Sacace Molecular Genetics

Hemochromatosis Real-TM

Handbook

Real Time PCR kit for detection of Single Nucleotide Polymorphisms (SNPs) in HFE gene: 187 C>G (H63D), 193 A>T (S65C), 845 G>A (C282Y) with melting curve analysis

Σ **48**

REF HM-1-50FRT

NAME

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INTRODUCTION

A single nucleotide polymorphism (SNP pronounced "snip") is a DNA polymorphisms at the level of a single nucleotide, a single base mutation in DNA. SNPs are ‘conserved’ across the genome and represent the most simple form and most common source of genetic polymorphism in the human genome: 90% of all human DNA polymorphisms are associated with SNPs and variation frequency is about 1 every 1000bp in the human genome (Sachidanandam et al.,2001).

The SNPs in the genome can affect gene functions, protein structure or expression and for these reasons they are used as markers in genetic disease studies (Kim & Mishra, 2007).

It’s sometimes possible to correlate a SNP with a particular trait or disease: susceptibility to disease may also be described as an ‘unfortunate trait’ that can be assessed checking if the mutated (unfortunate) polymorphism is carried in both alleles.

SNPs testing can be applied to:

- Diagnostics / risk profiling
- Drug response prediction
- Gene function identification

Several SNPs have been associated to genetic susceptibility to different diseases and disorders like for example:

- Hypertension
- Fibrinolysis
- Myocardial infarction
- Ischemic stroke
- Cancer
- Metabolic disorders

In order to perform SNP genotyping, two specific probes labeled with different dyes are used, the first for the wild type allele and the second for the mutant allele. If the assay results in the emission of only the first fluorescent color, then the individual is homozygous wild type at that locus. If the assay results in the emission of only the second fluorescent color, then the individual is homozygous mutant. And finally, if both fluorescent colors are produced, then the individual is heterozygous.

INTENDED USE

Sacace Molecular Genetics Kits are intended for detection and allelic discrimination of genetic polymorphisms associated with inherited susceptibility to increased risk of disease, or to different response to drugs. The Hemochromatosis Real-TM genotyping Kit is intended for detection and allelic discrimination of mutations associated with hereditary hemochromatosis, an autosomal recessive iron metabolism disorder. (OMIM #235200). The genotype information obtained from the assay can be used to diagnose the probability of disease progression.

PRINCIPLE OF ASSAY

Real-time PCR followed by melting curve analysis.

Both alleles are detected simultaneously in single tube. PCR-Mix contains an internal control (IC). IC assures there is sufficient DNA in the sample to exclude the possibility of false results.

MATERIALS PROVIDED

Content:

Reagent	Quantity	
PCR-mix		
1. HFE: 187 C>G (H63D)	960 µl	1 tube
2. HFE: 193 A>T (S65C)	960 µl	1 tube
3. HFE: 845 G>A (C282Y)	960 µl	1 tube
PCR-buffer	1.44 ml	1 tube
Taq-AT-polymerase	72 µl	1 tube
Mineral oil	2.88 ml	1 tube

Dye label detection channels corresponding to allelic variants and IC

PCR-mix	Fam	Hex	Rox	Cy5	Cy5.5
HFE: 187 C>G (H63D)	C	G	-	IC	-
HFE: 193 A>T (S65C)	A	T	-	IC	-
HFE: 845 G>A (C282Y)	G	A	-	IC	-

MATERIALS REQUIRED BUT NOT PROVIDED

Zone 1: sample preparation

- DNA extraction kit
- Biological cabinet
- Desktop microcentrifuge for “eppendorf” type tubes
- Dry heat block
- Vortex mixer
- Pipettes
- Sterile pipette tips with filters
- 1,5 ml polypropylene sterile tubes
- Biohazard waste container
- Refrigerator, Freezer

Zone 2: Real Time amplification

- Real Time Thermal cycler
- Workstation
- Pipettes (adjustable)
- Sterile pipette tips with filters
- Vortex mixer
- Desktop centrifuge with rotor for 1,5/2,0 ml tubes
- Freezer, refrigerator
- Tube racks

STORAGE INSTRUCTIONS

Sacace Molecular Genetics kits must be stored at 2-8°C. The Taq-AT-polymerase must be stored at -20°C over the storage period. The kits can be shipped at 2-8°C and stored as indicated immediately on receipt.

STABILITY

Sacace Molecular Genetics kits are stable up to the expiration date indicated on the kit label. The product will maintain performance through the control date printed on the label. Exposure to light, heat or humidity may affect the shelf life of some of the kit components and should be avoided. Repeated thawing and freezing of these reagents should be avoided, as this may reduce the sensitivity. Components stored under conditions other than those stated on the labels may not perform properly and may adversely affect the assay results.

QUALITY CONTROL

In accordance with Sacace's ISO 13485-Certified Quality Management System, each lot is tested against predetermined specifications to ensure consistent product quality.

WARNINGS AND PRECAUTIONS

The user should always pay attention to the following:

- Use sterile pipette tips with aerosol barriers and use new tip for every procedure.
- Store extracted positive material (samples, controls and amplicons) away from all other reagents and add it to the reaction mix in a separate area.
- Use disposable gloves, laboratory coats and eye protection when handling specimens and reagents. Thoroughly wash hands afterwards.
- Do not eat, drink, smoke, apply cosmetics, or handle contact lenses in laboratory work areas.
- Do not use a kit after its expiration date.
- Dispose of all specimens and unused reagents in accordance with local authorities' regulations.
- Specimens should be considered potentially infectious and handled in a biological cabinet in accordance with appropriate biosafety practices.
- Clean and disinfect all sample or reagent spills using a disinfectant such as 0.5% sodium hypochlorite, or other suitable disinfectant.
- Avoid sample or reagent contact with the skin, eyes, and mucous membranes. If skin, eyes, or mucous membranes come into contact, rinse immediately with water and seek medical advice immediately.
- Material Safety Data Sheets (MSDS) are available on request.
- Use of this product should be limited to personnel trained in the techniques of DNA amplification.
- The laboratory process must be one-directional, it should begin in the Extraction Area and then move to the Amplification and Detection Areas. Do not return samples, equipment and reagents to the area in which the previous step was performed.

PRODUCT USE LIMITATIONS

All reagents may exclusively be used in in vitro diagnostics. Use of this product should be limited to personnel trained in the techniques of DNA amplification (UNI EN ISO 18113-2:2012). Strict compliance with the user manual is required for optimal PCR results. Attention should be paid to expiration dates printed on the box and labels of all components. Do not use a kit after its expiration date.

SAMPLE COLLECTION, STORAGE AND TRANSPORT

Sacace Molecular Genetics Kits can analyze genomic DNA extracted from:

- *whole blood* collected in EDTA tubes;
- *Buccal swab*: insert the swab into the nuclease-free 1,5 ml tube and add 0,2 ml of Transport medium. Vigorously agitate swabs in medium for 15-20 sec.

Specimens can be stored at +2-8°C for no longer than 24 hours, or freeze at -20°C to -80°C.

Transportation of clinical specimens must comply with country, federal, state and local regulations for the transport of etiologic agents.

DNA ISOLATION

Any commercial RNA/DNA isolation kit, if IVD-CE validated for the specimen types indicated herein at the "SAMPLE COLLECTION, STORAGE AND TRANSPORT" paragraph, could be used.

⇒ **Genomic column DNA Express** – spin column extraction kit (Sacace, REF K-1-1/E)

⇒ **SaMag Blood DNA extraction kit** (Sacace, REF SM001);

⇒ **QIAamp DNA Blood mini kit** (Qiagen, REF 51104);

⇒ **DNA-Sorb-A** (Sacace, REF K-1-1/A) for buccal swab;

Please carry out DNA extraction according to the manufacturer's instruction.

PROTOCOL

Sacace Molecular Genetics kits do not include reagents required for sample preparation and DNA extraction. Blood samples and biological materials must be processed by using the recommended kits or those with similar performances of quality and quantity of extracted DNA. Use of blood samples collected in tubes containing heparin is not recommended.

The analysis of the genomic DNA specimens using **Sacace Molecular Genetics** kits includes the following stages:

1. Preparing the Real Time PCR;
2. Real Time PCR analysis;
3. Data analysis with the software of Real Time PCR instrument;
4. Results analysis and conclusions.

EXPERIMENTAL PROTOCOL

1. Preparing your PCR

Important! The quantity of DNA to be analyzed must be greater than or equal to 1.0 ng per reaction (the Cp parameter for IC must not be less than 32). The violation of this requirement will affect the validity of analysis and void the manufacturer guarantee.

1.1. Mark the required number of 0.2 ml PCR-tubes for each polymorphism to be tested (one tube for each sample to be tested and one extra for negative control “C-“).

Example:

For simultaneous testing of 5 samples in one PCR run, mark 15 tubes for samples and 3 tubes for “C-“. The resulting number of tubes is 18.

	HFE: 187 C>G (H63D)	HFE: 193 A>T (S65C)	HFE: 845 G>A (C282Y)
Sample 1	√	√	√
Sample 2	√	√	√
Sample 3	√	√	√
Sample 4	√	√	√
Sample 5	√	√	√
K-	√	√	√

1.2. Vortex the tubes for 3-5 seconds, then spin for 1-3 seconds to precipitate drops.

1.3. Add 20 µl of corresponding PCR-mix into the marked tubes (use a new pipette tip for each type of PCR-mix).

1.4. Vortex the tubes with PCR-buffer and Taq-AT-polymerase for 3-5 seconds, then spin for 1-3 seconds to collect the drops.

Important! Taq-AT-polymerase must be stored at -20°C. Remove from freezer just prior to use and place on ice.

1.5. Prepare the mixture of PCR-buffer and Taq-AT-polymerase. Add into one tube:

- 10× (N+1) µl of PCR-buffer,
- 0.5 × (N+1) µl of Taq-AT-polymerase,

N — number of the marked tubes including “C-”.

1.6. Vortex the tube for 3-5 seconds, then spin for 1-3 seconds to collect the drops.

Important! The mixture of PCR-buffer and Taq-AT-polymerase must be prepared just prior to use.

1.7. Add 10 µl of PCR-buffer and Taq-AT-polymerase mixture into each PCR-tube.

Important! Follow the steps listed in pp 1.8-1.13 within two hours after addition of Taq-AT-polymerase solution to PCR-mix.

1.8. Add one drop (20 µl at avg.) of mineral oil to each PCR-tube. Close the tubes tightly.

1.9 Open only one tube of sample at a time, then close it prior to addition of the next sample to avoid contamination. Use filtered pipette tips to add the samples. Add 5 µl of DNA sample into corresponding PCR-tubes (3 for each sample).

1.10. Add 5.0 µl of negative control ("C-") which passed all steps of DNA extraction procedure into corresponding tubes.

1.11. Spin the tubes for 1–3 seconds to collect drops.

1.12. Set the tubes in the Real-time PCR instrument.

1.13. Launch the Real-Time_PCR application in "Device handling" mode. Upload the ini file «Hemochromatosis.ini» before the first run. Add «HFE_187_C>G », «HFE_193_A>T », «HFE_845_G>A » tests or use multiple test mode in subsequent runs. Specify the number and types of samples including negative controls. Define position of strips in software interface according to position they were set in the thermoblock (see p. 1.12). Run PCR.

Note. The type of the negative control tubes must be specified as "Sample"

2. The PCR and post-PCR analysis are operated by software and held in automatic mode. In samples containing sufficient DNA for correct analysis, the software defines the genotype. The samples containing an insufficient quantity of DNA (less than 1.0 ng per reaction or Cp≤32) will be analyzed as invalid.

Appendix.

Table 1.
Genotypes and melting temperatures (only for Sacycler-96 instrument)

Polymorphism	Homozygote			Homozygote			Heterozygote		
	Genotype	Fam, °C	Hex, °C	Genotype	Fam, °C	Hex, °C	Genotype	Fam, °C	Hex, °C
HFE: 187 C>G (H63D)	CC	49,2	38,4	GG	34,1	49,3	CG	48,8	48,8
HFE: 193 A>T (S65C)	AA	61,5	52,4	TT	54,0	62,1	AT	60,3	60,5
HFE: 845 G>A (C282Y)	GG	57,3	49,3	AA	50,3	56,1	GA	57,2	55,3

KEY TO SYMBOLS USED

REF	List Number		Caution!
LOT	Lot Number		Contains sufficient for <n> tests
RUO	For *Research* Use Only	VER	Version
	Store at	**NCA**	Negative Control of Amplification
	Manufacturer	**NCE**	Negative control of Extraction
	Consult instructions for use	**C+**	Positive Control of Amplification
	Expiration Date	**IC**	Internal Control

* SaCycler™ is a registered trademark of Sacace Biotechnologies

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