



Product Manual

Cystic Fibrosis Genemer™

7 CF Mutation Panel Genotyping Genemer™ Kit

Catalog No.: 40-2029-07K

Store at -20 °C

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

Cystic Fibrosis 7 Mutation Panel Genemer Genotyping Kit

Genemer™ Primer pair for amplification of 7 most common CF mutations listed below. The quantity supplied is sufficient for 800 regular 25 µl PCR reactions for each mutation

CF Mutations
Cystic Fibrosis ΔF508 Genemer™
Cystic Fibrosis G542X Genemer™
Cystic Fibrosis W1282X Genemer™
Cystic Fibrosis R553X Genemer™
Cystic Fibrosis G551D Genemer™
Cystic Fibrosis CT3849+10kb Genemer™
Cystic Fibrosis N1303K Genemer™



Material Supplied

Cystic Fibrosis Genemer™

7 CF Mutation Panel Genotyping Genemer™

Catalog No.: 40-2029-07K; Cystic Fibrosis Genotyping Genemer™ Kit; 7 mutation panel

Storage Instructions:

1. Shipped lyophilized at room temperature.
2. Store at -20°C upon receipt.
3. Store at -20°C after reconstitution.

Cystic Fibrosis 7 Mutation Panel Genemer Genotyping Kit

Genemer™ Primer pair for amplification of 7 most common CF mutations listed below.
The quantity supplied is sufficient for 800 regular 25 µl PCR reactions for each mutation

Kit Components				
	Tubes Supplied	Catalog No.	Product	Unit Size
<input type="checkbox"/>	1	40-2029-11	Cystic Fibrosis ΔF508 Genemer™	10 nmole
<input type="checkbox"/>	2	40-2029-12	Cystic Fibrosis G542X; G551D & R553X Genemer™	10 nmole
<input type="checkbox"/>	1	40-2029-13	Cystic Fibrosis N1303K Genemer™	10 nmole
<input type="checkbox"/>	1	40-2029-14	Cystic Fibrosis W1282X Genemer™	10 nmole
<input type="checkbox"/>	1	40-2029-15	Cystic Fibrosis CT3849+10kb Genemer™	10 nmole

*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.

Cystic Fibrosis

Background

Cystic Fibrosis (CF) is the most common recessive disorder affecting Caucasians of European descent with a carrier frequency of 1 in 25. The frequency of these mutations is given below in the table on the left. Mutations at the CF locus occur in other racial and ethnic groups as shown below in the table below. Genemer™ are available for the seven most frequent mutation listed.

Frequency of CF Carriers	
Caucasian Americans of European descent	1 in 25
Ashkenazic Jews	1 in 29
Hispanic Americans	1 in 45
African Americans	1 in 60
Asian Americans	1 in 150

CF Mutation Frequency			
Caucasian Non-Jewish		Ashkenazic Jews	
Mutation	Frequency	Mutation	Frequency
ΔF508	75.8%	W1282X	60%
G551D	3.2%	ΔF508	23%
G542X	2.7%	G542X	4%
R553X	1.4%	N1303K	4%
N1303K	1.4%	3849+10kb C→T	4%
Total	~84%	Total	~95%

Detection Methods

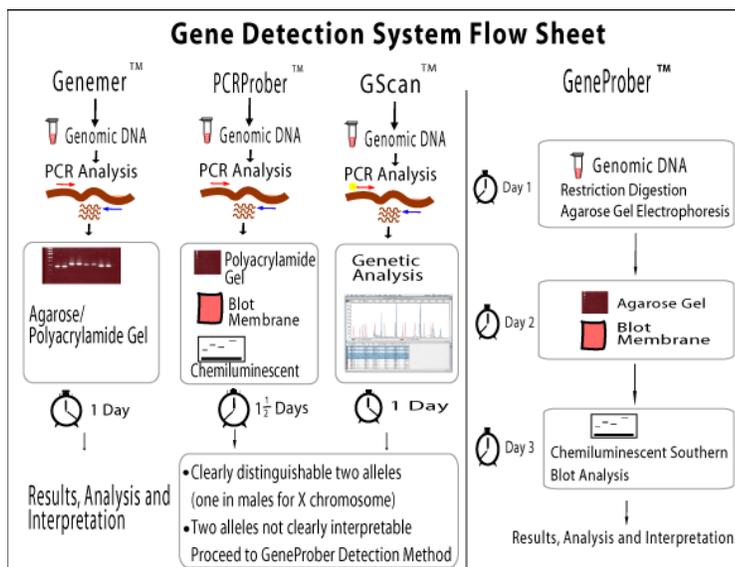
The choice of using methods for mutation analysis is restricted by our knowledge of the existence of a mutation and its type and in parallel to the development of safe and sensitive new detection methods. Several high throughput methods are available for simultaneous detection of 26 most common CF mutations by dot blot and hybridization, bead chip analysis, amplification refractory mutation system (ARMS), Holliday junctions etc.

Gene Link's Genemer™ primer pairs are for small scale educational CF mutation detection that are based on fragment analysis after amplification and restriction enzyme digestion.

Genemer™: Genemer™ is comprised of a primer pair for PCR amplification of the fragment of interest and visualization of the product by gel electrophoresis and ethidium bromide staining. This product contains one tube containing 10 nmol of forward and reverse lyophilized primer. The quantity supplied is sufficient for 400 regular 50 µl PCR reactions. The 10 nmol of primer when dissolved in 100µl water will give a solution of 100 µMolar i.e. 100 pmol/µl.

Genemer™ Kit: The Genemer™ kit is a set of 7 Genemer for the most common CF mutations.

Genemer™ Control DNA: These are cloned fragment of the mutation region of a particular gene for use with gene or mutation specific Genemer™ products. These control DNAs are ideal genotyping templates for optimizing and performing control amplification with unknown DNA. One tube is supplied containing 500 ng of lyophilized DNA segment of the specified region. The quantity supplied is sufficient for 1000 regular 50 µl PCR reactions.



Procedure

Mutation Analysis

This kit contains 5 Genemer™ primer pairs. Three mutations reside on Exon 11 and all of these will be amplified by the same Genemer™. A total of 6 amplification reactions will be required to detect these mutations. Mutation G551D/R553X is detected by a single amplification reaction. A list is given in the table below. It is essential to include negative, positive and blank control reactions for each mutation analysis.

PCR Reactions	Mutation	Genemer™
1	ΔF508 (Exon 10)	40-2029-11
2	G542X (Exon 11)	40-2029-12
3	G551D/R553X(Exon 11)	40-2029-12
4	N1303K (Exon 21)	40-2029-13
5	W1282X (Exon 20)	40-2029-14
6	CT3849 (Intron 19)	40-2029-15

Material Supplied

Each tube supplied contains 10 nmols lyophilized primer Genemer™ pair. Please refer to label on the specific tube. The quantity supplied is sufficient for 800 regular 25 μl PCR reaction.

A. Genemer™ Reconstitution

Stock Primer Mix: Dissolve the supplied 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.

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.

B. Thermal Cycler Files Amplification Profile

The following amplification profile has been optimized for specific product amplification using the supplied Genemer™. Hot start is recommended but is not essential.

Program the following thermal cycler files.

1. Hot Start

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.		

2. Amplification File

Amplification File			
Step	Temperature	Time	Cycles
Denaturation	94 °C	30 sec.	30
Annealing	55 °C	30 sec.	
Elongation	72 °C	60 sec.	
Fill in Extension	72 °C	7 minutes	1
Hold	4 °C	Infinity	Hold

C. PCR

1. PCR Premix Preparation (PP). Label tube "PP".

Determine the number of reactions to be performed and prepare sufficient PCR premix following the guideline below.

PCR Premix Preparation (PP)		
Component	1 X 25 µl Rxn.	10 X 25 µl Rxns.
Sterile Water	16 µl	160 µl
10 X PCR Buffer	2.5 µl	25 µl
2.0 mM dNTP	2.5 µl	25 µl
10 pmol/µl Genemer™ Mix	2 µl	20 µl
Template DNA (~500 ng)	1-2 µl	Add DNA to each tube
Total Volume	25 µl	
After adding template start 'Hot Start' PCR File		

Dispense 25 µl of the above PCR premix to individual PCR tubes for each amplification reaction and then add the template DNA. Start "Hot Start" thermal cycler file.

2. Taq Polymerase mix Preparation (EM). Label tube "EM"

Taq Enzyme Mix Preparation (EM)		
Component	1 X 25 µl Rxn.	10 X 25 µl Rxns.
Sterile Water	4 µl	40 µl
10 X PCR Buffer	0.5 µl	5 µl
Taq Polymerase	0.5 µl	5 µl
Add 5 µl to each reaction after holding after hot start		

While holding at 60°C add 5 µl of the Taq Enzyme Mix (EM). Start amplification file. Total reaction volume is 30 µl.

D. Restriction enzyme digestion (100 µl reaction)

Amplification reaction for ΔF508 is not digested by any restriction enzyme. All other amplification reactions are digested by a restriction enzyme as listed below. Total volume of amplification reaction is processed for restriction digestion. Follow the guideline below.

Restriction Enzyme Digestion	
Component	Volume
DNA; PCR Reaction	30 µl
10 X Buffer	10 µl
<i>Appropriate restriction enzyme</i>	30 units
Sterile Water	to 100 µl
Digest overnight at appropriate temperature	

Enzyme	Mutation	Incubation Temperature
BstN I	G542X and N1303K	60°C
Hind II	G551D/R553X	37°C
Mnl I	W1282X	37°C
Hph I	CT3849	37°C
Overnight digestion		

E. Polyacrylamide and Agarose Electrophoresis

1. Precipitate all overnight digestions and the Δ F508 amplified reaction. Dissolve pellets in 10 μ l 1x appropriate loading buffer; see table below.
2. Load 5 μ l samples to appropriate gel as listed in the table below.
3. Load positive, negative and blank control samples adjacent to unknown samples.
4. Electrophorese till the tracking dye is at the bottom of the gel.
5. Stain gel with ethidium bromide.
6. Record gel picture.

Mutation	Loading Buffer	Gel
Δ F508	Xylene Cyanol	16% PAGE- TAE
G542X	Xylene Cyanol	16% PAGE- TAE
G551D/R553X	Bromophenol Blue	16% PAGE- TAE
N1303K	Bromophenol Blue	16% PAGE TAE
W1282X	Xylene Cyanol	1.5 Agarose-TBE
CT3849	Xylene Cyanol	1.5 Agarose-TBE

Results and Interpretation

1. Based on the electrophoretic mobility pattern and the table below determine the genotype of the unknown samples.
2. Blank control samples should not yield an amplified fragment. If blank samples yield amplified fragment this is due to contamination. All samples should be repeated paying particular attention to prevent cross contamination.
3. Samples with heterozygous and homozygous mutations should be repeated to ascertain unequivocally.

Mutation	PCR Product, bp	Restriction Enzyme	N/N fragment size(s), bp	N/M fragment size(s), bp	M/M fragment size(s), bp
ΔF508	79	none	79/79	79/76	76/76
G542X	114	BstN /	90+24	114+90+24	114
G551D/R553X	114	Hind //	59+55	114+59+55	114
N1303K	60	BstN /	40+20	60+40+20	114
W1282X	473	Mnl /	178+172+123	301+178+172+123	301+ 172
CT3849	437	Hph /	349+88	349+222+127+88	222+127+88

References:

Kerem et al. (1990) PNAS. 87: 8447-8451 (caucasian mutation primers)
 Abeliovich et. al (1992) AJHG 51:951-956 (CT+10kb mutation)
 Shoshani et al. (1992) AJHG 50: 222-228 (W1282X,mutation)
 Ng et al. (1991) Hum. Genet. 87:613-617

Appendix

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
Rapid DNA Purification Protocol for 300 µl Whole Blood

Catalog Number: 40-4010-01

A. Initial Preparation

1. Label two sets of eppendorf 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 till 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 1-2µl.
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.

PCR Components and Analysis

Buffer Condition

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 indicated.

Standard Gene Link PCR Buffer	
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%

dNTP Concentration

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

Recipe	
2.0 mM dNTP Stock Solution Preparation*	
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	

MgCl₂ Concentration

The concentration of Mg⁺⁺ will vary from 1-5 mM, depending upon primers and substrate. Since Mg²⁺ ions form complexes with dNTPs, primers and DNA templates, the optimal concentration of MgCl₂ has to be selected for each experiment. Low Mg²⁺ ions result in a low yield of PCR product, and high concentrations increase the yield of non-specific products and promote mis-incorporation. Lower Mg²⁺ concentrations are desirable when fidelity of DNA synthesis is critical. The recommended range of MgCl₂ 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 MgCl₂ concentration of 1.5 is used in most cases. If the DNA samples contain EDTA or other chelators, the MgCl₂ concentration in the reaction mixture should be raised proportionally. Given below is an MgCl₂ concentration calculation and addition table using a stock solution of 25 mM MgCl₂.

MgCl ₂ Concentration & Addition Table								
Final concentration of MgCl ₂ in 50µl reaction mix, (mM)	1.0	1.25	1.5	1.75	2.0	2.5	3.0	4.0
Volume of 25mM 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.

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.

Primer Mix: Prepare a 10 pmols/μl Primer Mix solution by a ten fold dilution of the stock primer mix.

Example: Add 180 μl sterile TE to a new tube, to this tube add 20 μl of primer stock solution. Label this tube as Primer Mix 10 pmols/μl.

Amplification Thermal Cycling

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

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			

Typical Reaction Premix

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 ₂ O	37.5 µl
Total Volume	50 µl

Typical PCR Reaction Mix

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

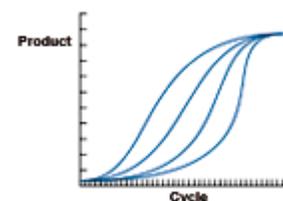
Taq Premix Preparation

Taq Premix (/50µl)	
Component	Volume
PCR Premix	6 µl
Taq polymerase (5 u/µl)	0.25µl
Add 5µl/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	

Yield and Kinetics

The target will be amplified by up to 10⁶ fold in a successful reaction, but the amplification will usually plateau at 1-10µg. 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.



Gel Electrophoresis of PCR Products

Gel electrophoresis of PCR products is the standard method for analyzing reaction quality and yield. PCR products can range up to 10kb in length, but the majority of amplifications are at 1kb and below. Agarose electrophoresis is the classical method to analyze amplification products from 150 bp to greater than 10kb. Polyacrylamide gel electrophoresis should be used for resolution of short fragments in the range of 100 bp to 500 bp when discrimination of 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.

Purification of PCR Product

Various purification methods are available for the purification of PCR products. The selection of a particular method over other 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 application 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. 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 eppendorf 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 eppendorf 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 20µl of H₂O.
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 700µl of hydrated Sephadex G-50 to each spin column, initiate flow using rubber bulb or any other method.

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

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 eppendorf tube appropriately labeled with sample name.
7. Apply up to 50 μ l 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 eppendorf tube is free of salts and primers shorter than 35-40mer.

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 use 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 T_m and the complex secondary structure thus the duplex stability (1-5). Tetramethyl ammonium chloride (TMAC) actually increases the specificity of hybridization and increases the T_m . 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 T _m 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 0.01µg/µl to 0.1µg/ 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 <i>Taq</i> 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 <i>Taq</i> polymerase and may also suppress 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 <i>Taq</i> 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 T _m and minimizes mis-pairing.	TMAC is generally used at a final concentration of 15-100mM to eliminate non-specific priming.

References:

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3. Daniel S. Mytelka, D.S., and Chamberlin, M.J.,(1996) Analysis and suppression of DNA polymerasepauses associated with a trinucleotide consensus. Nuc. Acids Res., 24:2774-278.
4. Keith, J. M., Cochran, D.A.E., Lala, G.H., Adams, P., Bryant, D.and Mitchelson, K.R. (2004) Unlocking hidden genomic sequence. Nucl. Acids Res. 32: e35.
5. Owczarzy, R., Dunietz, I., Behlke, M.A., Klotz, I.M. and Joseph A. Walder. (2003) Thermodynamic treatment of oligonucleotide duplex-simplex equilibria. PNAS, 100:14840-14845.

Ordering Information

Product	Size	Catalog No.	Price, \$
Cystic Fibrosis ΔF508 Genemer™	10 nmole	40-2029-11	100.00
Cystic Fibrosis G542X, G551D & R553X Genemer™	10 nmole	40-2029-12	100.00
Cystic Fibrosis N1303K Genemer™	10 nmole	40-2029-13	100.00
Cystic Fibrosis W1282X Genemer™	10 nmole	40-2029-14	100.00
Cystic Fibrosis CT3849+10kb Genemer™	10 nmole	40-2029-15	100.00
Cystic Fibrosis Genotyping Genemer™ Kit; 7 mutation panel	10 nmole	40-2029-07K	450.00

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Genemer™ control DNA is a cloned fragment of the mutation region of a particular gene. These control DNA are an ideal genotyping template for optimizing and performing control amplification with unknown DNA.

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

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GLDM CTG Genemer Control DNA; Myotonic Dystrophy (12, 45, 93, 129 & 194 CTG repeats available)	500 ng	40-2026-0X	175.00

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Genemer™ (Selected List) Primer pair for gene or mutation specific amplification. Special optimized conditions may be required for certain amplifications

Product	Size	Catalog No.	Price, \$
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Huntington Disease (spanning CAG triple repeat region)	10 nmols	40-2025-10	100.00
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