CT). If one or more of these failures occurs in the positive control, it invalidates the run as a Batch QC failure. The NTC is expected to give a Low Signal (LS) error.

Controls

Fragile X Positive Control and Diluent (for use as an NTC) are included in the kit. A single positive and no template control must be included in every run for the analysis software to interpret sample results correctly. The NTC should not generate any peak calls from the Reporter output (Figure 5). The Positive Control includes alleles at 18 ±1, 30 ±1, 32 ±1, 56 ±1, 85 ±3, 114 ±3, and > 200 CGG repeats, and yields a full mutation genotype (Figure 1).

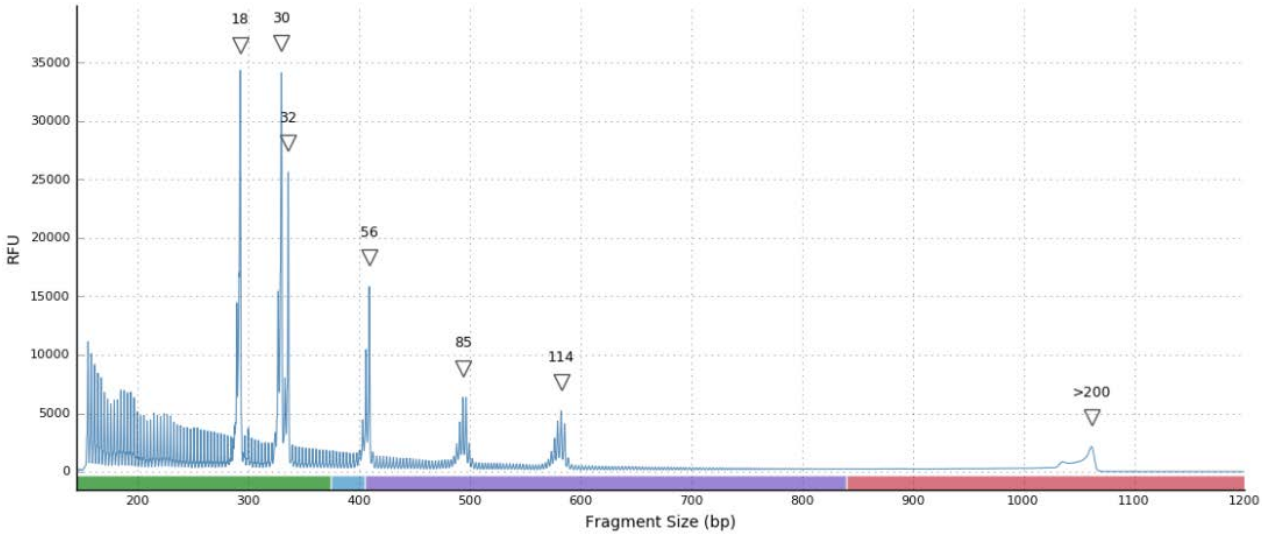

Figure 1. An electropherogram of a positive control (PC)

For a valid run, the AmplideX Fragile X Reporter output will identify each of these alleles within the expected precision range (Table 2) for their respective CGG repeat length.

Table 2. CGG Repeat Size Precision

CGG Repeat Range	Precision
1–70	±1
71–120	±3
121–199	±5%
≥ 200	N/A

Interpretation of Results

AmplideX Fragile X Reporter software provides automated analysis of the resulting electropherogram to identify and size *FMR1* alleles. It then categorizes the genotype of the sample into one of the following categories based on the size of the repeats as a Normal (NOR), Intermediate (INT), Premutation (PM), or Full mutation (FM) as shown in Table 3:

Table 3. *FMR1* CGG repeat length ranges and their corresponding genotypes

Genotype	Size (CGG) repeats
Normal	1-44
Intermediate	45-54
Premutation	55-199
Full Mutation	≥200

Individuals with normal (< 45 CGG repeats) or intermediate (45–54 CGG) FMR1 alleles are currently thought to be asymptomatic for disorders associated with the FMR1 gene. However, individuals who are carriers of a premutation allele (55–199 CGG) are at risk for passing on a full mutation to their offspring. This risk is proportional to the number of CGG repeats and to the AGG status of the expanded allele. Individuals with the FMR1 full mutation (≥ 200 CGG) typically have FXS (Monaghan et al. 2013). Since fragile X syndrome is an X-linked disorder, only mothers can pass on a full mutation to male offspring. For females, either father or mother can pass on the full mutation.

Premutation carriers can develop fragile X-associated tremor/ataxia syndrome (FXTAS) or fragile X-associated primary ovarian insufficiency (FXPOI) (Wheeler, A et al. 2017, Gallagher, A et al 2012, and Hantash, F et al. 2011). A general illustration of the biological significance of the repeat length is shown in Figure 2.

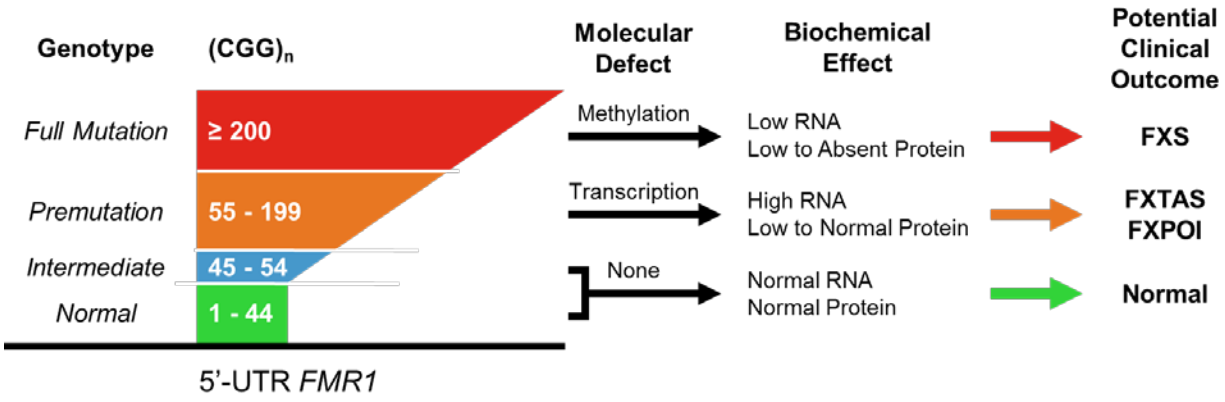

Figure 2. *FMR1* CGG repeat length ranges and their corresponding genotypes and outcomes.

Mosaicism

Mosaicism can result when a new genetic alteration arise after an embryo is formed. Both size mosaicism and methylation mosaicism occur in fragile X syndrome. The kit may also detect low levels of genetic mosaicism in CGG repeat length. The AmplideX® Fragile X Dx & Carrier Screen Kit reports all detected FMR1 alleles for each sample and determines the associated genotype category based on the longest detected allele. The assay does not distinguish between mosaic and non-mosaic alleles. The kit is not designed to detect or report methylation status.

Due to the challenges associated with size mosaicism, (e.g., due to both accurate or false results) when the assay detects more than one (1) peak for a male or more than two (2) peaks for a female, users are informed in the instructions for use to carefully review the electropherogram; and recommended to confirm the presence of size mosaicism and considered in conjunction with other laboratory and clinical information (e.g., sex of patient, diagnostic testing or carrier screening, patient symptoms) and should include consultation with a genetic professional..

L. Performance Characteristics:

1. Analytical performance:

   a. *Precision (Repeatability and Reproducibility)*
   The precision of the AmplideX Fragile X Kit was assessed based on both agreement for the categorical call (i.e., normal, intermediate, premutation, and full mutation) and for precision of the size of the CGG repeats (i.e., measurements of fragile X allele lengths). Within the precision studies, specific target criteria around CGG repeat length were applied, as shown in Table 2 above.

      i. Precision (3 site reproducibility)
      Between-laboratory precision, or reproducibility, was assessed using a panel of 11 samples consisting of 7 clinical whole blood specimens and 4 contrived samples that collectively represent allele sizes spanning all FXS genotype categories, including those at or near category boundaries. Contrived samples were genomic DNA extracted from cell lines with known gender, genotype, and expected allele sizes, spiked into leukocyte-depleted whole human blood. Table 4

describes the panel members.

Table 4. Sample Panel used in precision and reproducibility study

Sample ID	Sex	Source	Expected Number CGG Repeats	Genotype Category
ASGN-109	(b)(4)			Normal
ASGN-112				Normal
ASGN-005				Intermediate
ASGN-111				Intermediate
ASGN-103				Premutation
ASGN-113				Premutation
ASGN-101				Premutation
ASGN-016				Premutation
ASGN-018*				Full Mutation*
ASGN-023				Full Mutation
ASGN-104				Full Mutation

* ASGN-018 has a full-mutation mosaic allele at >200 repeats and therefore this is the call made by the software, a normal allele at 20 repeats, and a premutation allele at 197 repeats. Mosaic results that do not lead to categorical change not listed above.

The study was executed at three laboratories over 5 non-consecutive days utilizing a single kit lot, two operators per site, and a different 3500Dx instrument at each site. For each run, there were 6 PCR replicates per specimen. The study yielded 1980 total sample measurements excluding controls (3 sites x 2 operators x 6 replicates x 5 days = 180 replicates per sample). Of these, 30 replicates failed QC metrics (1.1% invalid rate) and were further evaluated for genotype category agreement and allele peak precision. The agreement was determined based upon the majority call.

The reproducibility of the assay for categorical call is shown in Table 5. For 10 of 11 samples, >97% genotype agreement was observed. Sample ASGN-005, ASGN-111 and ASGN-113 were not 100% due to the appearance of an extra allele which resulted in different categorical calls for 11 replicates because the assay software is designed to yield an interpretation based on the largest repeat fragment observed. These results were not observed for these samples in the lot-to-lot precision study. ASGN-018 has a full-mutation mosaic allele at >200 repeats, a normal allele at 20 repeats, and a premutation allele at 197 repeats. The mosaic allele was not found in 81/179 replicates. A limitation statement in the instructions for use alerts users to carefully review and confirm mosaic results.

Table 5. Three sites reproducibility– Categorical Genotype Agreement by Sample Excluding Invalids.

Sample	Genotype Category	Total number of Replicates (of 180)	Number of Replicates in Agreement	Agreement (%)	CI (95%)
ASGN-109	Normal	178	178	100	97.9, 100
ASGN-112	Normal	178	178	100	97.9, 100
ASGN-005	Intermediate	179	177	98.9	96, 99.7
ASGN-111	Intermediate	175	170	97.1	93.5, 98.8
ASGN-103	Premutation	178	178	100	97.9, 100
ASGN-113	Premutation	179	177	98.9	96, 99.7
ASGN-101	Premutation	174	174	100	97.8, 100
ASGN-016	Premutation	178	178	100	97.9, 100
ASGN-018	Full Mutation	179	98	54.7	47.4, 61.9
ASGN-023	Full Mutation	177	177	100	97.9, 100
ASGN-104	Full Mutation	175	175	100	97.9, 100

The reproducibility of the assay for allele size (number of repeats) is shown in Table 6 for each allele in the sample. Reproducibility was based on identifying the mode for each repeat. Replicates results were considered to agree if they fell within the precision specifications described in Table 2. As noted in the reproducibility for categorical calls, 11 replicates had artifacts and are shown below. The reproducibility of the expected allele lengths with the exception of the alleles identified as "mosaic" showed >98% reproducibility based on the pre-specified precision criteria. The samples with contaminants/artifacts are listed for information purposes.

Table 6. Three sites Reproducibility– Percent of Allele Replicates within Target Precision Range Excluding Invalids.

Sample ID	Mode Expected CGG Length (± Target Precision)	Measured CGG Length	No of replicates at that size	Total Number of replicates	% within Target Precision Range
ASGN -109	30 ± 1	29	1	178	100.0
		30	177		
ASGN-112	30 ± 1	30	177	178	100.0
		31	1		
	29 ± 1	29	177	178	100.0

Sample ID	Mode Expected CGG Length (± Target Precision)	Measured CGG Length	No of replicates at that size	Total Number of replicates	% within Target Precision Range
		30	1		
	Contamination/ artifact	34	1	1	0.6%
ASGN-005	29 ± 1	29	179	179	100.0
	45 ± 1	45	141	179	100.0
		46	38		
	Contamination/ artifact	> 200	2	2	1.1
ASGN-111	29 ± 1	29	173	175	100.0
		30	2		
	50 ± 1	50	87	175	98.9
		51	86		
		52	1		
		53	1		
	Contamination/ artifact	74	1	5	2.9
		> 200	4		
ASGN-103	30 ± 1	30	177	178	100.0
		31	1		
	56 ± 1	55	81	178	100.0
		56	96		
		57	1		
ASGN-113	30 ± 1	30	179	179	100.0
	103 ± 3	102	23	179	100.0
		103	82		
		104	73		
		105	1		
	Mosaic Result*	84	1	15	8.4
		88	7		
		89	7		
	Contamination/ artifact	>200	2	2	1.1
ASGN-101	35 ± 1	35	174	174	100
	93 ± 3	92	30	174	100.0
		93	132		

Sample ID	Mode Expected CGG Length (± Target Precision)	Measured CGG Length	No of replicates at that size	Total Number of replicates	% within Target Precision Range
		94	12		
	Mosaic result*	97	2	9	5.2
		98	7		
ASGN-016	18 ± 1	18	178	178	100.0
	114 ± 3	113	37	178	99.4
		114	115		
		115	23		
		116	2		
		119	1		
ASGN-018	20 ± 1	20	179	179	100.0
	198 ± 5% (10 CGG)	195	12	179	100.0
		196	20		
		197	57		
		198	65		
		199	22		
	Mosaic result	> 200	98	109	60.1
		184	1		
		183	1		
		182	2		
		181	6		
		180	1		
	Contamination/ artifact	30	1	2	1.1
		103	1		
ASGN-023	> 200	> 200	177	177	100.0
ASGN-104	24 ± 1	24	175	175	100.0
	> 200	> 200	175	1	100.0

*Represents potential mosaic allele. Mosaic results may be below the limit of detection for mosaicism.

ii. Precision (lot-to-lot reproducibility)

Within-laboratory precision was assessed running the same panel in the 3-site reproducibility study and described in Table 4. Each sample was tested in duplicate with 3 kit lots, 3 operators, and for over 12 non-consecutive days. The study was executed on 3 instruments and yielded 2376 total sample measurements excluding controls (2 replicates X 3 lots X 3 operators X 12 days = 216 per sample). Of the 2376 total sample measurements, 60 were invalid (Invalid rate 2.5%). Samples were evaluated for genotype category agreement and allele peak precision. The agreement was determined based upon the majority call. Excluding invalids, greater than >99%

categorical genotype agreement was observed for all samples as shown in Table 7.). Samples ASGN-112 and ASGN-016 were not 100% due to the appearance of third alleles (3 replicates) which resulted in different categorical calls. These results were not observed in the 3-site reproducibility result ASGN-018 has a full-mutation mosaic allele at >200 repeats, a normal allele at 20 repeats, and a premutation allele at 197 repeats. The mosaic allele was not found in 100/212 replicates. Allowing this sample to have a categorical call of a pre or full mutation results in 100% (212/212) agreement between replicates (95% CI: 98.2%-100%) and overall agreement of 99.9% (2313/2316, 95% CI: 99.6%-100%).In addition, as shown in Table 8, the reproducibility of the all alleles with the exception of the alleles identified as "mosaic" showed 100% reproducibility with the specifications). Precision across 3 lots was acceptable. Specimens with artifacts are listed for information purposes and calculated in the categorical section.

Table 7. Lot to lot reproducibility - Genotype Category Agreement by Sample Excluding Invalids.

Sample	Genotype Category	Total Number of Replicates from 216	Replicates in Agreement	Agreement (%)	CI (95%)
ASGN-109	Normal	200	200	100	98.1, 100
ASGN-112	Normal	214	213	99.5	97.4, 100
ASGN-005	Intermediate	209	209	100	98.2, 100
ASGN-111	Intermediate	213	213	100	98.2, 100
ASGN-103	Premutation	213	213	100	98.2, 100
ASGN-101	Premutation	212	212	100	98.2, 100
ASGN-113	Premutation	208	208	100	98.2, 100
ASGN-016	Premutation	213	211	99.1	96.6, 99.7
ASGN-018	Full Mutation	212	112	52.8	46.1, 59.4
ASGN-023	Full Mutation	212	212	100	98.2, 100
ASGN-104	Full Mutation	210	210	100	98.2, 100

Table 8: Lot to lot reproducibility – Percent of Allele Replicates within Target Precision Range Excluding Invalids.

Sample ID	Mode Expected CGG Length (± Target Precision)	Reported CGG Length	No of replicates at that size	Total Number of Replicates	% within Target Precision Range
ASGN-109	30 ± 1	29	4	200	100.0
		30	196		
ASGN-112	29 ± 1	28	2	214	100.0

Sample ID	Mode Expected CGG Length (± Target Precision)	Reported CGG Length	No of replicates at that size	Total Number of Replicates	% within Target Precision Range
		29	212		
	30 ± 1	29	2	214	100.0
		30	212		
	Contamination/ artifact	114	1	1	0.05
ASGN-101	35 ± 1	35	212	212	100.0
	92 ± 3	92	187	212	100
		93	24		
		94	1		
	Mosaic result*	96	10	22	10.4
		97	12		
	Contamination/ artifact	30	1	3	1.4
		55	1		
		79	1		
ASGN-005	29 ± 1	29	209	209	100.0
	45 ± 1	45	209		
ASGN-111	29 ± 1	29	213	213	100.0
	50 ± 1	50	208	213	100.0
		51	5		
ASGN-103	30 ± 1	30	213	213	100.0
	55 ± 1	55	207	213	100.0
		56	6		
ASGN-101	92 ± 3	92	187	212	100.0
		93	24		
		94	1		
ASGN-113	30 ± 1	30	208	208	100.0
	102 ± 3	101	5	208	100.0
		102	123		
		103	70		
		104	9		
		105	1		
	Mosaic result*	87	25	45	21.6
		88	20		
ASGN-016	18 ± 1	17	21	213	100.0
		18	192		
	113 ± 3	113	176	213	100.0
		114	32		
		115	4		
		116	1		
	Contamination/ artifact	> 200	2	2	9.4
ASGN-018	20 ± 1	19	3	212	100.0

Sample ID	Mode Expected CGG Length (± Target Precision)	Reported CGG Length	No of replicates at that size	Total Number of Replicates	% within Target Precision Range
		20	209		
	196 ± 5% (10 CGG)	195	19	212	100.0
		196	112		
		197	60		
		198	17		
		199	3		
		> 200	1		
	Mosaic allele	178	2	131	52.8
		179	6		
		180	5		
		181	5		
		182	1		
		>200	112		
ASGN-023	> 200	> 200	212	212	100.0
ASGN-104	24 ± 1	23	1	210	100.0
		24	209		
	> 200	> 200	210	210	100.0

*Represents potential mosaic allele. Mosaic results may be below the limit of detection for mosaicism.

iii. Precision (Mosaicism)

Mosaicism is well known for Fragile X and was observed in 6 panel members out of 11 in the precision study. These results were not present in 100% of the replicates. To assess the reproducibility at the claimed limit of detection for mosaicism, a mosaicism reproducibility/LOD study was conducted with minimal DNA input (see below in the Detection of Limit Section).

A sample panel representing different mosaic/major allele combinations was generated by mixing DNA from 7 clinical specimens of different repeat sizes. The targeted MAFs are at or higher than the estimated LoD. The samples were tested at the lowest total DNA input level (20 ng). Note, one clinical specimen used to create the panel was later determined to be an outlier due to reproducibly unusual peak pattern. Subsequently. panel members involving this specimen were removed from data analysis. Each panel member was tested by 3 lots of AmplideX Fragile X Dx & Carrier Screen Kit in duplicates by 2 operators across 3 days. The study was executed on 2 instruments (one 8-capillary and one 24-capillary) and yielded a total of 36 replicate measurements per specimen (2 replicates x 2 operators x 3 lots x 3 days = 36 replicates per sample). Greater than 95% hit rate were demonstrated for the claimed LoD listed in Table 9.

Table 9. Reproducibility of Mosaic Samples

Allele Combination		LOD (%MAF)	Valid Replicates (n)	% Detected	95% CI
Mosaic	**Major**				
Intermediate	Normal	2.0	34	100.0	90.0-100.0
Premutation	Normal	2.0	36	94.4	82.0-98.0
			36	100.0	90.0-100.0
Full Mutation	Normal	6.1	36	100.0	90.0-100.0
Full Mutation	Premutation (Low)	7.0	36	100.0	90.0-100.0
			35	100.0	90.0-100.0
	Premutation (High)	7.0	36	100.0	90.0-100.0
Premutation (Low)	Full Mutation	7.0	36	100	90.0-100.0
Premutation (High)		10.0	36	100	90.0-100.0
Premutation (Low)	Premutation (High)	5.0	34	97.1	85.0-99.0
			36	100.0	90.0-100.0
Premutation (High)	Premutation (Low)	5.0	36	100.0	90.0-100.0

iv. DNA extraction reproducibility/equivalence

Three common commercial DNA extraction method for extracting genomic DNA from whole human blood were assessed using a 5-member sample panel representing all FMR1 genotype categories. The methods evaluated included: (b) (4) solution precipitation, manual silica spin column, and manual magnetic bead. Each extraction method was performed [redacted] times per panel member. The extracted DNA was amplified in duplicate and analyzed on a single instrument.

All three methods yielded sufficient concentrations of DNA (on average) to meet the recommended input (b)(4) for the test kit. Categorical genotype agreement was 100% across all samples and 100% of all measured alleles were within target precision range. These results support the claim that the test can be used on gDNA generated using generally available DNA extraction methods capable of yielding at least 20ng/µL and A260-280 1.6-2.0.

v. Thermal Cycler equivalence

A 5-member sample panel representing all FMR1 genotype categories was amplified on three separate thermal cyclers and then sized on a single CE instrument. Four PCR runs were tested on each thermal cycler. In each PCR run

four replicates of each sample were tested.

For all sample replicates, across and within all thermal cycler types, genotype category calls were in 100% agreement. 100% of all identified independent alleles were within their respective target precision ranges. The study results support the use of commonly available commercial thermal cyclers that can run the specified protocol for the test kit.

b. Linearity/assay Reportable Range:

Not applicable.

c. Detection Limit

i. Limit of Detection

The *FMR1* gene is present on the X chromosome with one copy in males and two copies in females. The detection of the repeats is influenced by the DNA concentration and the size of the repeats. The "Limit of Detection" for detecting minor or mosaic alleles was assessed in combination with the reproducibility study.

The limit of detection (LoD) for mosaic alleles in different mosaic/major allele combinations were first estimated by testing mixed cell line DNA with known genotypes at 6 mosaic allele frequencies (MAF 10, 5, 2, 1, 0.1, and 0.01%). The allele frequency demonstrating >(b)(4) correct categorical call rate within (b)(4) replicates were identified. The estimated LoD were then confirmed by testing a panel of 30 samples representing different mosaic/major allele combinations. The panel was generated by mixing DNA from clinical specimens of different repeat sizes with targeted MAFs at or higher than the estimated LoD. The samples were tested at the lowest total DNA input level (20 ng). Note, one clinical specimen used to create the panel was later determined to be an outlier due to an unusual peak pattern. Subsequently 6 panel members involving this specimen were removed from data analysis. Each panel member was tested by 3 lots of AmplideX Fragile X Dx & Carrier Screen Kit in duplicates by 2 operators across 3 days. The study was executed on 2 instruments (one 8-capillary and one 24-capillary) and yielded a total of 36 replicate measurements per specimen (2 replicates X 2 operators x 3 lots x 3 days = 36 replicates per sample). Greater than 95% hit rate were demonstrated for the claimed LoD listed in Table 10.

Table 10. Limit of Detection for mosaic alleles in different major allele backgrounds

Mosaic Allele	Major Allele	LoD for Mosaic Allele (%MAF)
Intermediate	Normal	2.0
Premutation	Normal	2.0
Full Mutation	Normal	6.1

Normal	Premutation	2.0
Premutation	Premutation	5.0
Full Mutation	Premutation	7.0
Normal	Full Mutation	2.0
Premutation	Full Mutation	10.0

ii. DNA Input

The DNA input range for the assay is 20-80ng total (or 10-40 ng/uL). A study was conducted to assess a range of DNA input to span (b) (4) fold below and (b) (4) fold higher than this range to determine the robustness of the assay to imprecision of DNA measurements that might occur in a laboratory. DNA concentration and purity were quantified using standard spectrophotometric methods. Input levels were established using a panel of 8 samples (6 clinical samples and 2 contrived), representing all *FMR1* genotype categories, with DNA mass inputs corresponding to 1, 10, 20, 40, 80, and 160 ng per reaction. Each reaction was performed in duplicate. The study was executed by a single operator using two kit lots, and generated 20 replicates per dilution level per sample, resulting in 160 replicates per dilution level, and a total of 960 sample measurements excluding controls.

Categorical genotype agreement was 100% for all valid samples and all input levels. All expected allele peaks were within the target precision range. The QC failure rate at 1 ng, 10 ng, 20 ng, 40 ng, 80 ng, and 160 ng were 6.25%, 1.88%, 0.6%, 1.3%, 0%, and 0.63% respectively. These results support the recommended input range of 20–80 ng of gDNA for the assay as determined by standard absorbance or spectrophotometric methods.

d. Traceability

The assay uses molecular standards for use with fragment analysis on the instrument, and positive control that includes a range of CGG repeats. The assay is not traceable to any international standard, however an assessment of the accuracy of the molecular ladder used in the assay was conducted by testing the international standards. Refer to Clinical Section below.

e. Interfering Substances

Endogenous Interference

Endogenous interference was established by testing the interferents in accordance with CLSI EP07-A2 guidelines on a panel of (b)(4) samples ((b)(4) clinical and (b)(4) contrived). Interferents including conjugated bilirubin, unconjugated bilirubin, triglycerides, hemoglobin, and EDTA were tested. Each interferent, at its appropriate concentration, was added directly to an aliquot of a blood sample. Sodium hydroxide (diluent for hemoglobin and EDTA) as well as water were also tested to observe any solvent effects. An aliquot of untreated blood (no interferents added) was also extracted. Each condition was tested in triplicate.

Effects on DNA concentration yield were observed with hemoglobin (reduced yield) and EDTA (enhanced yield). Technical issues with the interference included low volume, low yield, sample loss, high screening QC failure rate, and instrument deviations, leading to some samples not completing processing. A limitation regarding the impact of excess hemoglobin and EDTA on the assay failure rate is included in the instructions for use. For samples that could be processed, the genotype category agreement was ≥ 95% and the number of alleles within the expected precision range was 100%.

f. Carry-Over and Cross- Contamination

(b)(4)

g. Stability

i. Specimen Handling Stability

The ability of the assay to utilize DNA extracted from whole human blood stored at 2 to 8 °C for up to 2 weeks was determined by testing a panel of 14 clinical specimens representing all *FMR1* genotype categories. For all samples, triplicate extractions were performed using independent aliquots on the day each sample was received to generate a T0 measurement. For subsequent timepoints, duplicate extractions were done. Extracted samples were stored at 2 to 8 °C for the duration of the study. A single operator using a single kit lot executed the study.

Genotype category agreement was 100% across all timepoints for each sample and all expected allele peaks were within target precision for all timepoints across all samples. These results support the use of sample DNA extracted from whole human blood that has been stored for up to 2 weeks at 2 to 8 °C.

ii. DNA freeze/thaw Stability

DNA freeze/thaw stability was tested using a (b)(4) contrived sample panel across (b)(4) freeze/thaw cycles. (b) (4) (b)(4) (b) (4) This study was executed by a single operator using a single lot of kit reagents and a single instrument.

Genotype category agreement was 100% across all timepoints for each sample

and all expected allele peaks were within target precision for all timepoints across all samples. These results support that extracted DNA is useable for at least (b) (4) freeze-thaw cycles to support (b) (4) .

iii. PCR & Capillary Electrophoresis (CE) Reagent Product Stability

PCR &CE product stability was assessed in two phases using a 5-member sample panel (4 clinical + 1 contrived) representing all FMR1 genotype categories. The first phase evaluated post-amplification DNA stability at 4 °C across (b)(4) time points for up to (b)(4) days ((b)(4) hours). With (b)(4) replicates per sample at each time point, a total of (b)(4) measurements were collected.

The second phase evaluated the on-instrument, ambient temperature stability of samples prepared for CE analysis. CE deck time stability was established by preparing a (b) (4)
(b) (4)

For PCR product stability, categorical genotype agreement was 100% for all timepoints across all samples, and ≥ 95% of all measured alleles were within target precision range. The study results demonstrated that PCR products generated by the assay were stable for up to 3 days when stored at 2 to 8 °C.

For CE product study, categorical genotype agreement was 100% for all timepoints across all samples and 100% of all measured alleles were within target precision range. The study results further demonstrated that samples prepared for CE analysis were stable at ambient temperature for up to 24 hours.

iv. Real Time Kit Stability

The real time kit stability was assessed using a 5-member sample panel representing all FMR1 genotype categories. Three lots of reagents were stored at -30°C to -15°C (b) (4)
(b) (4)

(b) (4) The study is still ongoing for (b) (4) timepoints.

Categorical genotype agreement was 100% for all timepoints across all samples and 100% of all measured alleles were within target precision range. The study results support at least a 1-year storage stability at this time. The study will carry on until 25-month to support 24-month stability.

v. Freeze/Thaw Kit Stability

The freeze/thaw kit stability was assessed using the same 5-member sample panel representing all FMR1 genotype categories as in real time kit stability study.
(b) (4)

(b) (4)

Categorical genotype agreement was 100% for all timepoints across all samples and 100% of all measured alleles were within target precision range. The study results support the stability of the kit for up to eight freeze/thaw cycles.

vi. Kit Shipping Stability

The kit shipping stability was assessed using the same 5-member sample panel representing all FMR1 genotype categories as in real time kit stability study. The temperature profile tested was based on the International Safe Transit Association (ISTA) 7D, 24-hour summer temperature profile. This profile (Table 11) was repeated for (b) (4) days to create the domestic and international shipping simulations, respectively.

Table 11. Shipping simulation based on the ISTA 7D 24-hour "Summer Profile".

Summer Profile [24-hr cycle]
(b) (4)

(b) (4) All the kits tested across all the different shipping configurations showed 100% of their measured alleles within the target precision range. In addition, all sample replicates showed 100% genotype category agreement.

2. Clinical Performance

Two multi-center clinical validation studies were conducted to validate a diagnostic claim and a carrier testing claim for the AmplideX Fragile X Dx & Carrier Screen Kit on the Applied Biosystems® 3500 Dx series of capillary electrophoresis instruments.

a. Diagnostic Performance

i. Full Mutation

Diagnostic performance was assessed for Full Mutation classification using Southern

Blot as the reference method. Leftover specimens were obtained from patient samples representing the intended use population submitted for routine FMR1 5'UTR mutation testing across (b)(4) linical sites. Specimens were selected using a specific protocol so as to minim e bias. Sixty-nine (b)(4) specimens were enrolled into each of the three categories (full mutation, premutation, and normal/intermediate) based on available Southern Blot result, and then tested on the AmplideX Fragile X Dx & Carrier Screen Kit. A total of 207 specimens were evaluated, including 111 specimens from female patients and 96 from male patients. The results demonstrated agreement for full mutation positive percent agreement (PPA), 95.71%, negative percent agreement (NPA) 99.3%, and overall percent agreement (OPA) 97.6%. One of the 3 discordant samples was borderline sample, 194-repeat by AmplideX and >200 peak by Southern Blot.

The agreement between the AmplideX and Southern blot for full mutation are shown in Table 12.

Table 12. Agreement comparing the AmplideX Fragile X Dx & Carrier Screen Kit with Southern Blot Analysis to identify full mutation alleles (≥ 200 CGGs).

		Reference Method (Southern Blot)		
		Positive (≥ 200)	**Negative (< 200)**	**Total**
AmplideX Fragile X Dx & Carrier Screen Kit	**Positive (≥ 200)**	67	1	68
	Negative (< 200)	3*	137	139
	Total	70	138	208
		Percent	**Lower 95% CI**	**Upper 95% CI**
	PPA	95.7	88.1	98.5
	NPA	99.3	96.0	99.9
	OPA	97.6	94.5	99.0

*One of the 3 discordant samples was borderline sample, 194 repeat by AmplideX and >200 peak by Southern Blot.

ii. Premutation vs. Normal or Intermediate assessment

Agreement between Southern blot analysis for premutation and normal/intermediate were also compared to the Amplide X. The results demonstrated PPA 100%, NPA 97.1% and OPA 98.6%, all exceeded 95% and all two-sided Wilson score 95% confidence intervals were at or above 90%. The results are shown in Table 13.

Table 13. Premutation vs. Normal or Intermediate assessment comparing the AmplideX Fragile X Dx & Carrier Screen with Southern Blot Analysis

		Reference Method (Southern Blot)		
		Positive (55-199)	**Negative (< 55)**	**Total**
AmplideX Fragile X Dx & Carrier Screen Kit	**Positive (55-199)**	69	2	71
	Negative (< 55)	0	67	67
	Total	69	69	138
		Percent	**Lower 95% CI**	**Upper 95% CI**
	PPA	100.0	94.7	100.0
	NPA	97.1	90.0	99.2
	OPA	98.6	94.9	99.6

b. Carrier Screening Performance

Carrier screening performance was assessed by determining the percent agreement between the AmplideX Fragile X Dx & Carrier Screen Kit and a reference method for categorization into Full Mutation/Premutation, Intermediate, or Normal genotypes. A total of (b)(4) specimens from females ((b)(4) years of age) were enrolled across (b)(4) clinical sites. (b)(4) evaluable subjects were obtained.

Because Southern blot is not expected to have accurate sizing in the premutation range, an alternate orthogonal independently validated PCR assay (FMR1 Dual-PCR Test) was used as the comparator. The assay is comprised of two different sets of primers that target the FMR1 gene for amplification. The FMR1 Dual-PCR Reference Method result is the average of the longest CGG repeat counts from each of the two sets of primers. The results are shown in Table 14 and Table 15.

Table 1. Classification results comparing the AmplideX Fragile X Dx & Carrier Screen with *FMR1* Dual-PCR

		Reference Method (*FMR1* Dual-PCR)			
		Premutation / Full mutation (> 54)	**Intermediate (45–54)**	**Normal (< 45)**	**Total**

AmplideX Fragile X Dx & Carrier Screen Kit	**Premutation / Full mutation**	68	10*	1	79
	Intermediate	0	60	0	60
	Normal	0	0	68	68
	Total	68	70	69	207

* Note: Eight of these samples had allele peaks of 54 and 55 by the FMR1 Dual-PCR Reference Method and the AmplideX Fragile X Dx & Carrier Screen Kit, respectively. Note the established precision of the assay in this range is ±1 CGG repeat.

Table 2. Agreement of the AmplideX® Fragile X Dx & Carrier with *FMR1*Dual-PCR

Category	Percent Agreement	Lower 95% CI	Upper 95% CI
Premutation / Full mutation	100.0	94.7	100.0
Intermediate*	85.7*	75.7*	92.1*
Normal	98.6	92.2	99.7

*** Note:** When taking into account the assay precision and count the 8 borderline samples as concordance, the percent agreement of the Intermediate category is 97.1%, 95% CI 90.2-99.2%.

The agreement of Premutation/Full Mutation, Intermediate and Normal categories are 100%, 85.7%, and 98.6% with 95% confidence intervals of 94.7-100%, 75.7-92.1%, and 92.2-99.7%. Eight (8) of the [(b)(4)] discordant samples were borderline samples, 55 repeats by AmplideX and 54 by [(b)(4)] R1 Dual-PCR Reference Method.

A secondary objective was to compare methods of reporting CGG repeat length between the AmplideX test and the reference method. Method comparison was done using a Passing-Bablok regression analysis. The analysis yields a slope of 1.0 with a 95% confidence interval of 1.0–1.0 (i.e., no measurable deviation from the line of identity) and an intercept of 0 with a 95% confidence interval of 0.0–0.0. The AmplideX Fragile X Dx and Carrier Kit detected an additional peak with low signal strength for 3 samples which may not be consistently detected and annotated by an alternate analysis method as noted in Figure 3.

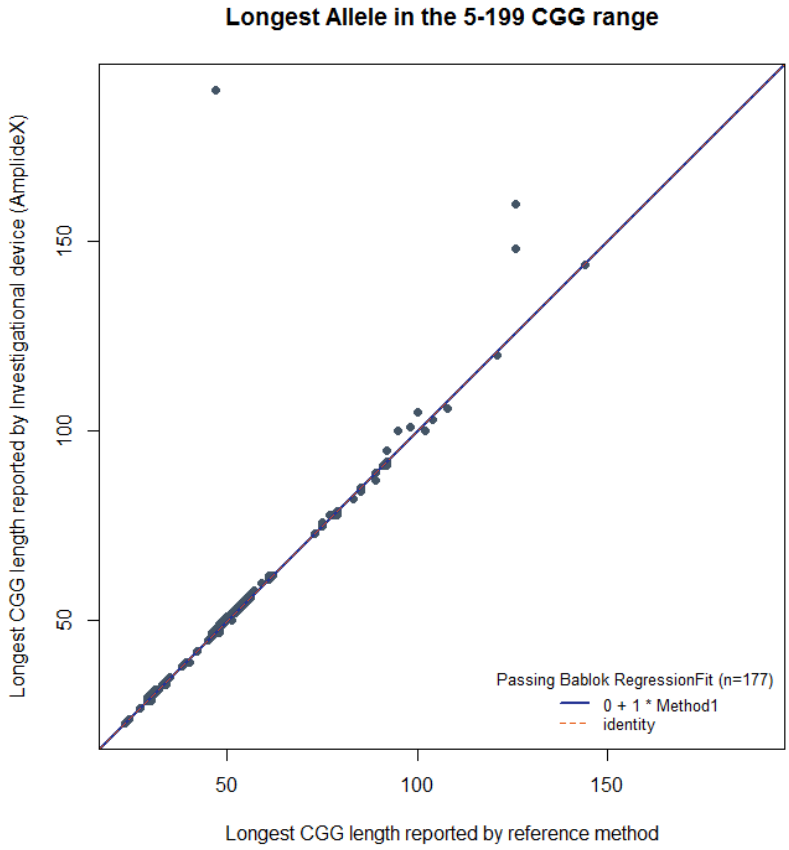

Figure 1. Scatter plot of the longest CGG repeat lengths reported from the investigational device (AmplideX) test (y-axis) compared to those reported by the *FMR1* Dual-PCR Reference Method.

The regression analysis above truncated the data set at (b)(4) length due to the poor resolution in (b)(4) range. A contingency table for allele greater than (b)(4) length is provided below to further demonstrate the agreement between AmplideX Fragile X Dx and Carrier Kit with FMR1 Dual-PCR.

Table 16: Dual FMR1 Ref PCR vs AmplideX Contingency Table

(b)(4)

c. *Mosaic allele calling in clinical studies and further analysis.*

Accuracy of the mosaic alleles

Mosaicism was detected in 49 specimens by Amplide X in the two clinical studies, 27 out of 207 (13%) in the diagnostic study and 22 out of 207 (~11%) in the carrier screening study. The mosaic results were not reported for 35 of the specimens by the orthogonal PCR method. To determine the accuracy for calling mosaic results, the results of the orthogonal PCR method were manually evaluated for mosaicism. Upon manual examination of the 35 cases, 31 mosaic cases detected by Asuragen, were also detected by the orthogonal method. Three (3) are suspected to have been contaminated during PCR setup, and 1 was due to a capillary electrophoresis artifact.

To ensure an unbiased resolution of the mosaic results, 40 randomly selected samples evaluated. After eliminating the cases already concordant for mosaicism or evaluated in the discordant resolution above, there were 34 samples: of these 31 showed no evidence of mosaicism by either method and three yielded mosaic results by both methods. Of the 83 independent specimens, 4 were discordant after manual evaluation.

d. *Testing of the International World Health Organization (WHO) Standards*

Accuracy was determined by running a panel of 5 samples (Table 17) from the WHO International Standard Fragile X Syndrome Reference Panel and the National Institute for Biological Standards and Control (NIBSC code: 08/158) (Hawkins *et al*. 2011). A single operator performed each reaction in triplicate across three different kit lots. The study yielded 135 sample measurements excluding control (b)(4) replicates per sample). Of these measurements, 134 passed QC metrics and were further analyzed to determine consensus in size ranges for the standards. The accuracy results for the WHO panel are summarized in Table 18.

Table 17. The sample panel as characterized by the WHO study consortium

Sample ID	Gender	Genotype	Mean Repeat Length (CGG)	Range of Repeat Lengths (CGG)
07/120	Female	Normal	22, 31	19–24, 28–33
07/122	Female	Premutation	33, 113	30–36, 100–132
07/168	Female	Full Mutation	38, 346	33–41, 300–401
07/170	Male	Full Mutation	754	353–960
07/174	Male	Premutation	114	97–127

Table 18. Summary of Results for the WHO Sample Panel

Sample ID	Expected CGG Range	Measured CGG Length	Allele Count	Total Alleles	Total Measurements	% in Expected Range
07/120	19–24, 28–33	22	27	27	54	100.0
		31	27	27		
07/122	30–36, 100–132	34	27	27	109	96.3
		102	1			
		103	23	27		
		104	2			
		105	1			
		113	2	27		
		114	23			
		115	2			
		125	18	24		
		126	4			
		127	2			
		87	1	2		
		88	1			
		> 200	2	2		
07/168	33–41, 300–401†	39	26	26	52	100.0
		> 200	26	26		
07/170	353–960†	> 200	27	27	27	100.0
07/174	97–127	110	24	27	54	100.0
		111	2			
		112	1			
		117	12	27		
		118	11			
		119	4			

† Expected allele size range is greater than 200 CGG; AmplideX Fragile X Reporter calls any peak larger than 200 CGG as ">200".

The test correctly classifies and sizes the international Fragile X reference standards from the World Health Organization (WHO) and the National Institute for Biological Standards and Control (NIBSC).

e. *Fragile X-associated tremor/ataxia syndrome (FXTAS) or fragile X-associated primary ovarian insufficiency (FXPOI)*

Literature was used to support the clinical validity of the claims associated with fragile X-associated tremor/ataxia syndrome (FXTAS) or fragile X-associated primary ovarian insufficiency (FXPOI) (Wheeler, A et al. 2017, Gallagher, A et al 2012, and Hantash, F et al. 2011).

M. Instrument Name

- Programmable thermal cycler capable of executing the assay-specific PCR protocol
- Applied Biosystems 3500 Dx Series Genetic Analyzer (8-capillary or 24-capillary)

N. System Descriptions

1. Modes of Operation:

   Applied Biosystems 3500 Dx Series Genetic Analyzer (8-capillary or 24-capillary) with the following reagents:
   - POP-7™ Polymer
   - Hi-Di Formamide
   - Dye Set D (DS-30) Matrix Standard Kit

   AmplideX Fragile X Reporter Software

2. Software:

   FDA has reviewed applicant's Hazard Analysis and software development processes for AmplideX Fragile X Reporter Software:

   Yes X or No _____

3. Specimen Identification:

   Specimen identification is entered manually.

4. Specimen Sampling and Handling:

   Blood may be stored for up to 14 days at 2–8 °C prior to extraction. Extracted DNA is stable for at least (b) freeze-thaw cycles.

5. Calibration:

   The ROX fluorescent dye is part of the ROX 1000 Size Ladder included in the kit. This ladder must be added to each sample to allow for proper fragment sizing analysis by the AmplideX Fragile X Reporter. Spectral calibration of the 3500 Dx instrument with DS-30 Matrix Standard Kit (Dye Set D) (P/N TBD) is required in order to properly detect and analyze samples containing ROX dye, and should be performed prior to any assay runs.

6. Quality Control:

The AmplideX Fragile X Reporter performs a series of QC checks on the batch controls (NTC and PC) as well as on the individual samples. The software assesses three general modalities of failure: Ladder Error (LD), Low Signal (LS), and Contamination (CT). If one or more of these failures occurs in the positive control, it invalidates the run as a Batch QC failure. The NTC is expected to give a Low Signal (LS) error. Rerun the flagged sample if one or more of these failures occurs in a sample.

Refer to the AmplideX Fragile X Reporter Software User Guide for more detailed description of the QC check parameters and error codes.

O. Other Supportive Instrument Performance Characteristics Data Not Covered In The "Performance Characteristics" Section above:

Not applicable

P. Patient Perspective

This submission did not include specific information on patient perspectives for this device.

Q. Identified Risks to Health and Identified Mitigations

Identified Risks to Health and Identified Mitigations:

Identified Risks to Health	Mitigation Measures
Incorrect test results	Certain design verification and validation Certain labeling information
Incorrect interpretation of test results	Certain design verification and validation Certain labeling information

R. Benefit/Risk Summary:

SUMMARY	
	The AmplideX® Fragile X Dx & Carrier Screen Kit is an in vitro diagnostic device that uses polymerase chain reaction (PCR) and capillary electrophoresis to detect and identify the number of cytosine-guanine-guanine (CGG) repeats in the fragile X mental retardation-1 (FMR1) gene using genomic DNA isolated from peripheral whole blood specimens. The probable benefit of the device is significant as it can aid in the diagnosis of Fragile X Syndrome and can be used for carrier testing in adults of reproductive age, for reproductive and genetic consultation. In

Summary of Benefits	addition, there is also significant benefit in that it can aid in the diagnosis of Fragile X-Associated Tremor/Ataxia Syndrome (FXTAS) and Fragile X-Associated Primary Ovarian Insufficiency (FXPOI), along with other clinical-pathological factors.
Summary of Risks	There is risk associated with the use of this device mainly due to incorrect test results (false positives, false negatives, and failure to provide a result) and incorrect interpretation of test results by the user, in the context of an aid in diagnosis for Fragile X Syndrome, FXTAS and FXPOI, and for carrier testing. The probable risk associated with the use of this device is that false positives, false negatives, and failure to provide a result or incorrect interpretation of test results by the user, can result in significant negative clinical consequences. Erroneous device results could adversely influence clinical consultation when used an aid in diagnosis for Fragile X Syndrome, FXTAS or FXPOI and reproductive consultation for carrier testing. In addition, it is important to note that this test can report out mosaic results. Mosaicism detected in blood may not reflect mosaicism in other tissues. The significance of reported mosaicism should be interpreted with caution in conjunction with other laboratory and clinical information (e.g., sex of patient, diagnostic testing or carrier screening, patient symptoms) and should include consultation with genetic professionals. In summary, there are potential risks associated with erroneous results or incorrect interpretation by the user of this test due to the analytical and clinical evidence provided and due to the test reporting out mosaic results, which need to be interpreted with caution.
Summary of Other Factors	The risks of false positive and false negative results are mitigated by the supportive analytical validation for this device. In addition, the risk is partially attenuated because the assay results are intended to be interpreted by a qualified molecular geneticist and the results of this device are to be used with other clinical and diagnostic findings, consistent with professional standards of practice. In addition, an important mitigation is that this test is intended to be used with reflex testing, clinical genetic evaluation, and genetic counseling, which should be offered as appropriate. In addition, the risks of this test are mitigated by the indication that states that this test excludes its use for fetal diagnostic testing, newborn screening or for stand-alone diagnostic purposes.

S. **Benefit/Risk Conclusion**

Given the device's indications for use, required general controls and special controls established for this device, the probable benefits outweigh the probable risks.

T. Conclusion

The De Novo request for the Amplide X Fragile X Dx and Carrier Screen Kit device is granted, and the device is classified under the following and subject to the special controls identified in the letter granting the De Novo request:

Product Code:	OYV
Device Type:	AmplideX Fragile X Dx & Carrier Screen Kit
Class:	II (special controls)
Regulation:	21 CFR 866.5970