

Detecting HFE Mutations in Human Genomic DNA: Student Laboratory Manual

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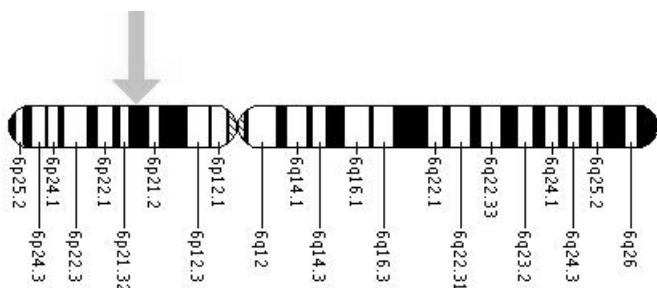
I. Purpose

The purpose of this laboratory is to screen human genomic DNA samples for mutations in HFE gene that are linked to hereditary hemochromatosis. You will use PCR to amplify exons 2 and 4 from the HFE gene and look for restriction fragment length polymorphisms associated with the mutations. The mutations analyzed include the C187G change in exon 2, which eliminates an MboI restriction site, and G845A change in exon 4, which creates a novel RsaI restriction site.

II. Introduction

Hereditary hemochromatosis is an autosomal recessive disorder of iron metabolism in which the body accumulates too much iron, leading to iron overload. In 1996 two mutations were identified in the HFE gene, that when present in the homozygote or compound heterozygote, can lead to the presentation of the disease. The HFE gene is located on the short (p) arm of Chromosome 6. The gene encodes a protein that is found on the surface of epithelial cells and some immune cells. The HFE protein appears to be involved in regulating the absorption of iron by the intestinal cells; it also may influence the expression of a second iron-regulatory protein, hepcidin.

The most commonly found mutations in the HFE gene that are associated with the disease are the C187G nucleotide change in exon 2, which leads to the substitution of an aspartic acid residue for the normally encoded histidine at position 63 in the protein (H63D), and the G845A nucleotide change in exon 4, which leads to the substitution of a tyrosine for the normally encoded cysteine at position 282 (C282Y).



The human HFE gene is located on the short arm of Chromosome 6. Figure reprinted from *Genetics Home Reference*, National Library of Medicine.

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A **compound heterozygote** is an organism that contains two copies of a gene, each copy having a different recessive mutation that together can confer the null (or disease) phenotype.

The **penetrance** of a phenotype refers to the percent of individuals with a particular genotype who also exhibit the associated phenotype. In this case not all individuals who are homozygous for one of the two mutations (or compound heterozygotes) will actually develop disease symptoms. Hemochromatosis is said to show *incomplete penetrance*.

Homozygosity for the mutation that results in the C282Y substitution is considered the most common genotype of hereditary hemochromatosis. Approximately 1 in 10 Caucasians in the United States are carriers of an HFE mutation, and 4.4 out of 1,000 Caucasians in the United States are homozygotes.

However the actual penetrance of the disease is not as high as the genotype frequencies would predict. Additionally, males are almost twice as likely to present with symptomatic disease as females. The difference between disease prevalence and genetics is thought to be due in part to environmental factors such as menstruation, pregnancy, frequent blood donation, alcohol consumption, diet and other accompanying disease, such as viral hepatitis.

Symptoms of hereditary hemochromatosis generally present in adults aged 30–60, with presentation occurring later in affected females. In the disease, excess iron is deposited in the liver, pancreas, heart, joints and endocrine glands. Early symptoms can include hyperpigmentation, enlarged liver, impotence, testicular atrophy, joint swelling and tenderness and fatigue. The disease is managed in its early and intermediate stages by phlebotomy (blood letting).

References

1. Emery, J. *et al.* (2007) Genetics and preventive health care. *Australian Family Physician* **36**, 808–11.
2. Whitlock, E.P. *et al.* (2006) Screening for hereditary hemochromatosis: a systematic review for the U.S. preventive services task force. *Annals of Internal Medicine*. **145**, 209–23.
3. Brissot, P. and de Bels, F. (2006) Current approaches to the management of hemochromatosis. *Hematology 2006* 36–41.
4. National Library of Medicine (2006) HFE. *Genetics Home Reference*. <http://ghr.nlm.nih.gov/gene=hfe> (accessed 2/13/2008).

III. Protocol for PCR Amplification of Fragments from the HFE Gene

- ⚠ Contamination of pipets and laboratory surfaces with DNA is a significant issue with PCR. Wear gloves and use sterile, barrier pipet tips. Use sterile, thin-walled reaction tubes for the PCR, and store the Master Mixes in sterile tubes as well.
- ⚠ Wear gloves, lab coats, closed-toe shoes and protective eyewear whenever you are working in a laboratory.

Materials Required

- vortex mixer
- primer pair mix I for exon 2
- primer pair mix II for exon 4
- Nuclease-Free Water (Cat.# P1193)
- dNTP Mix (Cat.# U1511)
- GoTaq® Hot Start DNA Polymerase (Cat.# M5001)
- 5X Green GoTaq® Flexi Buffer (included with enzyme)
- 25 mM MgCl₂ (included with enzyme)
- genomic DNA homozygous for C187G mutation
- genomic DNA from compound heterozygote for C187G and G845A
- wildtype genomic DNA for C187 and G845
- genomic DNA homozygous for G845A mutation
- Mineral Oil (Cat.# DY1151)
- ice bucket
- Barrier pipet tips (Cat.# A1491, A1521 and A1541)
- thin-walled PCR tubes
- thermal cycler

Protocol for PCR

1. Thaw, vortex and briefly centrifuge all reagents (except for the DNA polymerase) to collect liquid at the bottom of the tubes. Place reagents on ice.

Note: Do not vortex the DNA polymerase.

2. Label eight 0.5 ml PCR tubes. (Label the tubes 1–8 plus your group letter and the letter “P” for PCR. For example: 1AP, 2AP, etc.).
3. Prepare master mixes I and II by combining the components listed in Tables 1 and 2 in the order listed. Prepare enough of each master mix for 10 reactions to allow for pipetting errors (use the volumes in the third column of the tables to prepare your master mixes). Be sure to clearly label the master mix tubes so that you will easily be able to tell which tube contains master mix I and which one contains master mix II.

Table 1. PCR Master Mix I Components.

Components	Volume Per Individual Reaction	Volume for 10 Reactions (Master Mix I)
Exon 2 primer pair	1.0 µl	10.0 µl
5X GoTaq® Green Flexi Buffer	10.0 µl	100 µl
MgCl ₂ Solution, 25 mM	5.0 µl	50 µl
dNTP Mix	1.0 µl	10.0 µl
GoTaq® Hot Start DNA Polymerase	0.25 µl	2.5 µl
Nuclease-Free Water	7.75 µl	77.5 µl
final volume	25 µl	250 µl

Protocol for PCR (continued)

Table 2. PCR Master Mix II Components.

Components	Volume Per Individual Reaction	Volume for 10 Reactions (Master Mix II)
Exon 4 primer pair	1.0 µl	10.0 µl
5X GoTaq® Green Reaction Buffer	10.0 µl	100.0 µl
MgCl ₂ Solution, 25 mM	0.25 µl	2.5 µl
dNTP Mix	1.0 µl	10.0 µl
GoTaq® Hot Start DNA Polymerase	0.5 µl	5.0 µl
Nuclease-Free Water	<u>7.75 µl</u>	<u>77.5 µl</u>
final volume	25 µl	250 µl

4. Add the components in the order listed in Table 3 to the labeled 0.5 ml PCR amplification tubes.

Note: When adding the DNA sample, place the pipet tip at the bottom of the tube so that the tip is submerged under the water level. Centrifuge the tubes briefly to collect liquid at the bottom of the tube. The final total volume for each reaction will be 50 µl.

Table 3. Components and Volumes for Individual PCR Amplifications and Controls.

Reaction Number	Nuclease-Free Water	DNA Sample* (Coriell Cat.#)	PCR Master Mix I	PCR Master Mix II
1 (negative control)	25.0 µl	—	25.0 µl	—
2 (negative control)	25.0 µl	—	—	25.0 µl
3 (exon 2)	24.0 µl	1.0 µl (NA14656)	25.0 µl	—
4 (exon 2)	24.0 µl	1.0 µl (NA14690)	25.0 µl	—
5 (exon 2)	24.0 µl	1.0 µl (NA14620)	25.0 µl	—
6 (exon 4)	24.0 µl	1.0 µl (NA14656)	—	25.0 µl
7 (exon 4)	24.0 µl	1.0 µl (NA14650)	—	25.0 µl
8 (exon 4)	24.0 µl	1.0 µl (NA14686)	—	25.0 µl

***Note:** Make sure that your genomic DNA concentration is 50 ng/µl. The final concentration of each component in the reaction is: 1X Reaction Buffer, 1.5 mM MgCl₂, 0.5 units GoTaq® DNA polymerase, 200 µM each dNTP, 50 pmol of each PCR primer, and 50 ng of human genomic DNA.

5. Add one drop of mineral oil the side of each reaction tube unless you are using a thermal cycler with a heated lid. Store your reactions on ice until they are placed into the thermal cycler.

6. Process the amplifications using the profile below. The cycling profile will take approximately 1.5–2 hours to complete.

Table 4. PCR Cycling Profile.

95 °C for 2 minutes.	1 cycle
94 °C for 30 seconds; 60 °C for 30 seconds; 72 °C for 30 seconds	35 cycles
68 °C for 7 minutes	1 cycle
4 °C soak	1 cycle

IV. Protocol for Detection of Hemochromatosis RFLPs

Materials Required

- MboI Restriction Enzyme (Cat.# R6711)
- RsaI Restriction Enzyme (Cat.# R6371)
- 10X Restriction Enzyme Buffer C
- BenchTop PCR Markers (Cat.# G7531)
- TAE Buffer, 10X (Cat.# V4271)
- 5.0 µg/ml ethidium bromide
- staining trays
- Agarose, LMP, Preparative Grade for Small Fragments (10 to 1,000 bp) (Cat.# V3841)
- Agarose, LE, Analytical Grade (Cat.# V3121)
- ultraviolet light box, camera and film and/or digital imager
- power supplies for agarose gel electrophoresis

Warning: Ethidium bromide is a carcinogen. Please consult the Material Safety Data Sheet or your institution's chemical safety officer for appropriate usage and disposal instructions.

IV.A. Restriction Digestion

1. Label ten 1.5 ml tubes. (Label the tubes 1–10 plus your label group letter and the letter “D” for digests. For example: 1AD, 2AD).
2. Assemble the restriction enzyme digests according to Table 5 below. If you used mineral oil in your PCR, be sure to remove the aliquot of PCR product from BELOW the mineral oil.
3. Vortex mix and then briefly centrifuge the reactions to collect the liquid at the bottom of the tube. Incubate reactions at 37 °C for 3 hours.

Table 5. Restriction Enzyme Digestions of Exons 2 and 4 from the HFE Gene.

Restriction Digest Reaction Number	PCR Product to Use	Volume of PCR Product	Volume of 10X Buffer C	Volume of Mbo I	Volume of RsaI
1D	#1	26 µl	3 µl	1 µl	—
2D	#2	26 µl	3 µl	—	1 µl
3D (uncut control)	#3	8 µl	—	—	—
4D	#3	26 µl	3 µl	1 µl	—
5D	#4	26 µl	3 µl	1 µl	—
6D	#5	26 µl	3 µl	1 µl	—
7D (uncut control)	#6	8 µl	—	—	—
8D	#6	26 µl	3 µl	—	1 µl
9D	#7	26 µl	3 µl	—	1 µl
10D	#8	26 µl	3 µl	—	1 µl

Agarose Gel Electrophoresis

Note: If you used the GoTaq® Green Flexi Buffer you will not need to add any loading dye to your samples because this buffer contains loading dyes. The BenchTop PCR markers are also ready-to-use and do not need additional loading dyes.

1. Prepare a 3.0 % agarose gel in 1X TAE buffer (2.5% LMP Preparative Agarose for Small Fragments and 0.5% Agarose, LE, Analytical Grade).

Note: The gel running buffer is 1X TAE. Be sure to dilute any TAE stock to a 1X working solution.

2. Load 12 µl of each reaction and markers in the following order:

Lane	1	2	3	4	5	6	7	8	9	10	11	12
	BenchTop PCR Marker	Rxn 1	Rxn 2	Rxn 3	Rxn 4	Rxn 5	Rxn 6	Rxn 7	Rxn 8	Rxn 9	Rxn 10	BenchTop PCR Marker

3. Run the agarose gel at the voltage recommended by the maker of the power supply and gel apparatus that you are using. Allow the yellow dye to migrate to 5 mm from the bottom of the gel. The yellow dye migrates at approximately 50 bp.
4. Stain the gel in 5.0 µg/ml ethidium bromide for 10 minutes. Destain for 10 minutes in water.

Note: The destaining water will contain ethidium bromide and should be disposed of properly.

5. View the stained gel on a UV light box and photograph.