

HAEMOCHROMATOSIS H63D TEST KIT

This kit provides the components needed to detect the Haemochromatosis polymorphisms 175G-A, 187C-G and 193A-T in Exon 2 of the HFE gene (Chromosome 6p21.3) resulting in Val59Met, His63Asp and Ser65Cys amino acid substitutions respectively.

Store at -20°C

For in vitro use only



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Heteroduplex Analysis

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1. PRINCIPLE

Heteroduplex Analysis Test Kits contain DUPLEXER™ reagents. These are synthetic oligonucleotides that mimic naturally occurring gene segments of human DNA, but their structures may be manipulated and defined.

When amplified gene segments derived from human genomic DNA and the DUPLEXER™ reagent are mixed the resultant heteroduplexes possess unique 3-D structures specific to a particular genetic allele.

The genotype of clinical samples may be determined by comparison of the electrophoretic mobility patterns of heteroduplex bands from test DNA with control DNAs provided with the kit.

There are three main stages involved in distinguishing between wild homozygous, mutant homozygous and heterozygous alleles;

1. Amplification of Control DNAs, DUPLEXER™ and test genomic DNA in a PCR reaction.
2. Crossmatching of the DUPLEXER™ PCR product with the PCR products from the control and test DNAs.
3. Separation of the resulting heteroduplexes by polyacrylamide gel electrophoresis or other appropriate acrylamide based electrophoretic separation technique.

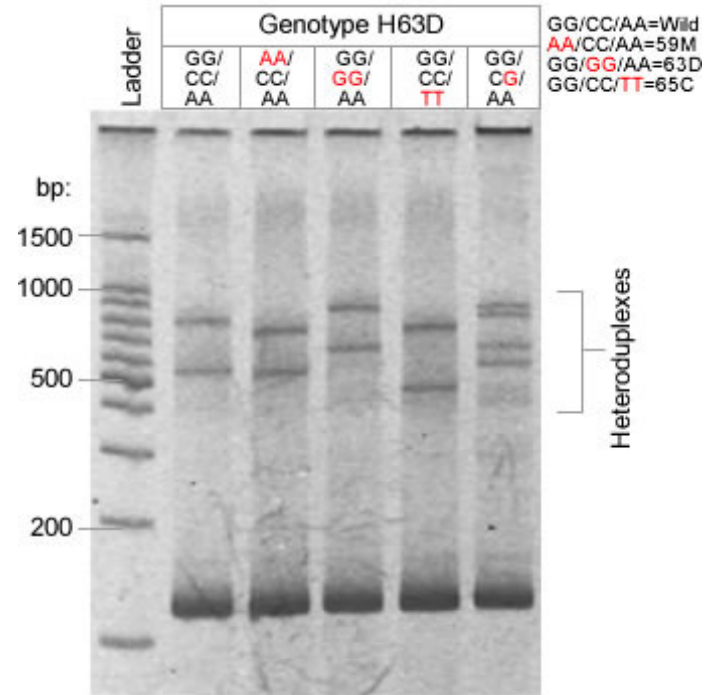
The principle of genotyping using DUPLEXER™ reagents is fully outlined on the Heteroduplex Analysis web site. It is recommended that the user studies the principle and examples provided on the web pages if unfamiliar with DUPLEXER™ technology.

CAUTION

DUPLEXER™ reagent is more easily amplified than genomic DNA. To minimise cross contamination ALWAYS open the DUPLEXER™ tube after completing the set-up of the rest of your PCR reaction tubes.

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STEP 4: Analysis of the Stained Gel



Genotypes of clinical samples may be determined by comparison of electrophoretic mobility patterns of heteroduplex bands with the control genomic DNAs provided with the kit.

The patterns are;

- Homozygous wild type DNA (175 GG, 187CC, 193 AA)
- Homozygous 59M mutated or polymorphic DNA (175 AA)
- Homozygous 63D mutated or polymorphic DNA (187 GG)
- Homozygous 65C mutated or polymorphic DNA (193 TT)
- Heterozygous wild type plus 63D mutated or polymorphic DNA (187 CG)

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STEP 3: Electrophoresis of Heteroduplexes

Electrophoresis may be performed in a range of electrophoresis equipment:

Large format gel (Nominal length 16cm)

- 15% (37.5:1) non-denaturing polyacrylamide gel
- 1x TBE running buffer
- Constant voltage of 500V
- Gel cooling to maintain a temperature of 10-15°C. Cold tap water cooling is sufficient

Midi format gel (Nominal length 9cm)

- 15% (37.5:1) non-denaturing polyacrylamide gel
- 1.5x TBE running buffer
- Constant voltage of 300V
- Gel cooling to maintain a temperature of 10-15°C. Cold tap water cooling is sufficient

An appropriate molecular weight marker, for example a 100 base pair ladder, should be included.

An appropriate amount of gel loading buffer should be added to each crossmatch reaction before loading the complete reaction mixture onto the gel. We recommend a gel loading buffer containing xylene cyanol.

For good resolution of heteroduplexes gels should be run until the xylene cyanol marker reaches the bottom of the gel.

Gels may be stained using ethidium bromide or SYBR Green.

NOTE: Heteroduplex Analysis kits may also be used in conjunction with most other acrylamide-electrophoretic based separation techniques.

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2. KIT CONTENT

The Haemochromatosis H63D Test Kit contains sufficient reagents for 10 tests.

Clear cap	'Buffer'
White cap	'H63D Test'
Green cap	'H63D DUPLEXER'
Red cap	'H63D Wild'
Black cap	'H63D Mutant 59M'
Orange cap	'H63D Mutant 63D'
Blue cap	'H63D Mutant 65C'

Kits in their lyophilized form may be stored for up to 6 months at -20°C.

Reconstitution of Reagents

Prior to use the lyophilized reagents must be reconstituted by addition of buffer solution as follows;

H63D Test	Add 264µl Buffer
H63D DUPLEXER	Add 336µl Buffer
H63D Wild	Add 336µl Buffer
H63D Mutant 59M	Add 336µl Buffer
H63D Mutant 63D	Add 336µl Buffer
H63D Mutant 65C	Add 336µl Buffer

The contents of the tubes should be thoroughly mixed by vortexing (10 seconds) and quick spinning at least three times.

Reconstituted reagents may be stored for up to 3 months at -20°C.

NOTE: It is recommended that stored reagents are mixed as above after being thawed completely.

Reagent to be supplied by user

Taq DNA polymerase (0.5 units/µl)

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3. DNA SAMPLE PREPARATION

This kit is intended for use with genomic DNA. Samples prepared using commercial DNA purification kits or by phenol/chloroform extraction may be used.

It is recommended that DNA is suspended in DNase-free water although the use of buffers supplied with kits will have no adverse effect.

Samples should be stored at 4°C or at -20°C for longer periods.

Genomic DNA should be diluted to a concentration of 10-100ng/μl before use.

4. PROCEDURE

STEP 1: PCR reaction

Tube	Mix for 1 PCR reaction (μl)					DUPLEXER *
	Wild Control	Mutant Control 59M	Mutant Control 63D	Mutant Control 65C	Test DNA	
Red cap	28	-	-	-	-	-
Black cap	-	28	-	-	-	-
Orange cap	-	-	28	-	-	-
Blue cap	-	-	-	28	-	-
White cap	-	-	-	-	22	-
Patient DNA	-	-	-	-	6	-
Green cap	-	-	-	-	-	28
Taq (0.5 units/μl)	2	2	2	2	2	2

*To run a complete set of controls (without heterozygotes) plus:

- 1 DNA test use 1 DUPLEXER reaction tube.
- 2 to 6 DNA tests use 2 DUPLEXER reaction tubes.
- 7 to 10 DNA tests use 3 DUPLEXER reaction tubes.

If including heterozygotes: 2 DUPLEXER reaction tubes for 1 to 3 DNA tests, 3 DUPLEXER reaction tubes for 4 to 8 DNA tests, and 4 DUPLEXER reaction tubes for 9 to 10 DNA tests.

Thermal Cycling Parameters;

Initial Denaturation (94°C, 5 min) – 1 cycle

Denaturation (94°C, 30 sec) Primer annealing (62°C, 30 sec) Primer extension (72°C, 30 sec)	}	28 cycles
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Final primer extension (72°C, 5 min) – 1 cycle

If the PCR products are not to be processed immediately store at -20°C.

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STEP 2: ‘Crossmatching’ - Formation of Heteroduplexes

In this step, aliquots of the test DNA and control DNA PCR products are mixed with aliquots of the DUPLEXER™ PCR product. The mixtures are placed in a thermal cycler and subjected to heating and cooling to induce the formation of heteroduplexes, a process referred to as crossmatching. Note: the control heterozygote genotypes are generated by mixing wild control and mutant control PCR products in equal proportions: wild + control 175AA to generate hetero Wild/59M (175GA), wild + control 187GG to generate hetero Wild/63D (187CG), and wild + control 193TT to generate hetero Wild/65C (193AT).

To ensure optimal heteroduplex formation, approximately equal molar amounts of PCR product from the DUPLEXER™ and test DNAs should be used in the crossmatch mixtures. Therefore before crossmatching we recommend testing of each test DNA, control DNA, and DUPLEXER™ PCR product to compare band intensities. This can be easily achieved by electrophoresis of 5μl of each DNA in 15% polyacrylamide gels or 2% agarose minigels.

Adjustment of the PCR product concentration can be made based on visual comparison of the band intensity.

Mix contents of the PCR tubes by briefly vortexing and centrifuging and prepare crossmatch reactions.

PCR Product	Mix for 1 Crossmatch reaction (μl)					Test DNA
	Control Wild 175GG 187CC 193AA	Control Mutant 59M 175AA	Control Mutant 63D 187GG	Control Mutant 65C 193TT	Control Hetero Wild/63D 187CG	
DUPLEXER	5	5	5	5	5	5
Wild	5	-	-	-	5	-
Control 59M (175 A)	-	5	-	-	-	-
Control 63D (187 G)	-	-	5	-	5	-
Control 65C (193 T)	-	-	-	5	-	-
Test DNA	-	-	-	-	-	5

Thermal Cycling Parameters;

Increase temperature to 95°C, hold for 2 minutes
 Cool to 60°C at 1°C per second, hold for 1 minute
 Cool to 45°C at 0.1°C per second, hold for 1 minute
 Cool to 6°C

If the crossmatched products are not to be processed immediately store at -20°C.

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