



GT FXS Detector

Product Instruction Manual

CAT#11011

CGG repeat expansion detection kit for Fragile X Syndrome

Produced by

GENETEK BIOPHARMA GmbH

Research Use Only

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1. GT FXS Detector Overview

- Accurate detection and sizing of CGG repeats in the 5' untranslated region (5'-UTR) of the FMR1 (NM_002024.4) gene responsible for Fragile X syndrome.
- Built-in panel makes repeat interpretation an easy task.
- Sizing ladder will help to interpret the results faster and better.
- Can be used on extracted DNA from blood, CVS, amniotic fluid, etc.
- Fragment analysis using capillary electrophoresis incorporating size standard gives precise size for each allele including full mutation 320 repeats (1200 bp).
- Compatible with Applied Biosystems® 3130/3130xl,3500/3500xL and SeqStudio Genetic Analyzers from Thermo Fisher Scientific Co.

1.1. Intended Use

The GT FXS Detector kit is intended to be used for the detection of Fragile X carriers and affected individuals with CGG repeat expansion in the 5'-UTR of FMR1 gene responsible for Fragile X syndrome.

1.2. Introduction

Fragile X syndrome (FXS) (FXS, OMIM # 300624), is a trinucleotide repeat disease caused predominantly by the expansion of the CGG sequences in the 5' untranslated region of the Fragile X Mental Retardation 1 (FMR1) gene [1]. FMR1 gene inactivation leads to non-expression of the fragile X mental retardation protein (FMRP), which is predominantly expressed in neurons and is involved in mRNA stability, transport and translation [2]. The shutoff of the FMRP production, results in the manifestation of FXS, which is the most prevalent type of heritable intellectual disability in men [3].

The clinical manifestation of the FXS is determined by the genotypes of the FMR1 alleles, which are categorized into **normal, intermediate, premutation, and full mutation** genotypes based on the types of the mutation [4]. Individuals with full mutations (>200 CGG repeats) often present classic FXS, characterized by mental retardation, autism, and emotional and psychiatric challenges [5]. Carriers with premutation alleles (55 to 200 repeats) do not develop FXS but are at risk for of developing other adverse health problems in adulthood such as fragile X-associated primary ovarian insufficiency (FXPOI) in females and tremor/ataxia syndrome (FXTAS) in both genders [5]. Importantly, female premutation carriers are at risk of transmitting a full mutation allele to their children. Indeed, it has been shown that repeat expansion causing premutation gene to convert to full mutation is mainly seen in females carrying premutation gene who have full mutation offspring.

Thus, a wide spectrum of people of all ages with various mental and physical health concerns may be impacted by fragile X syndrome and related illnesses. Genetic testing is crucial to establish the clinical diagnosis of FXS and its related illnesses and to identify carriers of expanded premutation alleles in family planning and genetic counselling.

For proper genetic counselling and result interpretation and report, it is highly suggested that the user of GT FXS Detector kit becomes fully familiar with molecular genetics of FMR1 gene structure, expansion mechanism, expansion inheritance, methylation, etc. The following guidelines and reviews are good sources of information. However, they may not suffice, and one has to resort to further information. We have provided several references at the end of this manual, and they are usually available online. ACMG guideline [6], a revised version [7] and a mini-review describing periodicity can be found as well [8].

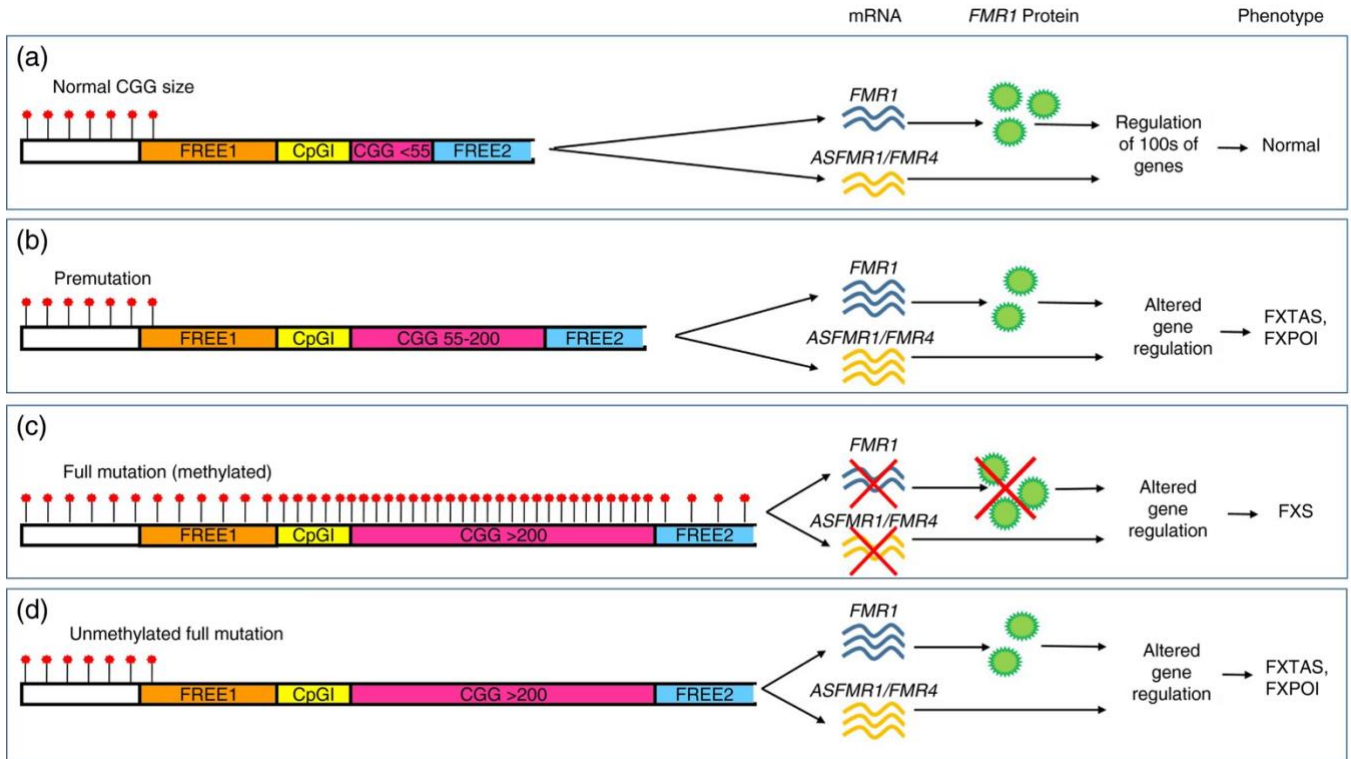


Figure 1: Different types of gene alterations of the FMR1 gene (from: Kraan CM, Godler DE and Amor DJ. Epigenetics of fragile X syndrome and fragile X-related disorders. Dev Med Child Neurol, 2019, 61(2):121-127).

The association between epigenotype and phenotype in fragile X-associated disorders. (a) Normal FMR1 alleles with CGG repeat sizes of less than 44 repeats are associated with the absence of promoter methylation and normal transcription of FMR1 and ASFMR1/FMR4, which in turn regulate the expression of hundreds of genes associated with normal neurodevelopment. (b) Premutation CGG repeats (55–200 repeats) are not associated with methylation of the promoter, but lead to increased transcription of FMR1 mRNA, which is thought to lead to premutation phenotypes such as FXTAS and fragile X-associated primary ovarian insufficiency (FXPOI) via the mechanism of RNA toxicity. Paradoxically, in carriers of large premutation, production of FMR1 protein is reduced. Transcription of ASFMR1/FMR4 is also increased in premutation carriers and may contribute to premutation phenotypes. (c) Full mutation CGG repeats (>200 repeats) are associated with methylation of the FMR1 promoter and reduced or abolished transcription of FMR1 RNA and translation of FMR1 protein. Transcription of ASFMR1/FMR4 is also reduced, which may contribute to the phenotype of fragile X syndrome. (d) In rare individuals with full mutation CGG repeats, the FMR1 promoter remains unmethylated, allowing transcription of FMR1 and ASFMR1/ FMR4. Similar to the case with premutation CGG expansions, transcription of these expanded repeats is associated with increased production of RNA, which may lead to FXTAS and FXPOI through the mechanism of RNA toxicity. Conversely, production of FMRP is reduced. In females, the promoter region between the 50 and 30 boundaries is subject to X-chromosome inactivation, and variability in X-inactivation and related methylation of the promoter region has been correlated with premutation-related phenotypes in females. CpG, CpG island.

Interpretation of FXS allelic classification

Risk assessment and clinical interpretation of FXS and related disorders are defined by the number of CGG repeats and methylation status of the gene. Based on the number of CGG repeats it is possible to distinguish four types of alleles according to current American College of Medical Genetics guidelines [6]:

- Normal (NL), approximately 5 to 44 repeats.
- Intermediate (IM), approximately 45 to 54 repeats.
- Premutation (PM), approximately 55 to 200 repeats.
- Full mutation (FM), more than 200 repeats.

Full mutation alleles can range from 200 to greater than 1000 repeats. Above 200 repeats, the FXS phenotype is associated with the methylation status of the allele and not necessarily the exact number of repeats exceeding 200 CGG [9]. In premutation alleles (~60 to 200 repeats), the risk of expansion to a full mutation increases with size specially in females. Above 100 repeats, there is a systematic risk of expansion in the next generation [10].

1.2.1. Fragile X testing

The analysis of the CGG repeats in the FMR1 gene typically relies on PCR with size resolution by capillary electrophoresis (CE), or agarose gel though exact sizing is not possible. The CGG repeats, resulting in large permutations and full mutations usually are undetected since the smaller alleles are preferentially amplified. FMR1 Southern blot analysis is used to characterize samples with CGG repeat numbers too large to amplify by PCR, and to determine the methylation status of the gene [11]. Southern blot analysis, on the other hand, is laborious, time-consuming, and necessitates large amounts of DNA. Resolution of Southern blot analysis is also low. These methods are thus unsuitable for routine testing and/or screening large numbers of samples for FMR1 CGG expansions. CE has proven to be more accurate, high throughput, and more reliable particularly for carrier detection.

Another issue is presence of mosaicism which can't easily be detected using agarose gel but can be seen by CE when the sizes of fragments are less than 1200 bp or smaller than 320 repeats.

The GT FXS Detector kit is designed to address the issues for sizing analysis of the FMR1 CGG repeat region. CGG repeats in FMR1 are amplified by triplet-primed PCR followed by fragment analysis using capillary electrophoresis (CE). The GT FXS Detector kit provides accurate sizing of alleles up to 320 CGG, identification of full mutation alleles >200 CGG by presenting particular patterns unique to expansion of repeat units (see figure 2 for an example and table 1 below).

1.3. Test Principle

The GT FXS Detector kit uses a TP or CGG repeat primed (RP) PCR to produce stutter peaks which are related to periodicity of CGG repeats. The results are run on any of Thermo Fisher Genetic Analyzers (i.e. 3130/xl, 3500/xl, SeqStudio). The GT FXS Detector kit is designed to produce fragment sizes which is easily converted to repeat numbers.

To help the kit users and facilitate allele interpretation, we have devised an easy interpretation methodology by use of bin set. This bioinformatic tool helps the user to determine the exact repeat number or allele size during result analysis using GeneMapper (Thermo Fisher Scientific), or GeneMarker (SoftGenetics) software. We have also provided a panel to ease visual interpretation and rough sizing of the results (an example is given below in figure 2).

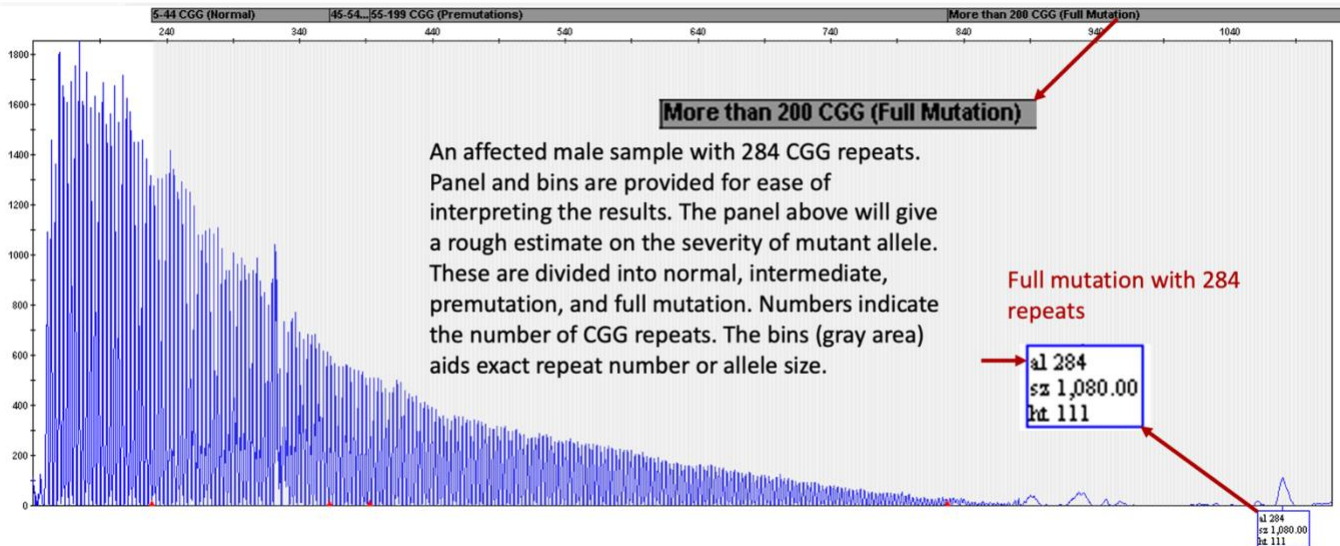


Figure 2: The figure shows an affected male result with 284 CGG repeats and panel for result interpretation. We have provided bins (the gray area) and panel for quick and reliable interpretation of results. The stutter or periodicity peaks are visible which indicate CGG expansion. If there was no expanded (mutant) allele visible, then the presence of these periodicity peaks indicate that there is an expansion and absence of the mutant allele indicate expanded mutant allele is more than 323 repeats since the size standard is about 1200 bp.

Table 1. Fragment sizes used for designing panel for the GT FXS Detector Kit

No.	Panel	Size Range	Disease severity
1	<45 CGG	(229-361)	Normal
2	45-54 CGG	(364-391)	Intermediate
3	55-200 CGG	(394-826)	Premutation
4	>200 CGG	(829-1200)	Full mutation

Following amplification, the PCR amplicons are subjected to capillary electrophoresis, as outlined in section 6.2 below.

The CGG-primed and gene-specific peaks will be produced in the electropherogram for uninterrupted alleles, corresponding to the numbers of FMR1 CGG repeats present in the sample. Due to the triplet-repeat nature of the CGG repeat tract, the CGG-primed amplicon peaks in the electropherogram exhibit a 3-basepair periodicity (Figure 3). The electropherogram generated from the capillary electrophoresis will also indicate continuity of the CGG repeat tract. Essentially, the AGG sequences interruption which are interspersed within the CGG repeat tract at the 5' end of the CGG repeat, will produce clusters of peaks separated by gaps with no or very little amplification (Red arrows in the Figure 3). Most people have one or two of these AGG interruptions. It is claimed that presence of these interruption has counter effect on expansion during transmission of mutated allele [8].

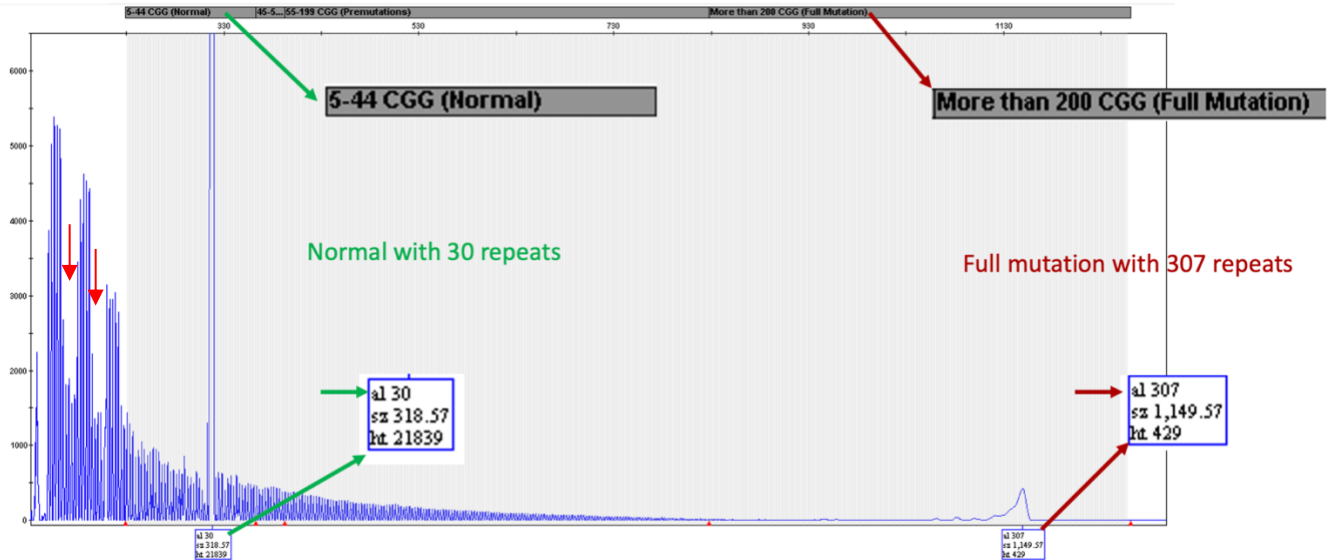


Figure 3. An example of a sample from a carrier female with full mutation with 30 & 307 CGG repeats. The peaks illustrate a 3-base pair periodicity. Interrupting AGG sequences are typically located at the 5' end of the repeat tract and are usually arrayed with a periodicity of 9-10 CGG repeats. Approximately 95% of normal individuals have one or two AGG interruption (Down facing Red arrows). Presence and quality of these peaks prior to the main normal peaks indicate how well PCR has amplified the CGG repeats.

It is recommended to include positive and negative controls with every run to ensure integrity of reagents and performance of the CE instrument. A negative, no-template, control will include all reagents except the genomic DNA sample. A heterozygote female with full mutation is recommended as a positive control.

2. Storage and Handling








- The GT FXS Detector kit comes in two boxes (A and B).
- Reagents in box A of the kit should be kept in -20°C freezer.
- Reagents of box B of the kit should be kept at a temperature of 2 to 8°C or -20°C.
- Keep the primer mix in a dark place (because of fluorescently labeled primers).
- Avoid frequent freeze and thawing (store the materials in small aliquots if you are going to use the kit content in several different days in an expansion of long time).
- Low-quality result may be obtained after the expiration date (12 months).
- Prior to opening, mix tubes contents by vortexing and briefly centrifuge each tube to collect any content on the wall or lid of the tubes at the bottom of the tube.
- Proper thawing of tube content is essential for proper function of the kit. Therefore, allow tubes to stand at room temperature enough to allow proper thawing, then mix the tube content and quick spin. We have freeze-thawed tube contents for more than 100 times with little effect. So, do not worry about tubes being left at room temperature for proper thawing.
- Assay setup should be prepared at room temperature.

3. Materials

3.1. Reagents provided by GT FXS Detector Kit

- Provided with the Kit with Box A and Box B. They should be kept separately since Box B contains PCR products though there is no risk of PCR carry over since these fragments are not from human genome.
- The Control DNA **GT QCDM102 (Control DNA-100ng/μl)** is a genomic DNA from a normal person.
- GT M5 is a matrix standard which is required only once for the calibration of Genetic Analyzer (please follow the manufacturer recommendations for the instrument calibration). For more information, see the 5-color calibration manual on the Genetek website and user guides from Thermo Fisher Genetic Analyzers for the relevant machine.

Table 3. Reagents provided with the Kit in Box A and Box B

BOX-A			BOX-B		
	Tube Label	Tube cap color		Tube Label	Tube cap color
1	PCR Mix		1	GT1200 Size Standard	
2	Primer Mix-1		2	GTM5 (Optional)	
3	Primer Mix-2				
4	GT Taq DNA Polymerase				
	GT QCDM102 (Control DNA-100ng/μl)				

3.2. Number of reactions

- The kit content is sufficient for at least 10, 25, 50 or 100 reactions (please see the label on the box to see which kit size you are using by looking at the last digits on the cat number of the kit in use [e.g., GT-11011-**100** is a 100 reactions kit]) for gene-specific PCR or CGG RP PCR. Therefore, the actual number of tests depends on how many controls you are using with the actual test samples. It is essential to use airtight microtubes since evaporation during PCR hampers proper amplification particularly longer fragments will not be generated properly. Genetek multiplex PCR kits have validated using microtubes from Sarstedt, Germany, other brands may do well.

3.3. Reagents and equipment required but not provided

- Use of positive control DNA is highly recommended: A female full mutation sample (such as NA07537 from Coriell Cell Repositories or other sources can be obtained).
- Reagents and equipment for DNA extraction.
- Equipment and consumable for amplification (i.e., Thermal Cycler, Micropipette, Filter Tips, etc.).
- Applied Biosystems Genetic Analyzer (ABI 3130/xl, 3500/xL or SeqStudio) with Data Collection software for 5-dye system detection.
- Performance optimized polymers (POP-7) and 36 or 50 cm Capillary Array or equivalent.
- Hi-Di™ Formamide or equivalent.
- GTM5 Matrix Standard for Spectral calibration (GT-41101-48).

4. Warnings and Precautions

- The GT FXS Detector kit can't be used to make a clinical diagnosis; it is only meant to be used as a sizing test for the number of CGG repeats. The result should be interpreted by specialist and following best practice guidelines (see reference section at the end of this manual). The kit is for **research use only** until it obtains IVD mark.
- Test administrators should have PCR and Capillary Electrophoresis setup prior experience to obtain the full advantage of this kit.
- All spent and unused chemicals must be disposed in accordance with local laws governing biosafety, chemical safety, and environmental protection.
- All DNA samples should be treated with caution since they may be biohazard. Throughout the setup, protective equipment and gloves should be worn. Abstain from skin contact and spillage. If either occurs, wash your hand with handwash detergent and plenty of water and clean surface area with either DNA Cleanser from Genetek Biopharma or 10% bleach to avoid possible DNA contamination. Use of DNA Cleanser is recommended since it does not have any PCR inhibitor property as bleach usually do. Protecting cuts and wounds is necessary. Hands need to be well cleaned both before and after setup.
- Don't use out-of-date reagents, as you may not be able to get proper and reliable results.
- Reagents from various kits and batches should not be mixed.
- Ascertain that the equipment is calibrated and utilized in accordance with the manufacturer's instructions.
- Refrain from repeated freeze-thaw cycles of the kit components.
- Make sure chemicals are stored in a dark place.
- After opening, the kit has a 12-months shelf life if properly used as recommended above.

5. Pre-Analytical Steps

- Genomic DNA extracted via common sample preparation methodologies from whole blood collected in EDTA is compatible with the GT FXS Detector kit. However, high quality DNA is essential for best performance of the kit as well the amount used. Therefore, please check the kit performance on known samples prior to patients' samples.
- After DNA extraction, the purified genomic DNA should be evaluated for concentration (Optical Density, OD 260) and purity (OD 260/280). The DNA samples should be stored below -15°C.
- Input about 25-100 ng into each reaction (1 µL of control DNA is about 100 ng/µL). Internal validation in each laboratory is recommended for optimizing and getting the best results.

6. Protocol of GT FXS Detector Kit

- Protocol of the GT FXS Detector kit involves three key steps:
 - I. PCR Master Mix Setup and Thermal Cycling
 - II. Capillary electrophoresis
 - III. Fragment sizing analysis
- The following instructions are written for a single reaction; preparation of master mixes is recommended for multiple reactions.
- The reagents provided in each kit are sufficient for at least 10, 25, 50 or 100 reactions (depending on the kit size).

- The workflow should proceed in a unidirectional manner starting with a dedicated pre-amplification area and moving to a segregated post-amplification area. Amplified product should remain in the post-amplification area to minimize risk of amplicon contamination.
- For each run performed, two controls should be included: a female full mutation sample as a positive control, and a no template control (NTC).

6.1. PCR Master Mix Setup and Thermal Cycling

1. Thaw all reagents except GT Taq DNA Polymerase at room temperature. Briefly vortex all tubes except the DNA Polymerase and then spin the content down.
2. Add the appropriate amount of each component into a 1.5 mL microfuge tube in the exact order specified in Table 4. Try to compensate for pipetting error by making an extra reaction. For example, if you are setting up for 6 reactions, make the mixes for 7 reactions.

Table 4. PCR Master Mix Setup

	Component	Volume per reaction (µl)
1	PCR Mix	17.85
2	Primer Mix-1	0.75
3	Primer Mix-2	0.4
4	GT Taq	1

Table 5. PCR Program. The PCR product at the end of cycling should be kept at 4-8°C or at -20°C. For extension

Initial step	Cycling			Final Extension	Storing in Cycler
	Denaturation	Annealing	Extension		
Start with 95 °C for 5 min then follow cycling program	97 °C	62 °C	68 °C	-	-
	35 sec	35 sec	4 min	-	-
	Above program for 10 Cycles				
-	97 °C	62 °C	68 °C	72 °C	4 °C
-	35 sec	35 sec	4 min*	20 min	∞
	Above program for 20 Cycles				

*After the 10th cycle, increase the extension time 20 seconds for each cycle and continue for 20 cycles (e.g., for 15th cycle it should be 5min and 40 sec. and 16th cycle should be 6 min, etc.).

- Note 1: All steps must be done in Pre-PCR environment and negative control should be used.
- Note 2: Preparation of a master mix is recommended for setup of multiple reactions. The master mix will include all components except for template DNA.

- Note 3: Thoroughly vortex master mix prior to aliquoting into PCR micro tubes, PCR plate or strip-tubes and dispense 20.00 µL master mix into each tube.
3. Add 100 ng (1.00 µL of 100 ng concentration) of the appropriate DNA sample into each tube.
 4. Pipette up/down at least twice to ensure adequate mixing or gently vortex and spin down the PCR tube.
 5. Transfer the sealed PCR tubes to a thermal cycler and run the appropriate cycling protocol (Table 5):

*Follow the instruction manual of the thermal cycler to add 20 *After

*After the 10th cycle, increase the extension time 20 seconds for each cycle and continue for 22 cycles seconds extension time per cycle for this step (e.g., for the 15th cycle it should be 5min and 40 sec. and for the 16th cycle it should be 6 min, so on).

6. After completion of PCR, it is recommended to run 10 ul of the PCR product on a 1-1.5% agarose gel to see if PCR had worked (and if affected male sample is included in your reaction), to see if the full expansion band can be seen (see figure 4 for some results on an agarose gel).
7. Transfer PCR products for CE analysis or store them at -15 to -30°C away from light until analyzed. PCR product stability at -15 to -30°C has been verified for at least 10 days storage.

6.2. Capillary Electrophoresis

We highly recommend that the user perform spectral calibration on their Genetic Analyzer since our size standard uses different fluorescent dyes. If needed, please ask us to provide you with GTM5 user manual.

To perform CE, thaw the HI-Di formamide or equivalent, and GT 1200 Size standard to room temperature. Thoroughly vortex (15 seconds) and spin tubes before use. Quality of formamide is very important and poor quality can hamper CE. A disadvantage of formamide is its tendency to decompose into formic acid over time or through exposure to moisture in the air. Whenever, high quality formamide is obtained, it should be dispensed into small aliquots and frozen.

1. Prepare a master mix solution by adding components in the order listed below:

Hi-Di Formamide	9 µL
GT 1200 Size standard	0.5 µL

Total Volume per well 9.5 µL

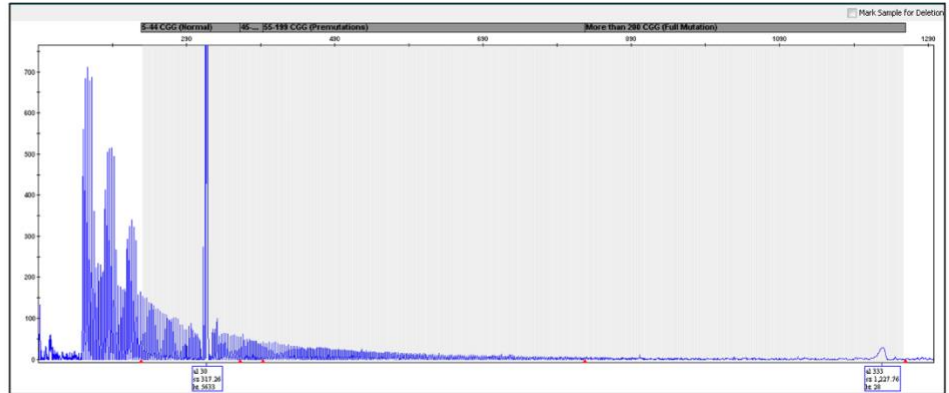
2. Mix all the added reagents by vortexing and spinning them down briefly to collect them at the bottom of the tube.
3. Aliquot 9.5 µL of Formamide/size standard solution to each well of a new CE analysis plate. If number of samples is less than capillaries (i.e., 4, 8, 16, 28, etc.) the empty wells must be filled with 13.5 µl Hi-Di Formamide.

4. Transfer 1-2 μ L of PCR products into each well in the CE plate, pipetting up and down 2 to 3 times to mix. A multi-channel pipette is recommended for transfer and mixing.
5. Seal the plate, vortex, centrifuge to remove air bubbles and transfer to a thermal cycler or heat block.
6. Denature for 2 min at 95°C followed by 4 °C until ready for injection on the CE instrument. Alternatively, the products may be stored on ice and protected from light after the denaturation step. This step is very important. We have tested this step, and the results are shown below (figures 4 and 5).

Hot and Ice effect on results

A

With Hot & ice



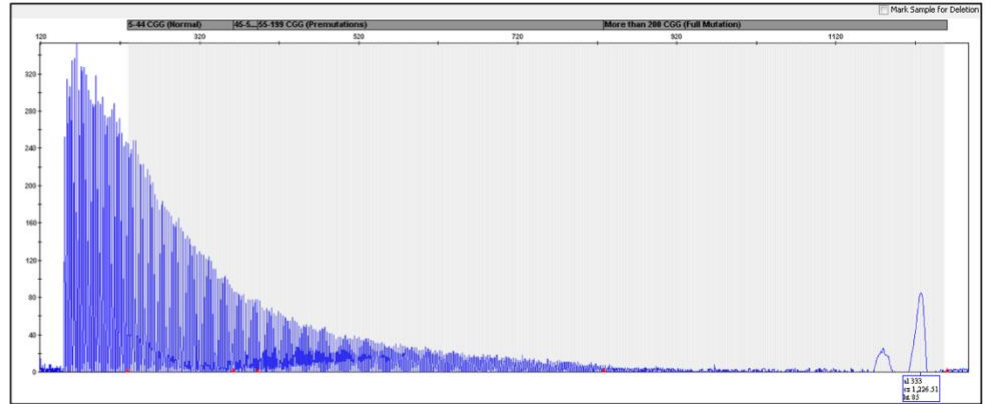
Without Hot & ice



Figure 4: Effect of hot (denaturation) and ice (cooling) on normal sample. The hot and ice treatment prior to electrophoresis has a positive effect on results not related to PCR. In the no “Hot & Ice” treatment, the normal allele is hardly visible (in both cases the fragment sizes are about 318 bp).

B

With Hot & ice



Without Hot & ice

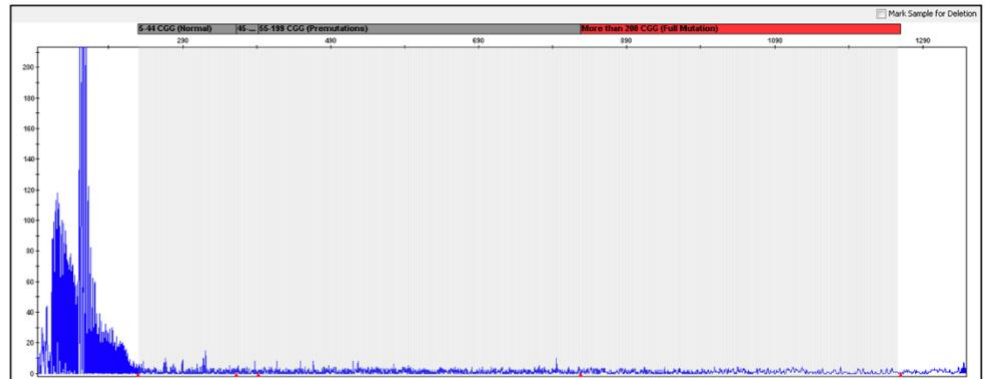


Figure 5: Effect of hot (denaturation) and ice (cooling) on a sample from a carrier female (A) and an affected male persons (B). The “hot and ice” treatment prior to electrophoresis has positive effect on results not related to PCR. In the no “Hot & Ice” treatment, the expansion can’t be interpreted properly. Even the expanded allele is not visible.

7. Prepare Genetic Analyzer for data acquisition according to manufacturer’s directions. Final injection and run conditions must be validated by the end user and may differ between instruments. The following considerations apply:
 - The instrument must be calibrated for the detection of both 6-FAM and GT 1200 size standard fluorescent dyes. Therefore, it is necessary to calibrate the Genetic Analyzer prior to first use to avoid shadows of GT 1200 sizes in the 6-Fam (blue) channel.
 - Use factory installed Fragment Analysis Protocol for POP-7 polymer and capillary length for your instrument as a base protocol.
 - Adjust the injection conditions and run time according to the instrument configuration and capillary length. Recommended starting values are listed in Table 6.

Table 6. Injection and Run Time adjustments for instrument classes and capillary lengths running POP-7 polymer.

Instrument	Capillary Length	Injection	Run Time
3130,3130xl	36 cm	2.5 kV, 20 s	4200 s
3500, 3500xL	50 cm	2.5 kV, 20 s	4200 s

8. After the run, the data may be analysed for amplicon size and conversion to CGG repeat length.

6.3. Fragment sizing analysis

Interpretation of the results of the GT FXS Detector kit can be done using agarose gel electrophoresis on a 1-1.5% agarose gel and capillary electrophoresis using **Genetic Analyzer** as outlined above.

6.3.1. Result interpretation based on gel electrophoresis

Interpretation of the results of the GT FXS Detector kit can be done using agarose gel electrophoresis. Figure 4 shows image of a 1.5% agarose gel for PCR product of the GT FXS Detector kit for normal male (lanes 2, 8, and 12), premutation female (lanes 3, 5, 9, and 11), full-mutation male (lanes 4 and 10), and negative control (lanes 6 and 13). This figure indicates that the result of affected individuals with a higher number of repetitions has a higher band compared to the normal individual. Full mutation with size close to 1200 can be seen with ease. AGG interruption related peaks are visible for samples 2, 3, 5, 8, 9, 11 and 12 below the normal allele around 200 bp marker.

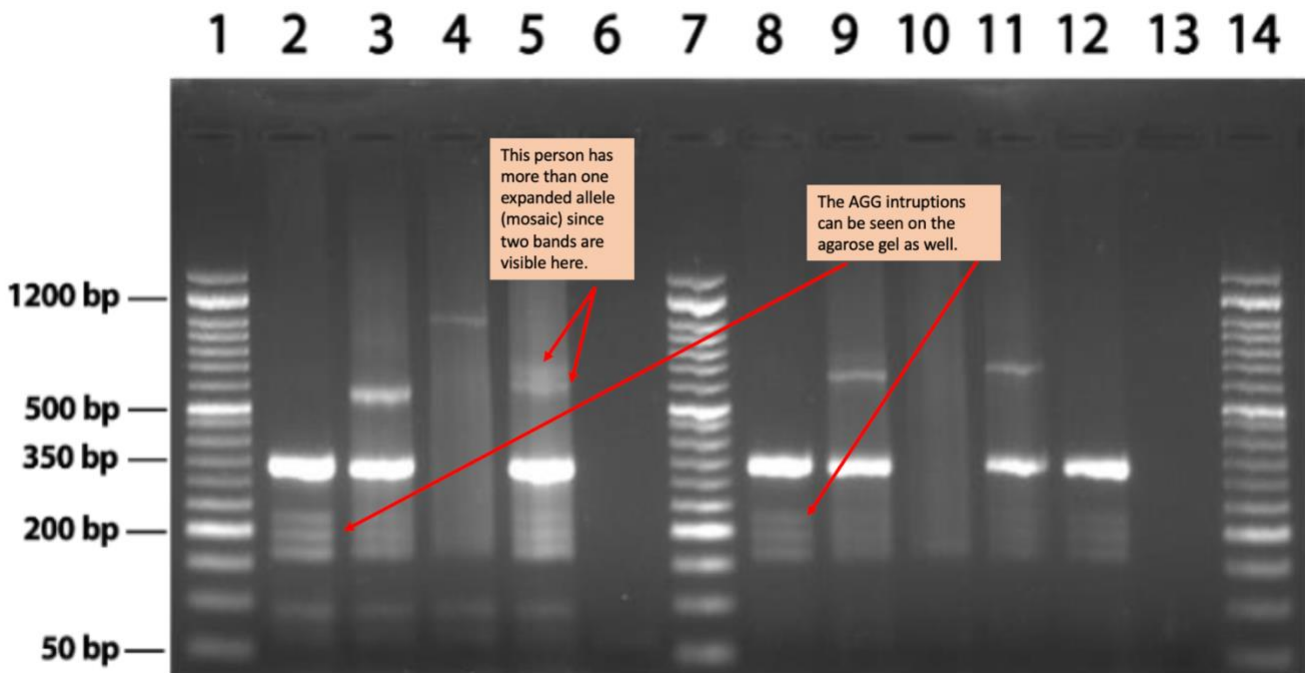


Figure 6. An agarose gel image of PCR product generated using the GT FXS Detector kit for a normal male sample (lanes 2, 8, and 12), premutation females (lanes 3, 5, 9, and 11), full-mutation male (lanes 4 and 10), and negative control (lanes 6 and 13). Visualizing the PCR product on agarose gel will provide further information when results are compared with CE results.

6.3.2. Result interpretation based on capillary electrophoresis

Interpretation of the results of the GT FXS Detector kit can also be done using capillary electrophoresis (CE). The GT FXS Detector kit can be used with ABI's Fragment Analysis software, suitable for Genetic Analyzer devices or software GeneScan® Analysis, Genotype Analysis, GeneMapper® and GeneMarker® analysis.

Fragment sizing analysis of the results of GT FXS Detector kit involves a series of steps to obtain the size of full-length product peaks and identify features in the run for interpretation of the data. These results are converted to CGG repeat length as described in Test Principle (section 1.3). An overview of the fragment sizing analysis workflow is shown in Figure 5.

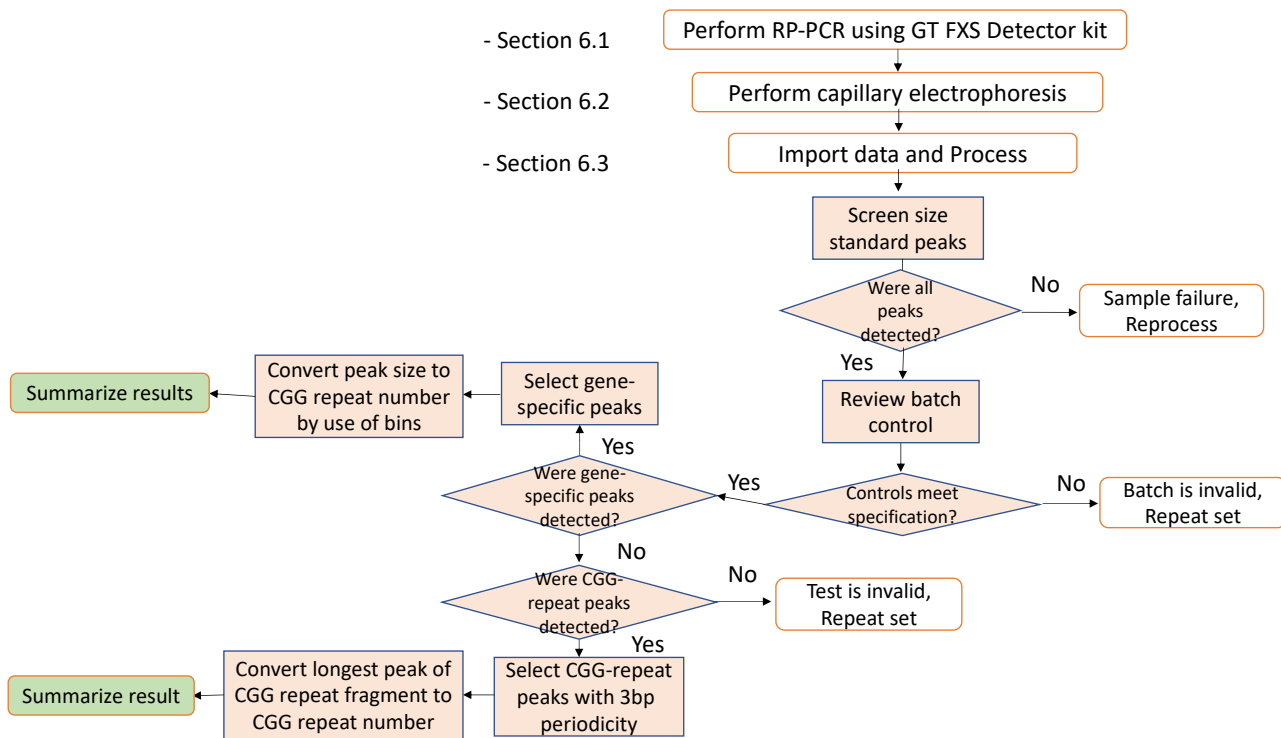


Figure 7. Schematic workflow for analysis of results and interpretation of the data.

6.3.2.1. Criteria for Interpretations of CE data

- “Size” shows the fragment size. The size may differ between individuals but are usually constant within a person and his/her parents particularly when allele size in is normal range, however, expansion from one generation to the next may be seen.
- Allele (al) under a peak shows the number of CGG repeats. This is facilitated by incorporation of bins in the result interpretation process using the above-mentioned software and the files we provide to be incorporated during data analysis and interpretation.
- The area under each peak in electropherogram represents the amount of amplified PCR product or accumulated florescent dye emission.
- The height of each peak represents the activity of each fluorescent component which shows the quantity of the fluorescent compartment of each marker.
- These results are shown as electropherograms in the analysis software. Height and the area related to each peak are observable in this software.
- Negative control should not show any fragment between 100 to 1200 bp.

6.3.2.2. Qualify validity of the run

- Review GT1200 size standard peaks: The GT1200 Size Standard produces characteristic size standard peaks (refer to Figure 6). Irregularities in the intervals between these size standard peaks will affect analysis. Samples with size standard issues (for instance, the peaks do not appear at the expected base pair sizes) must be excluded from further analysis.

GT FXS Detector Markers

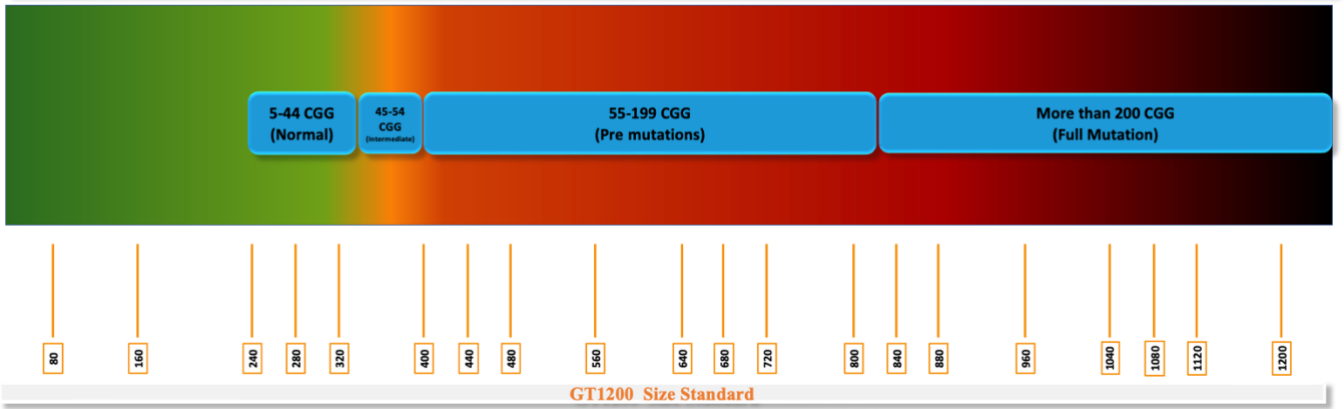


Figure 8. Representative profile of GT1200 Size Standard. Panel and repeat number effects on each individual are shown.

- Review negative control: Ensure no signal is present in the blue (FAM) channel for negative no template controls.
- Review positive control (female full mutation): Ensure the positive control meets the following specifications (Figure 7).

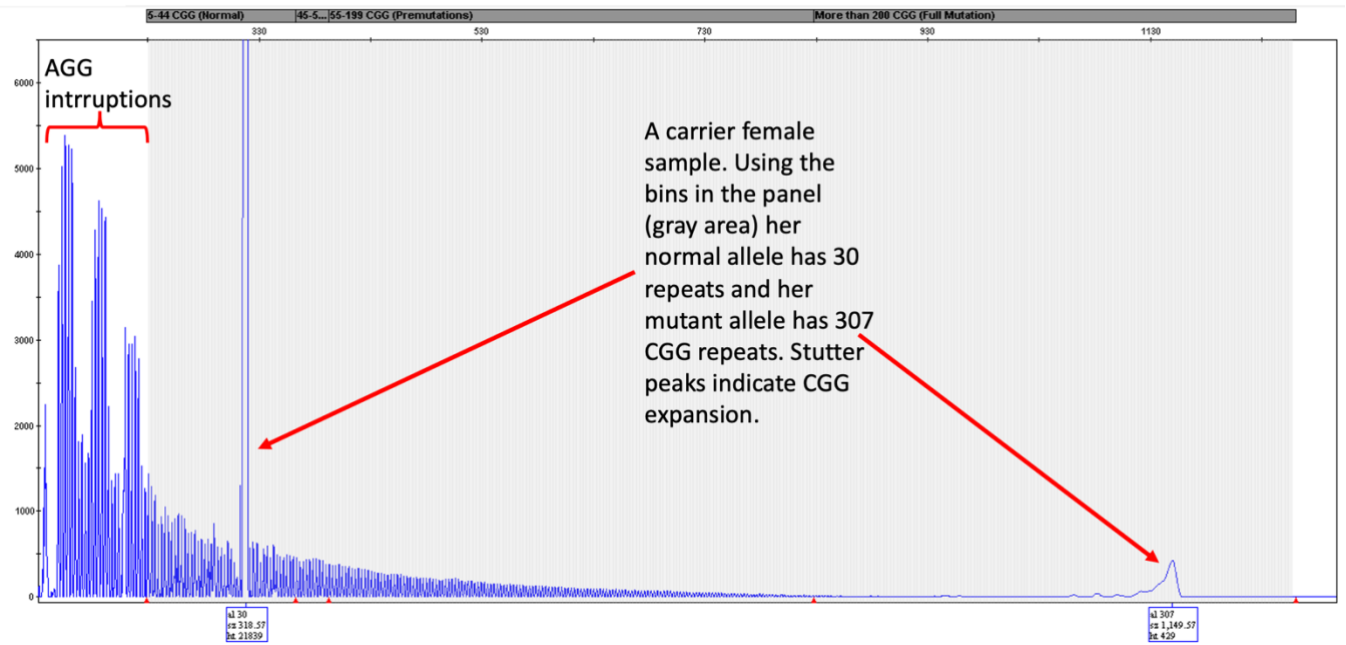


Figure 9. Representative profile of a carrier female. The first peak in the electropherogram has a size of about 318 bp or 30 CGG repeats, and her mutant allele has 307 CGG repeats. There AGG interruptions can be seen before her normal allele before the start of bins or 230 bp. This person has two AGG interruptions.

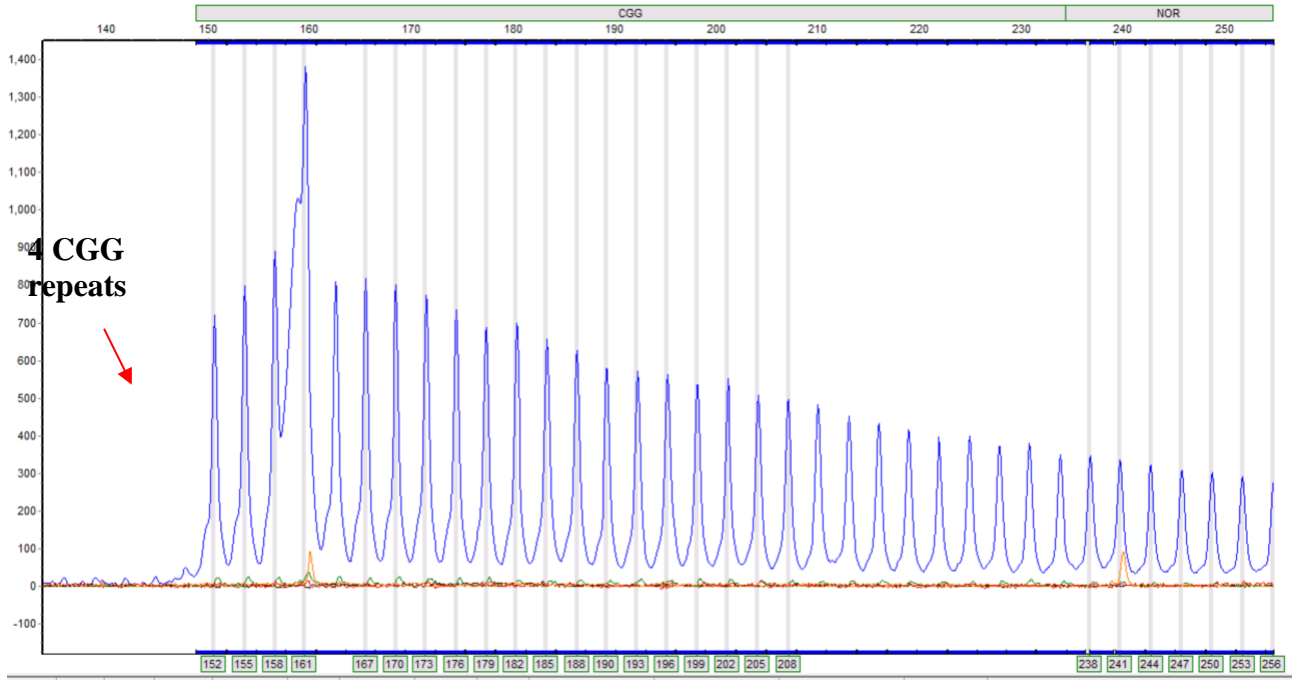


Figure 10. Electropherogram of a full mutation male. The peaks illustrate a 3-base pair periodicity. The first peak in this electropherogram is a fragment with a size of 4 CGG repeats and the flanking regions between the two Forward and Reverse primers. Presence and quality of these peaks prior to the main normal peaks indicate how well PCR has amplified the CGG repeats.

7. Sample cases

Electropherogram of a full-mutation male with 284 CGG repeats using ABI,3500xL genetic analyzer

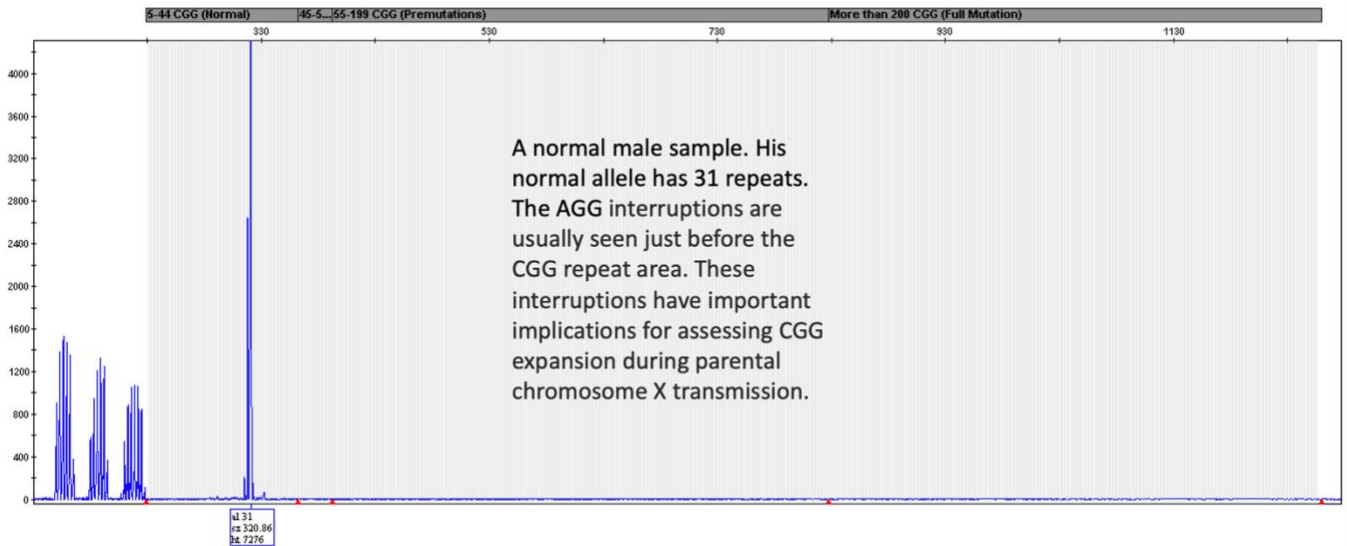


Figure 11: An Electropherogram of a normal male with 31 CGG repeats using ABI,3500xL genetic analyzer. Since the normal allele (number of CGG repeats) is not long, no stutter peaks have been generated and are visible.

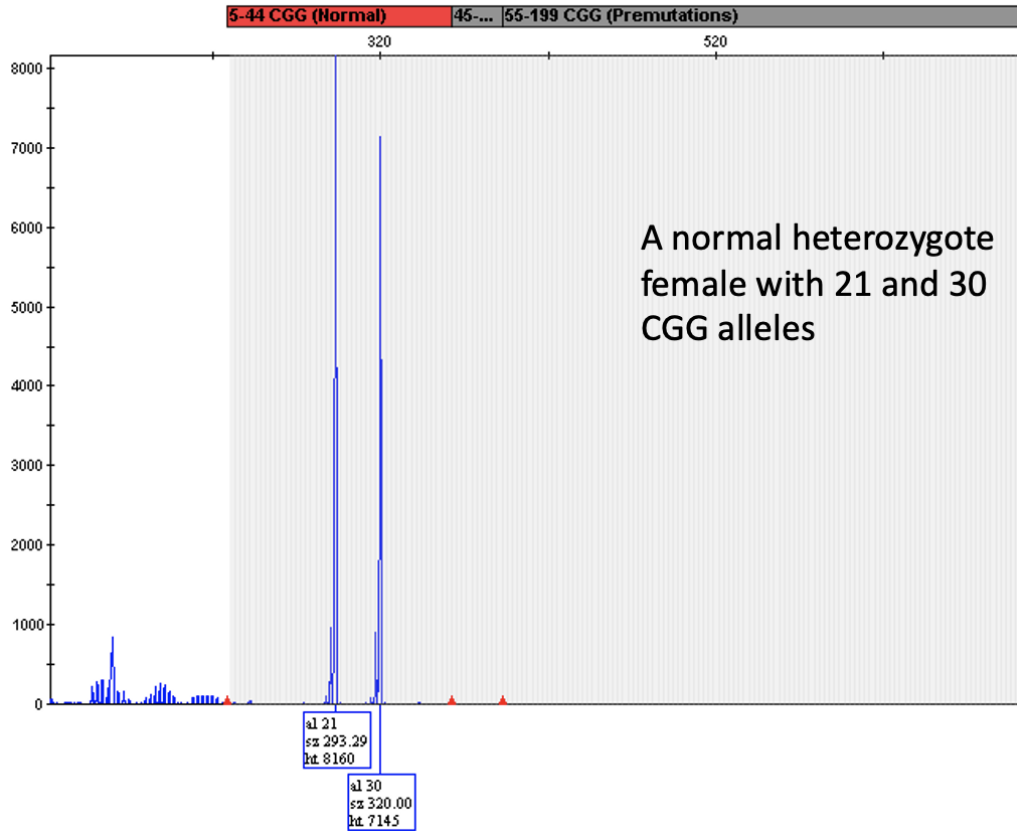


Figure 12: An Electropherogram of a heterozygote normal female with 21 and 30 CGG repeats.

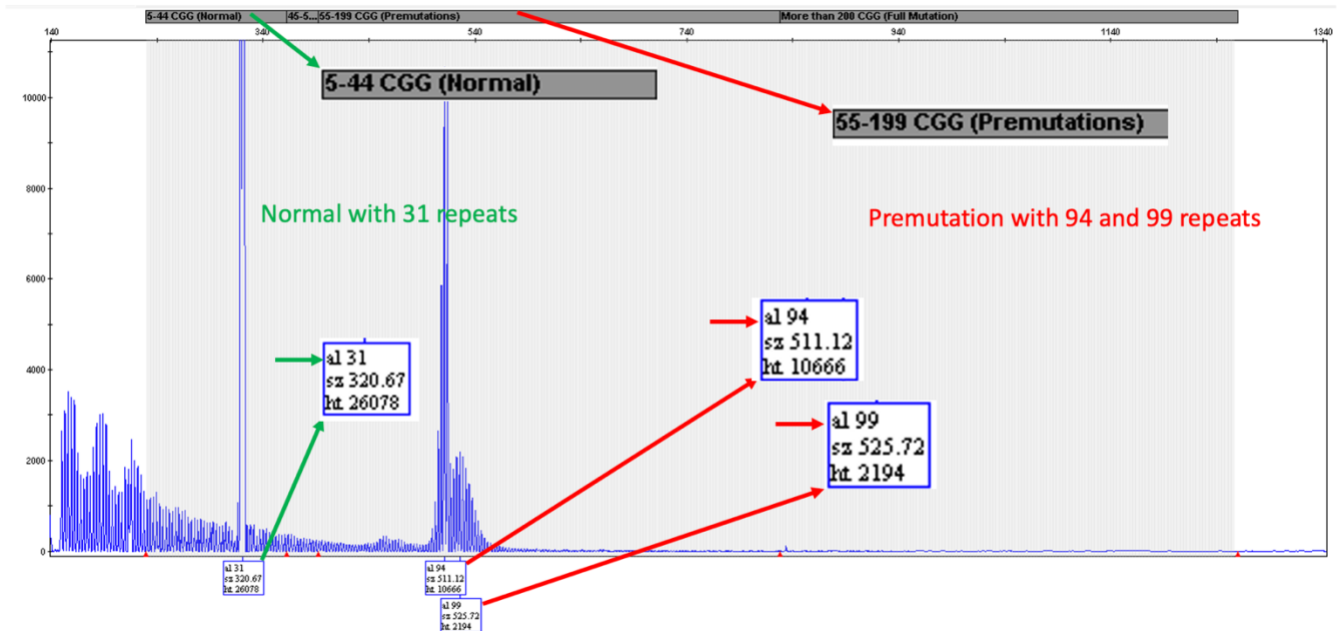


Figure 13: An Electropherogram of a premutation female with mosaicism with 31 (normal allele, 94 main mutant allele and 99 mosaic premutation alleles). Mosaicism (i.e., about 99 alleles) can be seen as minor peaks and sometimes cause confusion on deciding which one is the mutant allele. One has to be careful in interpreting the results particularly when the mosaic allele is longer than

the main mutant allele. When interpreting please consult best practice guidelines (see reference section). Usually, the main mutant allele determines the severity of the disease, but the mosaic allele may be transmitted to the offspring.

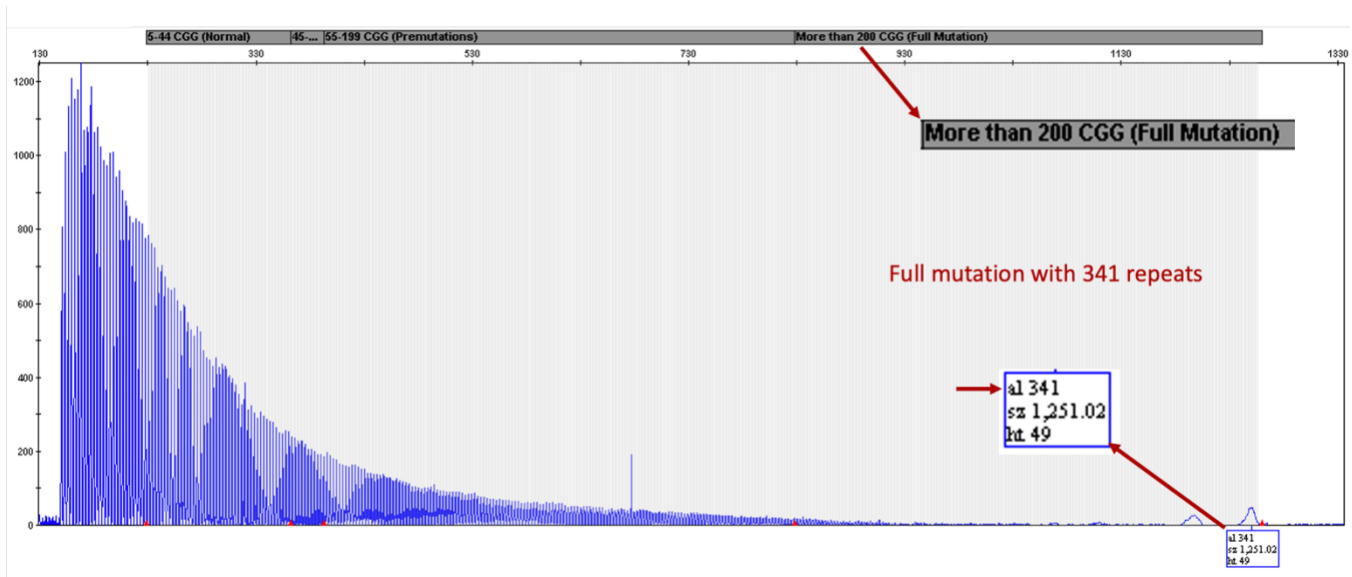


Figure 14: The figure shows result of sample from an affected male individual who is severely affected by having an expanded allele of 341 CGG repeats. Stutter or periodicity peaks are hallmark of the repeat expansion and are visible even before 240 bp.

8. Troubleshooting Guide

○ Issues related to Negative Control

Issue	Problem	Possible solution
Presence of peaks with a 3-bp periodicity in CE profile of negative control	Contamination of master mix with DNA template	Reperform the test, taking care to pipette DNA samples last to avoid contamination of the master mix.
	Contamination of pipettes with PCR amplicons	Decontaminate pipettes prior to reperforming the test. Ensure that pre-amplification and post-amplification work areas are segregated .

○ Issues related to positive control

Issue	Problem	Possible solution
No CE profiles obtained OR CE profiles obtained do not resemble the reference profiles provided (see Figure 5)	Error in reaction mix setup.	Reperform the test, checking that all reagents are added in the correct volumes.
	Insufficient / Degraded DNA template	Check the concentration and quality of DNA template used. If necessary, obtain fresh DNA samples (purchase new lot of reference DNA) and reperform the test.
	Wrong PCR settings	Check the run report from the instrument to ensure that the correct run settings were used. Reperform the test, ensuring the correct settings are used.
	Wrong CE settings	Check the run report from the instrument to ensure that the correct run settings were used. If the correct PCR setting was used, repeat the CE protocol on the amplicons (stored at 4 °C). If both PCR and CE settings were incorrect, reperform the test.
	Expired reagents	Check the expiry date. If necessary, reperform test with a new unexpired kit

○ Issues related to samples







Issue	Problem	Possible solution
CE profiles obtained do not resemble the reference profiles provided OR No CE profiles obtained	Insufficient / Degraded DNA template	Check the concentration and quality of DNA template used. If necessary, obtain fresh DNA samples (repeat the genomic DNA extraction procedure) and reperform the test. If repeated null/ atypical results are obtained despite having ruled out technical errors, samples may have deletions in the <i>FMR1</i> sequence. Such samples should be tested using alternative methods.

- For further information or enquiries please contact us at support@genetek.de

9. Limitations and Disclaimer

Any result obtained from GT FXS Detector kit or any other diagnostic Kit should be used and interpreted by qualified person. GT cannot bear any responsibilities for false use and interpretation being made by any lab.

10. Symbols used on labels and packaging

<u>Description</u>	<u>Symbol</u>
Read Instructions before Use	
Do not use after the year, month and date mentioned	
Manufacturer name and address	
Storage temperature limit – Upper and Lower	
Manufacturer's Catalogue number	
Manufacturer's Batch code or Lot number	

11. References

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