Non-radioactive Fragile X Genotyping by Southern Blot Analysis
Methylation Pattern and CGG Repeat Detection

GLFX Dig1 GeneProber™

Catalog No.: 40-3202-01 **110 µl (5 blots)** **Store at -20°C**

Digoxigenin labeled probe spanning the Fragile X CGG trinucleotide repeat region.
Ready to use for non-radioactive southern analysis.

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

Instruction Manual

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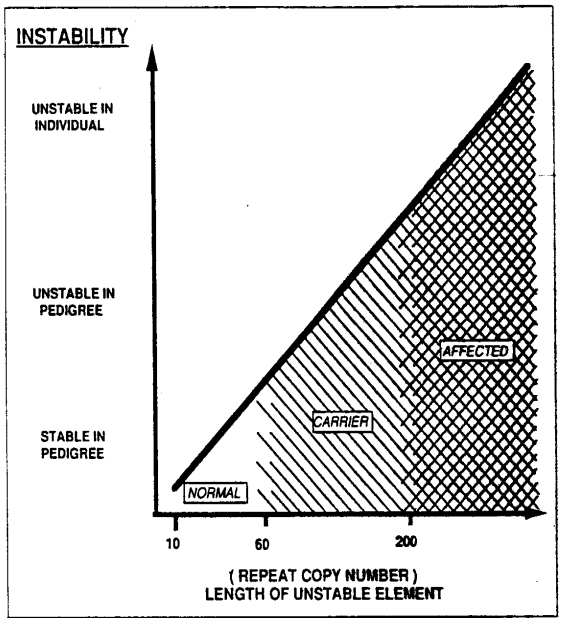
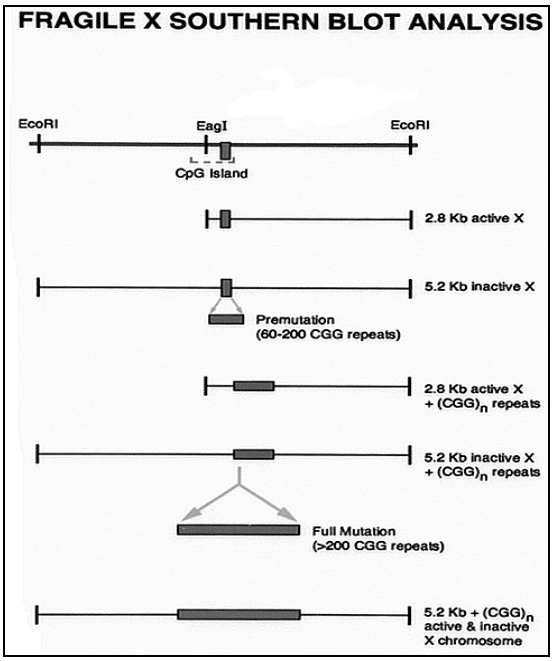
Fragile X Genotyping

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 (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).

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
Spincerebellar ataxia 1 (SCA1)	$(CAG)_n$	6-39	…	40-81 (Pure)
Spincerebellar ataxia 2 (SCA2)	$(CAG)_n$	14-31	…	34-59 (Pure)
Spincerebellar ataxia 3 (SCA3)/Machado Joseph disease (MJD)	$(CAG)_n$	13-44	?	60-84
Spincerebellar ataxia 6 (SCA6)	$(CAG)_n$	4-18	?	21-28
Spincerebellar ataxia 7 (SCA7)	$(CAG)_n$	7-17	?	38-130
Haw River syndrome (HRS; also DRPLA))	$(CAG)_n$	7-25	?	49-75
Friedreich ataxia (FRDA)	$(CAA)_n$	6-29	? (>34-40)	200-900

[a] Typically, repeats tracts contain sequence interruptions. See Pearson and Sinden (1998*a*) for a discussion of the sequence interruptions.
[b] No. of trinucleotidet repeatss.
[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 site in the region. Full mutation has been shown to methylate the active gene too and thus it prevents Eag I restriction of DNA. Hybridization of southern blots of Eco RI and 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 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 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).

Reliable non-radioactive PCR based and Southern based detection can be accomplished by using Gene Link products listed at the end of this manual.

Procedure: Fragile X Chemiluminescent Southern Protocol

Material Supplied

One tube containing 110 μl of *GeneProber™* GLFXDIG1 probe at a concentration of ~40ng/ul. This probe is digoxigenin labeled for non-radioactive detection. The quantity supplied is sufficient for at least 5 20x20 cm blots using 20μl for each blot as probe.

A. Chromosomal DNA digestion

DNA (5 to 10 μg)	X μl
10x Eag I Buffer	5 μl
10x EcoR I Buffer	5 μl
Eag I (10 U/μl)	4 μl
EcoR I (10 U/μl)	4 μl

	H_2O to 100 μl

♦ **Incubate over night at 37°C**

♦ **Precipitate the digests**

-To 100 μl DNA add 10 μl of 3M NaAc

-Add 2 volumes (250 μl) of 100% ethanol

-Put in the freezer (-20 °C) for 20-30 minutes

-Spin at -10 °C for 5 minutes

-Discard the supernatant

-Add 100 μl of 70% ethanol

-Spin again at -10 °C for 5 minutes

-Dry samples

♦ **Dissolve the pellets in 10 μl of 1x loading buffer**

B. Electrophoresis and Transfer

1. Load samples to 0.8% agarose gel , run over night at 45mA for 20-24 hours. (1.6 kb fragment on the bottom of the gel).

2. 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 room temperature (RT), neutralize with 1.5M NaCl/0.5M Tris (pH 7.5) for 30 min. at RT, transfer to the positively charged nylon membrane using 10xSSC and 10 pieces of SIGMA QuickDraw blotting paper overnight. Wash the membrane with 2x SSC, bake at 80°C for 2 hours.

C. Hybridization

Gene Link recommends using Roche Biochemicals (Boehringer Mannheim) Digoxigenin based washing and detection system.

1. Perform prehybridization at 55°C for 3 hours in 10 ml of Easy Hyb buffer (Roche Biochemicals).
2. Boil 20μl *GeneProber™* GLFXDIG1 probe in 500μl of Easy Hyb for 10 minutes. Chill directly on ice. Add the above probe to10ml of Easy Hyb. Discard the prehybridization buffer and replace it with the hybridization buffer containing the boiled probe. Hybridize overnight at 55°C.
3. Washing. Wash the membrane in 2xSSC/0.1% SDS at RT twice (5 min/wash), 0.5xSSC, 0.1%SDS twice at 60°C (15 min/wash), warm the blocking reagent at this point. (e.g. add 20 ml of 10% blocking reagent to 80 ml of Buffer A, use 80 ml for blocking, the rest of 20 ml for making Anti-DIG-AP conjugate.)

D. Anti-Dig Alkaline Phosphatase Binding

1. Equilibrate the membrane in 100ml of 1x washing buffer I for 1 minute.
2. Incubate the membrane in 100ml of 2% blocking solution at RT for 30 min. Prepare 1:10000 Anti-DIG-AP conjugate at this point. e.g. add 2 μl to 20 ml Buffer B.
3. Incubate the membrane in 20ml of Anti-DIG-AP conjugate solution at RT for 30 min.
4. Wash the membrane twice, 15 min/wash in 200ml of 1x washing buffer I at RT.
5. Equilibrate the membrane in 50ml of 1x Detection buffer for 2 min. Repeat 2 times. Total of three washes.

E. Detection

Detection with CDP star(Tropix) as substrate will yield reliable result by exposing to Kodak X-OMAT or XAR X-ray film for 1 hour to overnight at room temperature.

1. 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.
2. 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.
3. 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.

F. Stripping

Wash the membrane in water to remove the substrate. Then wash the membrane in 0.2N NaOH/0.1% SDS at 37°C for 30 minutes. Rinse the membrane in 2XSSC. Air dry.

Required reagents with recommended suppliers

Roche Biochemicals (Boehringer Mannheim) http://www.roche.com/diagnostics/ **Tel:1-800-262-1640**

1. Dig Easy Hyb, (Hybridization solution) Catalog No. 1603 558
2. Anti-DIG Alkaline phosphate Catalog No. 1093 274
3. DIG Wash and Block buffer set Catalog No. 1585 762
4. Nylon membrane positively charged Catalog No. 1209 272

Tropix Tel:1-800-542-2369

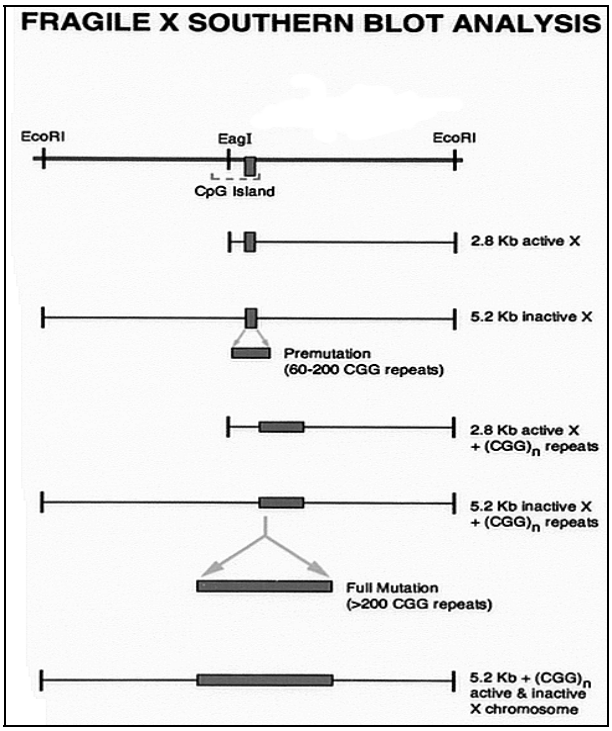
1. CDP-Star (ready to use substrate) Catalog No. MS050R

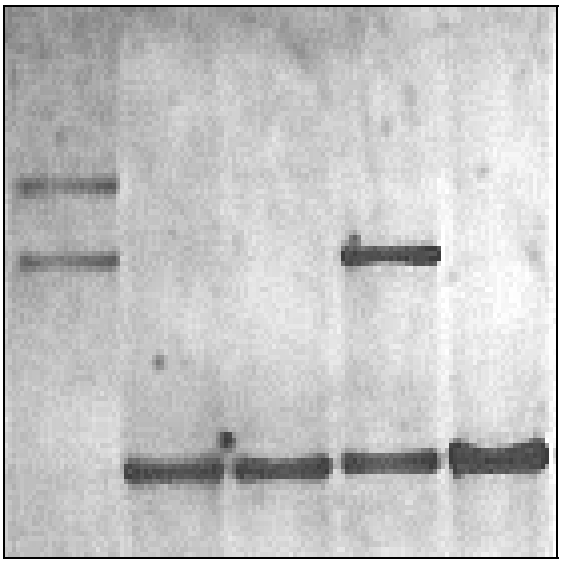
Preparation of additionally required buffers and solution

DNA dilution buffer	**N-lauroylsarcosine stock solution**	**Depurination solution**
10mM Tris-HCl, 1mM EDTA pH 8.0, 50µg/ml herring sperm DNA	10% (w/v) in water	0.25M HCl
Denaturation solution	**Neutralization solution**	**20X SSC**
0.5M NaOH, 1.5M NaCl	0.5M Tris-HCl pH 7.5, 1.5M NaCl	3M NaCl, 0.3M sodium acetate
SDS stock solution	**Detection buffer**	**Probe stripping solution**
20% (w/v) in water	100mM Tris-HCl pH 9.5, 100mM NaCl, 50mM $MgCl_2$.	0.1-0.5N NaOH, 0.1% SDS
Standard hybridization buffer	**Standard hybridization buffer with 50% formamide**	**10% Blocking reagent stock solution (5X)**
5XSSC, 0.1% N-lauroylsarcosine, 0.02% SDS, 1% blocking reagent using the 10% Blocking reagent stock solution	5XSSC, 50% deionized formamide, 0.1% N-lauroylsarcosine, 0.02% SDS, 2% blocking reagent using 10% Blocking reagent stock solution.	Blocking reagent is dissolved in Buffer I to a final concentration of 10% (w/v) with shaking and heating. **AVOID** boiling.
	Buffer 1/Maleic acid buffer	
	0.1M Maleic acid, 0.15M NaCl pH 7.5 (adjust pH with solid or concentrated NaOH)	

Results and Analysis

Southern blot analysis for Fragile X mutation detection involves the cleavage of DNA with enzyme Eco R I and 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 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).





Fragile X southern blot.. Lane 1 affected female. Lanes 2, 3 & 5 are normal males. Lane 4 normal female.
Non-radioactive detection, ~2hr. exposure

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 fragment	18-135	138-207	210-258	260-600	120-600	>600	120-600/ >600
PCR amplified Fragment Size	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.
2. 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. 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 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.

FRAGILE X PRODUCT LIST AND ORDERING INFORMATION

Product	Description	Size	Catalog No
GLFX1 GeneProber™	Probe spanning the fragile X trinucleotide repeat region. Suitable for random primer labeling for southern labeling for southern analysis. Contains probe only.	500 ng	40-3201-01
GLFXDig1 GeneProber™	Digoxigenin labeled probe spanning the Fragile X CGG trinucleotide repeat region. Ready to use for non-radioactive southern analysis. Contains probe only.	110 µl	40-3202-01
GLFX PCRProber™	Probe for non-radioactive detection of Fragile X CGG trinucleotide repeat region amplified PCR product. Contains probe only.	5 blots	40-3101-01
GLFX PCRProber™ Kit	Kit for amplification and non-radioactive detection of Fragile X CGG trinucleotide repeat region amplified PCR product.	5 blots	40-3102-00
GLFX Genemer™ kit	Kit for amplification and radioactive detection of Fragile X CGG trinucleotide repeat region amplified PCR product using ^{32}S or ^{32}P.	100 reactions	40-3103-00
GLFX Genemer™	Primers for Fragile X CGG trinucleotide repeat region amplification. Contains primers only.	10 nmoles	40-2004-10

Other Related Products

Product	Size	Catalog No.
Sickle Cell SC2/SC5 primer pair	10nmoles	40-2001-10
RhD (Rh D gene exon 10 specific)	10nmoles	40-2002-10
Rh EeCc (Rh Ee and Cc exon 7 specific)	10nmoles	40-2003-10
Sry (Sex determining region on Y)	10nmoles	40-2020-10
X alphoid region	10nmoles	40-2021-10
Y alphoid region	10nmoles	40-2022-10
STS (Steroid Sulfatase)	10nmoles	40-2023-10
HGH (Human growth hormone)	10nmoles	40-2024-10
Fragile X (spanning triple repeat region)	10nmoles	40-2004-10
Gaucher 1226G mutation specific	10nmoles	40-2005-10
Gaucher 1448C mutation specific	10nmoles	40-2006-10
Gaucher 84GG mutation specific	10nmoles	40-2007-10
Gaucher IVS2 mutation specific	10nmoles	40-2008-10
Cystic Fibrosis ΔF508	10nmoles	40-2009-10
Cystic Fibrosis G542X	10nmoles	40-2010-10
Cystic Fibrosis W1282X	10nmoles	40-2011-10
Cystic Fibrosis G551D/R553X	10nmoles	40-2012-10
Cystic Fibrosis N1303K	10nmoles	40-2013-10
Cystic FibrosisCT3849	10nmoles	40-2014-10