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Preparation and validation of the first WHO international genetic reference panel for
Fragile X syndrome

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Abstract

Fragile X syndrome is the most common inherited form of mental retardation. It is caused by expansion of a trinucleotide (CGG)_n repeat sequence in the 5' untranslated region of the *FMRI* gene, resulting in promoter hypermethylation and suppression of *FMRI* transcription. Additionally, pre-mutation alleles in carrier males and females may result in Fragile X Tremor/Ataxia Syndrome and primary ovarian insufficiency respectively. Fragile X is one of the most commonly requested molecular genetic tests worldwide. Quality assessment schemes have identified a wide disparity in allele sizing between laboratories. It is therefore important that clinical laboratories have access to characterized reference materials (RMs) to aid accurate allele sizing and diagnosis. With this in mind, a panel of genotyping reference materials for Fragile X syndrome has been developed which should be stable over many years and available to all diagnostic laboratories. Immortalised cell lines were produced by Epstein-Barr virus transformation of lymphocytes from consenting patients. Genomic DNA was extracted in bulk and reference material aliquots were freeze-dried in glass ampoules. Twenty one laboratories from seventeen countries participated in a collaborative study to assess their suitability. Participants evaluated the samples (blinded, in triplicate) in their routine methods alongside in-house and commercial controls. The panel of five genomic DNA samples was endorsed by the European Society of Human Genetics and approved as an International Standard by the Expert Committee on Biological Standardization at the World Health Organization.

Key words: Fragile X syndrome, *FMRI*, certified reference materials, genetic reference panel

Introduction

Fragile X syndrome is the most common heritable cause of mental retardation, affecting approximately 1 in 5,000 males and 1 in 10,000 females. It is caused by the expansion of a tract of CGG repeats in the 5'-untranslated region of the Fragile X mental retardation 1 (*FMRI*) gene located at Xq27.3, with consequent hypermethylation of promotor regions and silencing of gene expression¹. As the phenotypic presentation and frequency of clinical signs are variable, clinical diagnosis is challenging and definitive diagnosis in suspected individuals requires molecular measurement of the (CGG)_n allele size. The size of the (CGG)_n repeat tract is highly polymorphic in the general population, containing from 6 to about 50 repeats and most commonly 29-30 repeats²⁻⁴. Individuals with alleles of 59-200 repeats, known as pre-mutations, are considered to be carriers as alleles of this length are highly unstable in maternal transmission and at very high risk of expansion into 'full mutation' alleles of >200 repeats which are causative of the Fragile X syndrome.

Selected screening is highly justified among mentally retarded individuals, not only to provide the benefits of early clinical intervention, but also to enable prenatal diagnosis to be offered to related carriers^{5,6}. Genetic testing for Fragile X syndrome is widespread but it has been acknowledged by leading laboratories in the field to be one of the most technically challenging genetic tests. Performance in proficiency testing has been shown to be sub-optimal in several EQA schemes^{7,8}. Any assay using PCR amplification to determine (CGG)_n must overcome the problem of amplifying long sections of repetitive CG-rich sequence. Validation of the assays used is difficult because of the lack of reliable

reference materials. Accurate allele sizing becomes essential when distinguishing between alleles around the normal/pre-mutation and pre-mutation/full mutation thresholds. Most laboratories use DNA samples from patients which have been characterised by their own laboratory as controls. As such, allele sizing accuracy may vary between laboratories. Laboratories that are new to the field often rely on small finite amounts of materials supplied by other genetic reference laboratories, for example as part of external quality assessment schemes. However, these control materials are in short supply because of the relatively large amount needed for investigation (by Southern blotting) and sample distribution restrictions in Europe imposed by the IVD Directive (*Directive 98/79/EC of the European Parliament and of the Council of 27 October 1998 on in vitro diagnostic medical devices*).

The high frequency of male and female carriers of *FMR1* pre-mutation alleles (~1/813 to 1/251 and ~1/259 to 1/113 respectively)⁹⁻¹³ and the occurrence of pre-mutation phenotypes such as Fragile X Tremor Ataxia Syndrome (FXTAS)¹⁴ and Fragile X-associated primary ovarian insufficiency (FXPOI)¹⁵ will doubtless lead to increased *FMR1* genotyping and possibly to screening of the general population. The case for certified reference materials to aid accuracy in *FMR1* genotyping is therefore increasing in strength.

There are no internationally certified genetic reference materials available for *in vitro* diagnosis of Fragile X syndrome. A panel of nine PCR amplicons with a variety of *FMR1* trinucleotide repeat length standards are available from the US National Institute of Standards and Technology (NIST). However, the panel does not contain any full mutations and the materials can only be used in PCR and not in the more definitive

Southern blot assay. Genomic DNA (gDNA) reference materials would be preferable as these more closely resemble patient specimens. Well-characterized genomic DNA materials are available from Coriell Cell Repositories but are not approved or intended for *in vitro* diagnostic use¹⁶. Furthermore, there may be differences in DNA quality and performance (and also possibly even differences in allele sizes due to somatic cell mutation¹⁷ [& KEW, JRH unpublished data]) in future batches.

We have therefore chosen to produce in bulk a panel of gDNA materials which could be certified and, when eventually required, can be replenished from the same source. Bulk genomic DNA was extracted from immortalised cell lines produced by Epstein-Barr virus (EBV) transformation of lymphocytes from donors who were known to carry the wild type, pre-mutation and full mutation genotypes. Following validation by an international collaborative study, a panel of five gDNAs was endorsed by the European Society of Human Genetics and then approved as an International Standard by the Expert Committee on Biological Standardization at the World Health Organization (WHO) in November 2008.

Materials and Methods

Patients and Cell Lines

A protocol for approaching patients, obtaining informed consent and anonymizing samples was approved by a local research ethics committee. Patient blood samples were collected from six consenting donors as part of 'CRMGEN', an EU 5th framework project to evaluate and develop certified reference materials for genetic testing. One donor was a normal female individual and the remainder had been identified after previous molecular genetic investigation. Classifications of these patients were: female pre-mutation, male

pre-mutation, male full mutation, female full mutation (x2). Lymphoblastoid cell lines were established after EBV transformation, ensuring continued future supply. Each cell line was tested for Hepatitis A, Hepatitis B, Hepatitis C, Parvovirus B19 and HIV1. Master and working cell banks were established and stored to ensure continual future supplies of the same cells. Safety of storage was guaranteed by distribution to multiple liquid nitrogen vessels at multiple locations. Cell banks were identity-tested by DNA profiling with the Applied Biosystems 11-locus SGM+ kit. *FMRI* (CCG)_n repeat genotypes of the cell lines were confirmed to match those of the donor patients, except for the 2 female full mutation cell lines which prior to the collaborative study, remained unconfirmed. For that reason, two female full mutation samples were included in the panel for the collaborative study, in case one proved more suitable than the other for use as a reference material.

DNA Extraction and Freeze Drying

Cells were bulk cultured (ECACC, UK) to approximately 10^{10} cells and frozen as pellets of 10^8 cells. Genomic DNA was extracted from cell pellets using the PureGene DNA purification and Autopure LS equipment (Qiagen, UK). The integrity of the extracted DNA was confirmed by electrophoresis in agarose gels. Each of the DNA samples were dissolved at a concentration of approximately 29 μ g/ml in 0.8mM Tris/0.08mM EDTA buffer with 2.5mg/ml Trehalose. 0.8ml of this solution was dispensed into a minimum of 1200 glass ampoules and freeze-dried. Sealed ampoules were stored at -20°C.

Homogeneity Testing

Samples were tested for homogeneity by determining DNA concentration by spectrophotometric absorbance analysis (Nanodrop, USA). After reconstitution in

distilled water, the samples were allowed to equilibrate to room temperature for 1 hour with mixing by pipetting after 30 minutes before quantitation. Accuracy and precision of the spectrophotometer was determined by performing multiple reads on a control sample of commercial genomic DNA (Promega, UK).

Accelerated Degradation Study

Ampoules were tested for degradation by agarose gel and quantitative PCR comparison of samples stored at +56°C and -20°C with samples stored at -150°C. For agarose gel electrophoresis, the size marker was Hyper Ladder VI (Bioline, UK). For Q-PCR, the PCR primers sequences (5' to 3'): Forward- TGCCCAGTGCTTAACAAGACCA and Reverse- CTTGAAGGAAATGCCCCATTA, were used at 10µM to amplify a 486bp sequence of *F2* on chromosome 11. Cycling conditions were 95°C 10 mins then 45 cycles of 95°C for 10s, 60°C 20s and 72°C 30s using the LightCycler FastStart DNA Master plus SYBR Green I kit on a LightCycler 480 thermal cycler (Roche, UK).

Methylation Analysis

For analysis of methylation status at the *FMR1* promoter, Methyl Primer Express v1.0 was used to design bisulphite specific sequence primers for the amplification a 192bp region, covering 22 CpG's, of the *FMR1* gene promoter region (position 3435241-3437401 of NT_011681 of Chromosome X, NC_000023), upstream of the CGG repeat region. Primers used were forward 5'-TTGAGTGTATTTTTGTAGAAATGG-3' and reverse 5' – CCCTCTCTCTCAAATAACCT-3'. Ampoules were reconstituted in 40µl water and left to reconstitute for 60 min. Approximately 50ng of bisulphite converted DNA was used in the final HRM reaction mix, containing 1U Hot Star Taq (Qiagen, UK), 3mM MgCl₂, 5pmol primers, 200µM dNTPs and 5µM Syto9. PCR was performed

on the Rotorgene 6000, and consisted of an initial 10 minute 95°C denaturation step, followed by 40 cycles of 30 s at 95°C, 30 s at 55°C and 45 s at 72°C with a final 7 minute extension at 72°C . This PCR was followed by a melting step for HRM which consisted of 95°C for 5 s, followed by 50°C for 5 minutes and a temperature ramping from 70°C to 95°C at a rate of 0.2°C/sec. Fluorescence was acquired on the HRM channel. HRM data was analysed using Rotorgene 6000 software. The leading and trailing edges were normalised at 72-74°C and 83-86°C respectively. The assay was performed on 3 ampoules of each sample.

Collaborative Validation Study

Each of the six gDNA materials were sent in triplicate and blinded to each laboratory with instructions for reconstitution and storage. Each laboratory was asked to perform their routine test(s) for Fragile X syndrome on each of the 18 coded samples and to genotype the samples in groups of six on three separate days as indicated on the results sheet, using different lots of reagents or different operators if possible. Laboratories were asked to report the number of trinucleotide repeats. Overall findings of each sample and raw data *e.g.* images of autoradiographs, were to be returned together with full details of techniques used, any in house or commercial controls and reasons for failure of any of the samples tested.

Results

Each cell line was found negative for Hepatitis A, Hepatitis B, Hepatitis C, Parvovirus B19 and HIV1. All EBV-transformed cell lines remain positive for the presence of the EBV genome. Whilst there is an unquantified risk of EBV infectivity in the cell lines, infective viral particles are unlikely to survive DNA extraction and have not been

detected in electron microscopy studies or in infectivity assays (JRH unpublished data). In all cases cell line DNA profiles matched the profile of the original blood samples. No samples showed evidence of cross-contamination. However, sample 07/174, showed a very small additional peak at just two short tandem repeat marker loci. These peaks were not detected in the original blood samples.

Bulk genomic DNA was filled into glass ampoules, freeze-dried and sealed. Upon reconstitution with 40µl sterile water the DNA concentration is approximately 580µg/ml in 16mM Tris; 1.6mM EDTA; 50mg/ml Trehalose. The pH of the reconstituted candidate materials was measured and was found to be within 7.4 and 7.8 in all six. Table 1 shows the product summary for the six materials. Residual moisture and oxygen levels were consistent with those obtained in previous fills and within the levels acceptable for International Standards by the WHO¹⁸.

Homogeneity

Random ampoules were weighed periodically during the ampoule filling of each material. The coefficient of variation for each fill (0.15% to 0.31%; Table 1) indicates a very low level of variation in volumes aliquotted during each fill run.

The materials were tested for DNA homogeneity after reconstitution both within ampoules and between ampoules by DNA quantitation. Ten randomly selected ampoules of each material were reconstituted in 40ul distilled water. The DNA concentration of each ampoule was measured in triplicate. The triplicate reads were highly consistent, giving a modal coefficient of variability of just 2.9%. The distribution of variability is displayed in Figure 1a. This data indicates that the material in the ampoules is highly homogeneous 1 hour after reconstitution.

DNA concentrations showed more variation between individual ampoules of each material and between the different materials (Figure 1). However, the intended purpose is independent of DNA concentration above a minimum level and the amount supplied in each ampoule is enough for several tests by Southern blotting. Mean concentrations of each material varied from 568 to 818 $\mu\text{g/ml}$, equating to a mean of at least 23 μg per ampoule. Coefficients of variability between ampoules of a single material ranged from 7.2 to 15.3%.

Stability

An accelerated degradation study on samples stored at +56°C was carried out. When compared with samples stored at -150°C, after 2 years storage there was no detectable degradation in samples 07/122; 07/168; 07/170 or 07/174 as assessed by Q-PCR (Figure 2a). Ampoule 07/120 showed an increase in C_p value of 0.54 between the -150°C and +56°C samples, indicating a 31% decrease in DNA yield. This difference however may be the result of ampoule to ampoule variability or due to a 'baking' effect resulting in incomplete solubilisation. The Q-PCR findings were confirmed by agarose gel electrophoresis, where there was a slight increase in band mobility for the +56°C sample as well as a slight decrease in band intensity, but no visible increase in low molecular weight DNA (Figure 2b). Densitometry analysis of the gel bands indicates an average signal reduction of 22% for the +56°C samples. Together the Q-PCR and electrophoresis data indicates some, but limited degradation at +56°C. However, the limited degradation at +56°C and the absence of degradation at the storage temperature of -20°C after 2 years indicates that the DNA should be stable for many years.

Validation

To validate that the materials are fit for purpose as Reference Materials in the laboratory diagnosis of Fragile X syndrome, laboratories were invited to participate in an international collaborative study. Thirty eight laboratories were invited to take part in the study, 23 laboratories agreed to participate and results were returned by 21 laboratories. Seventeen different countries were represented among participants returning results: 13 from Europe, 4 from North America, 3 from Australasia, 1 from Asia. Collaborative validation study participants were requested to test the 18 coded samples on 3 separate days using different lots of reagents or different operators if possible. The methods used by the participants are listed in Table 2. Seventeen laboratories used in-house assays and four laboratories used a commercial kit developed by Celera and available from Abbott Molecular (Des Plaines, Illinois, USA). Some laboratories usually refer selected samples to another laboratory to complete their investigation (laboratories 11, 15) and others were unable to carry out their usual testing protocol because of technical difficulties (laboratories 8, 14 & 19). Laboratory 14 could not obtain any useful Southern blotting results with the samples supplied, while their in-house controls gave the expected results, as did the study samples in their PCR test.

No two in-house methods were entirely identical. Some laboratories indicated that their method was based on a previous publication (see Supplementary Data). Eight laboratories were able to use more than one batch of reagents and the testing was carried out by more than one operator in five laboratories. There is no evidence of the study materials performing differently between different methods. The materials performed similarly to patient samples in Southern blotting, with a spread of bands from expanded alleles, although none of the spreads were as extreme as can be seen in some patient samples (Figure 3). The methylation states of the alleles are indicated in Figure 3. The normal female and female premutation samples gave a 2 peak melting profile, with the larger

peak indicating unmethylated, and the smaller peak indicating methylated. This melting profile corresponds with the two alleles present in these samples, one of which is X-inactivated and appears as the higher T_m methylated peak. The female full mutation-1 sample also gave the 2 peak melting profile, which indicates that the *FMR1* expansion lies largely on the inactivated X-chromosome. In contrast, the female full mutation-2 sample gave a single methylated melting peak profile indicating that the *FMR1* expansion lies largely on the active X-chromosome. Male samples however have a simpler melting profile as they possess only a single X allele. The male premutation sample gave a single low T_m peak, indicating that the Fragile X promoter is unmethylated. The male full mutation sample shows a single fully methylated peak, indicating the presence of only a fully methylated Fragile X promoter.

The controls used by participants were as follows: 17 laboratories used in-house materials (previously characterised clinical samples from patients and normal controls), six laboratories used DNA samples supplied by the Coriell Institute and one lab used NIST PCR product controls.

Tables listing the full genotyping results of the validation study are available as Supplementary Data. Summaries of the clinical interpretations are as follows: **Samples 1, 11 & 12 (normal female)**: 19 labs reported 'normal female' (or 'normal genotype'). Exceptions to this interpretation were: Lab.11 did not get a PCR product with 1 out of the 3 samples, reported normal female for the other 2 samples. Lab.19 obtained genotypes in all 3 samples but was unable to complete the investigation and made no interpretation. **Samples 2, 7 & 14 (female, pre-mutation)**: 16 labs reported 'female, pre-mutation'. Exceptions to this interpretation were: Lab.14 which detected the pre-mutation in only 1 out of 3 samples and was unable to make an interpretation for 2 samples;

Lab.11 would refer all 3 samples for Southern blotting; Labs.15 & 17 reported all 3 samples as 'normal female'; Lab.19 obtained genotypes in all 3 samples but was unable to complete the investigation and made no interpretation. **Samples 3, 8 & 17 (female, full mutation):** 14 labs reported 'female full mutation'. Exceptions to this interpretation were: Lab.16 reported 'female full mutation' with only 2 out of 3 samples and would refer the 3rd sample for Southern blotting; Labs.11, 14 & 15 would refer all samples for Southern blotting; Lab.17 reported a different result for all 3 samples (normal male, male or Turner syndrome, female full mutation); Lab.19 only tested 2 samples and obtained genotypes but was unable to complete the investigation and made no interpretation; Lab.21 reported only 'one allele detected by PCR' and made no clinical interpretation. **Samples 4, 10 & 18 (male full mutation):** 14 labs reported 'male full mutation'. Exceptions to this interpretation were: Labs.11, 15 & 16 suspected a full mutation and would refer all samples for Southern blotting; Lab.8 reported 'normal male' with all 3 samples; Lab.17 reported 'male pre-mutation' with 2 out of 3 samples and 'male full mutation' with the third sample; Lab.19 was unable to complete the investigation and made no interpretation. Lab.21 did not amplify a PCR product and made no interpretation. **Samples 5, 15 & 16 (female, full mutation):** 14 labs reported 'female full mutation'. Exceptions to this interpretation were: Lab.8 suspected a full mutation but was unable to obtain Southern blot data; Labs.11, 14 & 15 would refer all samples for Southern blotting; Lab.17 reported 'normal female' with 2 out of 3 samples and 'female full mutation' for the third sample; Lab.19 was unable to complete the investigation and made no interpretation; Lab.21 reported only 'one allele detected by PCR'. **Sample 6, 9 & 13 (male, pre-mutation):** 17 labs reported 'male pre-mutation'. Exceptions to this interpretation were: Labs.11 & 15 suspected a pre-mutation and would refer all samples for Southern blotting; Lab.17 reported 'normal male' for 2 samples and 'male full

mutation' for the third sample; Lab 19 was unable to complete the investigation and made no interpretation.

A total of 18 non consensus results were reported, giving an overall rate of non concordance of 4.9% (21 laboratories x 18 samples -7 samples not tested), although these were clustered in three laboratories. There was no correlation between the non concordant results and any particular sample or a specific method.

One laboratory reported 12 of the 18 non concordant results. This laboratory was contacted, and their testing protocol was changed. Genotyping results are presented in Figure 4. The sample testing mean allele sizes and ranges obtained are summarized in Table 3. Three outliers identified by Grubb's Test were omitted from the data analysis¹⁹. These were: female full mutation 1 – allele 2: 575 repeats; female full mutation 2 – allele 1: 26 repeats; male full mutation – 7 repeats.

The detection of a very small 7 repeat allele in the male full mutation samples by two laboratories was unexpected. Such minimal repeats are occasionally detected in full mutation clinical samples and it is unclear if the small repeat is an assay artefact or a true mosaic allele. In this study one laboratory (lab. 8) found the 7 repeats by PCR and interpreted the result as a normal male without carrying out Southern blotting, while the other lab (lab. 18) found a similar peak by PCR but noted it as a likely artefact and continued to identify a full expansion by Southern blotting.

Discussion

In the absence of reference materials, genetic tests are being performed without adequate controls and new assays are difficult to validate. The need for reference materials in Fragile X syndrome testing is particularly pressing as it is a technically challenging test. For use in *in vitro* diagnostic assays, reference materials in the EU must be CE-marked or

in the U.S. must be FDA-approved. Alternatively, according to the EU IVD directive (*Directive 98/79/EC of the European Parliament and of the Council of 27 October 1998 on in vitro diagnostic medical devices*), reference materials of “higher order” are exempted from CE marking. WHO international standards are accepted as internationally recognised “highest order” measurement standards.

Whilst aimed to promote the wider availability and safety of commercial products for *in vitro* diagnostics, the European IVD directive may have hampered reference material development and use by preventing the distribution of ‘in-house’ quality control samples between laboratories. This also hampers harmonization of quality between laboratories. Despite the existence of a PCR-product based certified reference material for Fragile X testing (NIST, USA), there was a need for a more commutable, *i.e.* cellular or genomic, certified reference material. As EBV-positive cell lines are classified as requiring handling in Category 2 facilities and their shipment must be handled as ‘infectious’, the use of EBV-established cell lines as reference materials is not practical. We therefore chose to produce a panel of genomic DNA’s derived from cell lines which could be certified as a ‘higher order’ reference material panel. Purified genomic DNA was freeze-dried in glass ampoules. Twenty one laboratories from seventeen countries collaborated in a validation study of the panel of proposed reference materials.

Analysis of material homogeneity showed a significant level of variability between fills and between ampoules. Reasons for this are not clear. That the source of the variation is not due to the spectrophotometer is demonstrated by the low level of variability within ampoules and within the control sample reads (n=10; %CV=1.84). Variation in DNA concentration between fills may be due to inaccurate quantitation prior to ampoule filling, or due to poor homogeneity of the bulk DNA prior to filling. Variation between ampoules of the same fill may also be due to poor homogeneity of the bulk DNA. It is difficult to

achieve complete homogeneity in solution whilst retaining the high molecular weight DNA required for this application. Because of this variability, users are requested to determine the DNA concentration of each ampoule themselves. The high level of homogeneity within the reconstituted ampoules however, means that the user can be confident that the sample concentrations will not significantly vary between aliquots from a single ampoule.

Analysis of material stability by Q-PCR and gel electrophoresis of accelerated degradation samples shows some degradation at +56°C after 2 years. However, the limited extent of degradation at elevated temperature and the absence of degradation at the storage temperature of -20°C after 2 years indicates that the DNA should be stable for many years. Further samples stored at these temperatures as well as others stored at intermediate temperatures will be monitored at regular intervals in the future as part of planned quality control testing.

DNA profiling of the materials showed very small additional peaks at two loci in one sample (07/174). These peaks were not detected in the original blood samples. As the participants of the collaborative study did not obtain any anomalous results specific to this material and the lack of additional peaks at other loci is suggestive that these very minor peaks are caused by microsatellite mosaicism rather than contamination.

The wide spread of allele sizes obtained in the collaborative validation study is typical of that obtained in Fragile X testing external quality assessment schemes. A total of 18 incorrect results were reported, giving an overall error rate of 4.9%. This error rate is similar to the 5% level found in an Italian 5-year quality assessment⁷ but higher than that observed in our previous studies with different panels (Factor V Leiden: 0.7%; Prothrombin mutation G20210A: 0.7%; Haemophilia A Intron 22 Inversion: 1.8%)²² [& E.G. unpublished data]. The high variability of the results, both in reported repeat

numbers and the interpretation of results, emphasise the difficulty of *FMRI* repeat sizing and the need for Fragile X syndrome reference materials. The finding by two laboratories of an unexpected 7-repeat PCR product is a concern. It is unclear if this small additional putative allele is representative of the clinical sample from which it was derived or is a cell culture or typing artefact. To avoid confusion in the use of this sample as a referencematerial, a caution on the possibility of detecting this putative allele is included in the 'Instructions for Use' for the panel. While the presence of this allele makes the sample unsuitable for use in external quality assessment, it does not detract from the sample's utility as a reference material, once the user is aware of it.

By producing the reference panel from a single large batch of pooled DNA derived from a single bulk cell culture batch, the need to repeatedly re-culture the cells for the production of multiple small batches is avoided. This is important as new size alleles can be generated over time in cell culture, particularly when allelic methylation is only partial. It is therefore possible that if this reference material batch is ever exhausted, that the genotype of the replacement batch may not be identical. However, at that stage a repeat collaborative validation study would be performed to characterise the materials fully.

Two female full mutation samples were included in the study as neither had been previously genotyped and demonstrated to be suitable for inclusion in a reference panel. Both samples performed well. To maintain the widest variety of allele sizes, sample 1 (07/168) was included in the reference panel of five samples (normal female, female pre-mutation, male pre-mutation, female full mutation, male full mutation).

All laboratories that participated in the collaborative study were sent a study report and their approval was sought. All laboratories agreed that the candidate material is suitable for use as a genetic reference panel for Fragile X syndrome. The most frequent comment

from the participants (n=6) was that the DNA was of good quality. Three laboratories reported that the DNA concentration varied more than expected and two laboratories did not like the glass ampoules. The report was then endorsed by the board of the European Society for Human Genetics and was finally submitted to the Expert Committee on Biological Standardization at the World Health Organization (WHO). In November 2008, the panel was established as a WHO International Standard and made available through the NIBSC product catalogue (<http://www.nibsc.ac.uk/products/catalogue.html> - code: 08/158).

The panel is the only certified set of genomic DNA reference materials for Fragile X testing and is available to all diagnostic laboratories worldwide to aid accuracy in Fragile X syndrome testing. The panel will be of particular importance for the validation of commercial diagnostic kits for Fragile X syndrome which are beginning to enter the market, for laboratories setting up new in-house methods such as methylation analysis and for validating existing techniques after a change of reagents, operator or equipment.

The Full data from the homogeneity study, the collaborative study and participant methods used is available as supplementary information at the European Journal of Human Genetics website

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Conflict of Interest Statement

A fee is charged for the panel by NIBSC to cover handling and shipping.

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Titles and Legends to Figures

Table 1. Ampoule product summaries of the six candidate materials

Figure 1. Homogeneity

Legend:

A. Variability (% CV) in triplicate DNA quantitation reads from 10 ampoules of each material. The modal CV of reads from a single amp is 2.9% indicating the material is highly homogeneous with ampoules.

B. Variability between fills and between ampoules. Columns show mean DNA concentration ($\mu\text{g/ml}$) from 10 ampoules from each fill. Error bars show DNA concentration variability (± 1 SD) between ampoules

Figure 2. Accelerated Degradation

Legend:

A. Accelerated degradation- Real-Time PCR

Crossing point (CP) values for quantitative PCR (Roche Lightcycler) after storage of ampoules at baseline (-150°C) and elevated temperatures for 2 years. CVs show the level of variability between triplicate runs.

B. Accelerated degradation- Agarose Gel

Legend: 0.7% agarose gel of 200ng of each sample. The size marker is Hyperladder VI (Bioline).

Table 2. Testing procedures used by the participants

Figure 3.

A. Southern blot of study samples

Southern blot of study samples showing male and female; normal, pre-mutation and full expansion banding patterns. The control samples are patient DNA samples extracted from blood. Consensus expanded triplet repeat numbers are indicated on the right.

B. High resolution melt analysis

The x-axis represents temperature from 70°C to 95°C. The y-axis represents dF/dT (difference in fluorescence over temperature) from zero to 1.4. Methylation status is indicated by the presence and relative size of the left peak (unmethylated) and the right peak (methylated). The melt curves for the 3 ampoules used for each sample are presented in different colours.

Figure 4. Distribution of allele repeat numbers reported by study participants.

Legend:

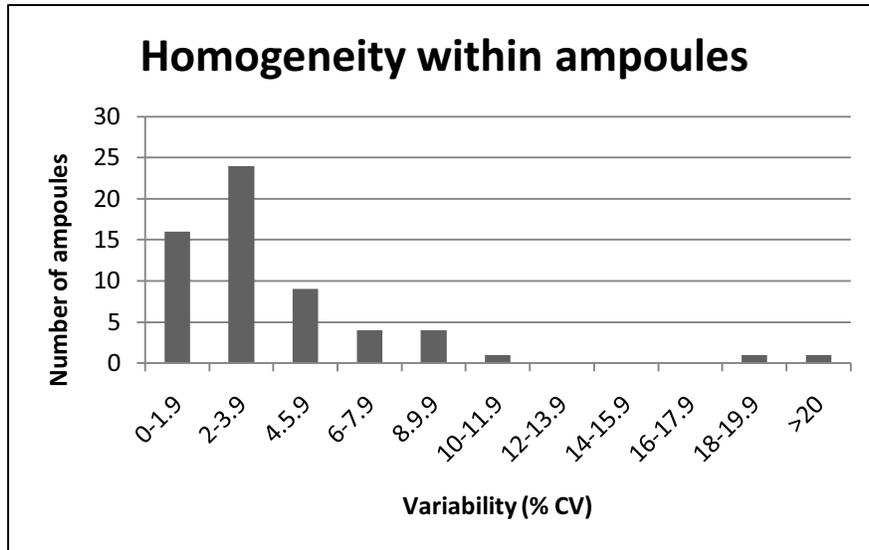
The graphs show the number of laboratories (Y axis) detecting each allele length (X-axis). The number of repeats was calculated for each sample in all laboratories, where possible, by taking a mean of all three replicates of the same sample tested in one or more techniques. Where a range of repeats was given the mean value was used. Values reported with 'greater than' or 'less than' symbols (>, <) were omitted. Where an approximate size was given, this was taken as definitive. All values have been rounded up to whole numbers for clarity. Sample outliers as identified by Grubbs Test have been omitted.

Table 3. Summary of allele sizes and ranges

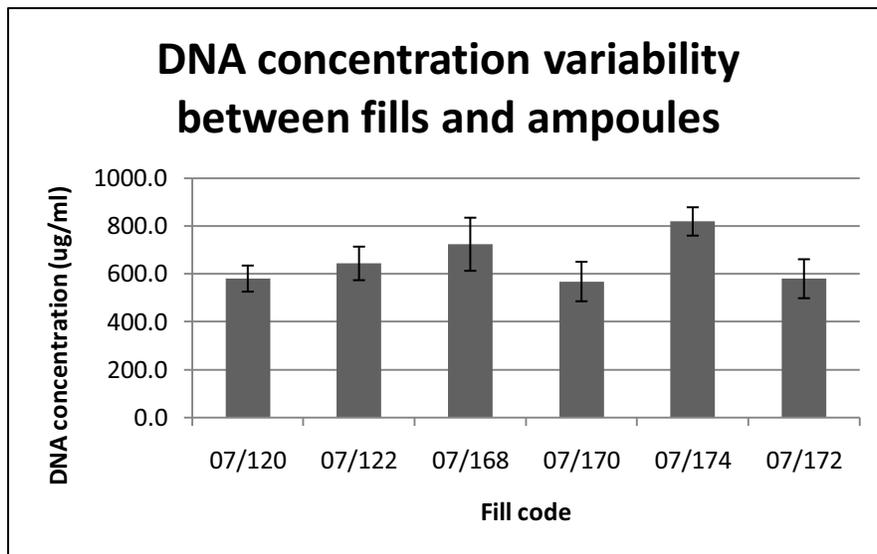
Table 1. Product summaries of the six candidate materials

NIBSC Code:	07/120	07/122	07/168	07/170	07/172	07/174
	Female, Wild-type	Female, Pre-mutation	Female, Full mutation 1	Male, Full mutation	Female, Full mutation 2	Male, Pre-mutation
Date filled	19.04.07	26.04.07	07.06.07	08.06.07	14.06.07	21.06.07.
Coefficient of variation of the fill (%) (n=39-44)	0.31	0.15	0.20	0.16	0.25	0.24
Residual moisture after lyophilisation (%) (n=12)	3.62	3.127	1.145	1.443	0.618	0.823
Mean dry weight (g) (n=6)	0.0025	0.0022	0.0019	0.0020	0.0021	0.0024
Residual oxygen% (n=12)	0.41	0.61	0.27	0.29	0.16	0.59
Mean OD ratio 260/280 nm (n=6)	1.85	1.79	1.88	1.83	1.86	1.89
Mean DNA conc. following resuspension in 40ul water ng/μL (n=6)	573.4	488.0	581.4	502.1	576.0	740.4
pH following resuspension	7.4	7.6	7.4	7.5	7.8	7.4
No of ampoules available	1728	2132	1758	2061	1774	1211
Presentation	Sealed, glass DIN ampoules					
Excipient	Trehalose 2.5mg/ml in Tris/EDTA buffer					
Storage temperature	-20°C					

A.



B.



A. Accelerated degradation- Real-Time PCR

Material	Storage Temperature					
	-150°C		-20°C		+56°C	
	Cp	CV%	Cp	CV%	Cp	CV%
07/120 (normal female)	20.51	0.11	20.71	0.20	21.05	0.07
07/122 (♀ pre-mutation)	21.23	0.03	20.57	0.10	20.80	0.13
07/168 (♀ full mutation 1)	20.97	0.06	20.61	0.13	20.77	0.36
07/170 (♂ full mutation)	21.13	0.08	20.98	0.10	20.99	0.10
07/172 (♀ full mutation 2)	N/D		N/D		N/D	
07/174 (♂ pre-mutation)	21.25	0.12	20.91	0.18	20.70	0.12

B. Accelerated degradation- Agarose Gel

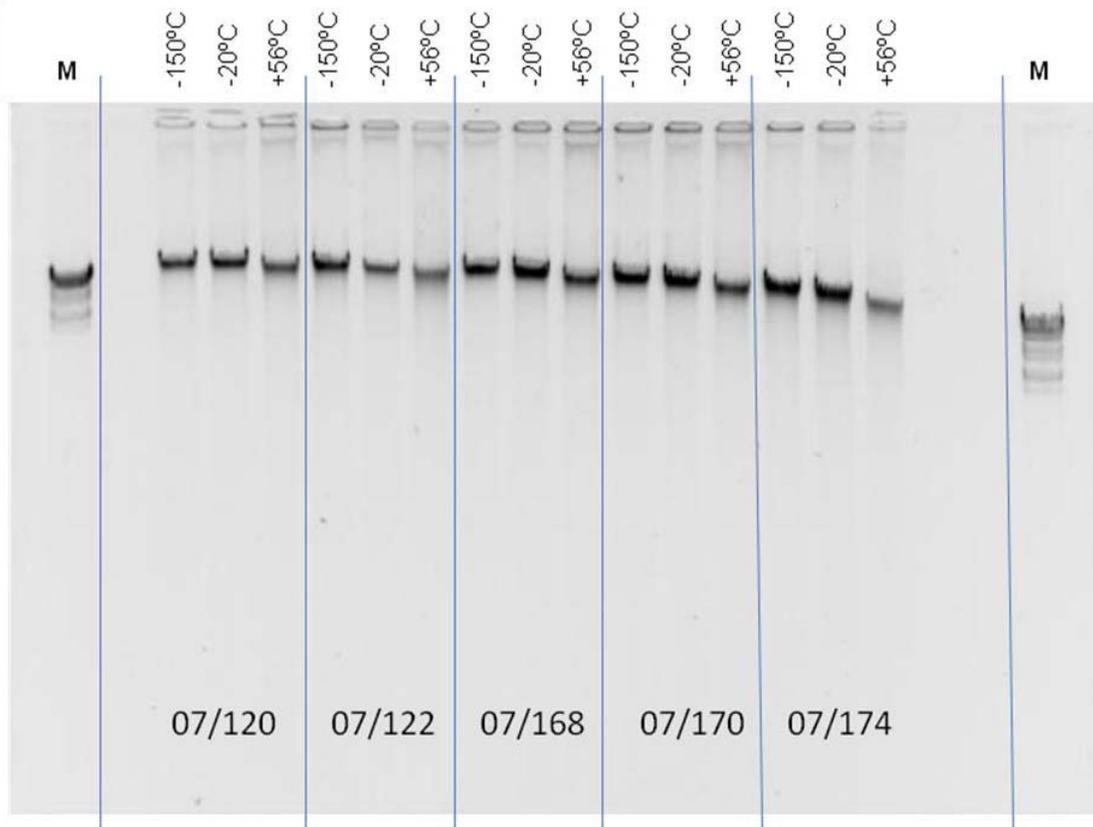
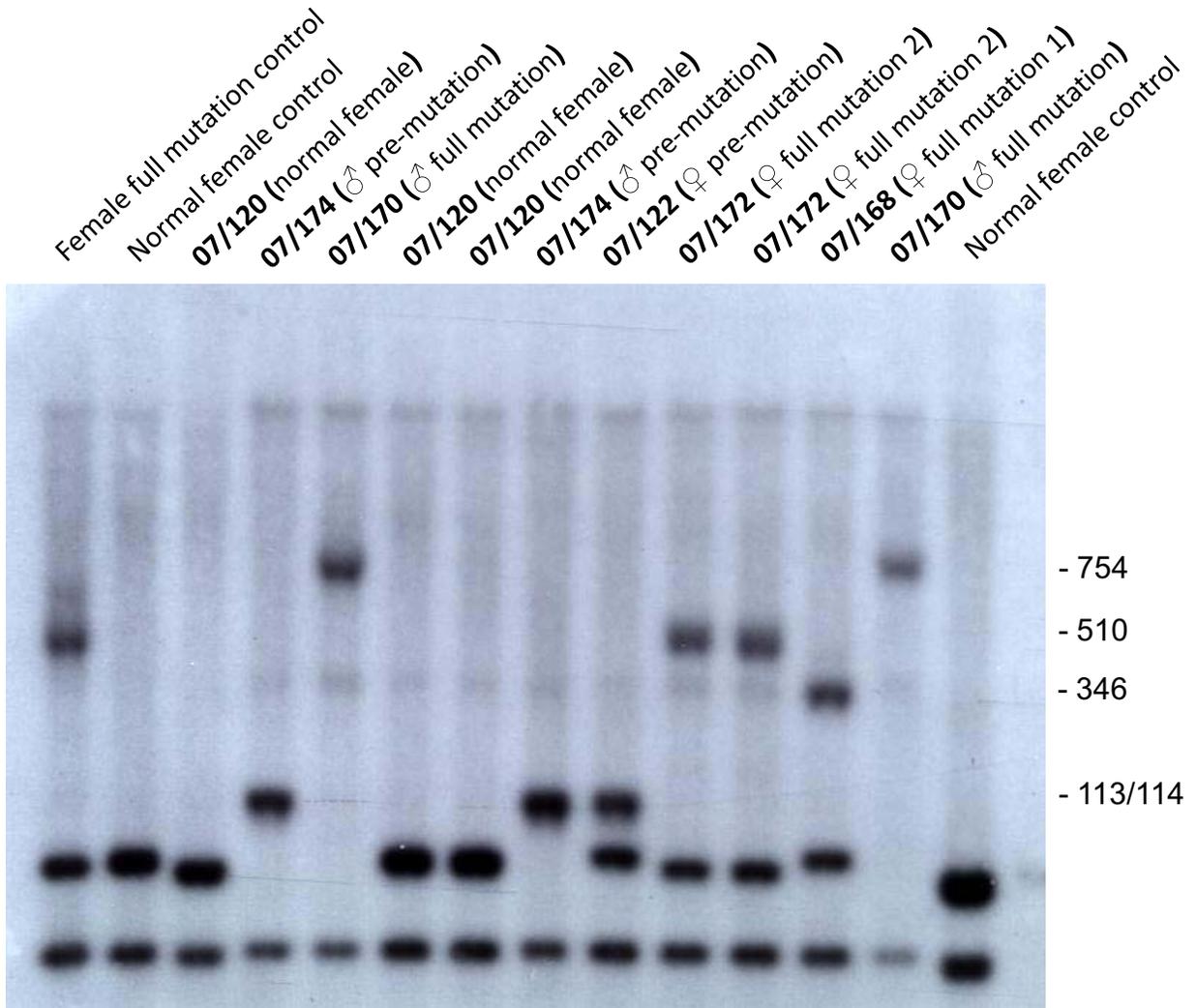
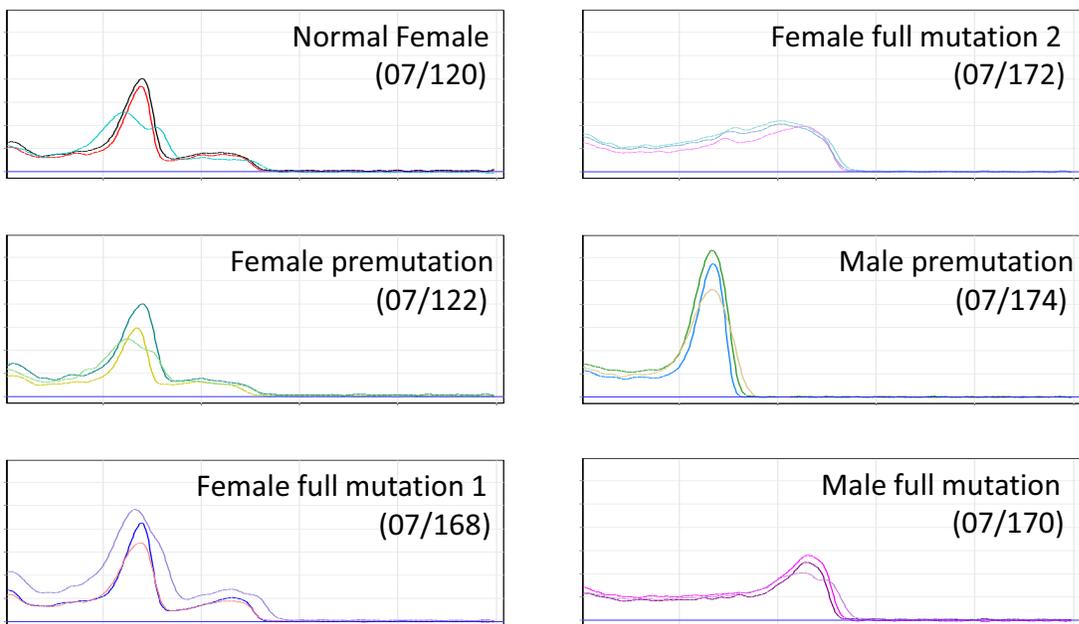


Table 2. Testing procedure used by the participants

Testing procedure	Participant laboratory code number	
	In-house assays	Abbott kit
PCR, then Southern blotting if required	4, 8, 17	
Southern blotting, then PCR if required	1	
All samples PCR and Southern blotting	6, 7, 9, 10, 12, 13, 14, 18, 20	3
PCR only	11, 15, 19	2, 5, 16
PCR and DNA sequencing	21	

A**B**

	Allele 1	Allele 2																																										
Female, Normal	<table border="1"> <caption>Allele 1, Female, Normal</caption> <tr><th>Allele</th><td>18</td><td>19</td><td>20</td><td>21</td><td>22</td><td>23</td><td>24</td><td>25</td></tr> <tr><th>Count</th><td>0</td><td>1</td><td>0</td><td>6</td><td>10</td><td>1</td><td>1</td><td>0</td></tr> </table>	Allele	18	19	20	21	22	23	24	25	Count	0	1	0	6	10	1	1	0	<table border="1"> <caption>Allele 2, Female, Normal</caption> <tr><th>Allele</th><td>27</td><td>28</td><td>29</td><td>30</td><td>31</td><td>32</td><td>33</td><td>34</td></tr> <tr><th>Count</th><td>0</td><td>1</td><td>1</td><td>5</td><td>10</td><td>1</td><td>1</td><td>0</td></tr> </table>	Allele	27	28	29	30	31	32	33	34	Count	0	1	1	5	10	1	1	0						
Allele	18	19	20	21	22	23	24	25																																				
Count	0	1	0	6	10	1	1	0																																				
Allele	27	28	29	30	31	32	33	34																																				
Count	0	1	1	5	10	1	1	0																																				
Female, Pre-Mutation	<table border="1"> <caption>Allele 1, Female, Pre-Mutation</caption> <tr><th>Allele</th><td>29</td><td>30</td><td>31</td><td>32</td><td>33</td><td>34</td><td>35</td><td>36</td><td>37</td></tr> <tr><th>Count</th><td>0</td><td>1</td><td>1</td><td>2</td><td>2</td><td>12</td><td>0</td><td>0</td><td>1</td></tr> </table>	Allele	29	30	31	32	33	34	35	36	37	Count	0	1	1	2	2	12	0	0	1	<table border="1"> <caption>Allele 2, Female, Pre-Mutation</caption> <tr><th>Allele Range</th><td>90-99</td><td>100-109</td><td>110-119</td><td>120-129</td><td>130-139</td><td>140-149</td></tr> <tr><th>Count</th><td>0</td><td>8</td><td>2</td><td>2</td><td>2</td><td>0</td></tr> </table>	Allele Range	90-99	100-109	110-119	120-129	130-139	140-149	Count	0	8	2	2	2	0								
Allele	29	30	31	32	33	34	35	36	37																																			
Count	0	1	1	2	2	12	0	0	1																																			
Allele Range	90-99	100-109	110-119	120-129	130-139	140-149																																						
Count	0	8	2	2	2	0																																						
Male, Pre-Mutation	<table border="1"> <caption>Allele 1, Male, Pre-Mutation</caption> <tr><th>Allele Range</th><td>80-89</td><td>90-99</td><td>100-109</td><td>110-119</td><td>120-129</td><td>130-139</td></tr> <tr><th>Count</th><td>0</td><td>1</td><td>4</td><td>4</td><td>5</td><td>0</td></tr> </table>	Allele Range	80-89	90-99	100-109	110-119	120-129	130-139	Count	0	1	4	4	5	0																													
Allele Range	80-89	90-99	100-109	110-119	120-129	130-139																																						
Count	0	1	4	4	5	0																																						
Female, Full Mutation -1	<table border="1"> <caption>Allele 1, Female, Full Mutation -1</caption> <tr><th>Allele</th><td>32</td><td>33</td><td>34</td><td>35</td><td>36</td><td>37</td><td>38</td><td>39</td><td>40</td><td>41</td><td>42</td></tr> <tr><th>Count</th><td>0</td><td>0</td><td>1</td><td>0</td><td>1</td><td>1</td><td>1</td><td>3</td><td>9</td><td>2</td><td>1</td></tr> </table>	Allele	32	33	34	35	36	37	38	39	40	41	42	Count	0	0	1	0	1	1	1	3	9	2	1	<table border="1"> <caption>Allele 2, Female, Full Mutation -1</caption> <tr><th>Allele Range</th><td>280-299</td><td>300-319</td><td>320-339</td><td>340-359</td><td>360-379</td><td>380-399</td><td>400-419</td><td>420-439</td></tr> <tr><th>Count</th><td>0</td><td>2</td><td>3</td><td>0</td><td>2</td><td>1</td><td>1</td><td>0</td></tr> </table>	Allele Range	280-299	300-319	320-339	340-359	360-379	380-399	400-419	420-439	Count	0	2	3	0	2	1	1	0
Allele	32	33	34	35	36	37	38	39	40	41	42																																	
Count	0	0	1	0	1	1	1	3	9	2	1																																	
Allele Range	280-299	300-319	320-339	340-359	360-379	380-399	400-419	420-439																																				
Count	0	2	3	0	2	1	1	0																																				
Female, Full Mutation -2	<table border="1"> <caption>Allele 1, Female, Full Mutation -2</caption> <tr><th>Allele</th><td>16</td><td>17</td><td>18</td><td>19</td><td>20</td><td>21</td><td>22</td></tr> <tr><th>Count</th><td>0</td><td>2</td><td>3</td><td>9</td><td>1</td><td>1</td><td>0</td></tr> </table>	Allele	16	17	18	19	20	21	22	Count	0	2	3	9	1	1	0	<table border="1"> <caption>Allele 2, Female, Full Mutation -2</caption> <tr><th>Allele Range</th><td>300-349</td><td>350-399</td><td>400-449</td><td>450-499</td><td>500-549</td><td>550-599</td><td>600-649</td><td>650-699</td></tr> <tr><th>Count</th><td>0</td><td>1</td><td>0</td><td>2</td><td>4</td><td>2</td><td>1</td><td>0</td></tr> </table>	Allele Range	300-349	350-399	400-449	450-499	500-549	550-599	600-649	650-699	Count	0	1	0	2	4	2	1	0								
Allele	16	17	18	19	20	21	22																																					
Count	0	2	3	9	1	1	0																																					
Allele Range	300-349	350-399	400-449	450-499	500-549	550-599	600-649	650-699																																				
Count	0	1	0	2	4	2	1	0																																				
Male, Full Mutation	<table border="1"> <caption>Allele 1, Male, Full Mutation</caption> <tr><th>Allele Range</th><td>250-349</td><td>350-449</td><td>450-549</td><td>550-649</td><td>650-749</td><td>750-849</td><td>850-949</td><td>950-1049</td><td>1050-1149</td></tr> <tr><th>Count</th><td>0</td><td>1</td><td>0</td><td>0</td><td>4</td><td>0</td><td>3</td><td>1</td><td>0</td></tr> </table>	Allele Range	250-349	350-449	450-549	550-649	650-749	750-849	850-949	950-1049	1050-1149	Count	0	1	0	0	4	0	3	1	0																							
Allele Range	250-349	350-449	450-549	550-649	650-749	750-849	850-949	950-1049	1050-1149																																			
Count	0	1	0	0	4	0	3	1	0																																			

Table 3. Summary of allele sizes and ranges

	Allele 1		Allele 2	
	Mean	Range	Mean	Range
Normal female	22	19-24	31	28-33
Female pre-mutation	33	30-36	113	100-132
Male pre-mutation	114	97-127		
Female full mutation 1	38	33-41	346	300-401
Female full mutation 2	19	17-21	510	355-600
Male full Mutation	754	353-960		