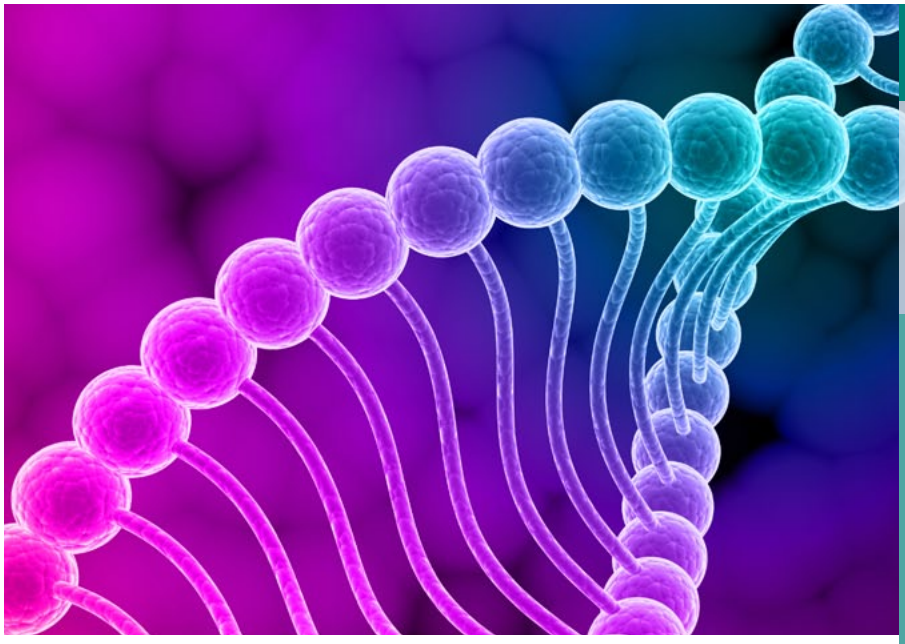


APPLICATION NOTE

Validation of a robust PCR-only assay for quantifying *FMR1* CGG repeats on clinical samples

Authors

J. Kent Moore
Galina Filippov
Karl Adler
Mack Schermer
PerkinElmer, Waltham, MA, USA

Monica J. Basehore
Michael J. Friez
Greenwood Genetic Center
Greenwood, South Carolina, USA

Heike Fiegler
PerkinElmer, UK

FragilEase - A New PCR-based Assay For Fragile X Testing

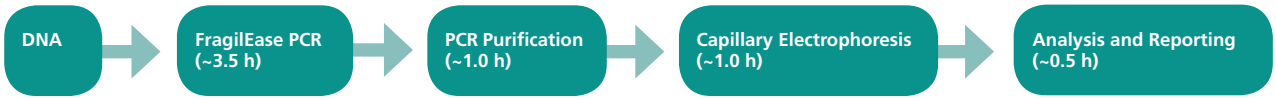
Introduction

Fragile X syndrome (FXS) is the most common form of inherited intellectual disability and is caused by an expansion of a CGG repeat in the 5′ end of the *FMR1* gene. Fragile X testing is traditionally performed using a *FMR1*-specific PCR followed by electrophoresis using a capillary sequencer. Typical laboratory developed PCR approaches have been hampered however by the ability to reliably amplify the CGG-rich region beyond about 100 – 130 repeats. Furthermore, electrophoresis with a standard capillary sequencer is capable of measuring *FMR1* PCR products only up to approximately 200 repeats with commercially available size standards.

An accurate measurement of the repeat size however is required for correct classification and diagnosis of FXS and other Fragile X-associated disorders. Thus, differentiating full mutations with greater than 200 CGG repeats from homozygous normal female samples, and confirming full mutations, has historically required labor-intensive measurement by Southern blot.

For this study we have used the FragilEase™ kit, a simple and robust PCR-based assay for quantification of CGG repeat alleles together with a low-cost benchtop electrophoretic instrument. The FragilEase™ PCR assay is designed to amplify the entire CGG repeat sequence in the *FMR1* promoter region thus allowing an accurate amplification and reliable detection of normal, intermediate, pre- and full mutations.

Workflow



Fragile X testing using FragilEase™

A blinded set of 200 archived DNA samples was analyzed by use of the FragilEase™ kit as previously described (1). The samples had previously been characterized by standard laboratory PCR and reflex testing by Southern analysis (2,3). The sample set included both male and female individuals with normal (n=167), intermediate (n=10), premutation (n=11), and full mutation alleles (n=12).

After PCR completion, the PCR fragments were purified using the PureLink Micro PCR purification kit (Invitrogen, USA). The purified PCR fragments were then analyzed using a 2100 Bioanalyzer (Agilent, USA) instrument. Two Coriell reference samples (Coriell, USA) with well characterized repeat sizes were included as standards in each set-up.

Analysis using FraXsoft™

Using FraXsoft™, the repeat number for each sample was calculated based on the measured fragment size. Including reference samples with well characterized repeat sizes in each experiment allows the construction of a linear regression standard curve. The measured fragment sizes of all test samples are then plotted against this linear regression curve, allowing an accurate determination of the repeat size of each test sample. According to the characterization guidelines chosen, the mapped repeat size will be displayed in its corresponding categories (i.e. normal, intermediate, premutation, full mutation).

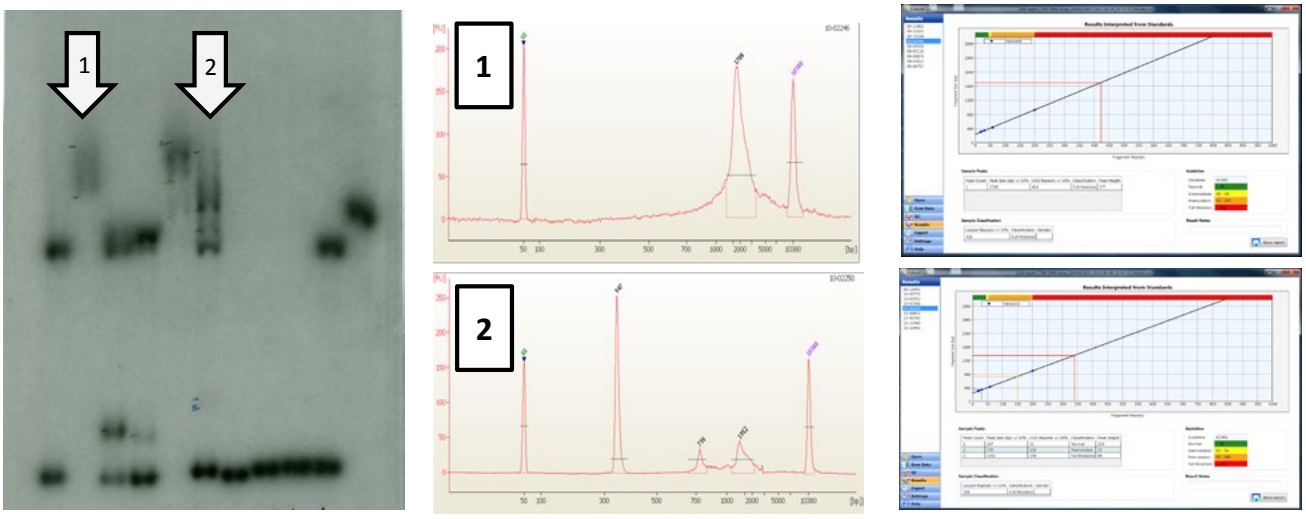


Figure 1. Two example cases representing representing full mutations samples are shown for a typical Fragile X Southern Blot together with the Bioanalyzer electropherogram and FraXsoft report after DNA amplification with FragilEase™.

Results

Of the 200 DNA samples tested, 199 samples were concordant with the previously determined classification. All twelve full-mutation samples were successfully classified, with the largest allele size measured at over 653 repeats. The first round PCR failure rate was 2% (4/200) with all four samples producing correct classification result on a subsequent second round of PCR.

The only one discordant sample was previously identified by Southern blot analysis to have 45 repeats and was subsequently classified as intermediate according to the ACMG guidelines. In this study, the calculated repeat number for the same sample was found to be 44 repeats. Using the ACMG guidelines this would classify as normal (normal <45 repeats). However, the precision for allele size detection using FragilEase™ together with the Bioanalyzer ranged from +/-1 to +/-2 repeats for alleles with repeat sizes of up to 58 repeats in our study. Hence, this sample is within the detection limits and we would recommend confirmation by an independent methodology in similar cases.

Male Sample Results

	New FragilEase™ PCR-only Method					
Classification	Normal	Intermediate	Premutation	Full Mutation	Total	**Reference Method**
Normal	87	0	0	0	87	
Intermediate	1	6	0	0	7	
Premutation	0	0	0	0	0	
Full Mutation	0	0	0	6	6	
Total	88	6	0	6	100	

Female Sample Results

	New FragilEase™ PCR-only Method					
Classification	Normal	Intermediate	Premutation	Full Mutation	Total	**Reference Method**
Normal	80	0	0	0	80	
Intermediate	0	3	0	0	3	
Premutation	0	0	11	0	11	
Full Mutation	0	0	0	6	6	
Total	80	3	11	6	100	

Summary

The FragilEase™ assay was used on 200 blinded samples enriched for a majority of normal samples. All samples were correctly classified including eleven premutation and twelve full mutation samples. Accurate measurement of the CGG trinucleotide repeat in the *FMR1* gene is part of the classification and diagnosis of the Fragile X syndrome and other Fragile X-associated disorders. While this assay doesn't determine methylation status of the patient sample, the ability to robustly amplify premutations and more importantly, full mutations, will reduce the requirement of reflex testing by Southern analysis. Furthermore, this assay fulfills an unmet need for a quick, low-labor and low equipment cost assay, allowing Fragile X testing to be performed in various laboratories within one working day.

References:

[1] Adler et al., 2011

[2] Basehore et al., 2012

[3] Basehore and Friez, 2009

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PerkinElmer, Inc.
940 Winter Street
Waltham, MA 02451 USA
Phone: (800) 762-4000 or
(+1) 203-925-4602
www.perkinelmer.com

PerkinElmer, Inc.
Wallac Oy
PO Box 10
20101 Turku, Finland
Phone: (+358) 22678-111
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