

# **FRAGILE X DNA TESTING: A GUIDE FOR PHYSICIANS AND FAMILIES**

**By Annette K. Taylor, M.S., Ph.D.  
Kimball Genetics, Inc.  
101 University Boulevard, Suite 350  
Denver, CO 80206  
800-320-1807**

The fragile X DNA test has revolutionized fragile X syndrome diagnosis and accompanying genetic counseling. It has been available since 1991 and provides definitive diagnosis of fragile X syndrome and extremely accurate carrier detection. Reliable for people of any age, it can also be performed prenatally. It has superseded the fragile X cytogenetic test due to its greater reliability and accuracy in diagnosis and its ability to identify unaffected carriers.

Although fragile X syndrome is the most common cause of inherited intellectual impairment, it is under diagnosed. Awareness of fragile X syndrome and the utility of the fragile X DNA test is growing among non-geneticist physicians but is not yet widespread. Knowledge of this disorder is particularly important in pediatrics, neurology, obstetrics/gynecology and general practice. Wide variability in the clinical presentation of the disorder is one reason the diagnosis may be missed. Although certain physical and behavioral features are often associated with fragile X syndrome, they are not always present. In at least 10% of cases in males, intellectual impairment is the only presenting sign. The classic triad of long face, prominent ears and macroorchidism is present in just 60% of cases. Mental retardation is not a constant, either. Approximately 15% of males with fragile X syndrome have an IQ above 70 (Hagerman et al., 1994). In cases such as these the possibility of fragile X syndrome may not be considered. Similarly, females with fragile X syndrome may not be correctly diagnosed because symptoms can be subtle.

Many asymptomatic carriers of fragile X syndrome are unaware they are carriers because there may not be a family history of fragile X syndrome, or because a relative with fragile X syndrome may not have been diagnosed. The carrier rate in females is quite high, at approximately 1/250. It is recommended that fragile X carrier testing be offered to all women of reproductive age who have a relative with mental retardation of unknown cause.

This article offers guidance in the following topics related to fragile X testing:

- Indications for testing
- Arrangements to make before testing
- Collecting and shipping the sample
- The fragile X DNA test: (i) The fragile X mutation (ii) The concept of the test
- From sample to result: A glimpse into the laboratory
- The report: What do the results mean?
- The importance of genetic counseling
- The benefits of fragile X testing

## **Indications for Testing**

There are three general types of circumstance in which fragile X testing should be considered: 1) a clinical presentation suggestive of fragile X syndrome, 2) a family history of fragile X syndrome or intellectual disabilities of unknown cause, or 3) risk of inheritance of fragile X syndrome by a fetus. Since the clinical presentation of fragile X syndrome is so varied, mental retardation, developmental delay, learning disabilities or autism are sufficient in males or females to warrant testing. Presence of one or more of the physical or behavioral features often associated with fragile X syndrome increases suspicion of the syndrome but is not necessary for testing to be appropriate.

### **Specific indications for testing:**

- Any male or female with mental retardation (borderline to severe), developmental delay or learning disabilities of unknown cause.
- Any male or female with autism or autistic-like characteristics.
- Any male or female with a relative who has fragile X syndrome or mental retardation of unknown cause.
- Anyone with a previous positive or equivocal result by the fragile X cytogenetic test. Confirmation of the presence of the FMR1 mutation is important.

### **In the obstetric setting:**

- Women or their spouses with a family history of fragile X syndrome or mental retardation of unknown cause.
- Women with a family history of premature menopause.
- Fetus of a pregnant woman known to be a fragile X carrier.

Guidelines to aid clinicians in making referrals for fragile X syndrome testing are also included in a policy statement on fragile X syndrome diagnostic and carrier testing produced by the American College of Medical Genetics (see references: American College of Medical Genetics, 1994).

In the search for a cause of mental retardation or developmental delay in an individual, it is important that routine chromosome analysis (cytogenetic testing) be ordered in addition to fragile X testing or as a follow-up if results of fragile X DNA testing are negative. Routine chromosome analysis checks the appearance of all chromosomes and can pick up abnormalities other than fragile X syndrome that could cause intellectual impairment.

### **Arrangements to Make before Testing**

Arranging for any special testing involves several steps and specific paperwork. Making arrangements prior to the date of the blood draw can prevent frustrations and helps the process proceed smoothly.

#### **1. Establish who will be the referring physician.**

A physician or other appropriate health care professional must refer (order) the test and be the recipient of the results from the laboratory. If you are a patient or relative and want testing for yourself or a family member, you should either obtain a referral to a clinical geneticist or discuss the test with your family practitioner or your child's pediatrician and arrange for him/her to be the referring physician. It is highly recommended that a genetic counselor be involved in each case (see the section, 'The Importance of Genetic Counseling', below).

#### **2. Choose a laboratory and ask for specific instructions.**

Special expertise and experience in fragile X testing are the most important criteria for selecting a laboratory for this testing. Interpretation of results is complex and can involve numerous subtleties. It is not necessary for the laboratory to be near you. It is common practice to mail samples and they are not hurt in any way by a day of transit. Before collecting a sample, call the laboratory to check exact sample requirements, shipping instructions and address.

#### **3. Establish who will be billed for the testing service.**

The laboratory may offer a choice of billing the referring institution (e.g., hospital), the physician's practice, Medicaid, Medicare, the insurance company, or the patient directly. The person filling out the requisition forms (see section on forms below) should indicate to the lab how the service should be billed. The fee for fragile X DNA testing is usually in the range of ~\$250 - \$300. Discounts are sometimes available if payment is received with the sample.

### **3. Check if the insurance policy covers this test.**

Rules about coverage differ between insurance companies (or HMOs) and between policies within the same insurance company. The specific indication(s) for testing (and corresponding ICD9 codes) should be mentioned when inquiring about coverage. In general, more policies cover testing for diagnostic than for carrier identification purposes. It may be necessary to state the lab procedure codes (CPT codes). For fragile X testing in most labs these are 83890, 83892, 83894, 83896, 83898 and 83912. If coverage is promised, be sure to ask for a 'preapproval number'. Using this number on the claim can shorten the time to reimbursement. It is also important to check for any restrictions the insurance policy places on which lab can be used, since sometimes a contract with a specific lab is involved.

### **4. Obtain all referrals required by the insurance company or HMO.**

In many cases when a specialist orders a test, a referral is necessary from the primary care physician in order to gain reimbursement. When a referral is requested for an office visit with a specialist, it is important to extend the referral to include any required testing.

### **5. Fill out all necessary forms.**

It is customary for laboratories to require a completed test request (requisition) form and often a consent form. These should be sent to the lab with the sample to be tested. Clinical indications for testing and any family history information should be included. If the laboratory is to make the claim, include all insurance, Medicaid or Medicare information.

## **Collecting and Shipping the Sample**

Always check for any specific requirements of the particular laboratory you will be using.

- **Sample requirements**

As a general rule, the sample requirements for fragile X testing are ~10 ml fresh blood drawn into a purple-top (EDTA) tube (~3 ml is a minimum requirement, eg. for infants). EDTA is a chemical that helps preserve the blood and prevents it from clotting. Many labs will send 'kits' (purple-top tubes, Styrofoam containers and paperwork) in advance if requested. For prenatal testing, phone the laboratory to determine specific requirements. Testing can be performed after amniocentesis or chorionic villus sampling on a direct sample and cultured cells.

- **Blood draw**

This is usually accomplished at the referring physician's office or convenient outpatient lab. Each tube should be labeled with the date and patient name. Blood should be drawn into the purple-top tubes and the tubes inverted to mix.

- **Handling and packaging of the sample**

Keep the sample at room temperature. Never freeze it. If shipment is not possible for a day or two (eg. over a weekend), store the tubes at room temperature in a safe place. Blood samples remain acceptable for testing for several days. Place the tubes in a Styrofoam box or other container offering protection and tape shut. Place the container in a zip lock plastic bag along with any paperwork required by the lab. Package in a mailer or larger box and attach mailing label.

- **Shipping**

Send the package by overnight mail (eg. Federal Express, Airborne etc.). Specifying morning delivery is often helpful to the laboratory. If the laboratory is local, sample pickup by a courier can usually be arranged.

## The Fragile X DNA Test

### **(i) The fragile X mutation**

Within the fragile X (FMR1) gene is a stretch of repeated trinucleotides with the sequence CGG. The nucleotides C and G are two of the four building blocks of DNA. Among people without the fragile X mutation, the number of these "CGG repeats" varies from 6 to about 40. The fragile X mutation involves an expanded number of the CGG repeats. Expansions with between 60 and 200 repeats, called premutations, are seen in unaffected carriers. Between 40 and 60 repeats is considered a 'gray zone' where normal and premutation size ranges overlap. An expansion with more than 200 repeats, called full mutations, are associated with methylation, which "turns off" the gene. Males with a full mutation are affected with fragile X syndrome. Approximately 50% of females with a full mutation have cognitive impairment and of the 50% with a normal IQ, 60% have some emotional or behavioral effects. An FMR1 premutation tends to increase in size when transmitted from a female to her children, and the risk for expansion to a full mutation increases with the size of the premutation. (For a review of the molecular biology of the fragile X mutation see Brown et al, 1996).

### **(ii) The concept of the test**

Testing for the fragile X mutation is based primarily on measuring the length of the FMR1 gene region containing the CGG repeat stretch and then calculating the CGG repeat number. Analysis of the gene's methylation status (i.e. whether the gene is turned 'off' or 'on') is often performed simultaneously. Categorization of the mutation type is based on CGG repeat number and in some cases also on the methylation status of the gene. Methylation information is useful for delineating premutations from full mutations when the repeat number is intermediate (~150-250) and can have prognostic value when a full mutation is methylated in only a small percentage of cells. (For a review of fragile X analysis, see Warren et al, 1994).

## From Sample to Result: A Glimpse into the Laboratory

Fragile X DNA testing is one of the more complex molecular genetic tests to perform and interpret. The procedure involves many steps performed over several days and requires a high degree of technical expertise. There are a wide variety of possible results, and interpretation requires experience. The time from the arrival of the sample in the laboratory to the completion of the written report is usually 10-14 days.

- **Sample Log-in**

After the sample reaches the lab, it is given a number for tracking and is logged into the patient database or accession notebook. All information about the sample is recorded and a patient folder is created to hold all paperwork relating to the case.

- **Isolation of DNA**

The first step in the laboratory is to isolate DNA (genetic material) from the sample.

**Blood samples:** Red blood cells (which do not contain DNA) are removed by adding a solution which causes them to burst. The white blood cells are then collected at the bottom of the tube and disrupted to release their DNA. The DNA is purified away from all other cellular material and in the last step it materializes as long white strands. These strands are dissolved in a small amount of water and the resulting clear, viscous liquid is the DNA used for testing.

**Amniocytes or chorionic villus cells:** The DNA isolation approach for prenatal samples is very similar to that for blood except that there are no red blood cells to remove and usually fewer cells are available.

- **DNA analysis**

There are two separate approaches to fragile X DNA testing, Southern blot analysis and PCR analysis, described below. They have different advantages. Southern blot analysis is the method of choice for identifying full mutations and large premutations and determining if the gene is methylated while PCR analysis allows accurate determination of CGG repeat number for normal, grey zone and premutation alleles. Fragile X testing is most accurate and reliable when both approaches are used in the laboratory. A recent study of labs offering fragile X testing indicated that about 38% of those surveyed do not perform PCR analysis.

As with many DNA tests, fragile X DNA analysis utilizes the fact that DNA is double stranded and one strand of a gene binds specifically to the other. The FMR1 gene of a person being tested can be 'seen' by sticking onto it a purified piece of the FMR1 gene with an attached radioactive label. This piece of gene is called a 'probe' and the mild radioactive signal the label emits allows the patient's bound gene to be visualized by exposure to X-ray film.

**Southern blot analysis:** A portion of the DNA isolated from the patient sample is cut into pieces of many sizes using certain enzymes, which recognize and cleave at specific DNA sequences. Among the millions of DNA fragments that result are some containing the section of the FMR1 gene with the CGG repeat. When there is no mutation, these FMR1 fragments are of a single known size. If a mutation is present, the fragments are longer. Furthermore, fragments from FMR1 genes that are methylated can be distinguished by size from those that are unmethylated because one of the enzymes utilized fails to cut if methylation is present.

All the DNA fragments in the mixture are separated according to size by using an electric current to draw them through a porous rubbery material (made from a component of seaweed) called a gel. Each patient DNA sample is applied to a slot at the top of the gel and runs down a narrow strip of the gel. Short fragments move faster and further than long fragments. The resulting long smear of DNA fragments can be visualized by staining with a dye that glows bright orange. At this point the FMR1 fragments are indistinguishable from all the other DNA fragments.

The smears of DNA fragments are transferred onto a thin sheet of nylon by blotting and then the sheet is bathed with a radioactively labeled FMR1 gene probe. The probe singles out the matching FMR1 gene fragments and binds tightly. Exposure of the nylon sheet to X-ray film results in short black horizontal lines on the film corresponding to the positions of the patient's FMR1 gene fragments. These lines ('bands') create a vertical pattern that is different depending on the presence or absence of a mutation, the type of mutation, the methylation status and the sex of the patient. Some examples of these patterns are shown in the diagram entitled 'Fragile X Analysis by Southern Blot' at the end of this article. Many unusual patterns not illustrated here can also be encountered.

Caution is required when interpreting a pattern suggesting a small premutation. Slight separation of two unmethylated bands may be seen when two normal alleles or a normal and a 'grey zone' allele differ significantly in size. Precise CGG repeat number determination for premutation and grey zone alleles requires PCR analysis.

**PCR analysis:** PCR (polymerase chain reaction) analysis for fragile X involves generating a million copies of a short section of the patient's FMR1 gene containing the CGG repeat. The resulting pieces of DNA (known as PCR products) are much shorter than those analyzed by Southern blot and much smaller size differences can be detected. After separation on a gel, pieces differing in length by as little as one CGG repeat can be distinguished. This is why quite accurate determination of CGG repeat number for normal alleles and premutations is possible by PCR analysis. An effective way of visualizing the FMR1 PCR product(s) is to use a probe that binds to the CGG repeat stretch. X-ray film exposure results in one, two or no bands. The diagram entitled 'Fragile X Analysis by PCR' at the end of this article shows the appearance of these bands and gives several examples of results.

As a rule, PCR analysis does not reliably detect full mutations, so absence of a band is suggestive of a full mutation in a male. Presence of the full mutation must be confirmed by Southern blot analysis. For a female with a full mutation, the PCR result looks the same (a single band) as for a female with two normal alleles of the same CGG repeat number. Again, complementary results from Southern blot analysis are essential.

### **The Report: What do the Results Mean?**

Reports include results, interpretation and often recommendations. The style of reports ranges from brief to detailed with explanations and relevant educational information. It is helpful if physicians send copies of the report to their patients. Never hesitate to call a laboratory to inquire about the meaning of a result or a statement in a report. Seeking the assistance of a genetic counselor is highly recommended for patients and their families.

#### **• Results**

Results typically include the following: presence or absence of the mutation, the mutation category (premutation, full mutation, both (mosaic), or 'grey zone'), and the CGG repeat number. The methylation status of full mutations may also be included.

**CGG repeat number:** The CGG repeat number should always be given for a mutation and is sometimes also given when it is in the normal range. CGG repeat number differences in the normal range have no clinical significance. Males with a normal FMR1 gene or a premutation have one CGG repeat number (one gene) and most females have two because the FMR1 gene is on the X chromosome. Some females without a mutation have the same CGG repeat number for both alleles. The most common CGG repeat number in the general population is 29 or 30. Slight variation in the exact repeat number determined for a particular person is expected between labs or between repeated tests by the same lab. Small differences are not clinically relevant and do not affect the interpretation of the result.

For full mutations, specific CGG repeat number(s) are sometimes given or the report may state the mutation has >200 repeats. Once a full mutation is over 200 repeats and methylated, the magnitude of the repeat number does not appear to significantly influence clinical outcome. In other words, a full mutation of 800 CGG repeats, for example, would not be expected to have more severe clinical consequences than a full mutation of 300 repeats.

For any given individual with a full mutation, there is often more than one CGG repeat number. This reflects the presence of different sized mutations in different populations of blood cells. In most cases, full mutations have one to three sizes, but some people have many more. Different sizes are thought to result from varying degrees of CGG repeat number increase in different cells when the mother's premutation expands to a full mutation during development of the embryo.

**Methylation status:** Methylation is a biological process by which extra molecules ('CH<sub>3</sub>') attach to gene control areas (promoters) and turn off gene function. Normal FMR1 genes and ones containing a premutation are unmethylated and functional, leading to normal production of the FMR1 protein, FMRP. FMR1 genes with full mutations are usually methylated (turned off) and therefore cannot produce FMRP. The lack of FMRP causes fragile X syndrome.

In most cases, full mutations are methylated in all cells tested and methylation is referred to as 'complete'. However, in rare cases the full mutations in a fraction of cells miss being methylated and so methylation is considered 'incomplete' or 'partial'. The methylation status (complete or incomplete) of full mutations is sometimes noted in reports. Research has shown that in some cases the degree of methylation can influence the severity of fragile X symptoms (McConkie-Rosell et al., 1993). Specifically, if methylation is present in less than 10% of cells tested in males with a full mutation, intellectual impairment tends to be milder than in males with complete methylation of the full mutation (Taylor et al., 1994).

- **Interpretation**

Standard interpretations are:

(i) No mutation: Rules out diagnosis of fragile X syndrome or carrier status (Note: in very rare cases, fragile X syndrome has been found to be caused by deletion of part or all of the fragile X gene or by a point mutation)

ii) Premutation: Establishes that the person tested is a carrier of fragile X syndrome, (iii) Full mutation: Establishes the diagnosis of fragile X syndrome in males and in females with clinical signs of the disorder and establishes carrier status in females. A few unusual results and interpretations are discussed below.

**The grey zone:** The most important point about an FMR-1 gene with CGG repeat number in the 'grey zone' (between approximately 40 and 60 repeats), is that it cannot be the cause of clinical problems. The gene is perfectly functional. Cognitive impairment in an individual with an FMR1 gene of this size must stem from another source. The only risk associated with a gene of CGG repeat number in the grey zone is that it may be unstable from generation to generation and could eventually expand to a full mutation. Therefore, there is a slight risk of fragile X syndrome in distant descendants. The risk for instability of a gene with less than 50 repeats is quite small.

**Mosaicism:** When referring to fragile X syndrome, the term 'mosaic' means the presence in one individual of both a full mutation and a premutation in the cells tested. This definition should not be confused with the term 'somatic mosaic' which is sometimes seen in reference to the presence of more than one size of full mutation. At least fifteen percent of males and approximately six percent of females with fragile X syndrome are mosaic for the full mutation and premutation. This mosaicism is thought to be established during embryogenesis when a mother's premutation expands to a full mutation in some but not all cells. It is believed that in female fragile X carriers the mutation in the oocytes is a premutation, even if a full mutation is present in the rest of the body. Although it is theoretically possible that presence of the premutation (which produces some FMRP) in the mosaic males could improve clinical outcome, most of these individuals are affected to a similar degree as males with only a full mutation. A study in collaboration with Dr. Randi Hagerman to determine whether levels of FMRP in male mosaics correlate with clinical severity is currently in progress in our laboratory. For more details on mosaicism in fragile X syndrome, see Nolin and Brown, 1996, available in this file set.

**Incomplete methylation of a full mutation:** This observation is discussed in the 'methylation status' section above. Incomplete methylation can have prognostic significance in certain cases.

- **Recommendations**

Reports may include recommendations for fragile X DNA testing of relatives, for other testing if fragile X is ruled out, and for genetic counseling to be offered to the extended family.

### **The Importance of Genetic Counseling**

It is highly recommended that families receive genetic counseling support after a diagnosis of fragile X syndrome is made. Call a genetics center or the National Society of Genetic Counselors at (610) 872-7608 to locate a genetic counselor in your vicinity. Some clinical molecular genetic laboratories have a genetic counselor that can help explain results to physicians and families. Contacting a counselor with special fragile X expertise can be particularly helpful. For example, Louise Staley-Gane at the Fragile X Treatment and Research Center in Denver works exclusively with fragile X families. She can be reached at (303) 837-2598. Aspects of genetic counseling for fragile X syndrome are discussed in depth in articles by Staley-Gane, 1996 (available in this file set) and Cronister, 1996.

### **The Benefits of Fragile X DNA Testing**

A diagnosis of fragile X syndrome influences treatment and intervention strategies which can contribute to improvement in outcome (Hagerman, 1996, available in this file set). For the family, the diagnosis marks the end of uncertainty about the cause of a child's difficulties and the end of what is often a long search for a diagnosis. Knowledge of the diagnosis can direct the family to appropriate information and to fragile X support groups.

Results of fragile X DNA testing allow accurate genetic counseling to be provided. Carrier testing for at-risk individuals and prenatal testing empowers families to make informed reproductive decisions.

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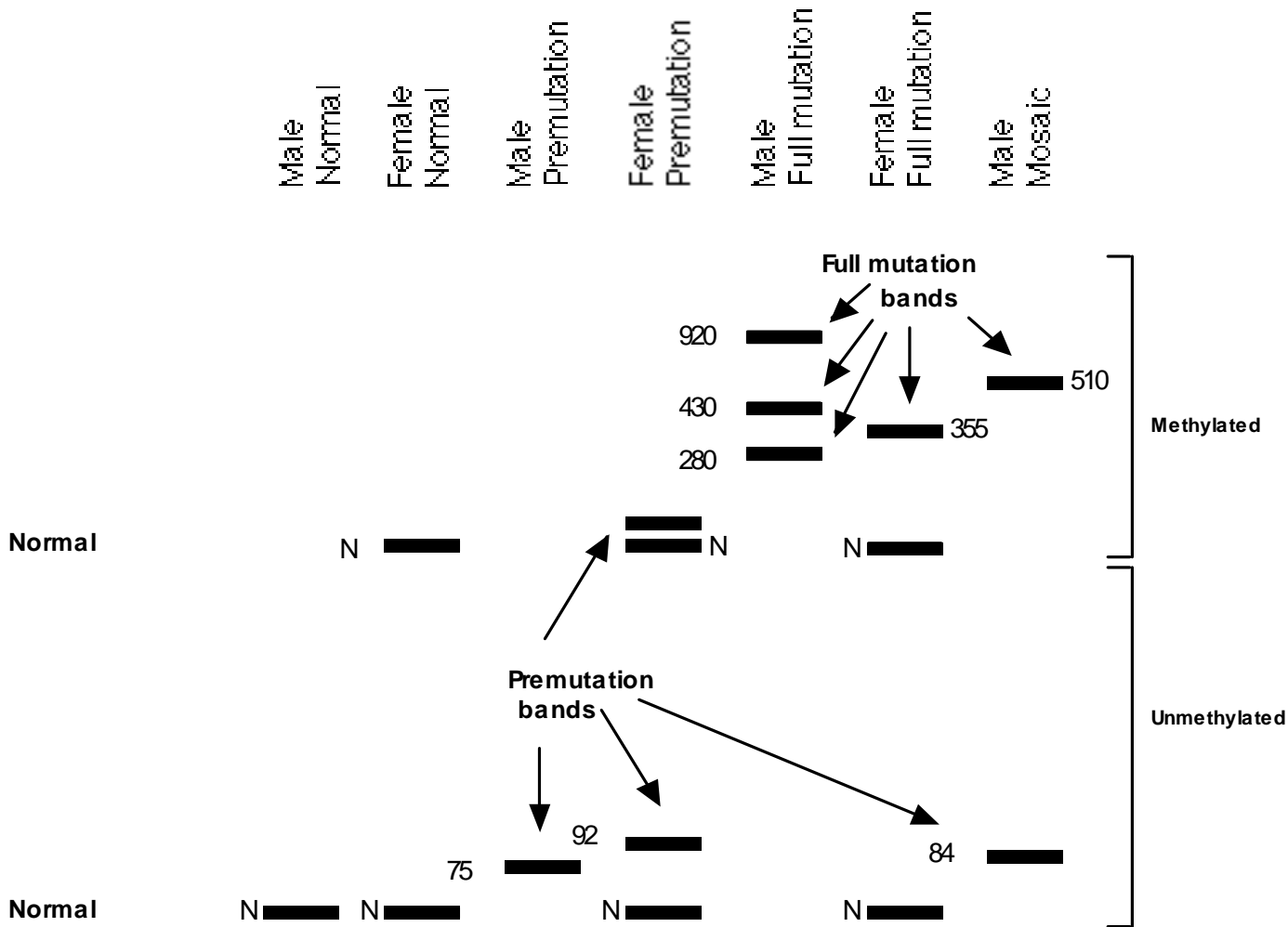
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## Fragile X Analysis by Southern Blot



**Diagram illustrating results of fragile X analysis by Southern blot**

'N' denotes normal bands.

Examples given are:

**Male, normal:** A single band of normal size, unmethylated

**Female, normal:** Two bands of normal size, one unmethylated (on the active X chromosome) and one methylated (on the inactive X chromosome).

**Male, premutation:** A single band of increased size, unmethylated. This premutation has 75 repeats (sized by PCR analysis).

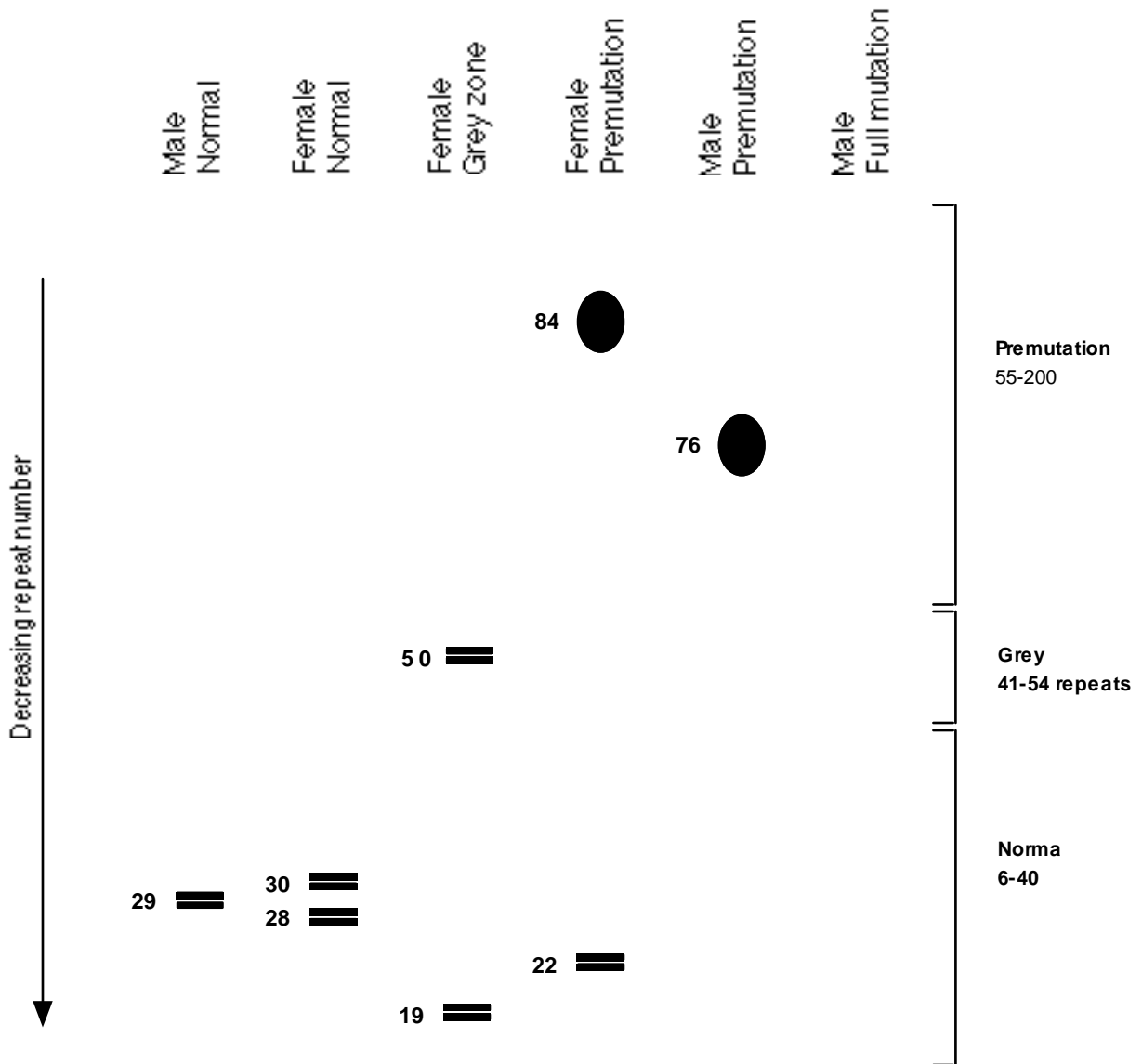
**Female, premutation:** A four-band pattern with an unmethylated (on the active X) and a methylated (on the inactive X) form of both the premutation and normal bands. This premutation has 92 repeats (sized by PCR analysis).

**Male, full mutation:** Band(s) with >200 repeats, methylated. Absence of a normal unmethylated band. In this case there are three different full mutation sizes, 280, 430 and 920 repeats (sized by Southern blot analysis).

**Female, full mutation:** Band(s) with >200 repeats, methylated. The two normal bands from the normal allele are also present. This full mutation has 355 repeats (sized by Southern blot analysis).

**Male, mosaic:** Full mutation and premutation. In this case the full mutation has 510 repeats (sized by Southern blot analysis) and the premutation has 84 repeats (sized by PCR analysis).

## Fragile X Analysis by PCR



### Diagram illustrating results of fragile X analysis by PCR

Bands are drawn to simulate the way they actually appear.

Examples given are:

**Male, normal:** 29 repeats

**Female, normal:** 30 and 28 repeats

**Female, grey zone:** 50 repeats (grey zone) and 19 repeats (normal allele)

**Female, premutation:** 84 repeats (premutation) and 22 repeats (normal allele)

**Male, premutation:** 76 repeats

**Male, full mutation:** No normal band. The full mutation must be confirmed by Southern blot analysis.