

# Detecting HFE Mutations in Human Genomic DNA: Instructor's Manual

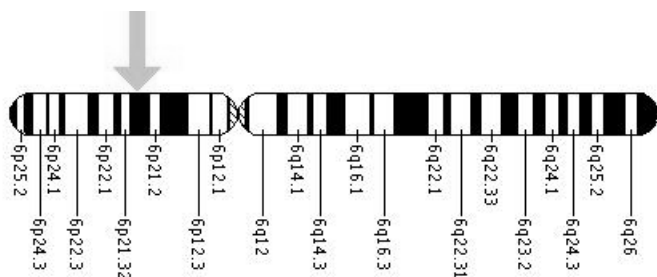
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## I. Purpose and Concepts Covered

The purpose of this laboratory is to screen human genomic DNA samples for mutations in the HFE gene that are linked to hereditary hemochromatosis. Students 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. Background on Hereditary Hemochromatosis

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 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*.

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

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 are present in adults aged 30–60, with presentation occurring later in 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. PCR Amplification of Exons 2 and 4 from the HFE Gene

#### 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 MgCl<sub>2</sub> (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

### III.A. Before you Begin

Section V of this manual provides a listing of reagent suppliers and ordering information for most of the materials required for this laboratory.

This protocol will require at least two laboratory sessions but can be divided into three sessions if necessary. We recommend setting up the PCR in the first session and then performing restriction digest and agarose gel electrophoresis in the second session. Alternatively, the PCR, restriction digests and electrophoresis can be performed in three separate sessions. PCR products and restriction digests may be stored at  $-20^{\circ}\text{C}$  until you are ready to perform the next step in the analysis.

We also recommend creating extra tubes of master mixes I and II to have on hand for spills and other student errors.

You may wish to perform the laboratory in advance of the students so that you have gel results that can be interpreted by student groups that were not successful in generating PCR product.

⚠ DNA contamination of pipettes and laboratory surfaces is a significant issue with PCR. Make sure that students wear gloves and use sterile barrier pipette tips. Use sterile, thin-walled reaction tubes for the PCR, and store the master mixes in sterile tubes.

⚠ Wear gloves, lab coats and protective eyewear whenever you are working in a laboratory.

### III.B. 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 $\mu\text{l}$	10.0 $\mu\text{l}$
5X GoTaq <sup>®</sup> Green Flexi Buffer	10.0 $\mu\text{l}$	100 $\mu\text{l}$
MgCl <sub>2</sub> Solution, 25 mM	5.0 $\mu\text{l}$	50 $\mu\text{l}$
dNTP Mix	1.0 $\mu\text{l}$	10.0 $\mu\text{l}$
GoTaq <sup>®</sup> Hot Start DNA Polymerase	0.25 $\mu\text{l}$	2.5 $\mu\text{l}$
Nuclease-Free Water	<u>7.75 <math>\mu\text{l}</math></u>	<u>77.5 <math>\mu\text{l}</math></u>
final volume	25.0 $\mu\text{l}$	250 $\mu\text{l}$

### III.B. 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 <sub>2</sub> 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	25.0 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 MgCl<sub>2</sub>, 0.5 units GoTaq® Hot Start 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 3–3.5 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

#### IV.B. 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

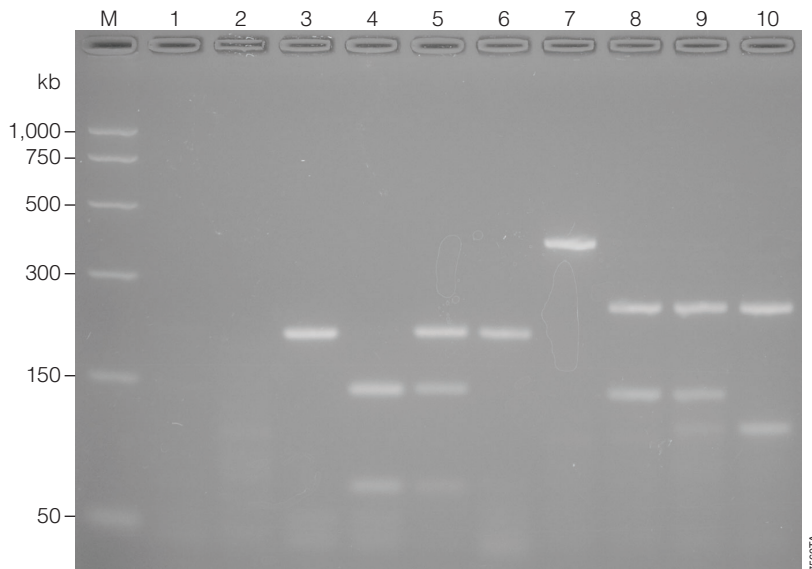
3. Run the agarose gel at the voltage recommended by the maker of the power supply and gel apparatus that you are using. Allow the yellow dye to migrate to 5 mm from the bottom of the gel. The yellow dye migrates at approximately 50 bp.
4. Stain the gel in 5.0 µg/ml ethidium bromide for 10 minutes. Destain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

#### IV.C. Expected Results and Discussion

The expected size of the PCR product from the amplification reaction using HFE primer pair mix I, which amplifies a region of exon 2, is 208 bp. For the wildtype allele of the HFE gene, this PCR product will be cut once with MboI, producing two fragments of 138 bp and 70 bp. The C187G mutation destroys the MboI site, and therefore product amplified from the C187G allele will not be digested and will remain at 208bp.

The expected size of the PCR product from the amplification reaction using HFE primer pair mix II, which amplifies a region of exon4, is 390 bp. For the wildtype allele of the HFE gene, this PCR product will be cut once with Rsa I, producing fragments of 248 bp and 142 bp. The G845A mutation introduces a second Rsa I digestion site, and therefore product amplified from a G845A allele will produce three RsaI fragments of 248, 113 and 29 bp. (Note: The 28 bp fragment will not be visible in your gel.)



Lane M: Marker

Reaction 1: Exon 2 negative control, no band expected

Reaction 2: Exon 4 negative control, no band expected

Reaction 3: Uncut Exon 2 fragment wildtype for C187

Reaction 4: MboI digest: homozygote wildtype (C187/C187)

Reaction 5: MboI digest: heterozygote (C187/C187G)

Reaction 6: MboI digest: homozygote (C187G/C187G)

Reaction 7: Uncut Exon 4 fragment wildtype for G845

Reaction 8: RsaI digest: wildtype for G845

Reaction 9: RsaI digest of compound heterozygote (C187G/G845A)

Reaction 10: RsaI digest of homozygote (G845A/G845A)

### Questions for Discussion

1. Given the difference between penetrance of the disease phenotype and presence of mutant alleles in the population, do you think a general screening test for adult hemochromatosis is warranted? Why or why not?
2. Explain what would happen if you left out the magnesium in the PCR?
3. If you had nonspecific contamination in your PCR, what would your results look like?
4. Can you propose a hypothesis about how the H63D and C282Y substitutions in the HFE protein affect its function?

Concepts that can be discussed against the backdrop of this laboratory include: autosomal recessive and dominant inheritance, phenotype penetrance, the relationship between genotype and phenotype, genetic code and codon usage, ethical considerations of genetic screening tests, iron metabolism and storage in cells and tissues, gene mapping, chromosome structure and nomenclature.

### IV.D. Troubleshooting

<b>Symptoms</b>	<b>Causes and Comments</b>
Low yield or no yield	<p>Template was degraded. Verify DNA integrity by electrophoresis after incubation in the presence of Mg<sup>2+</sup>.</p> <p>Thermal cycler was programmed incorrectly. Verify that times and temperatures are correct.</p> <p>Temperature was too low in some positions of thermal cycler. Perform a set of control reactions to determine whether certain positions in the thermal cycler give low yields.</p> <p>Top of thermal cycler was open. The top must be closed for correct heating and cooling.</p> <p>Mineral oil problem. The reaction must be overlaid with high-quality, nuclease-free light mineral oil when using thermal cyclers without a heated lid. Do not use autoclaved mineral oil.</p> <p>Degraded nucleotides or primers. Keep nucleotides and primers frozen in aliquots, thaw quickly and keep on ice once thawed. Avoid multiple freeze-thaw cycles</p> <p>Pipetting errors. Make sure that students understand how to set quantities on pipettors and how pipettors work (e.g., students should understand the volumes delivered at the different stops and positions).</p>
Multiple, nonspecific amplification products	<p>Contamination by another target DNA.</p> <ul style="list-style-type: none"> <li>•Use positive displacement pipettes or aerosol-resistant tips to reduce cross-contamination during pipetting.</li> <li>•Use a separate work area and pipettes for pre- and postamplification.</li> <li>•Wear gloves and change them often.</li> </ul>

## V. Supplier and Ordering Information

### V.A. Ordering Information

Product	Cat.#
GoTaq® Hot Start DNA Polymerase* (U.S. catalog number)	M5001
dNTP Mix*	M1511
Nuclease-Free Water*	P1193
Mineral Oil	DY1151
Promega 10 Barrier Tips	A1491
Promega 20 Barrier Tips	A1521
Promega 100 Barrier Tips	A1541
Mbol Restriction Enzyme*	R6711
Rsal Restriction Enzyme*	R6371
BenchTop PCR Markers*	G7531
TBE Buffer, 10X	V4251
Agarose, LMP, Preparative Grade for Small Fragments (10 to 1,000 bp)	V3841
Agarose, LE, Analytical Grade	V3121

\*For Laboratory Use.

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### V.B. Materials Required from Vendors Other Than Promega

Product	Vendor	Cat.#
Genomic DNA C187G homozygote	Coriell	NA14620
Genomic DNA C187G/wt (heterozygote)	Coriell	NA14690
Genomic DNA wildtype with respect to nucleotides 187 and 845	Coriell	NA14656
Genomic DNA G845A homozygote	Coriell	NA14686
Genomic DNA compound heterozygote (C187G, G845A)	Coriell	NA14650

Coriell Institute for Medical Research sells genomic DNA from B-cells obtained from affected and unaffected individuals from families with a history of hereditary hemochromatosis. Background for each of these genomic DNA preparations is available on their web site at: <http://ccr.coriell.org/>

Alternatively, if you have a relationship with a major research hospital you may be able to obtain genomic DNA preparations from the pathology laboratory.

### Primer Sequences and Concentrations:

#### Exon 2

5' ACATGGTTAAGGCCTGTTGC 3' Final concentration in PCR: 50 pmol

5' GCCACATCTGGCTTGAAATT 3' Final concentration in PCR: 50 pmol

#### Exon 4

5' TGGCAAGGGTAAACAGATCC 3' Final concentration in PCR: 50 pmol

5' CTCAGGCACTCCTCTCAACC 3' Final concentration in PCR: 50 pmol

Primers can be synthesized by a variety of microchemical facilities including Integrated DNA Technologies (<http://www.idtdna.com>).



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