Certificate of Analysis & Product Manual

Triple Repeat Disorders Genotyping
Fragile X, Myotonic Dystrophy, Friedreich's Ataxia, Huntington's disease
Fluorescent Probes, siRNA, Hybridization and Detection Reagents

FRAXE/FMR2/AFF2 CCG Repeat Genotyping GeneProber™

FMR2 CCG triple repeat non-radioactive Southern blot genotyping

Catalog No. 40-2054-41

Storage Condition: See Material Supplied List

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.

Material Supplied

FRAXE/FMR2/AFF2 CCG Repeat Genotyping GeneProber™

FMR2/FRAXE/AFF2 CCG triple repeat spanning region digoxigenin labeled probe for Southern blot genotyping

REF	Catalog No.	Description		Size
	40-2054-41	FMR2/AFF2/FRAXE GeneProber™ AFF2-AJ31Dig1 labeled	Digoxigenin	110 µL

Certificate of Analysis & Product Specifications

One tube containing 110 µL of GeneProber™ AFF2-AJ31Dig1 probe at a concentration of ~40ng/ µL. This probe is digoxigenin labeled for non-radioactive detection. The quantity supplied is sufficient for at least 5 20x20 cm blots using 20 µL for each blot as probe. Experienced users with optimized hybridization conditions can save the hybridization solution containing the probe and re-use at least one more time for hybridization.

The FMR2/FRAXE/AFF2 GeneProber™ AFF2-AJ31Dig1 probe supplied has been validated to hybridize to the CCG triple repeat spanning region of FMR2.

Appropriate nuclease free handling, dispensing and storage conditions required.

Product Label Information

RUO Research Use Only	-10°C -20°C	LOT
Research Use Only	**Storage** Store at -20°C to -10°C	**Lot Number** Stated on product tube and packing slip
Expiry One year from Date of Shipment	**Instructions** Consult product manual	**QR Code** Visit Gene Link website for product details

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. **Unlabeled GeneProber™ probes are also available for radio labeling and radioactive based detection.**

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
FRAXE/FMR2/AFF2 GeneProber™ AFF2-AJ31Dig1	110 µL	40-2054-41
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
Mitochondrial DNA deletion GeneProber™ GL557 Digoxigenin labeled	110 µL	40-2055-41

GScan™ Product Ordering Information

Gene Link's GScan™ gene detection products are safe, convenient and sensitive, and afford automated compilation of data for routine triple-repeat genotyping of greater than 100 repeats of all triple repeat disorders listed.

Product	Unit Size	Catalog No.
Fragile X GScan™ V2 Kit for fluorescent detection; 100 reactions kit	1 kit	40-2004-15XX
FRAXE/FMR2/AFF2 GScan™ Kit for fluorescent detection; 100 reactions kit	1 kit	40-2054-15FM
Huntington's Disease GScan™ V2 Kit for fluorescent detection; 100 reactions kit	1 kit	40-2025-15XX
Myotonic Dystrophy GScan™ Kit for fluorescent detection; 100 reactions kit	1 kit	40-2026-15XX
Friedreich's Ataxia GScan™ Kit for fluorescent detection; 100 reactions kit	1 kit	40-2027-15XX

Genemer™ Kits Product Ordering Information

Gene Link's Genemer™ kits contain optimized PCR amplification components for convenient agarose gel genotyping of triple repeat disorders and other genetic disorders. These are safe, convenient and sensitive, and afford rapid screening of samples of greater than 100 repeats of all triple repeat disorders listed.

Product	Unit Size	Catalog No.
Fragile X Genemer™ V2 Kit for gel based detection; 100 reactions kit	1 kit	40-2004-11
FRAXE/FMR2/AFF2 Genemer ™ Kit for gel based detection; 100 reactions kit	1 kit	40-2054-11
Huntington's Disease Genemer ™ V2 Kit for gel based detection; 100 reactions kit kit	1 kit	40-2025-11
Myotonic Dystrophy Genemer ™ Kit for for gel based detection; 100 reactions kit	1 kit	40-2026-11
Friedreich's Ataxia Genemer ™ Kit for gel based detection; 100 reactions kit	1 kit	40-2027-11

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

FRAXE/FMR2/AFF2 Genotyping

Background

FRAXE mental retardation (OMIM 309548) is a form of mild to moderate intellectual disability generally associated with learning difficulties, communication deficits, attention problems, hyperactivity and autistic behavior. FRAXE mental retardation is the cause of a non-syndromic X-linked mental retardation affecting 1/50,000 newborn males. The CCG repeat of FRAXE can either expand or contract and is equally unstable when transmitted through the male or the female germ line (1–3).

FRAXE (AFF2/ FMR2 gene) a folate-sensitive fragile site in Xq28 ~600 kb distal to the FRAXA (FMR1 gene) site is the most common form of inherited mental retardation (4-5). FMR2 is a large gene with a major 8.75-kb transcript in placenta, fibroblasts adult and brain and a longer 13.7-kb FMR2 isoform in fetal brain (6). The FMR2 gene is organized in 22 exons, showing several possibilities of alternative splicing for exons 2, 3, 5, 7 and 21. The longest of the FMR2 isoforms is composed of 1272 amino acids and contains two nuclear localization signal (NLS) sequences that are both able to direct GFP into the nucleus (6). Molecular characterization revealed that individuals expressing FRAXE had amplifications of a CCG repeat adjacent to a CpG island. Normal individuals showed 4–39 copies of the polymorphic FRAXE CCG repeat, while individuals expressing the fragile site had >200 copies and their CpG island was fully methylated. These findings are similar to those found for folate-sensitive fragile X site FRAXA. Reports of FRAXE full expansions and pre-mutations are rarely documented. In this respect, it has been very difficult to determine to what extent the alleles, with CCG repeats in the range of 36 and 199, have a pathogenic effect (7-8). Intellectually disabled individuals are primarily referred for FRAXA screening and individuals who are negative for FRAXA are possible candidates for FRAXE screening. Traditionally in some laboratories AFF2 molecular analysis is performed by PCR, it is known that CCG repeats in the range of ~80 and above are not reliably amplified. We embarked on an effort to supplement our PCR analysis by Southern blot and cloned a segment of the AFF2 gene that can be used by appropriate labeling as a probe to determine expansion of the CCG repeats in the AFF2 gene. We have developed a probe to be used for Southern blot analysis that reliably detects the AFF2 CCG triple repeat amplification. We have presented data of AFF2 molecular analysis in a subpopulation of 5,000 individuals referred for FRAXA screening (7-8). The presence of pre-mutated and fully expanded alleles in either gender, were confirmed by Southern blot analysis, which also enabled exclusion of methylation or repeat number mosaics as well as PCR failure. The use of this probe has been recommended as suitable for genotyping of pre-mutations, full mutations, and mosaics specifically for individuals presented for FRAXA screening with negative results to determine FRAXE status (7-8).

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

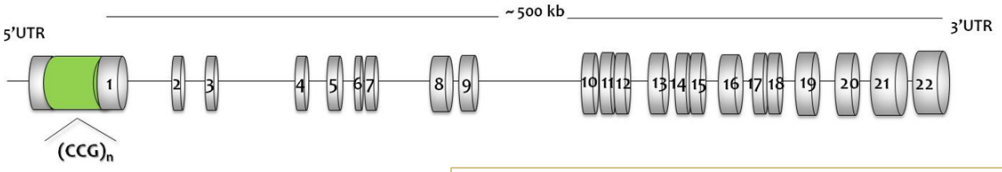
Disease	Repeat[a]	Normal Length[b]	Intermediate Length (Premulation)[a,b]	Full Disease Length[b]
Fragile XA (FRAXA)	$(CGG)_n$	6-52	59-230	230-2,000
Fragile XE (FRAXE)	$(CCG)_n$	4-39	? (31-61)	200-900
Fragile XF(FRAXF)	$(CGG)_n$	7-40	?	306-1,008
FRA16A	$(CCG)_n$	16-49	?	1,000-1,900
Jacobsen Syndrome (FRA11B)	$(CGC)_n$	11	80	100-1,000
Kennedy Syndrome (SMBA)	$(CAG)_n$	14-32	?	40-55
Myotonic Dystrophy (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

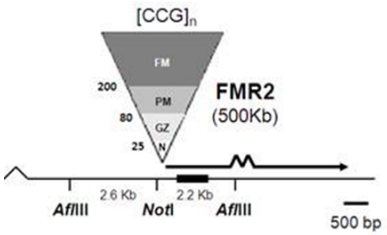
[a] Typically, repeats tracts contain sequence interruptions. See Pearson and Sinden (1998*a*) for a discussion of the sequence interruptions.
[b] No. of triplet repeats.
[c] A question mark (?) indicates potential mutagenic intermediate length, and an ellipsis (…) indicates none. Not all disease are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

Molecular Analysis

FRAXE/AFF2/FMR2 genotyping can be done by direct PCR amplification of the CCG 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 FRAXE gene region containing the CCG trinucleotide repeats is flanked by Not I and Afl III sites. Full mutation has been shown to methylate the active gene too and thus it prevents Not I restriction of DNA. Hybridization of southern blots Not I and Afl III double digested DNA clearly can distinguish between normal, premutation and full mutation genotypes

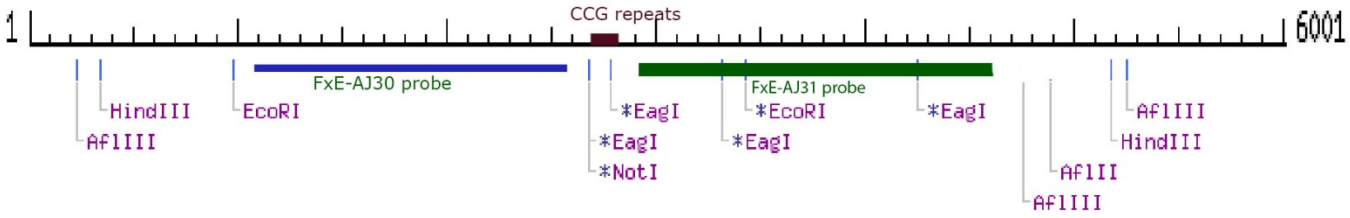
The size of the CCG repeats can be determined by PCR analysis and sizing preferably on sequencing gel. The PCR products can be either labeled with ^{35}S or ^{32}P followed by autoradiography. Another attractive alternate is to run a cold PCR reaction followed by blotting and hybridization with an alkaline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





Genomic Summary

- Approved symbol is AFF2
- Approved names are AF4/FMR2 family, member 2
- Previous symbol & names are FMR2, "fragile X mental retardation 2"
- HGNC:3776
- Synonym is FRAXE
- AFF2 gene is on the X chromosome at q28
- Comprises 22 exons spanning approximately 500 kb.
- The AFF2 CCG repetitive region is located in the 5'-UTR



FraxE-AJ28 6 kb
all approximate sizes refer to restriction table

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.

Southern blot analysis for FRAXE mutation detection involves the cleavage of DNA with enzyme Not I and Afl III. This method detects the size of CCG repeats region by hybridization of AFF2 AJ31-Dig1 GeneProber™ to DNA that has been double digested with restriction enzymes Not I and Afl III and blotted onto a membrane. In normal females two fragments are seen, a 2.2kb corresponding to the active X and a 4.8kb fragment corresponding to the methylated inactive X chromosome. Normal males exhibit only the 2.2kb banding pattern. Affected males will have an amplified CCG repeats region with methylation thus giving rise to fragments larger than the normal 4.8kb. Premutations in males and females will be seen as fragments from 2.3-3.3kb (normal 2.2kb) derived from the X chromosome. Premutations in females derived from the inactive X will give fragments from 4.9- ~6kb. Mosaicism is characterized by fragments appearing as a mixture of full mutation (methylated, larger than 4.8kb) and unmethylated premutation (2.2-3.3kb).

References

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2. Gecz,J., Gedeon,A.K., Sutherland,G.R. and Mulley,J.C. (1996) Identification of the gene FMR2, associated with FRAXE mental retardation. Nat. Genet., 13, 105–108.
3. Gu,Y., Shen,Y., Gibbs,R.A. and Nelson,D.L. (1996) Identification of FMR2, a novel gene associated with the FRAXE CCG repeat and CpG island. Nat. Genet., 13, 109–113.

4. Hillman,M.A. and Gecz,J. (2001) Fragile XE-associated familial mental retardation protein 2 (FMR2) acts as a potent transcription activator. J. Hum. Genet., 46, 251–259.
5. Didiot,M.C., Tian,Z., Schaeffer,C., Subramanian,M., Mandel,J.L. and Moine,H. (2008) The G-quartet containing FMRP binding site in FMR1 mRNA is a potent exonic splicing enhancer. Nucleic Acids Res., 36, 4902–4912.
6. Bensaid, M., Melko, M., Bechara, E.G., Davidovic, E., Berretta, A., Catania, M.V., Gecz, J., Lalli,E. and Bardoni, B. FRAXE-associated mental retardation protein (FMR2) is an RNA-binding protein with high affinity for G-quartet RNA forming structure (2009) Nucleic Acids Res., 37, 1269–1279.
7. Jorge, P., Marques, I.,Gonçalves, R.L., Gonçalves-Rocha, M. and Santos, R (2012) Study of FRAXE-MR in intellectuallydisabled individuals referred for Fragile-X Syndrome testing in Portugal. 2012-A-150-ESHG.
8. Javed, A., Ali, G. Caicedo, L., Marques, I., Santos,R . and Jorge, P. (2012) FRAXE molecular diagnosis in individuals referred for FRAXA screening. ASHG Abstract Control Number: 120121327.

Genemer™ Kit Agarose Gel Analysis

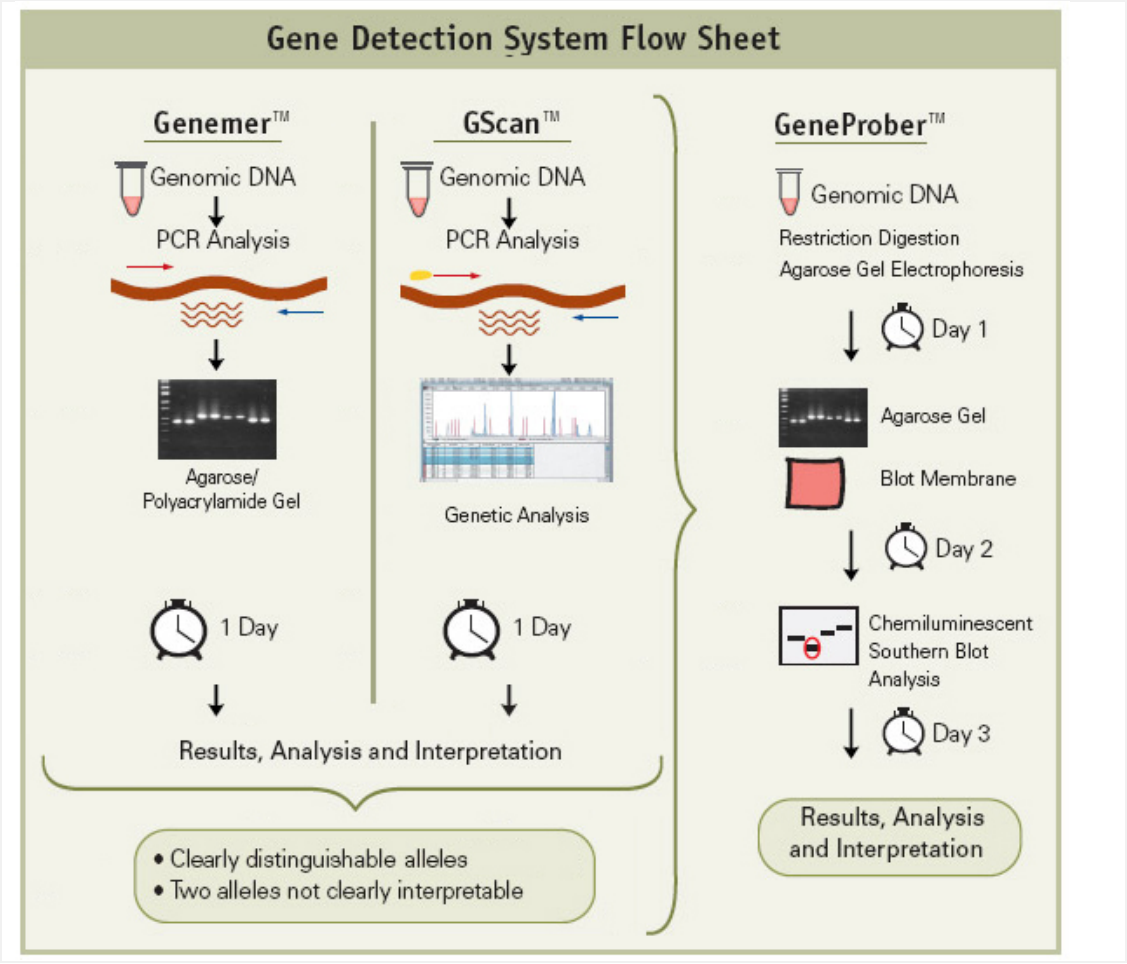
Genemer™ kit contains optimized components for PCR amplification of greater than 100 triple repeats using standard Taq polymerase. Amplified samples are resolved by agarose gel electrophoresis. This Genemer™ method or GScan™ fluorescent detection is recommended for initial screening of all samples.

GScan™ Kit

GScan™ kit contains optimized components for PCR amplification of greater than 100 triple repeats using standard Taq polymerase. Amplified samples are resolved by genetic analyzers capable of fluorescent detection or agarose gel electrophoresis. This Genemer™ Kit or GScan™ kit for fluorescent detection is recommended for initial screening of all samples.

GeneProber™ Probes for Southern Blot Analysis

Digoxigenin labelled probes for chemiluminescent Southern blot detection method or unlabeled probe for end user to perform radioactive label. Gene Link offers safe and reliable chemiluminescent detection methods as an alternate to radioactive based detection methods.



Procedure: AFF2/FRAXE/FMR2 Chemiluminescent Southern Protocol

Material Supplied

One tube containing 110 µl of *GeneProber™* probe at a concentration of ~40ng/µL. 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

AFF2-AJ31Dig1 and AFF2-AJ31 Probe Southern Blot Fragment Detection Double Digestion with Not I and Afl III				
Enzyme	**Specificity**	**CpG methylation Sensitive**	**Normal Male Fragment Size**	**Normal Female Fragment Size**
Not I and Afl III double digest				
Not I	GC▼GGCC▲GC	Yes (Blocked)	2.2kb	2.2 kb & 4.8 kb
Afl III	A▼CRYG▲T	No		

Important Note
-Double digest genomic DNA with Not I and Afl III restriction enzymes.

Restriction Digestion	
Component	Volume|Quantity
Genomic DNA	10µg
10x Not I Buffer	10 µL
Not I (10 u/µl)	4 µL
Afl III (5 u/µl)	4 µL
H_2O to	**100 µL**
Overnight digestion at 37^{O}C	

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

B. Electrophoresis and Transfer

1. Load samples to a 0.8% agarose gel. Electrophorese 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 500 ml H_2O) for 10 minutes.
3. Denature the DNA with 0.4N NaOH/0.6M NaCl for 30 min. at room temperature (RT).
4. Neutralize with 1.5M NaCl/0.5M Tris (pH 7.5) for 30 min. at RT.
5. Transfer overnight by Southern blot procedure to positively charged nylon membrane using 10xSSC.
6. Wash the membrane with 2x SSC and then bake at 80^{O}C for 2 hours.

C. Hybridization

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

1. Perform prehybridization at 55°C for 3 hours in 10 ml of Easy Hyb buffer (Roche Biochemicals).
2. Boil 20µL *GeneProber™* AFF2-AJ31DIG1 probe in 500µl of Easy Hyb for 10 minutes. Chill directly on ice.
3. Add the above probe to10ml of Easy Hyb.
4. Discard the prehybridization buffer and replace it with the hybridization buffer containing the boiled probe. Hybridize overnight at 55°C.
5. 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).
6. Warm the blocking reagent at this point. Prepare fresh 100 mL of Buffer MB by adding 10 mL of 10% blocking reagent [Gene Link Catalog no: 40-5026-10; Blocking solution for hybridization (10%)] and 10 mL of Maleic acid buffer 10X (Buffer M 10X) [Gene Link Catalog no: 40-5025-20; Maleic acid buffer 10X (Buffer M 10X)] to 80 mL of sterile water. 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 M for 1 minute.
2. Incubate the membrane in 80mL of Buffer MB (prepared in step 6 above) blocking solution at RT for 30 min.
3. Prepare 1:10000 Anti-DIG-AP conjugate at this point. e.g. add 2 µL to 20 mL Buffer MB (prepared in step 6 above).
4. Incubate the membrane in 20ml of Anti-DIG-AP conjugate solution at RT for 30 min.
5. Wash the membrane twice, 15 min/wash in 200ml of 1x washing buffer M at RT.
6. 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 Applied Science	http://www.roche-applied-science.com
Product Description	**Catalog Number**
Nylon Membranes, positively charged ; 20 sheets 10 x 15 cm	11209272001
DNA Molecular Weight Marker III, DIG-labeled ; 500 µl 10 µg/mL 5 µg	11218603910
DIG Easy Hyb ; 500 mL	11603558001
DIG Wash and Block Buffer Set ; 1 set 30 blots	11585762001
Anti-Digoxigenin-AP, Fab fragments from sheep; 200 µl 150 U	11093274910
CDP Star Ready to use; 2X 50 mL	12041677001

Gene Link	**http://www.genelink.com/geneprodsite/category.asp?c=44**	
Non-radioactive Southern Blot Reagents		
Product Description	**Catalog No.**	**Unit Size**
Agarose LE Molecular Biology Grade 100 gms	40-3010-10	100 gms
TAE Buffer 50 X Concentrate 1000 mL	40-3007-10	1 L
TBE Buffer 5 X Concentrate; 1L	40-3008-10	1 L
Loading buffer 10X BPB/XC non-denaturing; 1mL	40-3003-10	1 mL
Loading buffer 10X BPB/XC non-denaturing ; 15 mL	40-3003-15	15 mL
Lumisol II, Hybridization Solution; 200 mL	40-5023-20	200 mL
Depurination Solution (2X) for Southern Blotting; 1 L	40-5034-10	1 L
Denaturation Solution (2X) for Southern Blotting; 1L	40-5035-10	1 L
Neutralization Solution (2X) for Southern Blotting; 1L	40-5036-10	1 L
Hybwash A; Hybridization Wash Solution Concentrate (20X SSC); 250 mL	40-5020-25	250 mL
Hybwash B, Hybridization Wash Solution (10% SDS) ; 100 mL	40-5021-10	100 mL
Maleic acid buffer 10X (Buffer M 10X); 200 mL	40-5025-20	200 mL
10% Blocking Reagent; 100 mL	40-5026-10	200 mL
Detection Buffer 10X; Alkaline Phosphatase detection buffer; 100 mL	40-5031-10	100 mL
CDP-Star® Substrate; Ready-to-Use 0.25 mM in spray bottle; 10 mL	40-5010-10	10 mL

Reagent Preparation

Most reagents with composition listed below are available in a molecular biology laboratory or these can be prepared in house. Gene Link catalog numbers are also listed if you like to purchase these common reagents.

Depurination Solution (0.25M HCl)		
Product Description	**Catalog No.**	**Volume**
Depurination Solution (2X) for Southern Blotting	40-5034-10	150 mL
Sterile water		150 mL
Total Volume		**300 mL**

Denaturation Solution (0.5M NaOH, 1.5M NaCl)		
Product Description	**Catalog No.**	**Volume**
Denaturation Solution (2X) for Southern Blotting	40-5035-10	150 mL
Sterile water		150 mL
Total Volume		**300 mL**

Neutralization Solution (0.5M Tris-HCl pH 7.5, 1.5M NaCl)		
Product Description	**Catalog No.**	**Volume**
Neutralization Solution (2X) for Southern Blotting	40-5036-10	150 mL
Sterile water		150 mL
Total Volume		**300 mL**

Hybwash I (2xSSC, 0.1% SDS)		
Product Description	**Catalog No.**	**Volume**
Hybwash A; Hybridization Wash Solution Concentrate (20X SSC)	40-5020-25	35 mL
Sterile water		311 mL*
Hybwash B, Hybridization Wash Solution (10% SDS)	40-5021-10	4 mL*
Total Volume		**350 mL**
*** Volumes adjusted to whole numbers**		

Hybwash II (0.5xSSC, 0.1%SDS)		
Product Description	**Catalog No.**	**Volume**
Hybwash A; Hybridization Wash Solution Concentrate (20X SSC)	40-5020-25	9 mL*
Sterile water		337 mL
Hybwash B, Hybridization Wash Solution (10% SDS)	40-5021-10	4 mL*
Total Volume		**351 mL**
*** Volumes adjusted to whole numbers**		

1X Buffer M (Maleic Acid Buffer, 1X) (100 mM Maleic acid, 150 mM NaCl pH7.5)		
Product Description	**Catalog No.**	**Volume**
Buffer M 10X (Maleic acid buffer 10X)	40-5025-20	10 mL
Sterile water		90 mL
Total Volume		**100 mL**

Buffer MB (1 x Buffer M (Maleic acid buffer, 1X with Blocking Reagent) Always prepare fresh!		
Product Description	**Catalog No.**	**Volume**
Buffer M 10X (Maleic acid buffer 10X)	40-5025-20	10 mL
Sterile water		80 mL
10% Blocking Reagent*	40-5026-10	10 mL
Total Volume		**100 mL**

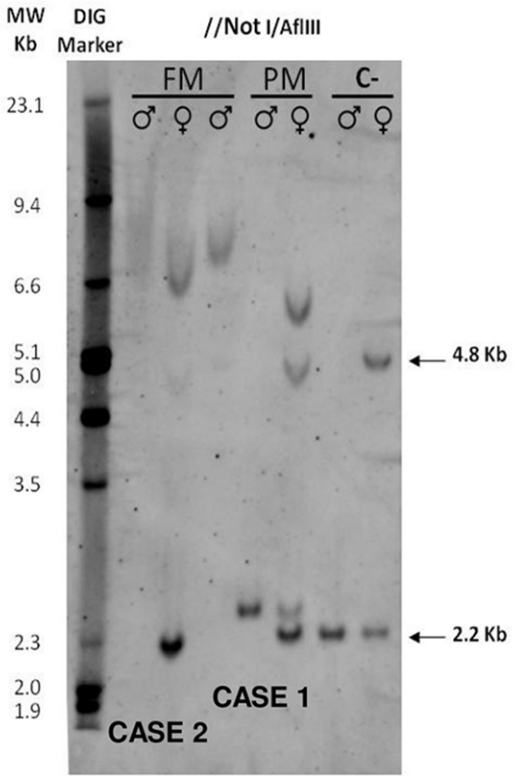
The prepared reagent will be turbid yellow in color

* The 10% Blocking Reagent is turbid yellow in color and will form precipitates on storage. Warm to 50°C and shake well before aliquoting. DO NOT SHAKE VIGOROUSLY

1X Detection Buffer, Alkaline phosphatase detection buffer (100mM Tris-HCl pH 9.5, 100mM NaCl)		
Product Description	**Catalog No.**	**Volume**
Detection Buffer 10X; Alkaline phosphatase detection buffer	40-5031-10	10 mL
Sterile water		90 mL
Total Volume		**100 mL**

Results and Analysis

Southern blot analysis for FRAXE genotyping involves the cleavage of DNA with enzyme Not I and Afl III. This method detects the size of CCG repeats region by hybridization of probe AFF2-AJ31 or AFF2-AJ31Dig1 GeneProber™ to DNA that has been double digested with restriction enzymes Not I and Afl III and blotted onto a membrane. In normal females two fragments are seen, a ~2.2kb corresponding to the active X and a ~4.8kb fragment corresponding to the methylated inactive X chromosome. Normal males exhibit only the ~2.2kb banding pattern. Affected males will have an amplified CCG repeats region with methylation thus giving rise to fragments larger than the normal ~2.2kb.



Southern Blot Restriction Pattern & Fragments

- Lane 1 – DNA Molecular Weight Marker
- Lanes 2, 3, and 4 are from the CASE 2 with a full mutation allele (FM) with ~500 CCG, mother , and brother.
- Lanes 5 and 6 represent results from the CASE 1 showing a “pure” premutation with ~68CGGs and his mother.
- Lanes 7 and 8 are the normal controls (C-).

Frequently Asked Questions/Troubleshooting

1. **General Comment** FRAXE genotyping is not easy. A lab 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** Background problems may be due to various reasons and has to be optimized in each lab. Here at Gene Link we use Roche positively charged nylon membrane and other products, a list of recommended products with catalog numbers is given in the methods. 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 dishes inherently have 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.

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 Gene Link 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.

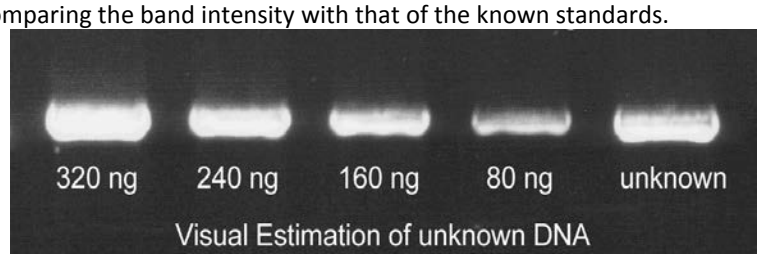
Recipe

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.

Fragile X Genotyping Product Ordering Information

Product	Unit 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 nmols	40-2004-10
Fragile X Genemer™ Kit Primers for amplification of CGG triple repeat spanning region. The quantity supplied is sufficient for 400 regular 25 µL PCR reactions.	100 rxns	40-2004-11
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 GScan Kits 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
Fragile X GeneProber™ GLFX1 Probe unlabeled Probe for radioactive labelling and Southern blot analysis	500 ng	40-2004-40
Fragile X GeneProber™ GLFX1 Probe Digoxigenin labeled Probe for non-radioactive chemiluminescent Southern blot analysis	110 µL	40-2004-41

FRAXE/FMR2/AFF2 Genotyping Product Ordering Information

Product	Unit Size	Catalog No.
FRAXE/FMR2/AFF2 GeneProber™ AFF2-AJ31Dig1 Probe Digoxigenin labeled Probe for non-radioactive chemiluminescent Southern blot analysis	110 µL	40-2054-41
FRAXE/FMR2/AFF2 GScan™ Kit for fluorescent detection Kit for performing fluorescent PCR amplification based detection, Fam labeled	1 Kit [100 rxns]	40-2054-15FM
FRAXE/FMR2/AFF2 Genemer™ Kit for agarose gel detection Kit for performing standard PCR amplification and gel based detection	1 Kit [100 rxns]	40-2054-11

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

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Southern Blot Buffers& Reagents

Product	Catalog No.	Unit Size
Agarose Tablets, 0.5 gm each; 100 tablets	40-3011-10	100 tablets
Agarose LE Molecular Biology Grade; 100 g	40-3010-10	100 g
Agarose LE Molecular Biology Grade; 500 g	40-3010-50	500 g
Hybwash A, Hybridization Wash Solution (20X SSC); 200 mL	40-5020-20	200 mL
Hybwash B, Hybridization Wash Solution (10% SDS); 100 mL	40-5021-10	100 mL
TAE Buffer; 50 X Concentrate; 100 mL	40-3007-01	100 mL
TAE Buffer; 50 X Concentrate; 1 L	40-3007-10	1 L
TBE Buffer; 5 X Concentrate; 1 L	40-3008-10	1 L
Buffer M 10X (Maleic Acid buffer); 100 mL	40-5025-10	100 mL
10% Blocking solution; 100 mL	40-5026-10	100 mL
Loading Buffer 2X BPB/XC Denaturing for Sequencing; 1 mL	40-5027-10	1 mL
10x AP Detection buffer (alkaline phosphatase detection buffer); 100 mL	40-5031-10	100 mL
Lumisol™ I Hybridization Solution; contains formamide; 200 mL	40-5022-20	200 mL
Lumisol™ II Hybridization Solution; for non-toxic hybridizations; 200 mL	40-5023-20	200 mL
Lumisol™ III Hybridization Solution; for oligo probes; 200 mL	40-5024-20	200 mL
CDP-Star® Substrate; Ready-to-Use 0.25 mM in spray bottle; 10 mL	40-5010-10	10 mL

Loading Buffers

Product	Catalog No.	Size
Gel Loading Buffer 5X BPB/XC non-denaturing; 1 mL	40-3002-10	1 mL
Gel Loading Buffer 5X BPB/XC non-denaturing; 15 mL	40-3002-15	15 mL
Gel Loading Buffer 10X BPB/XC non-denaturing; 1 mL	40-3003-10	1 mL
Gel Loading Buffer 10X BPB/XC non-denaturing; 15 mL	40-3003-15	15 mL
Gel Loading Buffer 5X Orange G/XC non-denaturing; 1 mL	40-3004-10	1 mL
Gel Loading Buffer 5X Orange G/XC non-denaturing; 15 mL	40-3004-15	15 mL
Gel Loading Buffer 2X BPB/XC Denaturing for Sequencing; 1 mL	40-5027-10	1 mL
Gel Loading Buffer 2X BPB/XC Denaturing for Sequencing; 15 mL	40-5027-15	15 mL
DNA SDS Gel Loading Buffer 5X BPB/XC DNA binding protein denaturing buffer ; 1 mL	40-5028-10	1 mL
DNA SDS Gel Loading Buffer 5X BPB/XC DNA binding protein denaturing buffer; 15 mL	40-5028-15	15 mL
RNA Gel Loading Buffer 2X BPB/XC with ethidium bromide; 1 mL	40-5029-10	1 mL
RNA Gel Loading Buffer 2X BPB/XC with ethidium bromide; 15 mL	40-5029-15	15 mL
RNA Gel Loading Buffer 2X BPB/XC without ethidium bromide ; 1 mL	40-5030-10	1 mL
RNA Gel Loading Buffer 2X BPB/XC without ethidium bromide; 15 mL	40-5030-15	15 mL

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Related Products Ordering Information

Omni-Pure™ DNA & RNA Purification Systems

Product	Catalog No.	Unit Size*(Purifications)
Omni-Pure™ Blood DNA Purification System	40-4010-01	100
Omni-Pure™ Blood DNA Purification System	40-4010-05	500
Omni-Pure™ Blood DNA Purification System	40-4010-10	1000
Omni-Pure™ Tissue DNA Purification System	40-4050-01	100
Omni-Pure™ Tissue DNA Purification System	40-4050-05	500
Omni-Pure™ Tissue DNA Purification System	40-4050-10	1000
Omni-Pure™ Plant DNA Purification System	40-4060-01	100
Omni-Pure™ Plant DNA Purification System	40-4060-05	500
Omni-Pure™ Plant DNA Purification System	40-4060-10	1000
Omni-Pure™ Viral DNA Purification System	40-3720-01	100
Omni-Pure™ Viral DNA Purification System	40-3720-05	500
Omni-Pure™ Microbial DNA Purification System	40-3700-01	100
Omni-Pure™ Microbial DNA Purification System	40-3700-05	500
Omni-Pure™ Viral RNA Purification System	40-3650-01	100
Omni-Pure™ Viral RNA Purification System	40-3650-05	500

*Sample volume for each purification system varies. Each purification yields sufficient quantity for desired applications.

Omni-Clean™ Gel DNA Purification and Concentration Systems

Product	Catalog No.	Unit Size*(Purifications)
Omni-Clean™ Gel DNA Beads Purification System	40-4110-10	100
Omni-Clean™ Gel DNA Beads Purification System	40-4110-50	500
Omni-Clean™ Gel DNA Spin Column Purification System	40-4120-10	100
Omni-Clean™ Gel DNA Spin Column Purification System	40-4120-50	500
Omni-Clean™ DNA Beads Concentration System	40-4130-10	100
Omni-Clean™ DNA Beads Concentration System	40-4130-50	500
Omni-Clean™ DNA Spin Column Concentration System	40-4140-10	100
Omni-Clean™ DNA Spin Column Concentration System	40-4140-50	500

*Sample volume for each purification system varies. Each purification yields sufficient quantity for desired applications.

Omni-Pure™ Plasmid DNA Purification Systems

Product	Catalog No.	Unit Size*(Purifications)
Omni-Pure™ Plasmid DNA Purification System	40-4020-01	100
Omni-Pure™ Plasmid DNA Purification System	40-4020-05	500

*Sample volume for each purification system varies. Each purification yields sufficient quantity for desired applications.

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