CarrierMax™ FMR1 Reagent Kit- A PCR/CE based assay for the determination number of CGG repeats in the FMR1 gene on chromosome X

Jane Law[1], Maryam Shariati[2], Jonathan Wang[1], GSD [1]Reproductive Health R&D, [2]CE Platform R&D, 3450 Central Express Way, Santa Clara, CA 95051

ABSTRACT

The occurrence of fragile X syndrome is closely related to the abnormality of the FMR1 gene. This kit uses quantitative PCR (Q-PCR) amplification combined with capillary electrophoresis (CE) detection method to determine the CGG repeat number in the FMR1 gene.

INTRODUCTION

Fragile X syndrome is the most common inherited mental retardation disorder. Over 98% of fragile X syndrome is due to an expansion of an unstable CGG repeat sequence located in the 5' untranslated region of the FMR1 gene on chromosome X. According to the National Fragile X Foundation (fragileX.org) shows that in the United States the incidence for men are about 1/3600 and for women are about 1/400 to 1/600. Carrier rate for fragile X is approximately 1 in 250 women in the general population.

When the CGG repeat number (noted as n) is greater than 200, it is defined as a full mutation of the FMR1 gene. In this situation, the CpG island of the FMR1 promoter region is highly methylated, suppressing the transcription of the FMR1 gene and then resulting in lack of functional protein. This finally disrupts relevant neural functions and leads to FXS. Individuals show typical characteristics of FXS, such as mental retardation and autism. When n is within the range of 55-200, it is called a pre-mutation of the FMR1 gene. Pre-mutation will produce excess mRNA, which in turn affects the regulation of expression of multiple proteins. Pre-mutations are considered to be a risk factor for fragile X-associated primary ovarian insufficiency (FXPOI) and fragile X-associated tremor and ataxia syndrome (FXTAS), on going research is still needed to determine the number of expansion expansion correspond to severity.

MATERIALS AND METHODS

This kit uses quantitative PCR (Q-PCR) amplification combined with capillary electrophoresis (CE) detection method to determine the CGG repeat number of FMR1 gene. As the Thermo Fisher CE systems is capable of single base pair resolution, it is the most precise way of measuring amplicon lengths.

This test consist of two PCR reactions: a full-length detection system and a repeat primed system. For the full-length system, PCR amplification is performed with two primers, located upstream and downstream of the CGG repeat region. The downstream primers is labeled with FAM (6-carboxyfluoroscein) for detection of the amplicon by the CE optical system. The size of the amplicon is then used to calculate the number of CGG repeats. For the repeat primed system, the PCR reaction consists of a downstream primer labeled with FAM, however unlike the full-length PCR the forward primer is designed to prime inside the repeat region. Because repeat primers can prime randomly in the repeat region, various sized amplicons will be produced resulting in many peaks in the electropherogram. In repeat reaction, CGG repeat number can be determined by the sizes of the largest amplicons. By combining the results of the full-length and repeat systems, the number of CGG repeats in a sample can be accurately determined.

In addition, we use a AF633 (Alexa Fluor 633) labeled size standards ranging from 70bps to 1200bps for precise sizing of the amplicons. The size standard comigrates with the FAM labeled amplicons in the same capillary to ensure precise sizing of the target amplicons.

This assay is compatible with the Applied Boisystems 3500 series and SeqStudio™ Genetic Analyzers. On the 3500 series we use 50cm capillary array and POP-7™ polymer; on the SeqStudio the V2 cartridge incorporated a 28cm capillary array and POP-1™ polymer. Both instrument utilize a custom long fragment analysis run module to achieve excellent fragment separation as the CGG expansion can result in fragment from normal size of several hundred bases to over a kilo bases long.

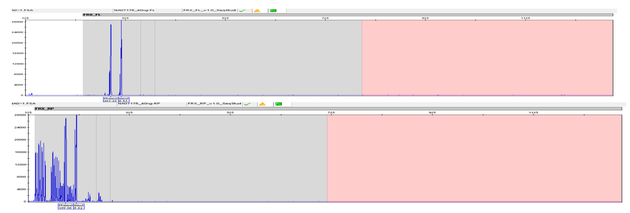
The resulting fragment data is then analyzed in GeneMapper 6.0 with specifically designed panel and bin (provided as downloadable files). The resulting genotype is then exported in csv format and then imported into the CarrierMax™ software for final report output that reports the CGG repeat numbers and the classification.

Figure 1. Instruments and Software Packages

3500 and SeqStudio instruments and the GeneMapper 6 and CarrierMax™ softwares.

RESULTS

Figure 2. Normal Samples Electropherogram

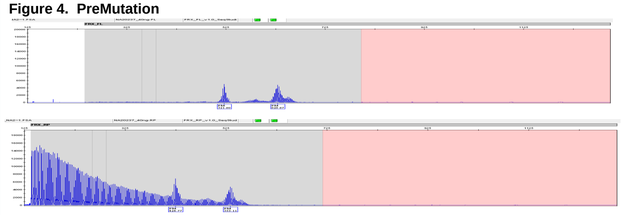


Normal sample with CGG repeats <45. Top panel is the full-length PCR. Two sharp peaks denotes this is a heterozygous sample with two X chromosomes.

Figure 3. Intermediate Electropherogram

Intermediate sample with CGG repeats 45 to 55. Figure showing single allele in the intermediate bin.

Figure 4. PreMutation



Premutation sample with CGG repeats between 55 to 200. This figure showing two alleles in the PreMutation bin.

Figure 5. Full Mutation

Full Mutation sample with CGG repeats >200. Since the large expansion of CGG repeat results in very long amplicon the PCR efficiency is greatly affected which result in full-length peak being very low in signal intensity. Zooming in is necessary to identify the signal peak above background.

Table 1. Coriell Normal and Intermediate Samples

No	Sample Name	Sex	FL1	FL2	RP1	RP2	CarrierMax™ CGG repeat	Coriell CGG repeat	CarrierMax™ Classification	Coriell Classification	Classification Concordance	Sizing Concordance
1	NA06889	F	23	30	23	30	23/30	23/30	Normal	Normal		
2	NA06893	F	23	30	23	30	23/30	23/30	Normal	Normal		
3	NA06904	F	24	29	24	29	24/29	23/29	Normal	Normal		
4	NA06911	F	29	30	29	30	29/30	30/Failed to amplify	Normal	Normal		
5	NA07175	F	23	30	23	30	23/30	23/30	Normal	Normal		
6	NA07538	F	28	29	28	29	28/29	29/29	Normal	Normal		
7	NA07540	F	23	29	23	29	23/29	23/29	Normal	Normal		
8	NA07543	F	20	29	20	29	20/29	20/29	Normal	Normal		
9	NA20238	F	29	30	29	30	29/30	29/30	Normal	Normal	100%	100%
10	NA20243	F	29	41	29	41	29/41	29/41	Normal	Normal		
11	NA06890	M	30		30		30	30	Normal	Normal		
12	NA06895	M	23		23		23	23	Normal	Normal		
13	NA07174	M	30		30		30	30	Normal	Normal		
14	NA07536	M	23		23		23	23	Normal	Normal		
15	NA07539	M	23		23		23	23	Normal	Normal		
16	NA07542	M	23		23		23	23	Normal	Normal		
17	NA20244	M	41		41		41	41	Normal	Normal		

No	Sample Name	Sex	FL1	FL2	RP1	RP2	CarrierMax™ CGG repeat	Coriell CGG repeat	MicroRead Classification	Coriell Classification	Classification Concordance	Sizing Concordance
1	NA13664	F	30	52	30	52	30/52	28(+/-3)..49(+/-3)	Intermediate	Intermediate		
2	NA20234	F	31	46	31	46	31/46	31/46	Intermediate	Intermediate		
3	NA20235	F	29	45	29	45	29/45	29/45	Intermediate	Intermediate	100%	100%
4	NA20236	F	31	54	31	54	31/54	31/53	Intermediate	Intermediate		
5	NA20230	M	54		54		54	53	Intermediate	Intermediate		
6	NA20232	M	46		46		46	46	Intermediate	Intermediate		

17 normal and 6 intermediate samples fully concordant with genotype information on Coriell data base.

Table 2. Coriell PreMutationa and Full Mutation Samples

No	SampleName	Sex	FL1	FL2	RP1	RP2	CarrierMax™ CGG repeat	Coriell CGG repeat	Publication CGG repeat	CarrierMax™ Classification	Coriell Classification	Classification Concordance	Sizing Concordance
1	NA06903	F	24	93	24	93	24/93	23/95		PM	PM		
2	NA06905	F	23	79	23	79	23/79	23/70	23/78	PM	PM		
3	NA06907	F	29	93	29	93	29/93	29/92	30/95	PM	PM		
4	NA09910	F	30	94	30	94	30/94	30/75-89		PM	PM		
5	NA06968	F	33	112	33	112	33/112	32/107		PM	PM		
6	NA20241	F	29	119	29	119	29/119	29/90-110	29/120	PM	PM		
7	NA20242	F	30	74	30	74	30/74	30/73		PM	PM		
8	NA06894	F	30	82	30	82	30/82	30/78		PM	PM		
9	NA20240	F	30	82	30	82	30/82	30/80		PM	PM	100%	100%
10	CD00014	M	56		56		56	56		PM	PM		
11	NA20231	M	78		78		78	76		PM	PM		
12	NA20233	M	119		119		119	117		PM	PM		
13	NA20237	M	100	137	100	137	100/137	100-104	139	PM	PM		
14	NA06891	M	108	121	121		108/121	118		PM	PM		
15	NA06892	M	79	93	93		79/93	93		PM	PM		
16	NA06906	M	101		101		101	96		PM	PM		

No	SampleName	Sex	FL1	FL2	RP1	RP2	CarrierMax™ CGG repeat	Coriell CGG repeat		MicroRead Classification	Coriell Classification	Classification Concordance	Sizing Concordance
1	NA04025	M	293		248		200+	645		FM	FM		
2	NA06897	M	122	93	260	93	200+	477		FM	FM		
3	NA07862	M	323	94	226	94	200+	501-550		FM	FM	100%	NA
4	NA09237	M	[redacted]		234		200+	931-940		FM	FM		
5	NA20239	F	20	205	20	206	20/200+	20/183-193	20/206	FM	FM		

16 premutation and 5 full mutation samples fully concordant with Coriell or publication. In one sample because of the high number of CGG repeat expansion the full-length PCR was not able to determine the size due to amplicon too large for the CE platform to resolve. However, the repeat PCR was able to make the correct classification call as a full mutation.

Table 3. 40 Clinical Research Samples

Sample ID	"Kit A" CGG number		CarrierMax™ CGG number		Classification
	CGG1	CGG2	CGG1	CGG2	
FX-C1	30	31	30	31	normal
FX-C2	29	>200	30	>200	full mutation
FX002	29		30		normal
FX004	29		29		normal
FX005	29	41	30	41	normal
FX007	41		41		normal
FX009	29		30		normal
FX011	29		29		normal
FX012	29		29		normal
FX018	>200		>200		full mutation
FX019	>200		>200		full mutation
FX020	>200		>200		full mutation
FX023	>200		>200		full mutation
FX025	36		36		normal
FX032	>200		>200		full mutation
FX033	>200		>200		full mutation
FX038	29		30		normal
FX039	29		29		normal
FX040	37		37		normal
FX041	29		29		normal
FX042	30		30		normal
FX043	29		30		normal
FX044	38		38		normal
FX045	30		30		normal
FX046	29	36	29	36	normal
FX047	29		29		normal
FX048	23	29	23	29	normal
FX049	30		30		normal
FX050	29		29		normal
FX051	29		29		normal
FX052	29		29		normal
FX053	29		30		normal
FX054	36		36		normal
FX055	36		36		normal
FX056	29		29		normal
FX057	29		30		normal
FX058	30		31		normal
FX059	29	39	29	39	normal
FX060	30	31	30	31	normal
FX107	30	69	31	70	pre-mutation

40 clinical research sample result fully concordant on the CGG repeat number and classification with another kit on the market.

CONCLUSIONS

We have shown the CarrierMax™ FMR1 Reagent Kit can reliably detect and calculate the CGG repeat numbers in different expansions ranging from normal (<45 repeats) to full mutation (>200 repeats). Using this kit we successfully determine and classify 44 Coriell samples and is 100% concordant with the genotype information on Coriell or compare to published data. We further characterize 40 clinical research samples and the repeat numbers as well are the classification are fully concordant with another research kits on the market.

CarrierMax™ FMR1 Kit's ability to determine CGG repeats makes it a valuable complement to the expanded carrier screening products: Thermo Fisher CarrierSeq™ ECS Kit on the Ion Torrent NGS platform or the Applied Biosystem CarrierScan™ Assay on the microarray platform.

REFERENCES

1. Chen, Liangjing, et al. "An Information-Rich CGG Repeat Primed PCR That Detects the Full Range of Fragile X Expanded Alleles and Minimizes the Need for Southern Blot Analysis." The Journal of Molecular Diagnostics, vol. 12, no. 5, 8 July 2010, pp. 589–600., doi:10.2353/jmoldx.2010.090227.
2. Grasso, Marina, et al. "A Novel Methylation PCR That Offers Standardized Determination of FMR1 Methylation and CGG Repeat Length without Southern Blot Analysis." The Journal of Molecular Diagnostics, vol. 16, no. 1, 29 Oct. 2013, pp. 23–31., doi:10.1016/j.jmoldx.2013.09.004.
3. Lim, Grace X. Y., et al. "Validation of a Commercially Available Test That Enables the Quantification of the Numbers of CGG Trinucleotide Repeat Expansion in FMR1 Gene." Plos One, vol. 12, no. 3, Sept. 2017, doi:10.1371/journal.pone.0173279.
4. Wilson, Jean Amos, et al. "Consensus Characterization of 16 FMR1 Reference Materials: A Consortium Study." The Journal of Molecular Diagnostics, vol. 10, no. 1, 28 Dec. 2007, pp. 2–12., doi:10.2353/jmoldx.2008.070105.
5. Zhou, Y. "Robust Fragile X (CGG)n Genotype Classification Using a Methylation Specific Triple PCR Assay." Journal of Medical Genetics, vol. 41, no. 4, Jan. 2004, doi:10.1136/jmg.2003.012716.

ACKNOWLEDGEMENT

We thank our OEM partner Beijing Microread Genetics Co.,Ltd. especially, YiFan Li who is the project coordinator at Microread. We also thank the Thermo Fisher CE-SMAX project core and extended team.

ThermoFisher SCIENTIFIC