Product Manual

Fragile X GLFX PCRProber™

Non-radioactive Fragile X Genotyping by PCR* Analysis

CGG Repeat Detection

Alkaline Phosphatase labeled probe non-radioactive detection of Fragile X CGG trinucleotide repeats region amplified PCR product

Catalog No. 40-2004-31 **12µl 5 blots**

Store at -20°C

For research use only. Not for use in diagnostic procedures for clinical purposes

Important Information

All Gene Link products are for research use only.
Not for use in diagnostic procedures for clinical purposes.
Product to be used by experienced researchers appropriately trained in performing molecular biology techniques following established safety procedures. Additional qualification and certification is required for interpretation of results.

*The polymerase chain reaction (PCR) process is covered by patents owned by Hoffmann-La Roche. A license to perform is automatically granted by the use of authorized reagents.

Storage instructions

Caution: DO NOT FREEZE. STORE AT 4°C.

Gene Link™

Material supplied

GLFX PCRProber™ AP labeled probe

Catalog No.: 40-2004-31 12µl (sufficient for 5 blots)

Alkaline Phosphatase labeled probe non-radioactive detection of Fragile X CGG trinucleotide repeats region amplified PCR product.

Storage instructions: STORE AT 4°C DO NOT FREEZE

Material Required But Not Supplied

Individual components of the kit can be ordered using the appropriate catalog number

Fragile X PCR reaction kit components Sufficient for 50 x 100 µl reaction				
Product	**Size**	**Description**	**Storage**	**Catalog No.**
FX PCR Component A	900 µl	FX PCR premix with primers	Store at -20 °C	40-2004-32A
FX PCR Component B	110 µl	FX PCR Component B	Store at -20 °C	40-2004-32B
FX PCR Component C	650 µl	FX PCR Component C	Store at -20 °C	40-2004-32C
Loading Buffer 2X BPB/XC Denaturing for Sequencing	1 ml	Seq loading buffer	Store at -20 °C	40-5027-10

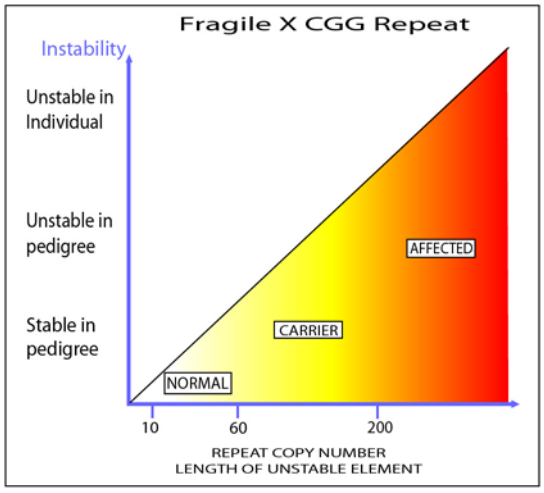
Hybridization and Detection kit components Sufficient for processing five 16 x 16 cm blots				
Product	**Size**	**Description**	**Storage**	**Catalog No.**
Hybwash Stock A	250 ml	Hybridization Wash Concentrate	Store at 15-25°C (Room Temperature)	40-5020-25
Hybwash Stock B	60 ml	Hybridization Wash Concentrate	Store at 15-25°C (Room Temperature)	40-5021-60
Lumisol ™ III hybridization buffer	80 ml	Hybridization Buffer	Store at 4 °C	40-5024-80
10 x AP detection buffer	60 ml	Alkaline Phosphatase Buffer	Store at 15-25°C (Room Temperature)	40-5031-60
CDP-star ready to use AP Substrate spray	10 ml	Alkaline Phosphatase Chemiluminescent Substrate	Store at 4 °C	40-5010-10

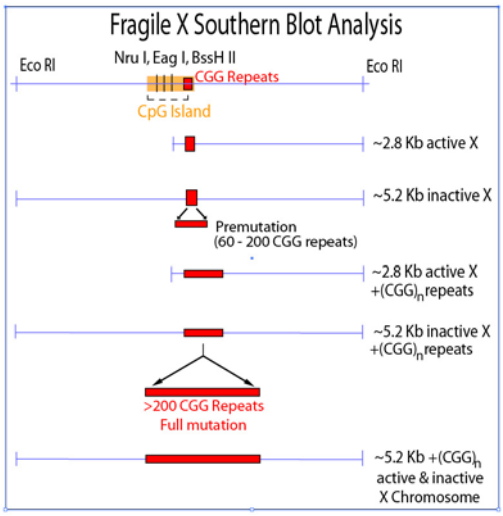
Fragile X Genotyping

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 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 repeats, which is located in the 5' region of the cDNA. The most common allele in the normal population consists of 29 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).

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 in the CpG region. Full mutation has been shown to methylate the active gene too and thus it prevents NruI and Eag I restriction of DNA. Hybridization of southern blots of Eco RI and Nru I 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

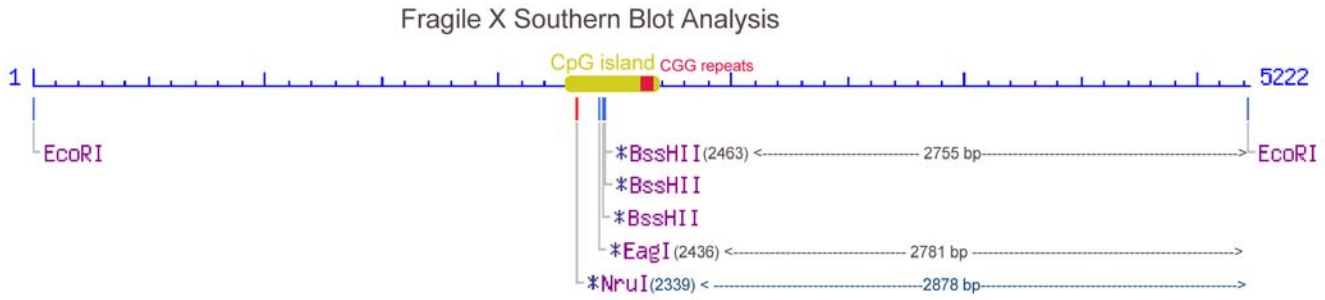
CGG	bp	Size	%		CGG	bp	Size	%
1	3	223			31	93	313	7.02
2	6	226			32	96	316	3.51
3	9	229			33	99	319	1.23
4	12	232			34	102	322	0.53
5	15	235			35	105	325	0.7
6	18	238			36	108	328	1.05
7	21	241			37	111	331	0.35
8	24	244			38	114	334	0.53
9	27	247			39	117	337	1.23
10	30	250			40	120	340	1.23
11	33	253			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 repeat 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 repeat expansion. In the case of Fragile X specifically, the nature of the mutation poses problems using normal PCR conditions. In Fragile X, the repeat 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 dGTP 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, Eag I or BssH II. 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).



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		

Procedure

Procedure: CGG Repeats Analysis by PCR

The procedure outlined below can be finished in less than 24hrs.
Day 1 afternoon start PCR and leave it to proceed overnight. Prepare a 6% polyacrylamide-7 M urea gel (15-well, 0.75mm, 16x16cm^2) and leave it overnight. Day 2, proceed with the rest of the procedure.

PCR Thermal Cycler Files

Program two PCR thermal cycler files as follows

Hot Start File		
Step	Time and Temperature	Cycles
Denaturation	5 minutes at 94°C	1
Hold	Hold for infinity at 60°C	Hold

Fragile X CGG Amplification File		
Step	Time and Temperature	Cycles
Denaturation	30 seconds at 94°C	30
Annealing	60 seconds at 65°C	
Extension	3 minutes at 72°C	
Fillup	7 minutes at 72°C	1
Hold	Hold for infinity at 4°C	Hold

Protocol:

PCR Amplification

A. Example: PCR Premix (for 10 reactions 100 µl each). Label tube as "PP" .

Given below is a protocol for preparing a PCR premix for ten 100 µl reactions. This can be scaled up or down as required.

Sufficient reagents are provided for performing 50 X 100 µl reactions. Experienced users can perform 100 X 50 µl reactions.

PCR Premix Preparation -PP	
Component	Volume
FX PCR Component A	170 µl
FX PCR Component B	20 µl
FX PCR Component C	100 µl
Sterile water	710 µl
Total	**1000 µl**

B. Enzyme Mix (for 10 reactions). Label tube as "EM".

Enzyme Mix Preparation-EM	
Component	Volume
PCR premix-PP (from A above)	100 µl
Taq. Polymerase*	5 µl
Total	**105 µl**

*The kit is optimized for regular Taq Polymerase. Any good quality Taq polymerase that is regularly used in the laboratory can be used.

C. Initial Denaturation. 'Hot Start' PCR.

For each sample add the following.

Hot Start PCR	
Component	Volume
PCR Premix-PP (from step A above)	90 µl
DNA Template (~100ng for chromosomal DNA)	1 µl

Transfer all sample tubes to thermal cycler and start "Hot Start" file.

D. PCR Amplification.

After initial denaturation while thermal cycler is 'holding' at 60°C add Enzyme Mix-EM as follows.

PCR Amplification	
Component	Volume
Hot Start PCR tubes (from step C above)	91 µl
Enzyme Premix-EM (from step B above)	10 µl

Start FX CGG amplification PCR file.

E. Post PCR Processing

1. Ethanol precipitate and dissolve pellet in 5 µl sterile water.
2. Add 5 µl loading buffer.
3. Sample is ready for loading to gel.

F. Electrophoresis & Electroblotting

1. Prepare a 6% polyacrylamide-7 M urea gel (15-well, 0.75mm, 16x16cm^2). Pre-electrophorese for 10 minutes at 25 Watts constant (~ 500 volts or 45 mAmps constant).

2. Heat the samples at 75°C for 5 min. Chill on ice. Load 5 µl to the gel. Run the gel constantly at 25 Watts constant (~ 500 volts or 45 mAmps constant) till xylene cyanol dye runs out. Electrophorese 10 minutes more after that. (Total electrophoresis time ~1hr.).

3. While gel is running, prepare for electroblotting. Soak positively charged nylon membrane in water for nearly 5 minutes, then keep soaked in 1 X TBE. Setup transfer following the electrotransfer apparatus manufacturer's directions. Electro-transfer at 400 mA for 1 hr. in 1xTBE.

G. Hybridization & Detection

Prepare for hybridization and detection while electroblotting.

Reagent Preparation

Hybwash I:

Add 35ml of Hybwash stock A, 312 ml of sterile deionized water, mix and then add 3.5 ml of Hybwash stock B.

Hybwash II

Add 7.5ml of Hybwash stock A, 340 ml of sterile deionized water, mix and then add 3.5 ml of Hybwash stock B.

1 x Detection buffer

To make 100ml of 1x Detection buffer, add 10 ml of 10x Detection buffer and 90 ml of sterile deionized water.

Procedure

1. After electrotransfer, wash the blot in 50 ml Hybwash I at 55°C for 5 min.
2. Prehybridize (roller bottle or bag) in 7.5 ml of pre-warmed Lumisol III at 55°C for 30 min.
3. Replace with 7.5ml fresh Lumisol III, (pre-warmed at 55°C) containing 2 µl of GLFX PCRProber™. Hybridize at 55°C for 30 min. DO NOT EXCEED 30 minutes.
4. Wash the blot in 75 ml of pre-warmed Hybwash I for 7 minutes at 55°C Repeat 3 times. Total of four washes.
5. Wash the blot in 150 ml pre-warmed Hybwash II for 5 min at 55°C. Repeat once. Total of two washes.
6. Wash the blot in 25 ml 1x Detection Buffer at room temperature for 5minutes. Repeat 3 times. Total of four washes.
7. Transfer blot to a plastic sheet, (sheet protector cut from two sides to open up) and drain off excess buffer. Wipe off edges with paper towel. Blot should not be allowed to dry.
8. Spray CDP-star ready-to-use substrate evenly to cover the blot. DO NOT OVER SPRAY. Cover the blot with plastic sheet and wipe entire surface of the covered blot to expel any excess substrate and air bubbles. Expose the film at room temperature for 1 hr. or for shorter or longer time as required.
9. Luminescence continues for at least 24 hours and signal intensity remains almost constant during the first few hours. Multiple exposures can be taken to achieve the desired signal strength.
10. 10. For re-hybridization the membrane can be stripped of the probe by washing in 50 ml of Hybwash II sol. at 65°C for 30 min. with gentle agitation.

Results and Interpretation

The PCR-Prober™ fragile X gene detection system is optimized to give results. Fragile X PCR based genotyping is considered difficult due to the extensive stretch of CGG repeats in fragile X mutation that causes a strong secondary structure in the DNA. This secondary structure prevents DNA polymerases, even Taq polymerase used at elevated temperature, from replicating the CGG repeat region. The PCR-Prober™ kit includes deaza GTP to reduce the secondary structure and increase the length of the CGG repeats that the Taq polymerase can copy. The PCR-Prober™system can reliably detect up to 50-60 CGG repeats. Detection of more than 60 repeats is not reliable. A DNA sample that does not yield a PCR product on duplicate analysis indicates either a failure of the PCR reaction, or a DNA sample with extensive CGG expansion. In the latter case, we recommend Southern analysis using the GeneProber™ Fragile X Detection System to clearly determine the genotype.

Using the reagents provided, the PCR-Prober™ kit will yield an amplified Fragile X CGG triple repeat spanning PCR product with 30 CGG repeats of ~310 base pairs. All other repeats can be tabulated based on this size. Please refer to Table 2 of this manual. Note that the amplified product can only be resolved on a suitable denaturing polyacrylamide gel followed by hybridization to the supplied alkaline phosphatase probe and chemi-luminescent detection. The mobility of the amplified samples can not be exactly correlated to the size markers due to the extensive CGG repeats. Based on sequenced CGG repeat DNA samples, we observe that the amplified CGG repeat fragments runs slower and is thus, when compared to the molecular weight markers run approximately 3 base or one CGG repeat shorter. Example, a 29 CGG repeat fragment is 307 bp and travels as a 304 bp fragment when compared to commercially available molecular weight markers. It is advisable to run several control samples of known CGG repeats to ascertain the approximate size by comparison.

All normal samples except female homozygous samples will yield interpretable results. This range will cover almost 90% of your routine samples, unless you are being referred real Fragile X cases. The normal pattern is one amplified fragment with CGG repeat size below 45 from male DNA samples and two amplified fragments with CGG repeat size below 45 from female DNA samples. Keep in mind that more than 40% of the population is homozygous for the 29-30 CGG allele and thus may give only one amplified fragment from female DNA in PCR results but are actually normal.

From our experience, less than 5% of the samples referred for Fragile X are actually Fx cases. PCR amplification and detection is a way to ascertain genotype of almost 90% of the samples. *It is essential to perform Southern blot analysis for ALL samples that does not give NORMAL results in duplicate PCR analysis.* PCR analysis is to screen bulk of the samples and then perform Southern blot analysis only on those samples that do not yield 'normal' amplification pattern.

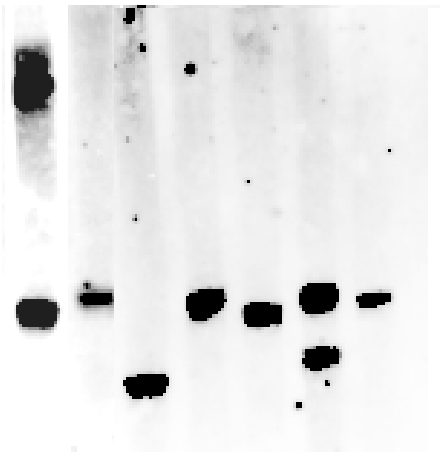
You should perform Southern blot analysis for the following sample types

a. Only one fragment amplified for female samples. Realize that more than 40% of the population is homozygous for the 29-30 CGG allele and thus may give only one fragment in PCR results but are actually normal with two alleles.
b. No amplification products on duplicate amplification.
c. Samples with amplification over ~45 CGG repeats.

PCR-Prober™ results should be easy to interpret based on the size of the fragment amplified, a guideline is provided in Table 3.

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 Mutatiom (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



Fragile X PCR blot.
Lane 1 pre-mutation female; 30/60 CGG repeats.
Non-radioactive detection, ~2 hr. exposure.

References

1. Nelson, D.L. (1993) Growth Genetics and Hormone. 9:1-4.
2. Rousseau, F. et al. (1991) NEJM 325:1673-1681.
3. Verkerk, A. et al. (1991) Cell 65:905-914
4. Fu, Y.H et al. (1991) Cell 67:1047-1058.
5. Oberle, I. et al. (1991) Science 252:1097-1102.
6. Yu, S. et al. (1991) Science 252: 1179-1181.
7. Nelson, D.L. (1996) Growth Gen. and Hormone. 12:1-4.
8. Richards, R and Sutherland, G.R (1992) TIG 8: 249-255.

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, especially running denaturing gel is important. Fragile X PCR based genotyping is difficult due to the extensive stretch of CGG. Even taq polymerase at elevated temperature can not replicate the long stretch of CGG repeats. The kit includes deaza GTP to reduce the strong secondary structure. Our kit is optimized to give results. 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.

3. Reliable Detection. The system will be able to detect reliably the CGG repeats up to 50-60 repeats. Detection beyond this is dependent on the PCR conditions and gel systems but is NOT reliable. Any DNA sample which does not give a reliable CGG repeat analysis on this system clearly indicates either a failure of the PCR reaction or a DNA with extensive CGG repeats. A particular DNA sample not yielding a PCR product on duplicate analysis clearly indicates the possibility of long CGG repeats. In cases like these we suggest that southern analysis should be done using the GeneProber™ Fragile X gene detection system to clearly determine the genotype. Southern analysis is also strongly advised when both the alleles are not reliably genotyped

Appendix: **Protocols**

Genomic DNA Purification

Genomic DNA is usually extracted from blood. A simple procedure is given below that purifies ~10 µg DNA from 300 µl blood using a 30 minute procedure.

Omni-Pure™ Genomic DNA Purification System Catalog Number: 40-4010-01
Rapid DNA Purification Protocol for 300 µl Whole Blood

A. Initial Preparation

1. Label two sets of 1.5 ml tubes per sample.
2. Add 900 µl GD-1 solution (RBC Lysis Solution) to one tube for each sample.
3. Add 300 µl Isopropanol (2-propanol) to one tube for each sample. Cap the tubes.

B. Cell Lysis

1. To the tube containing 900 µl GD-1 solution (RBC Lysis Solution) using a filter tip pipet transfer 300 µl whole blood. Cap and gently mix by inversion. Incubate for 1-3 minutes at room temperature. Mix by inversion a few times during this incubation period. Incubate longer for fresh blood cells as they are intact and not lysed already.
2. Centrifuge at 3 K rpm for 20 seconds to pellet the white blood cells. A reddish white pellet should be clearly visible. Decant and discard supernatant leaving behind the last few droplets. Do not totally remove the supernatant.
3. Completely resuspend the white blood cell pellet by vigorously vortexing the tube. Ensure that the pellet is completely resuspended.
4. To the resuspended cells add 300 µl GD-2 solution (Cell Lysis Solution). Mix by gentle vortexing. You will notice release of DNA by the thickening of the liquid in the sample. Samples may be stored at this stage for processing later. It has been shown that the samples are stable in Cell Lysis Solution for at least 2 years at room temperature.

C. Protein Precipitation

1. Add 100 µl GD-3 solution (Protein Precipitation Solution) to the sample in cell lysis solution.
2. Vortex vigorously at for 20 seconds. Small particles of brown color will be appear and be visible at this stage.
3. Centrifuge at 5 K rpm for 1 minute to pellet the precipitated proteins. A clearly visible brown pellet containing proteins should be collected at the bottom of the tube.

D. DNA Precipitation

1. Decant the supernatant containing the DNA to a new appropriately labeled tube (see initial preparation above) containing 300 µl 100% Isopropanol (2-propanol).
2. Mix the sample by inversion until a visible white floating DNA strand-particle is identified. 30-40 mixing by inversion is usually sufficient.
3. Centrifuge at 6 K rpm for 1 minute to collect the DNA as a pellet. A white DNA pellet should be clearly visible.
4. Decant supernatant and place tube inverted on a clean Kimwipe™ tissue paper to drain the remaining supernatant.
5. To remove residual salts, add 300 µl of 70% ethanol. Vortex gently.
6. Centrifuge at 6 K rpm for 1 minute to collect the DNA as a pellet. Gently take out the tubes so that the pellet is not dislodged. While holding the tube, rotate tube so that you can watch the pellet. Now carefully decant the ethanol, keeping an eye on the pellet so that it does not flow away.
7. Place tube inverted on a clean Kimwipe™ tissue paper to drain the remaining ethanol.
8. Air dry the DNA pellet. Do not use vacuum.

E. DNA Reconstitution & Use

1. Add 100 µl of GD-4 solution (DNA Reconstitution Solution). Vortex gently. Incubate at 60°C for 5 minutes to facilitate dissolution or keep overnight at room temperature.
2. Store DNA at 4 °C. For long-term storage, place sample at –20 °C or –80 °C.
3. Average yield of 10 µg is expected from 300 µl blood DNA. The range is between 5 µg to 15 µg.
4. The 100 µl of purified DNA obtained will have an average concentration of ~ 100 ng/µl.
5. For PCR amplification use 1-2 µl.
6. Use 100 µl for restriction digestion followed by Southern blot analysis.
7. It is convenient to perform multiple 300 µl blood DNA purification instead of scaling up the procedure.

Gel Electrophoresis of DNA

Gel electrophoresis of PCR products is the standard method for analyzing reaction quality and yield. PCR products can range up to 10 kb in length, but the majority of amplifications are at 1 kb and below. Agarose electrophoresis is the classical method to analyze amplification products from 150 bp to greater than 10 kb. Polyacrylamide gel electrophoresis should be used for resolution of short fragments in the range of 100 bp to 500 bp when discrimination of as small as a 10 bp difference is required.
PAGE gels for PCR products formulated with the amount of cross-linker chosen to give pore sizes optimal for the size of DNA fragment desired. Gels are most often stained in ethidium bromide, even though the fluorescence of this stain is quenched by polyacrylamide, which decreases sensitivity 2-5 fold. This decrease in sensitivity generally does not present a problem, because most PCR reactions yield product levels in the microgram range, and ethidium will detect as little as 1/10 of this amount. Polyacrylamide gels can be stained by silver staining for more sensitive detection.

Agarose Gel Electrophoresis of DNA

Agarose gels are typically run at 20 to 150V. The upper voltage limit is the amount of heat produced. At room temperature about 5 Watts is correct for a minigel (Volts x Amps = Watts). At low voltages migration is linearly proportional to voltage, but long DNA molecules migrate relatively faster in stronger fields. Migration is inversely proportional to the log of the fragment length; a log function also governs migration rate and gel concentration (0.5 to 2% for most purposes). Furthermore, supercoiled / circular DNA molecules migrate at different rates from linear molecules; single-stranded DNA and RNA migrate at similar rates, but usually faster than double-stranded DNA of the same length. Salt in the samples increases conductivity and, hence, migration rate.
The buffers used for most neutral agarose gels (the gel itself and the solution in which it lies) is 1 x TAE or 1 x TBE. Agarose powder is added to the buffer at room temperature, heated in a microwave and boiled slowly until the powder has dissolved. Cast the gel on a horizontal surface once the agarose has been cooled to ca. 60° C (just cool enough to hold) and add 0.1 µg of ethidium bromide solution for each ml of gel volume. At times, during removal of the comb, it is possible to tear the bottom of the sample wells gels, which results in sample leakage upon loading. This can be avoided by removing the comb after the gel has been placed in the running buffer.

• Use TAE buffer for most molecular biology agarose gel electrophoresis.

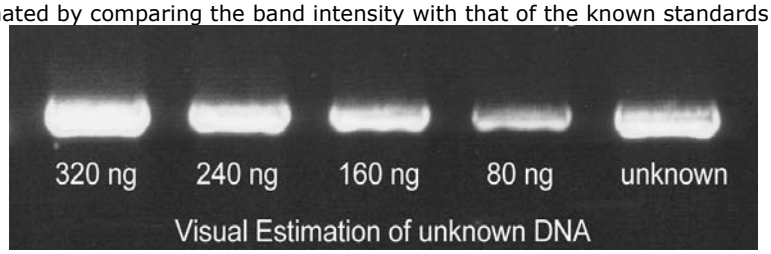
Recipe

1 X TAE Buffer
Agarose Gel Electrophoresis Buffer
40 mM Tris-Acetate pH 7.8
1 mM EDTA

1 X TBE
Agarose and Polyacrylamide Gel Electrophoresis Buffer
0.089 M Tris
0.089 M Boric Acid
0.002 M EDTA

Spectrophotometric Determination of DNA Concentration & Estimation by Agarose Gel Electrophoresis

Measuring the optical density (OD) or absorbance at 260 nm (A_{260}) in a UV spectrophotometer is a relatively accurate method for calculating the concentration of DNA in an aqueous solution if a standard curve is meticulously prepared. An A_{260} of 1, using a 1 cm path length, corresponds to a DNA concentration of 50 µg/ml for double stranded DNA, 40 µg/ml for RNA and 33 µg/ml for oligonucleotides. However, this method is not suitable for determining concentrations of dilute solutions of DNA, as the sensitivity of this method is not very high. For reliable readings, the concentration of double stranded DNA must be greater than 1 µg/ml. A simple, inexpensive method for the estimation of nanogram quantities of DNA is described in the following section. We recommend the use of agarose gel electrophoresis for routine approximate determination of DNA concentration.
The amount of DNA in sample may be estimated by running the sample alongside standards containing known amounts of the same-sized DNA fragment. In the presence of ethidium bromide staining, the amount of sample DNA can be visually estimated by comparing the band intensity with that of the known standards.



An unknown amount of a 4 kb DNA fragment (U) was run alongside known quantities (indicated in nanograms) of the same DNA fragment. As estimated by visual comparison with the known standards, the unknown sample contained 240-320 ng of DNA.

Ethidium bromide is a carcinogen. Follow Health and Safety Procedures established by your institution.
Follow proper Hazardous Material Disposal procedures established by your institution.

• Use 0.1 µg of ethidium bromide solution for each ml of gel volume.

Polymerase Chain Reaction

PCR Components and Analysis
PCR buffer conditions vary and it is imperative to optimize buffer conditions for each amplification reaction. At Gene Link most amplification reactions have been optimized to work with the following standard buffer condition, unless otherwise indicated.

dNTP Concentration
Standard dNTP concentration of 0.2 mM of each base is used. See section on PCR additives when dNTP concentration is changed.

$MgCl_2$ Concentration
The concentration of Mg^{++} will vary from 1-5 mM, depending upon primers and substrate. Since Mg^{2+} ions form complexes with dNTPs, primers and DNA templates, the optimal concentration of $MgCl_2$ has to be selected for each experiment. Low Mg^{2+} ions result in a low yield of PCR product, and high concentrations increase the yield of non-specific products and promote mis-incorporation. Lower Mg^{2+} concentrations are desirable when fidelity of DNA synthesis is critical. The recommended range of $MgCl_2$ concentration is 1-4 mM, under the standard reaction conditions specified. At Gene Link, using the standard PCR buffer with KCl, a final dNTP concentration of 0.2 mM, a $MgCl_2$ concentration of 1.5 is used in most cases. If the DNA samples contain EDTA or other chelators, the $MgCl_2$ concentration in the reaction mixture should be raised proportionally. Given below is an $MgCl_2$ concentration calculation and addition table using a stock solution of 25 mM $MgCl_2$.

Recipe

Standard Gene Link PCR Buffer Composition	
10 X PCR buffer	**1 X PCR buffer**
100 mM Tris-HCl pH 8.3	10 mM
500 mM KCl	50 mM
15 mM $MgCl_2$	1.5 mM
0.01% Gelatin	0.001%

Recipe

2.0 mM dNTP Stock Solution Preparation*	
Component	**Volume**
100 mM dGTP	100 µl
100 mM dATP	100 µl
100 mM dTTP	100 µl
100 mM dCTP	100 µl
Water	4.6 ml
Total Volume	5 ml
*Aliquot and freeze	

$MgCl_2$ Concentration & Addition Table								
Final concentration of $MgCl_2$ in 50 µl reaction mix, (mM)	1.0	1.25	1.5	1.75	2.0	2.5	3.0	4.0
Volume of 25 mM $MgCl_2$, µl	2	2.5	3	3.5	4	5	6	8

Primer Concentration

The final concentration of primers in a PCR reaction is usually 0.5 to 1 µM (micromolar). This is equivalent to 0.5 to 1 pmol/µl. For a 100 µl reaction you would add 50 to 100 pmols. At Gene Link we use 0.5 pmol/µl in the final PCR.

Always use filter barrier pipette tips to prevent cross contamination

Genemer™ Reconstitution
Stock Primer Mix: Dissolve the supplied 10 nmols of lyophilized Genemer™ in 100 µl sterile TE. The 10 nmols of primers when dissolved in 100 µl will give a solution of 100 µM i.e. 100 pmols/µl.

Recipe

TE Buffer pH 7.5 Composition
1 X TE Buffer pH 7.5
10 mM Tris-HCl pH 7.5
1 mM EDTA

Primer Mix: Prepare a 10 pmols/µl Primer Mix solution by a ten fold dilution of the stock primer mix.
Example: Add 180 µl sterile TE to a new tube, to this tube add 20 µl of primer stock solution. Label this tube as Primer Mix 10 pmols/µl.

Amplification Thermal Cycling
Hot Start: It is essential to have a 'Hot Start' profile for amplification of any fragment from a complex template like human genomic DNA. Taq polymerase has low activity at room temperature and it is essential to minimize any mis-priming in the first cycle of amplification. A typical hot start profile is given below. Various enzyme preparations are available which are activated by heat in the first cycle. A simple hot start protocol is given below that can be used with regular Taq polymerase. See the section on PCR additives for amplification of products from high GC content templates.

• Program your thermal cycler instrument with an amplification profile prior to beginning the amplification protocol. Consult your appropriate instrument manufacturer's manual.

Recipe

Typical PCR Premix (/50µl)	
Component	**Volume**
10 x PCR Buffer	5 µl

Hot Start		
Step	**Time & Temperature**	**Cycles**
Initial Denaturation	95 °C for 5 minutes	1
Annealing	60 °C Hold Infinity	Hold
Comments: Add Taq premix while on hold.		

Amplification File

The initial denaturation step at 94 °C for 30 seconds is sufficient for all templates. The number of cycles is usually set to 30 and is sufficient to amplify 1-10 µg of product depending on the initial concentration of template. A higher number of cycles from 35-45 cycles may be used, but internal priming on the product and over amplification of unwanted bands often result from over-cycling. Generally, it is better to focus on optimizing reaction conditions than to go beyond 35 cycles.

Typical Amplification File			
Step	**Temperature**	**Time**	**Cycles**
Denaturation	94 °C	30 sec.	30
Annealing	*	30 sec.	
Elongation	72 °C	30 sec.	
Fill in Extension	72 °C	7 minutes	1
Hold	4 °C	Infinity	Hold
Based on the Tm of the primers. Usually varies from 50 °C to 65 °C			

PCR Premix Preparation (PP)		
Component	**1 X 50 µl Rxn.**	**10 X 50 µl Rxns.**
Sterile Water	32 µl	320 µl
10 X PCR Buffer	4.5 µl	45 µl
2.0 mM dNTP	5 µl	50 µl
10 pmol/µl Primer Mix	2.5 µl	25 µl
Taq Enzyme Mix (EM)	5 µl	50 µl
Template DNA (~500 ng)	1-2 µl	Add 1-2 µl DNA to each tube
Total Volume	50 µl	
Keep on ice during set up. After adding template start PCR File		

2.0 mM dNTP mix (each)	5 µl
Primer Mix (10 pmol/µl each) or 2.5µl of 10 pmol/µl of individual primer (final 25 pmol of each primer/50µl)	2.5 µl
H_2O	37.5 µl
Total Volume	50 µl

Recipe

PCR reaction (/50µl)	
Component	**Volume**
PCR premix	45 µl
100ng/µl diluted DNA	1 µl
Hot start and then add	
Taq premix	5 µl

Recipe

Taq Premix EM (/50µl)	
Component	**Volume**
PCR Premix	6 µl
Taq polymerase (5 u/µl)	0.25µl
Add 5 µl/50 µl rxn after initial denaturation.	
Use 2.5 units of Taq for 100 µl reactions. Taq is usually supplied at a concentration of 5 units/µl	

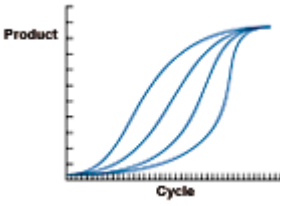
- The PCR premix preparation protocol is written considering that more than one amplification reaction will be performed at the same time. If only one reaction is planned then there is no need to prepare the Taq Enzyme Mix (EM).

Recipe

Gene Link PCR Buffer
1 X PCR Buffer
10 mM Tris-HCl pH 8.3
50 mM KCl
1.5 mM $MgCl_2$
0.001% Gelatin

Yield and Kinetics

The target will be amplified by up to 10^6 fold in a successful reaction, but the amplification will usually plateau at 1-10 µg. Thus, 1 pg of target sequence in the reaction is a good place to begin.



PCR reactions produce product in a nonlinear pattern. Amplification follows a typical exponential curve until some saturation point is reached. Generally products will not be further amplified once 1-5 µg has been generated. Saturation by one product of a reaction does not always prevent further amplification of other generally unwanted products. Over-cycling may decrease the quality of an otherwise good reaction. When first optimizing a reaction, it is advisable to take samples every 5 or 10 cycles to determine the number of cycles actually needed.

PCR Additives

DNA polymerases need to elongate rapidly and accurately to function effectively *in vivo* and *in vitro*, yet certain DNA regions appear to interfere with their progress. One common problem is pause sites, at which DNA polymerase molecules cease elongation for varying lengths of time. Many strong DNA polymerase pauses are at the beginnings of regions of strong secondary structure such as template hairpins (1). Taq polymerase used in PCR suffers the same fate and GC-rich DNA sequences often require laborious work to optimize the amplification assay. The GC-rich sequences possess high thermal and structural stability, presumably because the high duplex melting temperature that permits stable secondary structures to form, thus preventing completion of a faithful replication (2).

Nucleotide analog 7-deaza dGTP is effective in reducing the secondary structure associated with GC rich region by reducing the duplex stability (4). Betaine, DMSO and formamide reduces the Tm and the complex secondary structure, thus the duplex stability (1-5). Tetramethyl ammonium chloride (TMAC) actually increases the specificity of hybridization and increases the Tm. The use of TMAC is recommended in PCR conditions using degenerate primers.

These PCR additives and enhancing agents have been used to increase the yield, specificity and consistency of PCR reactions. These additives may have beneficial effects on some amplification and it is impossible to predict which agents will be useful in a particular context and therefore they must be empirically tested for each combination of template and primers.

PCR Additives		
Additive	**Purpose & Function**	**Concentration**
7-deaza-2'-deoxyguanosine; 7-deaza dGTP	GC rich region amplification. Reduce the stability of duplex DNA	Totally replace dGTP with 7-deaza dGTP; or use 7-deaza dGTP: dGTP at 3:1
Betaine (N,N,N-trimethylglycine = [carboxymethyl]trimethylammonium)	Reduces Tm facilitating GC rich region amplification. Reduces duplex stability	Use 3.5M to 0.1M betaine. Be sure to use Betaine or Betaine (mono)hydrate and **not** Betaine HCl.
BSA (bovine serum albumin)	BSA has proven particularly useful when attempting to amplify ancient DNA or templates, which contain PCR inhibitors such as melanin.	BSA concentration of 0.01 µg/µl to 0.1 µg/ µl can be used.
DMSO (dimethyl sulfoxide)	DMSO is thought to reduce secondary structure and is particularly useful for GC rich templates.	DMSO at 2-10% may be necessary for amplification of some templates, however 10% DMSO can reduce *Taq* polymerase activity by up to 50% so it should not be used routinely.
Formamide	Reduces secondary structure and is particularly useful for GC rich templates.	Formamide is generally used at 1-5%. Do not exceed 10%.
Non-ionic detergents e.g. Triton X-100, Tween 20 or Nonidet P-40 (NP-40)	Non-ionic detergents stabilise *Taq* polymerase and may also supress the formation of secondary structure.	0.1-1% Triton X-100, Tween 20 or NP-40 may increase yield but may also increase non-specific amplification. As little as 0.01% SDS contamination of the template DNA (left-over from the extraction procedure) can inhibit PCR by reducing *Taq* polymerase activity to as low as 10%, however, inclusion of 0.5% Tween-20 or -40 will effectively neutralize this effect.
TMAC (tetramethylammonium chloride)	TMAC is used to reduce potential DNA-RNA mismatch and improve the stringency of hybridization reactions. It increases Tm and minimizes mis-pairing.	TMAC is generally used at a final concentration of 15-100 mM to eliminate non-specific priming.

Purification of PCR Product

Various purification methods are available for the purification of PCR products. The selection of a particular method over another is based on the downstream application and the initial robustness of the amplification. Usually no further purification is required for most cloning experiments if a single fragment is amplified, whereas for sequencing applications the amplified product should be purified from the primers and any other minor amplification products.

The preferred method of purification of an amplified fragment is the excision of the fragment band after agarose gel electrophoresis. This method yields the purification of a single fragment; as such care should be taken to excise a gel piece containing a single electrophoretically resolved fragment. The Omni-Clean™ Purification System available from Gene Link can be used for this purpose. Catalog No. 40-4110-10 for bead based system; 40-4120-10 for spin column based system and 40-4130-10 for DNA concentration. Please refer to product insert for detailed protocol or visit **www.genelink.com**.

A. **Purification of DNA from gel slices using glass beads. Provides purified single fragment.**
[Omni-Clean™ Gel DNA Beads Purification System; Catalog No. 40-4110-10]

Protocol

1. By weight, determine the volume of the excised DNA fragment.
2. Add 3 volumes of NaI solution and heat to 55 °C. Visually determine the dissolution of gel pieces.
3. Add 1 µl of glass bead suspension per µg of DNA and vortex.
4. Centrifuge at 2K rpm for 20 seconds to pellet glass bead/DNA complex. Discard supernatant.
5. Re-suspend pellet in 400 µl Omni-Clean™ wash buffer. Centrifuge at 2K rpm for 20 seconds and discard wash buffer.
6. Pipet out any remaining buffer in the tube.
7. Add 25 µl water or TE; re-suspend pellet and centrifuge at 2K rpm for 20 seconds.
8. The supernatant contains the purified DNA. Using a pipet, collect the supernatant and transfer to a new appropriately labeled tube.

B. **Purification of DNA from gel slices using spin column. Provides purified single fragment.**
[Omni-Clean™ Gel DNA Spin Column Purification System; Catalog No. 40-4120-50]

Protocol

1. By weight, determine the volume of the excised DNA fragment.
2. Add 3 volumes of NaI solution and heat to 55 °C. Visually determine the dissolution of gel pieces.
3. Add the above solution to the spin column assembled on a collection tube.
4. Let the solution flow by gravity or centrifuge at 2K rpm for 20 seconds. Discard flow through collected in the collection tube.
5. Add 400 µl Omni-Clean™ wash buffer to the spin column. Centrifuge at 2K rpm for 2 minutes and discard wash buffer collected in the collection tube.
6. Replace the collection tube with a new appropriately labeled 1.5ml tube.
7. Add 25 µl water or TE to the spin column. Let sit for 3 minutes.
8. Centrifuge at 2K rpm for 2 minutes.
9. The collection tube contains the purified DNA.

C. **Purification of DNA from solution using glass beads. Provides removal of salts, primers and dNTP.**
[Omni-Clean™ DNA Beads Concentration System; Catalog No. 40-4130-10]

Protocol

1. Determine volume of DNA solution and add 3 volumes of NaI solution.
2. Add 1 µl of glass bead suspension per µg of DNA.
3. Centrifuge at 2K rpm for 20 seconds to pellet glass bead/DNA complex. Discard supernatant.
4. Re-suspend pellet in 400 µl Omni-Clean™ wash buffer.
5. Centrifuge at 2K rpm for 20 seconds and discard wash buffer.
6. Pipet out any remaining buffer in the tube.
7. Add 25 µl water or TE; re-suspend pellet and centrifuge at 2K rpm for 20 seconds.
8. The supernatant contains the purified DNA. Using a pipet, collect the supernatant and transfer to a new appropriately labeled tube.

D. **Purification of DNA from solution using spin column. Provides removal of salts, primers and dNTP.**
[Omni-Clean™ DNA Spin Column Concentration System; Catalog No. 40-4140-10]

Protocol

1. Determine volume of DNA solution and add 3 volumes of NaI solution.
2. Add the above solution to the spin column assembled on a collection tube.
3. Let the solution flow by gravity or centrifuge at 2K rpm for 20 seconds. Discard flow through collected in the collection tube.
4. Add 400 µl Omni-Clean™ wash buffer to the spin column. Centrifuge at 2K rpm for 2 minutes and discard wash buffer collected in the collection tube.
5. Replace the collection tube with a new appropriately labeled 1.5ml tube.
6. Add 25 µl water or TE to the spin column. Let sit for 3 minutes.
7. Centrifuge at 2K rpm for 2 minutes.

8. The collection tube contains the purified DNA.

PEG Precipitation

Primers and salts are efficiently removed by a simple PEG precipitation. This method is recommended for downstream DNA sequencing application. This method is generally used for plasmid DNA.

Protocol

1. To 50 µl of amplified PCR reaction add 6.0 µl of 5 M NaCl and 40 µl of 13% (w/v) PEG 8000. Incubate the mixture on ice for 20-30 minutes.
2. Collect the DNA precipitate by centrifugation at maximum speed for 15 minutes at 4 °C in a microfuge. Carefully remove the supernatant by gentle aspiration.
   The pellet of DNA is translucent and generally invisible at this stage.
3. Rinse the pellet with 500 µl of 70% ethanol.
   The precipitate changes to a milky-white color and becomes visible.
4. Carefully pour off the 70% ethanol. Rinse the DNA pellet once more with 70% ethanol. Store the tube in an inverted position at room temperature until the last visible traces of ethanol have evaporated.
5. Dissolve the DNA in 20 µl of H_20.
6. Run an aliquot on an agarose gel to confirm the presence of the correct amplified product. The purified DNA is sequence grade and can be used directly for sequencing.

Gel Filtration

Primers and salts are efficiently removed by gel filtration using Sephadex G-50. This method is recommended for downstream DNA sequencing application.

Protocol

1. Hydrate Sephadex G-50 ahead of time in sterile water or TE (10mM Tris pH 8, 1 mM EDTA). Take out from fridge if already stored hydrated. Bring to room temperature.
2. Assemble a spin column on a collection tube.
3. Add 700 µl of hydrated Sephadex G-50 to each spin column, initiate flow using rubber bulb or any other method.
4. Allow flowing by gravity till there is no more fluid left above the Sephadex G-50 bed. Discard flow through from the collection tube.
5. Spin the spin column placed inside the collection tube for 2 minutes at 3 K rpm.
6. Change collection tube to new 1.5 ml tube appropriately labeled with sample name.
7. Apply up to 50 µl sample gently to the G-50 bed of the column.
8. Spin for 2 minutes at 3 K rpm.
9. Purified sample is collected in the collection tube. The eluent collected in the 1.5 ml tube is free of salts and primers shorter than 35-40mer.

References

1. Kovarova, M; and Draber, P; (2000) New Specificity and yield enhancer for polymerase chain reactions (2000) Nucl. Acids. Res. 28: e70.
2. Henke, W., Herdel, K., Jung, K., Schnorr, D. and Stefan A. Loening, S. (1997) Betaine improves the PCR amplification of GC-rich DNA sequences. Nucl. Acids Res. 25: 3957-3958.
3. Daniel S. Mytelka, D.S., and Chamberlin, M.J.,(1996) Analysis and suppression of DNA polymerasepauses associated with a trinucleotide consensus. Nuc. Acids Res.,. 24:2774–278.
4. Keith, J. M., Cochran, D.A.E., Lala, G.H., Adams, P., Bryant, D.and Mitchelson, K.R. (2004) Unlocking hidden genomic sequence. Nucl. Acids Res. 32: e35.
5. Owczarzy, R., Dunietz, I., Behlke, M.A., Klotz, I.M. and Joseph A. Walder. (2003) Thermodynamic treatment of oligonucleotide duplex–simplex equilibria. PNAS, 100:14840-14845.

Fragile X Product Ordering Information

Product	Size	Catalog No.
Fragile X Genemer™ Primer pair Primers for amplification of CGG triple repeat spanning region. The quantity supplied is sufficient for 400 regular 50 µl PCR reactions.	10 nmole	40-2004-10
Fragile X GeneProber™ GLFX1 Probe unlabeled Fragile X CGG triple repeat spanning region unlabeled probe for radioactive labeling and Southern blot detection. Suitable for random primer labeling.	500 ng	40-2004-40
Fragile X GeneProber™ GLFXDig1 Probe Digoxigenin labeled Fragile X CGG triple repeat spanning region digoxigenin labeled probe for non-radioactive Southern blot detection.	110 µl	40-2004-41
Fragile X PCRProber ™ AP labeled probe Alkaline phosphatase labeled probe	12 µl	40-2004-31
Fragile X PCRProber ™ Kit for chemiluminescent detection Kit for performing PCR amplification and chemiluminescent based detection.	5 blots [50 rxns]	40-2004-32
Fragile X Genemer™ Kit for Radioactive Detection Kit for amplification and radioactive detection of Fragile X CGG triple repeat region amplified PCR products using ^{35}S or ^{32}P. 100 Reactions.	100 [rxns]	40-2004-20
Fragile X GScan™ Kit for fluorescent detection Kit for performing fluorescent PCR amplification based detection. Various dye kits. XX=FM for 6-Fam; HX for Hex; TT for Tet; C3 for Cy3 and C5 for Cy5.	1 Kit [100 rxns]	40-2004-15XX

Genemer™ control DNA Cloned fragment of the mutation region of a particular gene. These control DNA's are ideal genotyping templates for optimizing and performing control amplification with unknown DNA. The size of the triple repeats has been determined by sequencing and gel electrophoresis. The stability of size repeats upon cloning and amplification has NOT been determined. Thus, the size should be considered approximate and there is no claim for each fragment to contain the exact number of triple repeats. These control DNA's are sold with the express condition that these NOT be used for exact triple repeat size determination of DNA of unknown genotype. The control DNA should be used for determining the performance of specific Genemer™ and PCRProber™ Gene Link products.

Fragile X ~16 CGG repeat Genemer Control DNA	500 ng	40-2004-01
Fragile X ~29 CGG repeat Genemer Control DNA	500 ng	40-2004-02
Fragile X ~40 CGG repeat Genemer Control DNA	500 ng	40-2004-03
Fragile X ~60 CGG repeat Genemer Control DNA	500 ng	40-2004-04
Fragile X ~90 CGG repeat Genemer Control DNA	500 ng	40-2004-05

Please visit www.genelink.com for other Genemer™ control DNA not listed here

All Gene Link products are for research use only Current pricing are posted at http://www.genelink.com/

GeneProber™ Product Ordering Information

The GeneProber™ product line is based on the chemiluminescent Southern blot detection method. Gene Link's non-radioactive detection systems for genotyping of triple repeat disorders are rapid, reliable and as sensitive as the ^{32}P labeled southern blots. No more decayed probes and radioactive exposure. Kits are available for reliable genotyping of the fragile X, myotonic dystrophy and other triple repeat mutation group disorders.

Unlabeled GeneProber™ probes are also available for radio labeling and radioactive based detection. Gene Link strongly recommends the use of non-radioactive gene detection systems. Consider switching to Gene Link's product line of non-radioactive detection systems

Product	Unit Size	Catalog No.
Fragile X GeneProber™ GLFX1 Probe unlabeled	500 ng	40-2004-40
Fragile X GeneProber™ GLFXDig1 Probe Digoxigenin labeled	110 µl	40-2004-41
Huntington's Disease GeneProber™ GLHD14 Probe unlabeled	500 ng	40-2025-40
Huntington's Disease GeneProber™ GLHDDig2X Probe Digoxigenin labeled	110 µl	40-2025-41
Myotonic Dystrophy GeneProber™ GLDM1 Probe unlabeled	500 ng	40-2026-40
Myotonic Dystrophy GeneProber™ GLDMDig2 Probe Digoxigenin labeled	110 µl	40-2026-41
Friedreich's Ataxia GeneProber™ GLFRDA21 Probe unlabeled	500 ng	40-2027-40
Friedreich's Ataxia GeneProber™ GLFRDADig21 Probe Digoxigenin labeled	110 µl	40-2027-41

GScan™ Products Product Ordering Information

Gene Link's GScan™ gene detection products are safe, convenient and sensitive, and afford automated compilation of data. The kits contain optimized PCR amplification reagents and a wide array of fluorescent-labeled primers for genotyping after PCR using fluorescent genetic analyzer instrument(s). Included in these kits are ready-to-run control samples of various repeats of the triple repeat disorder kit. These control samples are for calibration with the molecular weight markers for accurate size determination of the amplified fragments.

The GScan™ kits are simple and robust for routine triple-repeat detection of greater than 100 repeats of all triple repeat disorders listed, except Fragile X. The CGG repeat in Fragile X can be detected up to ~50 repeats.

.

Product	Unit Size	Catalog No.
Fragile X GScan™ Kit for fluorescent detection; 100 reactions kit	1 kit	40-2004-15XX
Fragile X GScan™ Kit for fluorescent detection; 20 reactions kit	1 kit	40-2004-15FMS
Huntington's Disease GScan™ Kit for fluorescent detection; 100 reactions kit	1 kit	40-2025-15XX
Huntington's Disease GScan™ Kit for fluorescent detection; 20 reactions kit	1 kit	40-2025-15FMS
Myotonic Dystrophy GScan™ Kit for fluorescent detection; 100 reactions kit	1 kit	40-2026-15XX
Myotonic Dystrophy GScan™ Kit for fluorescent detection; 20 reactions kit	1 kit	40-2026-15FMS
Friedreich's Ataxia GScan™ Kit for fluorescent detection; 100 reactions kit	1 kit	40-2027-15XX
Friedreich's Ataxia GScan™ Kit for fluorescent detection; 20 reactions kit	1 kit	40-2027-15FMS

All Gene Link products are for research use only Current pricing are posted at http://www.genelink.com/