DATA SHEET

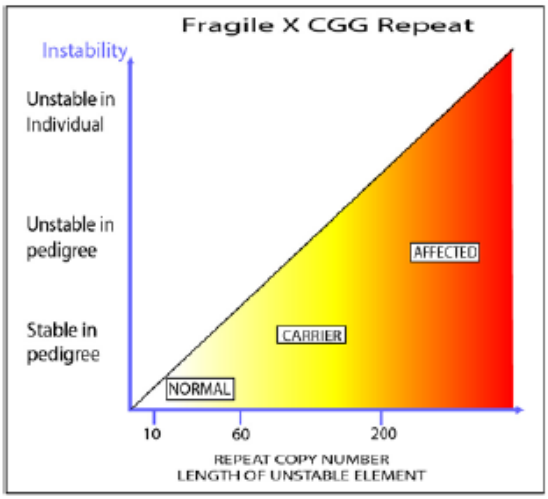
Fragile X Genotyping

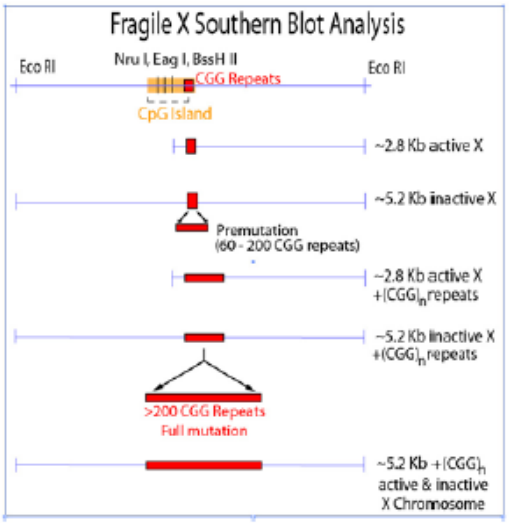
Cat. No. 40-2004-40

Background

Fragile X syndrome is the most common form of inherited mental retardation. It affects approximately 1 in 1200 males and 1 in 2500 females. As suggested by the name, it is associated with a fragile site under specific cytogenetic laboratory conditions at position Xq27.3 (1).

The inheritance pattern of fragile X puzzled geneticists, as it did not follow a clear X linked pattern. Approximately 20% of males who are carriers based on pedigree analysis do not manifest any clinical symptoms and are thus termed as Normal Transmitting Males (NTM), mental retardation is rare among the daughters of male carriers. Approximately 35% of female carriers have some mental impairment. Based on the above it has been proposed that there are two states of the mutation, one mutation range in which there is no clinical expression (premutation), which could change to the disease causing state predominantly when transmitted by a female (full mutation)(2).





The fragile X syndrome gene (FMR-1, fragile X mental retardation) was cloned in 1991 simultaneously by three groups (3-6). Soon the peculiar genetic mode of transmission was established and a new class of mutation came into existence- Trinucleotide Repeats (TNR's) amplification. This explained the clinical state of 'premutation' and 'full mutation' as well as 'anticipation'. The fragile X syndrome is caused by the amplification of CGG trinucleotide repeat, which is located in the 5' region of the cDNA. The most common allele in the normal population consists of 30 repeats, the range varying from 6 to 54 repeats. Premutations in fragile X families showing no phenotypic effect range in size from 52 to over 200 repeats. All alleles with greater than 52 repeats are meiotically unstable with a mutation frequency of one. In general repeats up to 45 are considered normal, repeats above 50 to 200 are considered as premutation and above 200 as full mutation (3-7). The range between 40-55 is considered even by most experienced clinical geneticists and molecular geneticists very difficult to interpret and is considered as a 'gray zone' with interpretations made on a case-by-case basis (8).

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Trinucleotide Repeats

To date, trinucleotide repeats expansion has been shown to be responsible for at least 15 different neuro degenerative disorders in humans. Table 1 lists these disorders. All share the instability of the repeats above a particular threshold. Once this threshold is exceeded the trinucleotide repeats become meiotically unstable and upon expansion exhibit the onset of disease symptoms.

Table 1. Trinucleotide Repeats in Human Genetic Disease

Disease	Repeat[a]	Normal Length[b]	Intermediate Length (Premulation)[a,b]	Full Disease Length[b]
Fragile XA (FRAXA)	$(CGG)_n$	6-52	59-230	230-2,000
Fragile XE (FRAXE)	$(CCG)_n$	4-39	? (31-61)	200-900
Fragile XF(FRAXF)	$(CGG)_n$	7-40	?	306-1,008
FRA16A	$(CCG)_n$	16-49	?	1,000-1,900
Jacobsen Syndrome (FRA11B)	$(CGC)_n$	11	80	100-1,000
Kennedy Syndrome (SMBA)	$(CAG)_n$	14-32	?	40-55
Myotonic Dstrophy (DM)	$(CTG)_n$	5-37	50-80	80-1,000; congenital, 2,000-3,000
Huntington disease (HD)	$(CAG)_n$	10-34	36-39	40-121
Spinocerebellar ataxia 1 (SCA1)	$(CAG)_n$	6-39	None Reported	40-81
Spinocerebellar ataxia 2 (SCA2)	$(CAG)_n$	14-31	None Reported	34-59
Spinocerebellar ataxia 3 (SCA3)/Machado Joseph disease (MJD)	$(CAG)_n$	13-44	None Reported	60-84
Spinocerebellar ataxia 6 (SCA6)	$(CAG)_n$	4-18	None Reported	21-28
Spinocerebellar ataxia 7 (SCA7)	$(CAG)_n$	7-17	28-35	38-130
Haw River syndrome (HRS; also DRPLA))	$(CAG)_n$	7-25	?	49-75
Friedreich ataxia (FRDA)	$(GAA)_n$	6-29	? (>34-40)	200-900

[a] Typically, repeats tracts contain sequence interruptions. See Pearson and Sinden (1998a) for a discussion of the sequence interruptions.
[b] No. of triplet repeats.
[c] A question mark (?) indicates potential mutagenic intermediate length, and an ellipsis (...) indicates none. Not all disease are associated with a permutation length repeats tract or permutation disease condition.

Molecular Analysis

Fragile X genotyping can be done by direct PCR amplification of the CGG trinucleotide repeats region or by southern analysis. In most cases both methods are used to complement the results. Full mutations usually cannot be identified by PCR by most investigators and southern analysis is the preferred method to distinguish full mutations. The FMR-1 gene region containing the CGG trinucleotide repeats is flanked by Eco RI sites; and an Eag I and Nru I site and three BssH II sites in the CpG region. Full mutation has been shown to methylate the active gene too and thus it prevents NruI, BssH II and Eag I restriction of DNA. Hybridization of southern blots of Eco RI and Nru I, BssH II or Eag I double digested DNA clearly can distinguish between normal, premutation and full mutation genotypes

The size of the CGG repeats can be determined by PCR analysis and sizing preferably on a sequencing gel. The PCR products can be either labeled with ^{35}S or ^{32}P followed by autoradiography. Another attractive alternate is to run a cold PCR reaction followed by blotting and hybridization with an alkaline phosphatase conjugated probe for non-radioactive detection.

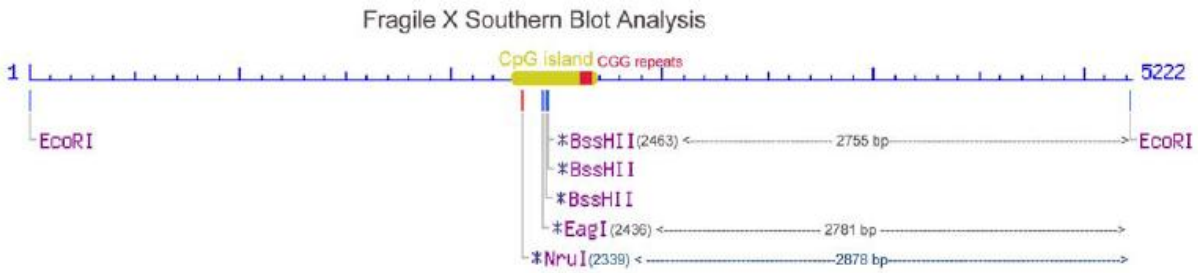
Table 2: CGG TRINUCLEOTIDE REPEATS PERCENTAGE AND FRAGMENT SIZE								
CGG	bp	Size	%		CGG	bp	Size	%
1	3	223			31	93	313	7.02
2	6	226			32	96	316	3.51
3	9	229			33	99	319	1.23
4	12	232			34	102	322	0.53
5	15	235			35	105	325	0.7
6	18	238			36	108	328	1.05
7	21	241			37	111	331	0.35
8	24	244			38	114	334	0.53
9	27	247			39	117	337	1.23
10	30	250			40	120	340	1.23
11	33	253			41	123	343	0.35
12	36	256	0.18		42	126	346	0.7
13	39	259			43	129	349	0.7
14	42	262			44	132	352	0.18
15	45	265	0.18		45	135	355	
16	48	268	0.35		46	138	358	
17	51	271			47	141	361	0.18
18	54	274			48	144	364	0.18
19	57	277			49	147	367	0.18
20	60	280	6.32		50	150	370	
21	63	283	0.18		51	153	373	
22	66	286	0.88		52	156	376	0.35
23	69	289	6.14		53	159	379	
24	72	292	2.63		54	162	382	
25	75	295	0.88		55	165	385	
26	78	298	1.4		56	168	388	
27	81	301	0.88		57	171	391	
28	84	304	2.28		58	174	394	
29	87	307	**18.78**		59	177	397	
30	90	310	**38.77**		60	180	400	

The detection of amplification/expansion of a region of DNA sequence can be detected by PCR and Southern, these methods can be used for all disorders involving increase in size of a region of DNA. DNA analysis for direct detection of fragile X mutation is based on enzymatic amplification of a fragment containing the CGG repeats sequence of the *FMR-1* gene. This test detects the fragile X mutation by the size of the amplified product; an increase in size is correlated with the corresponding number of CGG repeats and a risk factor calculated. The most common allele in the normal population consists of 30 repeats, the range varying from 6 to 54 repeats. Premutations in fragile X families showing no phenotypic effect range in size from 52 to over 200 repeats. All alleles with greater than 52 repeats are meiotically unstable with a mutation frequency of one.

PCR based methods are fundamentally similar. The two primers are constructed such that they span the region of trinucleotide repeats expansion. In the case of Fragile X specifically, the nature of the mutation poses problems using normal PCR conditions. In Fragile X, the repeats is of CGG which can be hundreds to thousands bases long. All DNA polymerases, including Taq DNA polymerase do not copy long stretches of G residues efficiently. An analog of G called 7-deaza GTP functions better and is partially replaced in the PCR reaction to achieve amplification. The use of 7 deaza GTP instead of G precludes the staining of gels with ethiduim bromide for visualization as 7 deaza GTP containing DNA does not stain well. This is resolved by using radioactively labeled nucleotide followed by autoradiography. Fragile X PCR still does not give accurate results for full mutations due to the inherent massive expansion and the inability of PCR to amplify very large fragments efficiently. All normal and

premutation PCR amplification are reliable, but still is coupled with a Southern blot analysis. In our laboratory PCR is performed in addition to Southern blot analysis. The PCR results are obtained in 2 days followed by Southern blot results. All results from PCR are verifiable by Southern except full mutations which are not reliable with PCR.

Southern blot analysis for Fragile X mutation detection involves the cleavage of DNA with enzyme Eco R I and Nru I, BssH II or Eag I. This method detects the size of CGG repeats region by hybridization of probe GLFX1 or GLFXDig1 GeneProber™ to DNA that has been double digested with restriction enzymes Eco RI and Nru I, BssH II or Eag I and blotted onto a membrane. In normal females two fragments are seen, a 2.8kb corresponding to the active X and a 5.2kb fragment corresponding to the methylated inactive X chromosome. Normal males exhibit only the 2.8kb banding pattern. Affected males will have an amplified CGG repeats region with methylation thus giving rise to fragments larger than the normal 5.7kb. Premutations in males and females will be seen as fragments from 2.9-3.3kb (normal 2.8kb) derived from the X chromosome. Premutations in females derived from the inactive X will give fragments from 5.3-5.7kb. Mosaicism is characterized by fragments appearing as a mixture of full mutation (methylated, larger than 5.7kb) and unmethylated premutation (2.9-3.3kb).



Procedure

Caution
Product to be used by experienced researchers properly trained in performing molecular biology techniques following established safety procedures. End user must be qualified and certified for research using radioactive materials.

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*Important information

Gene Link strongly recommends the use of non-radioactive gene detection systems. Consider switching to Gene Link's GLFX PCRProber™ non-radioactive detection system (Catalog Number 40-2004-32), GScan™ fluorescent based genotyping (Catalog Number 40-2004-15) and GLFXDig1 GeneProber™ Southern blot gene detection system (Catalog Number 40-2004-41).

Brief Product Protocol

The protocol given below can be substituted by your laboratory's established protocol for Southern blot analysis using random prime labeled probes.

Material Supplied
One tube containing 500 ng of lyophilized GLFX *GeneProber™* probe. The DNA probe is stable in dried state for an extended period at room temperature. Upon reconstitution it should be stored at -20°C. The quantity supplied is sufficient for at least 5 random prime labeling reactions using 100ng for each reaction. Gene Link recommends using 25ng for each labeling reaction

Fragile X Southern Protocol

1. Genomic DNA restriction digestion

GLFXDig1 and GLFX1 Probe Southern Blot Fragment Detection Double Digestion with Eco RI and Eag I or Nru I or BssH II				
Enzyme	**Specificity**	**CpG methylation Sensitive**	**Normal Male Fragment Size**	**Normal Female Fragment Size**
EcoRI and EagI double digest				
EcoRI	G^AATT_C	No	2781 bp	5216 & 2781 bp
EagI	C^GGCC_G	Yes		
EcoRI and NruI double digest				
EcoRI	G^AATT_C	No	2878 bp	5216 & 2878 bp
NruI	TCG_CGA	Yes		
EcoRI and BssH II double digest				
EcoRI	G^AATT_C	No	2755 bp	5216 & 2755 bp
BssH II	G^CGCG_C	Yes		

Important Note
-Double digest genomic DNA with Eco RI and Nru I, BssH II or Eag I.

Restriction Digestion	
Component	Volume|Quantity
Genomic DNA	10µg
10x Eco RI Buffer	10 µl
Nru I, BssH II or Eag I (10 u/µl)	4 µl
Eco RI (40 u/µl)	4 µl
H_2O to	**100 µl**
Overnight digestion at 37°C	

Ethanol precipitate the digests, dissolve the pellets in 10 µl of 1x Loading buffer.

2. Load samples to 0.8% agarose gel , run over night at 40mA for ~14 hours. (~1.6kb fragment on the bottom of the gel).

3. Depurinate with 0.25N HCl (add 10 ml HCl to 500ml H_2O) for 10 minutes, denature the DNA with 0.4N NaOH/0.6M NaCl for 30 min. at RT, neutralize with 1.5M NaCl/0.5M Tris (pH 7.5) for 30 min. at RT, transfer to the MagnaCharge Nylon membrane (MSI) by 10xSSC and 10 pieces of SIGMA QuickDraw blotting paper overnight.
Wash the membrane with 2x SSC, bake it at 80°C for 2 hours.

4. Perform pre-hybridization at 50°C for 3 hours in 10 ml of Lumisol I buffer (Gene Link).

5. Label the probe as following: (Any Random Primer DNA Labeling Kit)

Random Primer Labeling	
Component	Volume|Quantity
GLFX1 GeneProber™	25 -100 ng
H_2O	up to 9 µl
Boil 5 minutes, and put on ice. Then add	
Reaction mix	2 µl
dNTP w/o dCTP	3 µl
α^{32}PdCTP (3000 Ci/mmol)	5 µl (50 µCi)
Klenow (2 U/µl)	1 µl
Total	**20 µl**

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Incubate at 37°C for 30 minutes. Add 500 µl of 5 x SSC to the reaction tube, boil 5 minutes, then add to the 50 ml Falcon tube containing the membrane and Lumisol I solution, mix well, incubate in shaking water bath at 50°C overnight.

6. Wash the membrane in 2 x SSC/ 0.1% SDS at RT twice (5 min per wash), then wash with 0.1 x SSC/ 0.1% SDS at 60°C twice (30 min. per wash). Wrap the membrane and put X-ray film on it, expose at -80°C over night. Develop the film next morning.

If required, strip the membrane by incubating in 0.5 N NaOH for 1 hour at RT with constant agitation. Change the solution and incubate overnight if necessary. Rinse the membrane with 2x SSC, air dry.

Results and Analysis

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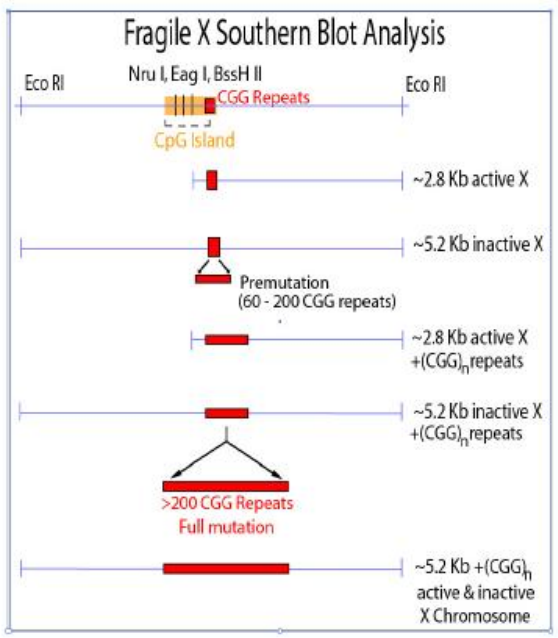

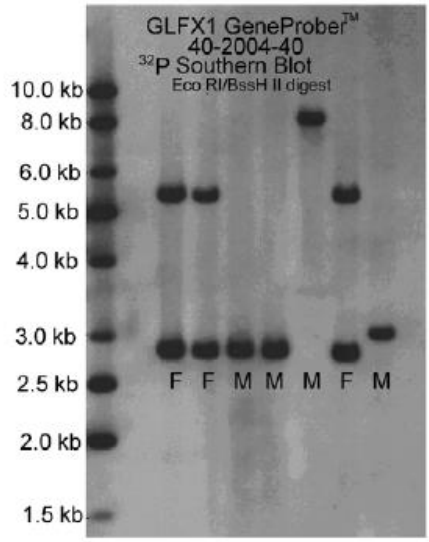

Fragile X southern blot analysis. Genomic DNA digested with Eco RI/BssH II and GLFX1 GeneProber labeled with ^{32}P as the probe. Normal pattern expected is as follows. Female: 5.2 kb and 2.7kb; Males; 2.7kb.

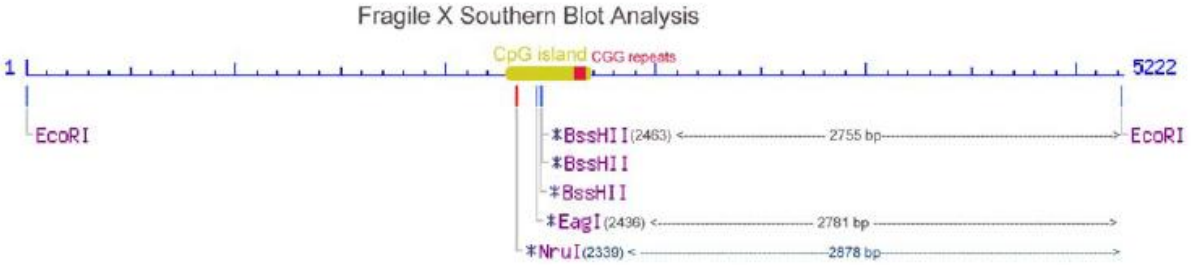

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Table 3: Fragile X Molecular Analysis Results Interpretation							
Clinical Category	**Normal (male/female)**	**Female Carrier with small amplification**	**Female carrier with significant amplification**	**Female carrier with Large amplification**	**Carrier male with premutation (NTM)**	**Full Mutation (Male/Female)**	**Carrier with Fragile X Mosaicism**
Risk mutation will become full mutation in next generation	0%	moderate	significant	high	0%	moderate to high	can vary from 0-100%
Number of CGG repeats	6-45	46-69	70-86	87-200	40-200	>200	40-200/ >200
Size of CGG repeats; bp	18-135	138-207	210-258	260-600	120-600	>600	120-600/ >600
Total Fragment Size; bp	221-338	341-410	413-461	464-803	323-803	>803	323-803/ >803

References

1. Nelson, D.L. (1993) Growth Genetics and Hormone. 9:1-4.
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4. Fu, Y.H et al. (1991) Cell 67:1047-1058.
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6. Yu, S. et al. (1991) Science 252: 1179-1181.
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Fragile X Frequently Asked Questions/Troubleshooting

1. **General Comment** Fragile X genotyping is not easy. A lab really has to optimize conditions. Following the protocol exactly works. A few initial rounds of optimization may be required. Once the investigator is experienced with all the manipulations, getting good results should be routine.

2. **High Background** The background problem may be due to various reasons and has to be optimized in each lab. Here at Gene Link we use Boehringer Mannheim products, the membrane is nylon positively charged catalog number 1209 272. Other positively charged membranes work but do not give consistently low background. The main reason for background is inadequate blocking and/or the membrane itself is curled, folded or has scratches and creases which trap the probe. We advise using glass trays or bottles for all washing and hybridization procedures. Plastic inherently has small surface variations and can scratch the membrane. We would also advise increasing the washing and stringency and exposure to x-ray film for one hour initially. Wash again if you observe too much background and no real signal in an hour. .Expose for longer time if the one hour exposure gives nearly no background. We get good signal in a 2 hr. exposure.

Again, to summarize, the background problem varies from lab to lab and has to be optimized. Once optimized, you will consistently get excellent signal in 1-2 hr. exposure.