Primer Design Ltd

Z-Path-GT-HFE

Detection of Haemochromatosis (HFE gene: C282Y, H63D, S65C)

Kit version: 1

SNPsig® real-time PCR mutation detection/allelic discrimination kit

50 tests

GENESIG

Kits by Primerdesign

For general laboratory and research use only

Kit contents

- **1x HFE-C282Y primer/probe mix (50 reactions, BROWN)**
  Wild-type ROX labelled, mutant VIC labelled
- **1x HFE-H63D primer/probe mix (50 reactions, BROWN)**
  Wild-type ROX labelled, mutant VIC labelled
- **1x HFE-S65C primer/probe mix (50 reactions, BROWN)**
  Wild-type ROX labelled, mutant VIC labelled
- **1x HFE-C282Y Wild-type positive control template (RED)**
- **1x HFE-C282Y Mutant positive control template (RED)**
- **1x HFE-H63D Wild-type positive control template (RED)**
- **1x HFE-H63D Mutant positive control template (RED)**
- **1x HFE-S65C Wild-type positive control template (RED)**
- **1x HFE-S65C Mutant positive control template (RED)**
- **1x RNase/DNase free water (WHITE)**
  for resuspension of primer/probe mixes
- **6x Template preparation buffer (YELLOW)**
  for resuspension and dilution of positive control templates

Reagents and equipment to be supplied by the user

Real-time PCR Instrument
Must be able to read fluorescence through the VIC and ROX channels.

Extraction kit
This kit is recommended for use with genesig® Easy DNA/RNA extraction kit or exsig®Mag. However, it is designed to work well with all processes that yield high-quality nucleic acid with minimal PCR inhibitors.

oasig® lyophilised, PrecisionPLUS® or PrecisionFAST® 2X qPCR Master Mix
This kit is designed to work well with oasig®, PrecisionPLUS® or PrecisionFAST® 2X qPCR Master Mix. Primer Design Ltd can only guarantee accurate genotyping results when oasig®, PrecisionPLUS® or PrecisionFAST® Master Mix is used.

Pipettors and filter tips

Vortex and centrifuge

1.5 ml microtubes

qPCR plates or reaction tubes

Principles of the test

Genotyping by real-time PCR using hydrolysis probes

The genotyping primer/probe mixes contain two labelled probes homologous to the two genotypes under investigation. During qPCR amplification of the target DNA, the probes will compete for binding across the variant region. The probe that is 100% homologous to the DNA binding site will preferentially bind and give a fluorescent signal as PCR proceeds. It follows that the wild-type sequence will give a strong amplification plot through one channel, whilst giving a very weak signal through the alternative channel. Homozygous variant samples will give an exactly inverse result. Heterozygote samples contain both probe binding sites on each of the two alleles and therefore give an intermediate signal through both channels. The three possible genotypes can be resolved by comparing end point fluorescence. Most hardware platforms can perform this analysis automatically.

Positive controls

The kit contains positive control templates for each of the genotypes. These can be run as parallel samples to give control signals for each genotype. In order to provide good positive control data that is directly relevant to the samples being tested, the control DNA should be used at a similar copy number to the sample DNA. This protocol contains guidelines for varied dilution of the positive control templates depending on the level of genomic DNA added to each sample. The optimum sample DNA level is 5ng. Positive control templates are a potential contamination risk to subsequent tests and therefore must be handled carefully.

Negative control

To validate any positive findings a negative control reaction should be included every time the kit is used. For this reaction the RNase/DNase free water should be used instead of template. A negative result indicates that the reagents have not become contaminated while setting up the run. It is also known as a No Template Control or NTC. If a positive result is obtained for the negative control, the sample test results should be ignored and the test repeated. Possible sources of contamination should first be explored and removed.

Thermocycling parameters

The optimum cycling parameters for Primer Design Ltd genotyping kits is a two-step cycling procedure. The first set of cycles are designed for optimal PCR amplification. Where the test samples contain very low levels of input gDNA (<1ng), additional first stage cycles can be added. The second set of cycles are optimal for probe discrimination and therefore cycle at a higher temperature. Fluorescent data is only collected during the second set of amplification cycles. These parameters can be programmed into most machines, but it should be noted that the reported Cycle threshold values produced by this program will be lower than usual and that this is expected.

Master mix compatibility

PrecisionPLUS® Master Mix, PrecisionFAST® Master Mix and oasig® Master Mix contain the enzyme, nucleotides, buffers and salts at precisely the correct concentration for this application. The annealing temperatures of the primer and probe have been carefully calibrated and any change in the reaction buffer can significantly alter the performance of the assay. For this reason, Primer Design Ltd can only guarantee accurate genotyping results when PrecisionPLUS®, PrecisionFAST® or oasig® Master Mix is used.

Resuspension protocol

To minimize the risk of contamination with foreign DNA, we recommend that all pipetting is performed in a PCR clean environment. Ideally this would be a designated PCR lab or PCR cabinet. Filter tips are recommended for all pipetting steps. The positive control templates are a significant contamination risk and should therefore be pipetted after negative control and sample wells.

1. **Pulse-spin each tube in a centrifuge before opening.**
   This will ensure lyophilised primer probe mix or template is in the base of the tube and is not lost upon opening the tube.

2. **Resuspend the primer/probe mixes in the RNase/DNase free water supplied, according to the table below.**
   To ensure complete resuspension, vortex the tube thoroughly.

Component - resuspend in water	Volume
Pre-PCR pack	
HFE-C282Y primer/probe mix (**BROWN**)	55 µl
HFE-H63D primer/probe mix (**BROWN**)	55 µl
HFE-S65C primer/probe mix (**BROWN**)	55 µl

3. **Resuspend the positive control templates in the template preparation buffer supplied, according to the table below:**
   To ensure complete resuspension, vortex each tube thoroughly.

Component - resuspend in template preparation buffer	Volume
Post-PCR heat-sealed foil	
HFE-C282Y Wild-type Positive Control Template (**RED**) *	500 µl
HFE-C282Y Mutant Positive Control Template (**RED**) *	500 µl
HFE-H63D Wild-type Positive Control Template (**RED**) *	500 µl
HFE-H63D Mutant Positive Control Template (**RED**) *	500 µl
HFE-S65C Wild-type Positive Control Template (**RED**) *	500 µl
HFE-S65C Mutant Positive Control Template (**RED**) *	500 µl

* This component contains high copy number template and is a VERY significant contamination risk. It must be opened and handled in a separate laboratory environment, away from the other components.

qPCR detection protocol

1. **For each DNA sample prepare a reaction mix according to the table below.**
   Each genotyping assay (C282Y, H63D or S65C) needs to be set-up and analysed individually and cannot be multiplexed.
   Include sufficient reactions for positive and negative controls for each genotyping assay.

Component	Volume
oasig®, PrecisionPLUS® or PrecisionFAST® 2X qPCR Master Mix	10 µl
HFE-C282Y or HFE-H63D or HFE-S65C primer/probe mix (**BROWN**)	1 µl
RNase/DNase free water (**WHITE**)	4 µl
Final Volume	**15 µl**

2. **Pipette 15µl of these mixes into each well according to your qPCR experimental plate set up.**

3. **Pipette 5µl of DNA template into each well, according to your experimental plate set up.**
   To obtain a strong signal, the recommended input DNA concentration is 0.2-2ng/µl (1-10ng in total), with the ideal total being 5ng. For negative control wells use 5µl of RNase/DNase free water. The final volume in each well is 20µl.

4. **Dilute positive control templates in template preparation buffer.**
   It is important that the input copy number for the positive control templates is matched to the amount of sample DNA being added to each reaction. The positive control templates must be diluted according to the table below:

Sample Input DNA	Positive control dilution factor
10ng	1:25
5ng	1:50
1ng	1:250

5. **Pipette 5µl of each positive control according to your experimental plate set up.**

qPCR Amplification Protocol

For machines that can be programmed to include 2 cycling stages, the following protocol is recommended for optimum resolution between genotypes.

If using oasig® Master Mix, DO NOT add ROX to the master mix as a passive reference. If using a machine that uses ROX as a passive reference, then the passive reference must be turned off or set to “none” indicating no passive reference.

Protocol for oasig®, PrecisionPLUS® or PrecisionFAST® 2X qPCR Master Mix:

	Step	Time	Temp
	Enzyme activation	2 min	95 °C
Cycling x10**	Denaturation	10 s	95 °C
	Extension	60 s	60 °C
Cycling x35	Denaturation	10 s	95 °C
	Extension DATA COLLECTION *	60 s	66 °C

* Fluorogenic data should be collected during this step through the ROX and VIC channels
** Where the sample amount is 1ng or less per well, add 5 cycles to the first stage (15 cycles total)

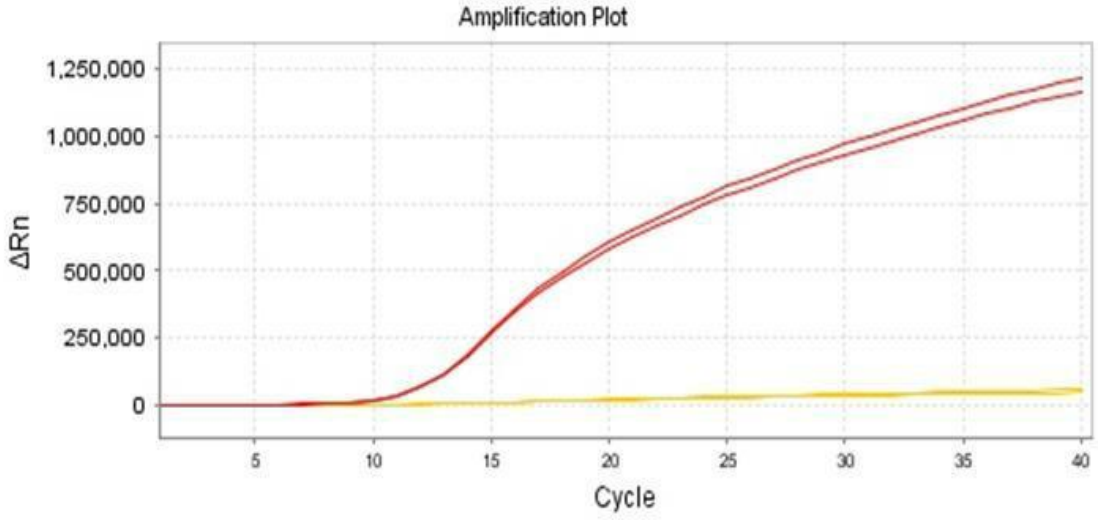
Interpretation of results

Each genotyping assay (C282Y, H63D or S65C) needs to be analysed individually. The wild-type probe is labelled to read through the ROX channel, whilst the mutant probe is labelled to read through the VIC channel. On wild-type sequences, the ROX channel will give a strong amplification plot and the VIC channel will give very low or no signal. The signals are reversed on homozygous variant samples. Heterozygote samples will give an intermediate signal through both ROX and VIC channels.

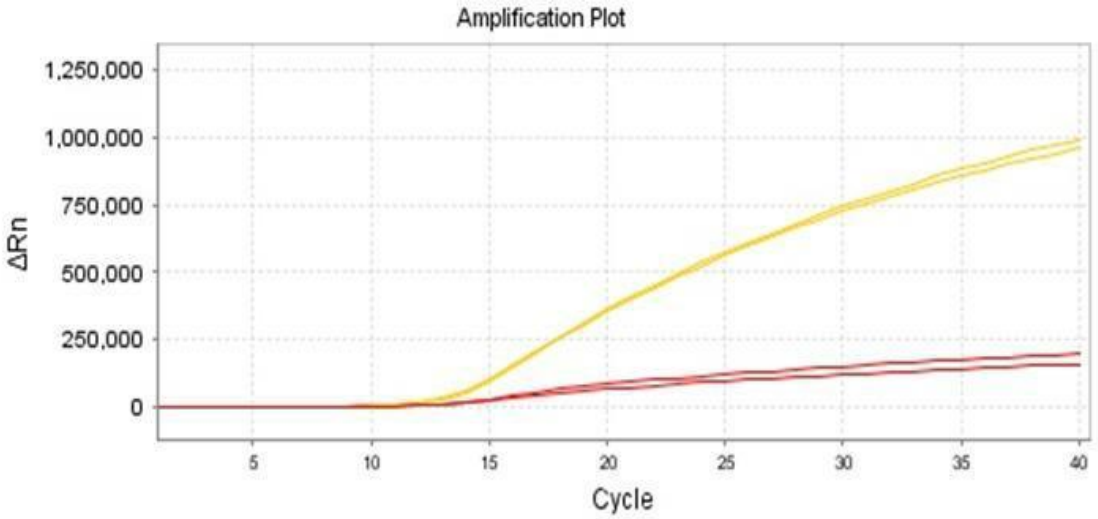
The genotype (wild-type, heterozygous, or homozygous variant) of each sample is calculated by comparing the ratio of signals between the two channels (ROX and VIC) for each genotyping assay (C282Y, H63D and S65C) individually.

Sample data

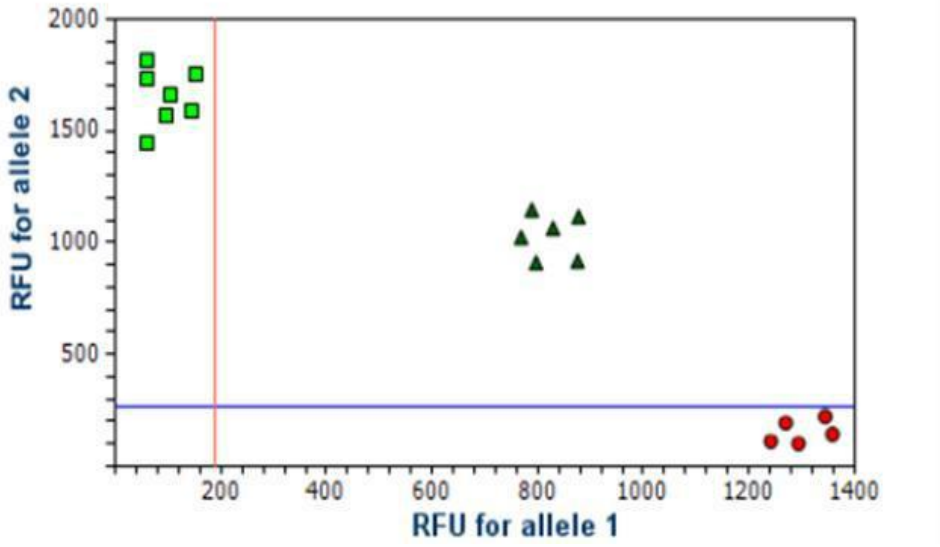
Wild Type sample (WT signal: ROX (red), Mutant signal: VIC (yellow))



Variant DNA sample (WT signal: ROX (red), Mutant signal: VIC (yellow))



The raw data above can best be visualised by using a cluster analysis; plotting the end point fluorescence data from the ROX channel on one axis and the end point fluorescence data from the VIC channel on the other axis. Most qPCR software platforms will perform this analysis automatically, so follow the manufacturer's instructions for your software. The data are quickly resolved into clusters corresponding to the wild-type, heterozygote, and homozygous variant samples.



Kit storage and stability

This kit is stable for shipping at ambient temperature but should be stored at -20°C upon arrival. Once the lyophilised components have been resuspended, they should not be exposed to temperatures above -20°C for longer than 30 minutes at a time and unnecessary repeated freeze/thawing should be avoided. The kit is stable for six months from the date of resuspension under these circumstances. Primer Design Ltd does not recommend using the kit after the expiry date stated on the pack.

Suitable sample material

All kinds of sample material suited for PCR amplification can be used. Please ensure the samples are suitable in terms of purity, concentration, and DNA integrity.

Notices and disclaimers

This product is developed, designed and sold for research purposes only. It is not intended for human diagnostic or drug purposes or to be administered to humans unless clearly expressed for that purpose by the Food and Drug Administration in the USA or the appropriate regulatory authorities in the country of use. During the warranty period, Primer Design Ltd genesig® detection kits allow precise and reproducible data recovery combined with excellent sensitivity. For data obtained by violation to the general GLP guidelines and the manufacturer's recommendations the right to claim under guarantee is expired. PCR is a proprietary technology covered by several US and foreign patents. These patents are owned by Roche Molecular Systems Inc. and have been sub-licensed by PE Corporation in certain fields. Depending on your specific application you may need a license from Roche or PE to practice PCR. Additional information on purchasing licenses to practice the PCR process may be obtained by contacting the Director of Licensing at Roche Molecular Systems, 1145 Atlantic Avenue, Alameda, CA 94501 or Applied Biosystems business group of the Applera Corporation, 850 Lincoln Centre Drive, Foster City, CA 94404. In addition, the 5' nuclease assay and other homogeneous amplification methods used in connection with the PCR process may be covered by U.S. Patents 5,210,015 and 5,487,972, owned by Roche Molecular Systems, Inc, and by U.S. Patent 5,538,848, owned by The Perkin-Elmer Corporation.

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