Non-radioactive Fragile X CGG repeat detection by PCR*

GLFX PCRProber™ Kit

Catalog No.: 40-3102-00 5 blots (50 reactions)

Kit for amplification and non-radioactive detection of Fragile X CGG trinucleotide repeats region amplified PCR product.

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

Instruction Manual

***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
Kit components require different storage conditions.
Immediately upon receipt store components as labeled.

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

140 Old Saw Mill River Road Hawthorne, NY 10532
Tel: 914.769.1192 www.genelink.com Fax: 914.769.1193

Material supplied

GLFX PCRProber™ Kit

Catalog No.: 40-3102-00 5 blots (50 reactions)

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

Storage instructions

Kit components require different storage conditions.
Immediately upon receipt store components as labeled.
Caution: DO NOT FREEZE COMPONENTS LABELED TO BE STORED AT 4°C.

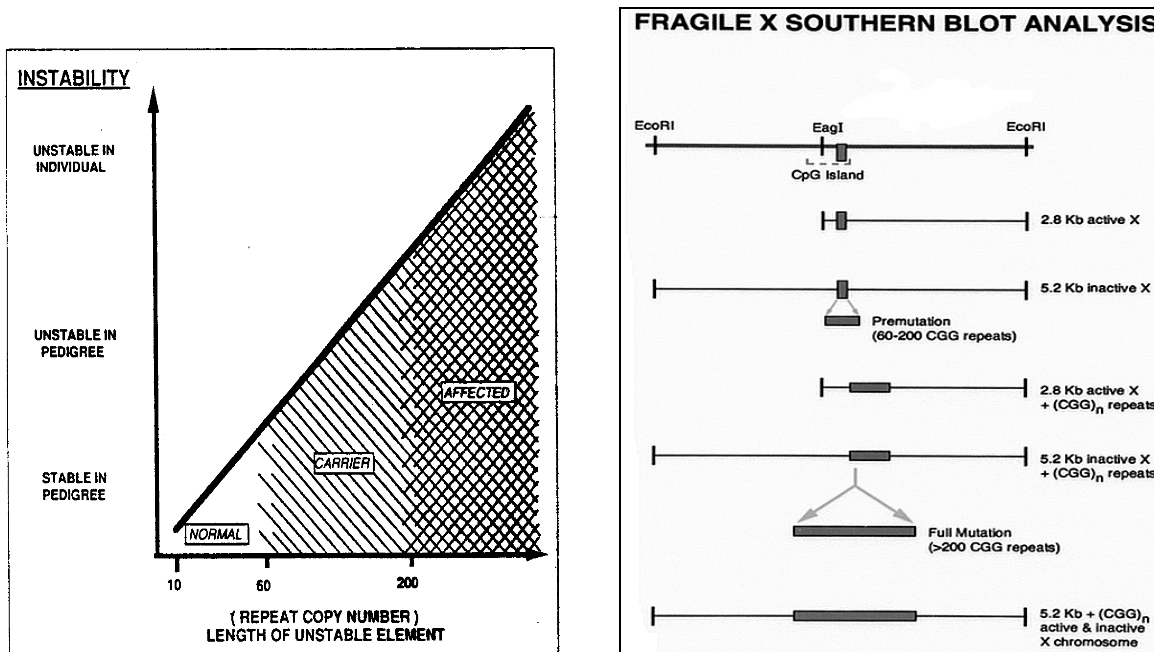
PCR reaction kit components Sufficient for 50 x 100 μl reaction				
Product	**Size**	**Description**	**Storage**	**Catalog No.**
Component A	900 μl	FX PCR premix with primers	Store at -20 ^{o}C.	40-5001-00
Component B	110 μl	10 mM deaza GTP	Store at -20 ^{o}C.	40-5002-00
Component C	650 μl	PCR additive	Store at -20 ^{o}C.	40-5003-00
Sequencing loading buffer	1 ml	Seq loading buffer	Store at -20 ^{o}C.	40-5027-00
Hybridization and Detection kit components Sufficient for processing five 16 x 16 cm blots				
Product	**Size**	**Description**	**Storage**	**Catalog No.**
GLFX PCRProber	12 μl	Alkaline Phosphatase labeled probe	Store at 4°C.	40-3101-01
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 ^{o}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 ^{o}C.	40-5010-10

Fragile X Genotyping

Background

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

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



The fragile X syndrome gene (FMR-1, fragile X mental retardation) was cloned in 1991 simultaneously by three groups (3-6). Soon the peculiar genetic mode of transmission was established and a new class of mutation came into existence- Trinucleotide repeats amplification. This explained the clinical state of 'premutation' and 'full mutation' as well as 'anticipation'. The fragile X syndrome is caused by the amplification of CGG repeats, which is located in the 5' region of the cDNA. The most common allele in the normal population consists of 29 repeats, the range varying from 6 to 54 repeats. Premutations in fragile X families showing no phenotypic effect range in size from 52 to over 200 repeats. All alleles with greater than 52 repeats are meiotically unstable with a mutation frequency of one. In general repeats up to 45 are considered normal, repeats above 50 to 200 are considered as premutation and above 200 as full mutation (3-7). The range between 40-55 is considered even by most experienced clinical geneticists and molecular geneticists very difficult to interpret and is considered as a 'gray zone' with interpretations made on a case-by-case basis (8).

Table 1: Trinucleotides Repeats in Human Genetic Disease

Disease	Repeat[a]	Normal Length[b]	Intermediate Length (Premulation)[a,b]	Full Disease Length[b]
Fragile XA (FRAXA)	$(CGG)_n$	6-52	59-230	230-2,000
Fragile XE (FRAXE)	$(CCG)_n$	4-39	? (31-61)	200-900
Fragile XF(FRAXF)	$(CGG)_n$	7-40	?	306-1,008
FRA16A	$(CCG)_n$	16-49	?	1,000-1,900
Jacobsen Syndrome (FRA11B)	$(CGC)_n$	11	80	100-1,000
Kennedy Syndrome (SMBA)	$(CAG)_n$	14-32	?	40-55
Myotonic Dstrophy (DM)	$(CTG)_n$	5-37	50-80	80-1,000; congenital, 2,000-3,000
Huntington disease (HD)	$(CAG)_n$	10-34	36-39	40-121
Spincerebellar ataxia 1 (SCA1)	$(CAG)_n$	6-39	…	40-81 (Pure)
Spincerebellar ataxia 2 (SCA2)	$(CAG)_n$	14-31	…	34-59 (Pure)
Spincerebellar ataxia 3 (SCA3)/Machado Joseph disease (MJD)	$(CAG)_n$	13-44	?	60-84
Spincerebellar ataxia 6 (SCA6)	$(CAG)_n$	4-18	?	21-28
Spincerebellar ataxia 7 (SCA7)	$(CAG)_n$	7-17	?	38-130
Haw River syndrome (HRS; also DRPLA))	$(CAG)_n$	7-25	?	49-75
Friedreich ataxia (FRDA)	$(CAA)_n$	6-29	? (>34-40)	200-900

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

Molecular Analysis

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

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

Table 2: CGG TRINUCLEOTIDE REPEATS PERCENTAGE AND FRAGMENT SIZE

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

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

PCR based methods are fundamentally similar. The two primers are constructed such that they span the region of trinucleotide repeat expansion. In the case of Fragile X specifically, the nature of the mutation poses problems using normal PCR conditions. In Fragile X, the repeat is of CGG which can be hundreds to thousands bases long. All DNA polymerases, including Taq DNA polymerase do not copy long stretches of G residues efficiently. An analog of G called 7-deaza GTP functions better and is partially replaced in the PCR reaction to achieve amplification. The use of 7 deaza GTP instead of G precludes the staining of gels with ethiduim bromide for visualization as 7 deaza GTP containing DNA does not stain well. This is resolved by using radioactively labeled nucleotide followed by autoradiography. Fragile X PCR still does not give accurate results for full mutations due to the inherent massive expansion and the inability of PCR to amplify very large fragments efficiently. All normal and premutation PCR amplification are reliable, but still is coupled with a Southern blot analysis. In our laboratory PCR is performed in addition to Southern blot analysis. The PCR results are obtained in 2 days followed by Southern blot results. All results from PCR are verifiable by Southern except full mutations which are not reliable with PCR.

Southern blot analysis for Fragile X mutation detection involves the cleavage of DNA with enzyme Eco R I and Eag I. This method detects the size of CGG repeats region by hybridization of probe StB12.3 to DNA that has been double digested with restriction enzymes Eco RI and Eag I and blotted onto a membrane. In normal females two fragments are seen, a 2.8kb corresponding to the active X and a 5.2kb fragment corresponding to the methylated inactive X chromosome. Normal males exhibit only the 2.8kb banding pattern. Affected males will have an amplified CGG repeats region with methylation thus giving rise to fragments larger than the normal 5.7kb. Premutations in males and females will be seen as fragments from 2.9-3.3kb (normal 2.8kb) derived from the X chromosome. Premutations in females derived from the inactive X will give fragments from 5.3-5.7kb. Mosaicism is characterized by fragments appearing as a mixture of full mutation (methylated, larger than 5.7kb) and unmethylated premutation (2.9-3.3kb).

Procedure

Procedure: CGG Repeats Analysis by PCR

The procedure outlined below can be finished in less than 24hrs.

Day 1 afternoon start PCR and leave it to proceed overnight. Prepare a 6% polyacrylamide-7 M urea gel (15-well, 0.75mm, 16x16cm^2) and leave it overnight. Day 2 proceed with the rest of the procedure.

PCR Premix Preparation

Thaw individual components. *Promptly store at -20°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 1ml premix following the volumes given below. Follow the same ratio for preparing other final volumes.

Component A	**170 µl**
Component B	**20 µl**
Component C	**100 µl**
Sterile deionized water	**710 µl**

Vortex gently.

'Hot start' PCR

Program two PCR thermal cycler files as follows

A. 'Hot start' 5' denaturation at 94°C, hold at 60°C

B. FX CGG amplification PCR file

94°C 30 sec, 65°C 60 sec., 72°C 2 min., 30 cycles. Hold at 4°C

Use 90 µl of the PCR premix for each reaction, add 50-100 ng DNA and start 'Hot start' denaturation for five minutes at 94°C.

While waiting for the five minute denaturation add Taq polymerase to the left over PCR premix. Add 2.5 units Taq polymerase per 10 µl of the left over PCR premix. *Label this tube as Enzyme Mix*

While the PCR is on hold at 60°C (after the initial 5' denaturation at 94°C) add 10 µl of the enzyme mix to each tube. After adding to all the tubes start the PCR file for cycling.

Precipitate PCR products by ethanol precipitation. Dissolve the pellets in 5µl of sterile H_2O 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 electrotransfer 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

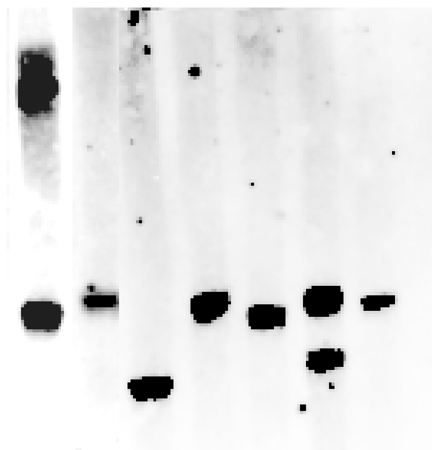
To make 100ml of 1x Detection buffer, add 10 ml of 10x Detection buffer and 90 ml of sterile deionized water.

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 GLFX PCRProber™ . Hybridize at 55°C for 30 min. DO NOT EXCEED 30 minutes.
4. Wash the blot in 75 ml of pre-warmed Hybwash I for 7 minutes at 55°C Repeat 3 times. Total of four washes.
5. Wash the blot in 150 ml pre-warmed Hybwash II for 5 min at 55°C. Repeat once. Total of two washes.
6. Wash the blot in 25 ml1x Detection Buffer at room temperature for 5minutes. Repeat 3 times. Total of four washes.
7. Transfer blot to a plastic sheet, (sheet protector cut from two sides to open up) and drain off excess buffer. Wipe off edges with paper towel. Blot should not be allowed to dry.
8. Spray CDP-star ready-to-use substrate evenly to cover the blot. DO NOT OVER SPRAY. Cover the blot with plastic sheet and wipe entire surface of the covered blot to expel any excess substrate and air bubbles. Expose the film at room temperature for 1 hr. or for shorter or longer time as required.
9. Luminescence continues for at least 24 hours and signal intensity remains almost constant during the first few hours. Multiple exposures can be taken to achieve the desired signal strength.

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



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

Table 3: Fragile X Molecular Analysis Results Interpretation

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

References

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

Fragile X Frequently Asked Questions/Troubleshooting

1. General Comment. Fragile X genotyping is not easy. A lab really has to optimize conditions. Following the protocol exactly works, especially running denaturing gel is important. Fragile X PCR based genotyping is difficult due to the extensive stretch of CGG. Even taq polymerase at elevated temperature can not replicate the long stretch of CGG repeats. The kit includes deaza GTP to reduce the strong secondary structure. Our kit is optimized to give results. A few initial rounds of optimization may be required. Once the investigator is experienced with all the manipulations, getting good results should be routine.

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

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

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

FRAGILE X PRODUCT LIST AND ORDERING INFORMATION

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

Other Related Products

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