Product Manual

Friedreich's Ataxia GLFRDA PCRProber™ Kit

Non-radioactive Friedreich's Ataxia Genotyping by PCR* Analysis

GAA Repeat Detection

Kit for Amplification and non-radioactive detection of Friedreich's Ataxia GAA trinucleotide repeats region amplified PCR product

Catalog No. 40-2027-32 5 blots [Sufficient for 50 x 50 μl reaction]

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

***Important information**

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 COMPONENTS LABELED TO BE STORED AT 4°C.

140 Old Saw Mill River Road | Hawthorne, NY 10532-1515 | tel: 914-769-1192 | fax: 914-769-1193 | www.genelink.com

Gene Link™

Material supplied

Friedreich's Ataxia GLFRDA PCRProber™ Kit

Catalog No. 40-2027-32 5 blots [Sufficient for 50 x 50 µl reaction]

Storage instructions: Store as labeled

Caution: DO NOT FREEZE COMPONENTS LABELED TO BE STORED AT 4°C.

GLFRDA PCRProber™ Kit components
Sufficient for 50 x 50 µl reaction

Product	Size	Description	Storage	Catalog No.
GLFRDA PCRProber™	12 µl	Alkaline Phosphatase labeled Probe	4 °C	40-2027-31
GLFRDA PCR Component A	1.1 ml	GLFRDA PCR premix with primers	-20 °C.	40-2027-32A
PCR Component M	600 µl	PCR additive	-20 °C.	40-3053-60
PCR Component N	300 µl	2.5 mM dNTP	-20 °C.	40-3021-30
Sequencing loading buffer	1 ml	Seq loading buffer	-20 °C.	40-5027-00

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

Friedreich's Ataxia Genotyping

Introduction

Friedreich's ataxia (FRDA [MIM 229300], NM_181425) is an autosomal recessive neurodegenerative disorder characterized by a progressive loss of voluntary muscle coordination (ataxia). The disorder affects upper and lower limbs, and the head and neck. FRDA is characterized clinically by progressive gait and limb ataxia; signs of upper motoneuron dysfunction including dysarthria, areflexia, and loss of the senses of position and vibration; cardiomyopathy; diabetes mellitus; and secondary skeletal abnormalities. Most patients develop hypertrophic cardiomyopathy and skeletal abnormalities, and some become diabetic (1,2). These symptoms progress with age, such that most patients become wheelchair-bound by their late twenties and die by their mid-thirties—most commonly of congestive heart failure. Some of the other symptoms include muscle weakness, loss of pressure and position sense in the arms and legs, speech problem and heart disease. Unlike some neurological diseases, FRDA does not affect mental capacity. See recent reviews (3,4).

Although rare, FRDA is the most prevalent inherited ataxia, affecting about 1-2 in every 50,000 individuals. It is usually diagnosed in childhood between the ages of 5 and 15. The majority (~98%) of patients with FRDA are homozygous for a GAA repeat expansion within the first intron of frataxin gene. The remaining patients are compound heterozygotes for the GAA expansion and for point mutations within the X25 gene. In normal alleles, the repeat varies in size between 7 and 30 units, whereas in mutated alleles the repeat length ranges from 100 to more than 1000 units. Generally, the milder forms or late onset of the disease are associated with shorter expansions.

FRDA is caused by degeneration of nerve tissue in the spinal cord and of nerves that extend to peripheral areas such as the arms and legs. The disorder is associated with an unstable expansion of GAA repeats in the first intron of the FRDA gene, called X25, on chromosome 9q13. The encoded protein, frataxin, is located in mitochondria and reduced in FRDA patients. It is suggested that FRDA is the result of mitochondrial iron overload leading to excess production of free radicals, which results in cellular damage and death.

The majority (>95%) of patients with FRDA are homozygous for large expansions of a GAA triplet–repeat sequence (66–1800 triplets) located within the first intron of the gene *X25*, which encodes the protein frataxin (2). The expansion causes a severe reduction in the levels of frataxin, a 210–amino acid protein that is targeted to mitochondrial matrix and that appears to play a crucial role in iron homeostasis. The severity of the disease is directly correlated with the length of the expansion. A very small minority of patients are compound heterozygotes for the GAA expansion and for point mutations within the *X25* gene. Chamberlain and coworkers have recently summarized all point mutations described to date (5).

Frataxin RNA levels were severely reduced lymphoblast cell lines of patients with FRDA who were homozygous for the GAA expansion. Several groups have demonstrated that the GAA-repeat expansion interferes with transcription. It has been show by various groups that the GAA Triplet–Repeat Expansion acts as an Impediment to Transcription (3).

Genetically, FRDA belongs to a class of neurodegenerative disorders in which the underlying gene, *FRDA*, carries an unstable trinucleotide-repeat sequence. At least eight other members of this class have been identified, including HD and many types of spinocerebellar ataxia. However, key genetic features separate FA from the other trinucleotide-repeat disorders. First, the sequence of the trinucleotide repeat in the *FRDA1* gene is GAA (2), whereas a CAG repeat occurs in the other

trinucleotide-associated ataxias, and other repeats (CTG or CGG) are seen in other trinucleotide diseases. Second, the GAA repeat of *FRDA* is located in the first intron and is therefore noncoding, whereas the CAG repeat in HD and the spinocerebellar ataxias always occurs within an exon and encodes glutamine.

The third difference is that FRDA is inherited in a recessive manner, and multiple lines of evidence suggest that loss of function leads to the disease. In contrast, in the other trinucleotide-repeat disorders, whether the repeat occurs in an expressed DNA sequence or in a 3' untranslated sequence, the mutation is inherited in a dominant manner, and it is a gain of function of the affected protein or RNA that perturbs cell physiology.

Table 1: Trinucleotide Repeats in Human Genetic Disease

Disease	Repeat[a]	Normal Length[b]	Intermediate Length (Premutation)[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	…	40-81 (Pure)
Spinocerebellar ataxia 2 (SCA2)	$(CAG)_n$	14-31	…	34-59 (Pure)
Spinocerebellar ataxia 3 (SCA3)/Machado Joseph disease (MJD)	$(CAG)_n$	13-44	?	60-84
Spinocerebellar ataxia 6 (SCA6)	$(CAG)_n$	4-18	?	21-28
Spinocerebellar ataxia 7 (SCA7)	$(CAG)_n$	7-17	?	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.-

The severity of the disease correlates with decreased *FRDA* expression and with the length of the hyperexpansive repeat. Normally, this gene, which encodes the protein frataxin, contains <39 GAA repeats, but in patients with FRDA, this locus contains 66–1,700 repeat units. This hyperexpansion results in marked decreases in frataxin mRNA levels, thought to result from the formation of an unusual non-β DNA structure inhibiting transcription (3). More than 95% of patients with FRDA are homozygous for the GAA hyperexpansion, although the alleles are polymorphic in the number of GAA

repeats. Studies have shown a correlation between the length of the GAA expansion on the smaller allele and severity of disease (1). An inverse correlation between GAA expansion size and frataxin protein levels has been observed in lymphoblast cell lines from patients with FRDA (3). Together, these findings suggest that lack of frataxin protein in critical tissues leads to FRDA. The remaining 5% of patients with FRDA are compound heterozygotes for the GAA expansion on one allele and carry point mutations within *FRDA1* on the other allele.

The most common disease-causing point mutation in frataxin is I154F (numbering based on the initiator methionine of the predicted open reading frame [ORF]), prevalent in some southern Italian families. Those individuals carrying this missense mutation on one allele, together with the hyperexpansion on the other allele, are indistinguishable in disease severity when compared with homozygous relatives who carry the GAA triplet expansion on both alleles (4). Another missense mutation in frataxin, G130V, compounded with a hyperexpansive allele, is associated with a milder and more slowly progressive disease course (3).

Meiotic instability and Somatic Variation in GAA Expansion Length

The GAA expansion shows intergenerational variation in length, with evidence for changes in the prezygotic and postzygotic stages. Studies have shown that the expanded alleles seen in patients arose from a small pool of uninterrupted "large normal" alleles referred to as "premutations." Interruptions within the pure GAA triplet repeats impeded these large normal alleles from expanding into disease-causing alleles. De Michele et al. (10) have noted that premutation alleles can undergo large expansions in a single generation. Expanded GAA repeats can expand or contract when transmitted through the female germline. In contrast, contractions are favored in male transmission. This is attributed to postzygotic mechanisms, because shorter expansions are seen in sperm DNA when compared with lymphocyte DNA (prezygotic mechanism). However, evidence for postzygotic variation in repeat number has also been suggested, because the degree of repeat contraction in the sperm is greater than that actually seen in intergenerational transmission and because the overall length of expanded alleles is shorter in homozygous versus heterozygous carriers. The formation of unexpected parallel duplex has been shown in GAA and TTC trinucleotide repeats of Friedreich's ataxia (11). This presumably interferes with normal transcription activity.

Number of GAA repeats	Clinical Condition	Symptoms
5-30 repeats	Unaffected	Normal
?34-40 repeats	Mild	Premutation
200-900 repeats	Severe	Full mutation

Genotyping

Molecular diagnosis of Friedreich's Ataxia is available. It involves a combination of direct PCR analysis and Southern blotting tests to determine the GAA-repeat number within the FRDA gene. PCR can identify GAA expansions between 5-200 GAA repeats.

With larger expansions, Southern blot analysis of restriction fragments can be used for an accurate measure of the repeat size. Genomic DNA is digested with BsiHKAI. The DNA blot is then hybridized with FRDA-GL DNA probe.

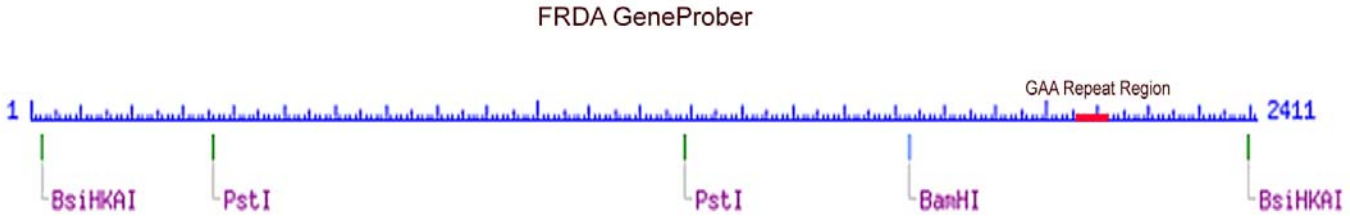
Molecular Analysis

The direct analysis of GAA repeats in the *FRDA* gene (chromosomal locus 9q13) is available. An increased number of GAA repeats is identified in essentially 100% of patients with FRDA. The number of GAA repeats ranges from 5 to <30 in normal alleles. GAA repeat lengths in the range from about >30-49 are considered "premutations." Persons with GAA expansions in the premutation range have not been reported as having developed severe symptoms, but their children are at risk of inheriting a larger repeat size. Persons with GAA repeat length greater than 50 are frequently symptomatic.

Friedreich's Ataxia genotyping can be done by direct PCR amplification of the GAA 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 and southern analysis is the preferred method to distinguish full mutations.

The size of the GAA 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

Southern blot analysis for Friedreich's Ataxia mutation detection involves the cleavage of genomic DNA with BsiHKAI enzyme. This method detects the size of GAA repeats region by hybridization of probe FRDA-GL to DNA that has been digested with the appropriate restriction enzyme and blotted onto a membrane. The GAA repeat in the normal range yields a ~2374 bp.



PCR Analysis

The following table lists the size of PCR fragment in base pairs (bp) that can be expected when using the GAA primer mix (F1B/R3C) that has been provided. The formula for determining PCR fragment size is 322 + 3n, where n= the number of GAA repeats.

GAA Repeat PCR Amplified Fragment Size Using Primer Set F1B/R3C*			
$GAA_{(n)}$	PCR Fragment (bp)	$GAA_{(n)}$	PCR Fragment (bp)
5	337	45	445
6	340	50	460
7	343	55	475
8	346	60	490
9	349	65	505
10	352	70	520
11	355	75	535
12	358	80	550
13	361	85	565
14	364	90	580
15	367	95	595
16	370	100	610
17	373	105	625
18	376	110	640
19	379	115	655
20	382	120	670
21	385	125	685
22	388	130	700
23	391	135	715
24	394	140	730
25	397	145	745
26	400	150	760
27	403	155	775
28	406	160	790
29	409	165	805
30	412	170	820
31	415	175	835
32	418	180	850
33	421	185	865
34	424	190	880
35	427	195	895
40	430	200	910
*The size of PCR fragment that can be expected when using the FRDA GAA primer mix (F1B/R3C) that has been provided in this kit. The formula for determining PCR fragment size is 322 + 3n, where n= the number of GAA repeats			

Number of GAA repeats	Clinical Condition	Symptoms
5-30 repeats	Unaffected	Normal
?34-40 repeats	Mild	Premutation
200-900 repeats	Severe	Full mutation

References

1. Durr, A, Cossee M, Agid Y, Campuzano V, Mignard C, Penet C, Mandel JL, et al (1996) Clinical and genetic abnormalities in patients with Friedreich's ataxia. New Engl J Med 335:1169–1175.
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5. Monrὸs, E. et al. (1997) Am. J. Hum. Genet. 61: 101-110.
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9. Ohshima K, Montermini L, Wells RD, Pandolfo M (1998) Inhibitory effects of expanded GAA.TTC triplet repeats from intron I of the Friedreich ataxia gene on transcription and replication in vivo. J Biol Chem 273:14588–14595
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12. De Michele G, Cavalcanti F, Criscuolo C, Pianese L, Monticelli A, Filla A, Cocozza S (1998) Parental gender, age at birth and expansion length influence GAA repeat intergenerational instability in the X25 gene: pedigree studies and analysis of sperm from patients with Friedreich's ataxia. Hum Mol Genet 7:1901–1906
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Procedure

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 1% Agarose gel; leave these covered overnight. Day 2 proceed with the rest of the procedure.

PCR Premix Preparation

Thaw individual components. *Promptly store at -20^{o}C after use*. Prepare ***fresh*** before use enough PCR premix for the number of reactions to be performed. Prepare 10% more for pipeting allowance. Prepare premix following the volumes given below. Follow the same ratio for preparing other final volumes.

Material Supplied: Please see page 2 of this manual.

PCR Thermal Cycler Files

Prepare the following PCR thermal cycler files

Hot Start File	
Step	**Time and Temperature**
Denaturation	5 minutes at 94°C
Hold	60°C

FRDA Amplification File		
Step	**Time and Temperature**	**Cycles**
Denaturation	30 seconds at 94°C	30 Cycles
Annealing	30 seconds at 58°C	
Extension	3 minute at 72°C	
Fillup	7 minutes at 72°C	1 Cycle
Hold	Hold for infinity at 4°C	Hold for infinity

Protocol:

PCR Amplification

A. PCR premix preparation

Given below is a protocol for preparing a PCR premix for 50 µl reactions. This can be scaled up as required. Experienced users can perform 25 µl reactions.

PCR Premix Preparation		
Component	**1 x 50 µl rxn.**	**10 x 50 µl rxns.**
FRDA PCRProber™ Component A	20 µl	200 µl
PCR Component M	8.0 µl	80 µl
PCR Component N	4.0 µl	40 µl
Sterile Water	18 µl	180 µl
Total	**50 µl**	**500 µl**

B. Enzyme premix preparation

Enzyme Mix Preparation		
Component	**1 x 50 µl rxn.**	**10 x 50 µl rxns.**
PCR premix (above)	5.0 µl	50 µl
Taq. Polymerase	0.5 µl	5 µl
Total	**5.5 µl**	**55 µl**

C. PCR reaction

'Hot Start' PCR

For each sample add the following

Hot Start PCR	
Component	**Quantity**
PCR premix (above)	44 µl
DNA Template (~100ng chromosomal DNA)	1 µl
Total	**45 µl**

Start "Hot Start" file.
After initial denaturation while thermal cycler is 'holding' at 60°C
Add 5 µl of Enzyme premix to each tube and start FRDA amplification PCR file.

D. Post PCR Processing

Step 1

For initial optimization and genotyping interpretation it is recommended to run a 10 µl sample aliquot on a 1% agarose gel to view the amplification fragments. Based on the results of agarose gel electrophoresis an initial genotyping interpretation can be made. For large amplifications over 60 GAA repeats (larger than 500 bp fragment) and appearance of smear above 500 bp fragments signifies large repeats that will not be resolved on a polyacrylamide gel electrophoresis. Southern blot analysis is recommended using Gene Link FRDA GeneProber (Catalog #: 40-2027-40 FRDA unlabeled probe and 40-2027-41 digoxigenin labeled probe).

Step 2

Fragment Resolution of ~400 bp to 500 bp (20 to 60 GAA repeats)

Based on the fragment size obtained from agarose gel electrophoresis proceed to resolve fragment size between 400 bp to 500 bp by polyacrylamide gel. Follow the protocol given below.

Ethanol precipitate the remaining 40µl. Dissolve pellet in 5 µl sterile water and then add 5 µl of seq. loading buffer.

Electrophoresis & Electroblotting

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).

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.).

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 electro-transfer apparatus manufacturers' directions. Electro-transfer at 400 mA for 1 hr. in 1xTBE.

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 GL-FRDA 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 5 minutes. 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.

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

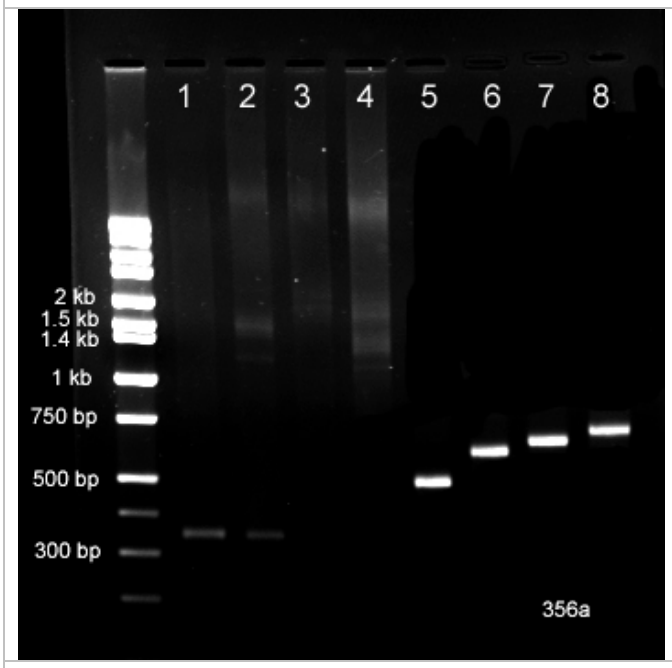
The results obtained from the electrophoretic separation will approximately show the fragment size amplified, based on these results an interpretation can be made about the genotype of the sample. It is known that there is an overlap between the normal and FRDA allele sizes. The repeat sizes obtained falling in the overlap region should be preferably repeated and possibly run with more samples from other family members. Refer to the table 2 for determining the GAA repeats and fragment size expected using the primers provided in this kit. The formula for determining PCR fragment size with the amplification primer F1B/R3C is 322 + 3n, where n= the number of GAA repeats.

Number of GAA repeats	Clinical Condition	Symptoms
5-30 repeats	Unaffected	Normal
?34-40 repeats	Mild	Premutation
200-900 repeats	Severe	Full mutation

Important Note

All samples that do not yield two fragments smaller than 400 bp and all samples that have smears of large fragments above 500 bp indicates large GAA amplification. Based on the results of agarose gel electrophoresis an initial genotyping interpretation can be made. Large amplifications over 60 GAA repeats (larger than 500 bp fragment) and appearance of smear above 500 bp fragments signifies large repeats that will not be resolved on a polyacrylamide gel electrophoresis. Southern blot analysis is recommended using Gene Link FRDA GeneProber (Catalog #: 40-2027-40 FRDA unlabeled probe and 40-2027-41 digoxigenin labeled probe).

FRDA GAA Repeat Genotyping with Primer Set F1B/R3C*



Lanes 1-4 human genomic DNA samples. Lane 1 normal FRDA DNA GAA repeat fragment sizes of ~335 bp (6 & 8 GAA repeats) ; lane 2 heterozygote FRDA DNA Coriell ID NA16213 of ~335 and 1579 bp fragments (6 GAA and 420 GAA repeats); lane 3 homozygote FRDA DNA Coriell ID NA 16203 of ~2239 and 2809 bp fragments (670 and 830 GAA repeats) and lane 4 homozygote FRDA DNA Coriell ID NA04079 of ~1339 and 1579 bp fragments (340 and 420 GAA repeats).

Lanes 5-8 cloned Gene Link FRDA Genemer™ control DNA.

Lane 5 (Catalog #: 40-2027-01) 64 GAA repeats ~502 bp fragment. Lane 6 (Catalog #: 40-2027-02) 102 GAA repeats ~616 bp fragment. Lane 7 (Catalog #: 40-2027-03) 110 GAA repeats ~640 bp fragment and lane 8 (Catalog #: 40-2027-04) 125 GAA repeats ~685 bp fragment.

***1% agarose gel electrophoresis of FRDA GAA repeats genotyping with primer set F1B/R3C. Long GAA repeats are not amplified with high fidelity as discrete fragments and appears as a broad smear.**

Frequently Asked Questions/Troubleshooting

1. General Comment. Initially the addition of more steps in the detection protocol as compared to radioactive detection may seem annoying, but is worth the patience. All the steps have to be optimized. Following the protocol exactly works, especially running denaturing gel is important. 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 that 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 GAA repeats up to 200 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 GAA repeat analysis on this system clearly indicates either a failure of the PCR reaction or a DNA with extensive GAA repeats. A particular DNA sample not yielding a PCR product on duplicate analysis clearly indicates the possibility of long GAA repeats. In cases like these we suggest that southern analysis should be done using the FRDA-GLDig21 GeneProber™ 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-XX
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 (Cell Suspension 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 (Cell Suspension Solution) using a filter tip pipette 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.

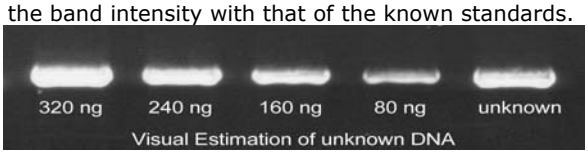
Recipe

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.



Visual Estimation of unknown DNA

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

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

Recipe

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.

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		

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

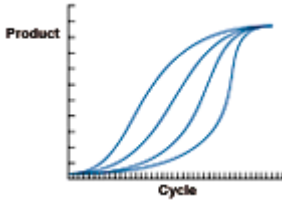
- The PCR premix preparation protocol is written considering that more than one amplification reaction will be performed at the same time. If only one reaction is planned then there is no need to prepare the Taq Enzyme Mix (EM).

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.

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

GScan™ Kits Product Ordering Information

Kits 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.

Product	Size	Catalog No.	Price, $
Fragile X GScan™ Kit for fluorescent detection; 100 reactions kit	1 kit	40-2004-15XX	650.00
Fragile X GScan™ Kit for fluorescent detection; 20 reactions kit	1 kit	40-2004-15FMS	250.00
Huntington's Disease GScan™ Kit for fluorescent detection; 100 reactions kit	1 kit	40-2025-15XX	650.00
Huntington's Disease GScan™ Kit for fluorescent detection; 20 reactions kit	1 kit	40-2025-15FMS	250.00
Myotonic Dystrophy GScan™ Kit for fluorescent detection; 100 reactions kit	1 kit	40-2026-15XX	650.00
Myotonic Dystrophy GScan™ Kit for fluorescent detection; 20 reactions kit	1 kit	40-2026-15FMS	250.00
Friedreich's Ataxia GScan™Kit for fluorescent detection; 100 reactions kit	1 kit	40-2027-15XX	650.00
Friedreich's Ataxia GScan™Kit for fluorescent detection; 20 reactions kit	1 kit	40-2027-15FMS	250.00
Kennedy Disease GScan™ Kit for fluorescent detection; 100 reactions kit	1 kit	40-2032-15XX	650.00
Kennedy Disease GScan™ Kit for fluorescent detection; 20 reactions kit	1 kit	40-2032-15FMS	250.00

Freidreich Ataxia Product Ordering Information

Product	Size	Catalog No.	Price, $
Friedreich Ataxia Genemer™ Primer pair for amplification of GAA triple repeat spanning region. The quantity supplied is sufficient for 400 regular 50 µl PCR reactions.	10 nmols	40-2027-10	$100.00
Friedreich Ataxia GeneProber™ GLFRDA Probe unlabeled Friedreich Ataxia GAA triple repeat spanning region unlabeled probe for radioactive labeling and Southern blot detection. Suitable for random primer labeling.	500 ng	40-2027-40	$350.00
Friedreich Ataxia GeneProber™ GLFRDA Probe Digoxigenin labeled Friedreich Ataxia GAA triple repeat spanning region digoxigenin labeled probe for Southern blot non-radioactive detection.	110 µl	40-2027-41	$425.00
Friedreich Ataxia PCRProber ™ AP labeled probe Alkaline phosphatase labeled probe	12 µl	40-2027-31	$400.00
Friedreich Ataxia PCRProber ™ Kit. Kit for performing non-radioactive PCR amplification based detection. 5 blots (50 rxns)	5 blots	40-2027-32	$650.00

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.

GLFRDA ~64 GAA repeat Genemer Control DNA	500 ng	40-2027-01	175.00
GLFRDA ~102 GAA repeat Genemer Control DNA	500 ng	40-2027-02	175.00
GLFRDA ~110 GAA repeat Genemer Control DNA	500 ng	40-2027-03	175.00
GLFRDA ~125 GAA repeat Genemer Control DNA	500 ng	40-2027-04	175.00
GLFRDA ~9 GAA repeat Genemer Control DNA	500 ng	40-2027-05	175.00

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

Genemer™ Control DNA (Selected List) Control DNA for use with gene or mutation specific Genemer™

Product	Size	Catalog No.	Price, $
Fragile X, various CGG triple repeat region control DNA	500 ng	40-2004-XX	175.00
Huntington Disease various CAG triple repeat region control DNA	500 ng	40-2025-XX	175.00
Myotonic Dystrophy various CTG triple repeat region control DNA	500 ng	40-2026-XX	175.00
Friedreich's Ataxia, various GAA triple repeat region control DNA	500 ng	40-2027-XX	175.00

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

**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.

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