LightMix® in-vitro diagnostics kit HFE H63D S65C C282Y

Cat.-No.: 40-0340-32

Detection of the HFE gene DNA variations encoding for p.H63D, p.S65C and p.C282Y

for use with the

Roche Diagnostics LightCycler® Instruments

Reagents for 96 reactions

Upon arrival:

Store Premixed PCR reagents and Controls protected from light at room temperature or cooled (do not freeze)

Store FastStart DNA Master HybProbe reagents frozen (-20°C) (if included)

Manual version V160626

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1. Product Information

1.1 Contents: LightMix® Kit HFE H63D S65C C282Y

Lyophilized premixed PCR reagents

⚠ Store cooled or at room temperature (4-25°C) in the dark

	Cap color	Label	Description content	Reaction / Tube status	Total
3 x	Red	PSR	Parameter-Specific Reagent (PSR) contains premixed and lyophilized primers and probes for 32 reactions each. <0,01pg unlabeled oligonucleotides <0,01pg SimpleProbe® 519 labeled probe (H63D, S65C); <0,01pg LightCyclerRed 640 labeled probe (C282); <0,01pg Fluorescein labeled probe (C282Y).	32 reactions green-blue pellet lyophilized	96 rxs

Standards (Control DNA)

⚠ Store cooled or at room temperature (4-25°C) in the dark

	Cap color	Label	Description content	Reaction Tube status	Total
1 x	Yellow	Standard 1	Positive Control HFE Mutant 282Y and wild type for all other positions <0,01pg plasmid target (synthetic) [about 10E4 genome equivalents]	32 reactions blue pellet lyophilized	32 rxs
1 x	Yellow	Standard 2	Positive Control HFE Mutant 63D and wild type for all other positions <0,01pg plasmid target (synthetic) [about 10E4 genome equivalents]	32 reactions blue pellet lyophilized	32 rxs
1 x	Yellow	Standard 3	Positive Control HFE Mutant 65C and wild type for all other positions <0,01pg plasmid target (synthetic) [about 10E4 genome equivalents]	32 reactions blue pellet lyophilized	32 rxs

Polymerase Mix: LightCycler® FastStart DNA Master HybProbe

⚠ Store at -20°C upon arrival

FastStart DNA Master HybProbe is included in kits supplied directly by TIB MOLBIOL for customers in Central Europe only [1].
The FastStart DNA Master HybProbe is not included in HFE kits supplied through Roche Diagnostics or its local distributor.

	Cap color	Label	Description content	Reaction Tube storage	Total
3 x	Red	1a	LightCycler® FastStart Enzyme	32 reactions frozen	96 rxs
3 x	White	1b	LightCycler® FastStart Reaction Mix HybProbe, 10x conc.	32 reactions frozen	96 rxs
3 x[2]	Color-less	Water	H_2O PCR grade	frozen	96 rxs
3 x[2]	Blue	$MgCl_2$	$MgCl_2$, 25 mM	32 reactions frozen	96 rxs

1) FastStart DNA Master HybProbe is shipped by TIB MOLBIOL at ambient temperature.
2) FastStart DNA Master HybProbe supplied through Roche Diagnostics contains only 2 tubes of H_2O and 1 tube of $MgCl_2$, nevertheless the quantity provided is sufficient for the use described for this kit.

1.2 Intended Use

This kit allows to detect common mutations in the HFE gene (OMIM: 235200) in genomic human DNA from nucleic acid extracts obtained from peripheral blood.

Hemochromatosis type 1 (HFE1) is hereditary and linked to various mutations in the HFE gene. Hemochromatosis results in multi-organ dysfunction caused by increased iron deposition.

This product is intended to help clinicians to analyze the genetical background of patients with hepatopathy of unknown ethiology, patients with liver cirrhosis, diabetes mellitus, bronze skin pigmentation in connection with elevated serum iron concentrations, elevated transferrin saturation and elevated serum ferritin levels.

The present test can be performed in addition or after a biochemical assay for iron overload using transferring saturation.

Results obtained using this kit are not intended to be the only basis for any therapy decision. The patient´s mutation status should be considered alongside other disease factors.

Note: The performance of the assay can be guaranteed only when used with Roche LightCycler® Instruments or cobas z 480 Analyzer (see 1.3.2 for details).

The present product is an *in-vitro* diagnostic device, which must be used by qualified personnel only.

1.3 Specifications

The *LightMix® Kit HFE H63D S65C C282Y* is an *in-vitro* diagnostic test which allows to detect and identify the clinical relevant single nucleotide polymorphism variants p.H63D, p.S65C and p.C282Y in the HFE gene.

The presence of other gene variants may interfere with the test. The following exon 4 rare variants will be also detected (see 3.1 and 7.7.3) but not 'identified' :

p.T281M	c.842C>T	no dbSNP	≈ ΔTm -15°C
p.T281T	c.843G>A	rs369354634	
p.Q283P	c.848A>C	rs111033563	≈ ΔTm - 8°C

1.3.1 Clinical Samples

The test requires 5 µl of purified genomic DNA in an aqueous solution extracted from clinical specimen (peripheral blood), containing from 3 ng/µl to 100 ng/µl of genomic DNA (15 ng – 500 ng total amount), which is the tenfold amount of the minimum amount working technically (1.5 ng, see section 3.3), with the DNA concentration determined by UV spectrophotometry (1 OD_{260} = 50 µg DNA/ml).

1.3.2 Instruments, Software and Productivity

One kit contains reagents for 96 reactions performed in a 20 µl volume.
Each run requires including three standards and one negative control.
The table below summarizes some features of the kit :

LightCycler® Instrument	Software Version (or higher)	Run Time (approx.)	Max Samples per run (2)	Maximum Productivity of the kit (3)	Minimum Productivity of the kit (4)
LC 1.2	4.10 (1)	60 min	28 + 4 ctrl.	81	18
LC 1.5	4.10 (1)	60 min	28 + 4 ctrl.	81	18
LC 2.0	4.05	60 min	28 + 4 ctrl.	81	18
LC480 (96 wells)	1.5	100 min	92 + 4 ctrl.	89	18
LC480 (384 wells)	1.5	100 min	380(5) + 4 ctrl.	89	18
z 480 (open channel)	1.5	100 min	92 + 4 ctrl.	89	18
Nano	1.0 (6)	60 min	28 + 4 ctrl.	84	18

1 Running the test with the LightCycler® 1.2 or 1.5 Instruments with the software version 3.5 yields comparable results. Instructions for software 3.5 programming, data analysis and interpretation of results are not described in this manual. **Upgrade to version 4.10 or higher when possible.** LightCycler® software 3.5.3 does not contain the automatic genotyping module; equivalent results can be obtained by trained personnel which must analyze each sample manually.
2 Each run must include 3 standards and one No-Target Control (NTC) for a total of 4 control reactions.
3 The first run of the kit requires including 7 controls (instead of 4) to teach the genotyping module. The maximum number of samples that can be processed is reduced accordingly. Depending on local regulations, all 7 genotyping controls may have to be included in each run, reducing the total number of patient's samples that can be analyzed.
4 Calculated considering a single clinical sample analyzed in each run.
5 It requires using four kits.
6 Nano LightCycler® software 1.0 does not contain the automatic genotyping module, therefore it is not necessary to add the Genotyping Standards; equivalent results can be obtained by trained personnel which must analyze each sample manually.

1.4 Storage and Stability

Note the **different storage conditions** for reagents and polymerase mix!

Storage Conditions

Reagents and Controls:

Store the lyophilized reagents (PSR and Standards) protected from light and at room temperature or cooled (4°C - 25°C).
Do not freeze these dried reagents. Expiration date is printed on the kit label.

Polymerase mix :

Store the LightCycler® FastStart DNA Master HybProbe at -15°C to -25°C.
See expiration date on the polymerase tube label.

Shipping:

Products are shipped at ambient temperature. Transport stability of reagents and enzyme components have been tested under shipping conditions.

2. Additional Devices and Reagents

2.1 Required

	TIB Molbiol
LightMix® Kit – Color Compensation HybProbe (not required for LightCycler® Nano Instrument)	Cat.-No. 40-0318-00
LightCycler® 2.0 Instrument	**Roche Diagnostics**
LightCycler® 2.0 Instrument	Cat.-No. 12 011 468 001
LightCycler® Software Version 4.05 or	Discontinued
LightCycler® Software Version 4.10 or higher	Cat.-No. 04 779 584 001
LightCycler® Capillaries (20 µl)	Cat.-No. 11 909 339 001
Or	
LightCycler® 480 Instruments	**Roche Diagnostics**
LightCycler® 480 Instrument (model I)	Discontinued
LightCycler® 480 II Instrument	Cat.-No. 05 015 278 001
cobas z 480 Analyzer	Cat.-No. 05 200 881 001
LightCycler® Software Version 1.5 or higher	Cat.-No. 04 994 884 001
LightCycler® 480 Multiwell Plate 96 white or	Cat.-No. 04 729 692 001
LightCycler® 480 Multiwell Plate 384 white	Cat.-No. 04 729 749 001
Or	
LightCycler® Nano Instrument	**Roche Diagnostics**
LightCycler® Nano Instrument	Cat.-No. 06 407 773 001
LightCycler® Software Version 1.0 or higher	Included with Instrument
LightCycler® Nano tubes	Cat.-No. 06 327 672 001
Or	
LightCycler® 1.x Instruments	**Roche Diagnostics**
LightCycler® 1.2 and 1.5 Instruments	Discontinued
LightCycler® Software Version 4.10	Cat.-No. 04 779 584 001
LightCycler® Capillaries (20 µl)	Cat.-No. 11 909 339 001

2.2 Optional

Instruments:

LC Carousel Centrifuge 2.0 (230 Volt)	Cat.-No. 03 709 582 001
Capping Tool	Cat.-No. 03 357 317 001

2.3 Sample Preparation

Manual Sample Preparation:	**Roche Diagnostics**
High Pure PCR Template Preparation Kit	Cat.-No. 11 796 828 001
Nuclease-free PCR grade water	any supplier
Ethanol p.a.	any supplier
Isopropanol p.a.	any supplier
Automatic Sample Preparation:	**Roche Diagnostics**
MagNA Pure Instrument	Discontinued
MagNA Pure LC DNA Isolation Kit I	Cat.-No. 03 003 990 001
MagNA Pure 2.0 Instrument	Cat.-No. 05 197 686 001
MagNA Pure LC DNA Isolation Kit I	Cat.-No. 03 003 990 001
MagNA Pure Compact Instrument	Cat.-No. 03 731 146 001
MagNA Pure Compact Nucleic Acid Isolation Kit I	Cat.-No. 03 730 964 001
MagNA Pure 96 Instrument	Cat.-No. 05 195 322 001
MagNA Pure 96 DNA and Viral NA Small Volume Kit	Cat.-No. 05 467 497 001
MagNA Pure 96 IVD Instrument	Cat.-No. 06 541 089 001
MagNA Pure 96 DNA and Viral NA Small Volume Kit	Cat.-No. 06 543 588 001

3. Background Information

3.1 Medical Background

Hereditary hemochromatosis is considered to be one of the most common hereditary diseases in population of Caucasian origin.

In European caucasians the prevalence for hemochromatosis is reported to be 1 : 400 (Feder et al., 1996)[1]. About 90% of the hemochromatosis patients bear a homozygous mutation resulting in amino acid 282 Cys changed to Tyr (282 Y/Y), while another 5% are compound heterozygote, having the 282 C/Y variation combined with a change from 63 His to Asp for the other allele (63 H/D), or have a homozygous 63 D/D mutation, causing a mild form of hemochromatosis only.

The heterozygote 282 C/Y variant alone shows no risk for hemochromatosis. Heterozygote mutations for 63 H/D or 65 S/C and combined heterozygotes 63/65 without a collateral amino acid change 282 C/Y show no increased risk. 63D and 282Y mutation on the same allele is extremly rare (Best et al., 2001[11]).

The HGVS nomenclature for mutations is related to **p.**rotein or **c.**DNA positions:

p.H63D	c.187C>G	**63 H/D**	(used here)
p.S65C	c.193A>T	**65 S/C**	
p.C282Y	c.845G>A	**282 C/Y**	

Clinical Manifestations

Hereditary hemochromatosis is characterized by an inappropriately high absorption of iron by the gastrointestinal mucosa, resulting in excessive storage of iron particularly in the liver, skin, pancreas, heart, joints, and testes.

A well-known manifestion of tissue damage caused by iron accumulation is liver cirrhosis that may lead to hepatocelllular carcinoma (Willis et al., 2000)[2]. Also common for hemochromatosis are arthropathy, hypogonadism, pancreas damage, heart failures, and insulin resistance (Diabetes) (Edwards et al., 1980[3], Pietrangelo, A., 2004[4], Mc Dermott et al., 2005[5], Franchini, M., 2006[6]).

Genetic Testing

In clinical studies it was found that HFE mutations were significantly more frequent in disease than in control specimens (Willis et al., 2000)[2].

The analysis of genes helps us to understand the genetic background for certain diseases; genetic testing alone will identify individuals which might develop a disease but not the disease itself.

Genetic variations can be detected for example by DNA sequencing, hybridization to immobilized probes on arrays or strips, or more convenient by Real-time PCR. Detection of HFE-related mutations by means of a melting curve analysis using fluorescent labeled probes has been published already 1999 (Mangasser et al.1999[7], Bollhalder et al. 1999[8]).

For details and more information see section 3.2.

3.2 Methodology and Assay Principle

Using PCR methodology, two fragments of the HFE gene are amplified simultaneously with specific oligonucleotide primers. Fluorescent labeled probes are used to identify the PCR product and to determine the genotype by performing a melting curve analysis.

The probe binds to a part of the amplified fragment spanning the mutation site. Any mismatch covered by the probe destabilizes the hybrid. During the melting curve analysis the temperature is slowly increased. The probe melts off at a specific melting temperatures causing the fluorescence to decrease.

Exon 4 : Variant p.C282Y

A 284 bp long PCR fragment containing the c.845G>A (C282Y) polymorphism is analized using a LightCycler® Red 640 red oligomer matching the mutant 282Y allele. In the melting curve analysis the 282Y samples display a higher temperature than the wild type C282 allele.

Other variants, eg. 281M or 283P yield different melting temperatures (see 7.8).

Exon 2: Variants p.H63D and p.S65C

A 163 bp long PCR fragment containing the c.187C>G (63 H/D) and c.193A>T (65 S/C) polymorphisms is analized using a SimpleProbe® 519 oligomer which matches the mutant 63D allele.

```
   F58                H63   S65
 TG TTC GTGTTCTATGAT CAT GAG AGT CGC
 AC AAG CACAAGATACTA GTA CTC TCA GCG
```

The probe binds to a part of the amplified fragment starting from amino acid 58 Phe and passing beyond the mutation sites.

In the melting curve analysis the 63D samples display a higher temperature than the wild type allele 63H, while the 65C samples have the lowest melting temperature. Other variants yield different melting temperatures (see 7.8).

Reading of the genotype results is based on the melting temperatures compared to the supplied standards. If permitted by the instrument software reading of the genotype results can be achieved by the automated genotyping (instrument-dependent: software module 'Melt Curve Genotyping').

Automated genotyping results must be reviewed by eye for deviating curves and different melting point temperatures. In case that automated typing fails to report consistent genotype results, the genotype must be deducted from the melting temperatures following the criteria described in chapter 7.

Automated genotyping might fail in case that the supplied standards generate much higher signals than average patient samples. It is allowed to use known (pre-typed) clinical DNA samples as references for teaching the genotypes.

See also section 7.8 for melting temperatures expected for other variants.

The kit contains DNA standards encoding for the 282 C/Y, 63 H/D and 65 S/C variants to enable a comparison with clinical samples.

3.3 Performance Characteristics

Analytical Specificity
The specificity to the target gene and the suitability of the PCR amplification employed in the present kit was demonstrated by comparison with the results obtained by direct sequencing of the amplicon for *HFE H63D S65C* and the amplicon for HFE C282Y.

Analytical Sensitivity
Detection of serial dilutions of several heterozygous human genomic DNA has revealed that the limit of detection of the present kit is 250 copies DNA (1.5 ng). The smallest amount allowed to be used for testing is 15 ng (see section 1.3.1).

Diagnostic Specificity and Sensitivity
A total number of 120 different genomic DNA samples from individuals of Caucasian origin were analyzed in parallel by sequencing and with the present kit. The study compared results obtained with the kit with ABI 3730xl DNA sequencing data obtained by LGC Genomics GmbH, Berlin.

Study results: Results for both analytical methods were in 100% concordance.

In particular:

HFE p.H63D S65C: 17 of the samples were heterozygous c.187 C/G (63 H/D) and homozygous wild type c.193 A/A (65 S/S), 93 homozygous wild type 187 C/C and 193 A/A (63 H/H 65 S/S), 4 homozygous wild type 187 C/C (63 H/H) and heterozygous 193 A/T (65 S/C), 6 homozygous mutants 187 G/G (63 D/D) and homozygous wild type 193 A/A (65 S/S); the double homozygous combination mutant 187 G/G and 193 T/T has been never observed.
HFE p.C282Y:118 samples were homozygous wild type 845 G/G (282 C/C), 2 were heterozygous 845 G/A (282 C/Y) while none was homozygous mutant 845 A/A (282 Y/Y).

Summary of results:

93 samples	homozygous 'wild type'	63 H/H	65 S/S	282 C/C
4 samples	heterozygous for 65	63 H/H	65 S/C	282 C/C
15 samples	heterozygous for 63	63 H/D	65 S/S	282 C/C
6 samples	homozygous mutant for 63	63 D/D	65 S/S	282 C/C
2 samples	heterozygous for 63 and for 282	63 H/D	65 S/S	282 C/Y

4. Precautions and Warnings

Handling Requirements

The present product is an *in-vitro* diagnostic device and therefore must be used by qualified personnel only.

General precautions for the handling of generic laboratory materials are required.

The laboratory work-flow must conform to standard practices. Due to the risk of contamination, PCR preparation and PCR amplification must be performed in physically separated areas.

Do not mix reagents from different lots.

Do not use the reagents after the expiration date.

Use the manual version which is delivered with the kit (see kit label).

Laboratory Procedures

All materials of human origin and related waste must be considered potentially infectious. Thoroughly clean and treat all work surfaces with disinfectants approved by local authorities.

Do not eat, drink or smoke in the laboratory working area.

Do not pipet by mouth.

Wear disposable protective gloves, laboratory coats and adequate eye protection during the handling of samples and set components.

Avoid microbial or nuclease contamination of the reagents while pipetting the aliquots. The use of disposable sterile tips with filters is essential.

Thoroughly wash your hands after handling the samples and the sets components.

Sample Preparation

Regarding proper handling and disposal refer to the safety instructions enclosed in the package insert of the product employed (see chapter 2.3).

Amplification and Detection

Before using this product, please read the LightCycler® Operator's Manual .

Save a sample file to identify each position for correct sample identification.

Check LightCycler® Instrument settings and make sure that they match those reported in the following section "Programming" specific for your Instrument.

Do not touch the capillary surface or plate cover without gloves.

Please refer to all the operative and safety instructions of the LightCycler® Instrument.

Handling of Waste Materials

Dispose of the unused reagents and waste materials according to the current laws.

5. Programming

5.1 Color Compensation

Color Compensation is required for the use of the *LightMix® Kit HFE H63D S65C C282Y.*
Analyze data with 'Color Compensation' (TIB Molbiol CC 530-640).
Its deactivation will generate invalid readouts of the results.
Color Compensation is not required for the LightCycler® Nano.

5.2 Capillary Based LightCycler® Instruments

For details see the LightCycler® Operator's Manual.
The protocol consists of four program steps (Tab.1):

1. **Denaturation** of sample and activation of the enzyme
2. **Cycling** PCR-amplification of the target DNA
3. **Melting** Identification of PCR amplified DNA sequence
4. **Cooling** of the Instrument

Step:	**1**	**2**			**3**			**4**
Parameter								
Analysis Mode	**None**	**Quantification mode**			**Melting Curves mode**			**None**
Cycles	1	45			1			1
Target [°C]	95	95	60	72	95	43	75	40
Hold [hh:mm:ss]	**00:10:00**	**00:00:05**	**00:00:10**	**00:00:15**	**00:00:20**	**00:00:20**	**00:00:00**	**00:00:30**
Ramp Rate [°C/s]*	**20**	**20**	**20**	**20**	**20**	**20**	**0.2**	**20**
Sec Target [°C]	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
Step Size [°C]	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
Step Delay [cycles]	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
Acquisition Mode	None	None	Single	None	None	None	Cont.	None

* For LightCycler 1.x Instruments using software version 3.5.3 read 'Temperature Transition Rate' [°C/s] instead of Ramp Rate.

Tab. 1:
Programming of capillary based Instruments.
Note:
While programming maintain default software values: channel = 530, max. samples = 32, seek temperature = 30°C and capillary size = 20 µl. Do not change the capillary size value to 100 µl.
Store the program and the default values as '**RUN Template'** which can be loaded to start every HFE LightCycler® run.
Number of cycles may be increased up to 50 cycles to increase the signals in channel 530.

Just before starting the run, modify max. samples (default = 32) to the number of samples plus controls included in the run to prevent stopping of the instrument due to missing capillaries.

5.3 Roche 480 Instruments

For details see the Operator's Manual.

Detection Format: TIB Molbiol 530-640
Please refer to the manual of:
LightMix® Kit- Color Compensation HybProbe.
Cat. No. 40-0318-00

Reaction Volume: 20 µl

Programming:
The protocol consists of four program steps (Tab.2):

1. **Denaturation** of sample and activation of the enzyme
2. **Cycling** PCR-amplification of the target DNA
3. **Melting** Identification of PCR amplified DNA sequence
4. **Cooling** of the Instrument

Step:	**1**	**2**			**3**			**4**
Parameter								
Analysis Mode	**None**	**Quantification mode**			**Melting Curves mode**			**None**
Cycles	1	45			1			1
Target [°C]	95	95	60	72	95	43	75	40
Acquisition Mode	**None**	**None**	**Single**	**None**	**None**	**None**	**Cont.**	**None**
Hold [hh:mm:ss]	**00:10:00**	**00:00:05**	**00:00:10**	**00:00:15**	**00:00:30**	**00:02:00**	**00:00:00**	**00:00:30**
Ramp Rate [C°/ s] **96**	**4.4**	**4.4**	**2.2**	**4.4**	**4.4**	**1.5**	**0.29**	**1.5**
Ramp Rate [C°/ s] **384**	**4.6**	**4.6**	**2.4**	**4.6**	**4.6**	**2.0**	**0.29**	**2.0**
Acquisitions [per °C]	**-**	**-**	**-**	**-**	**-**	**-**	**1**	**-**
Sec Target [°C]	**0**	**0**	**0**	**0**	**-**	**-**	**-**	**-**
Step Size [°C]	**0**	**0**	**0**	**0**	**-**	**-**	**-**	**-**
Step Delay [cycles]	**0**	**0**	**0**	**0**	**-**	**-**	**-**	**-**

Tab. 2: Programming of LightCycler® 480 Instruments (96 well and 384 well formats) and cobas z 480 Analyzer.

Note:

a) Store the program and the default values as '**RUN Template'** which can be loaded to start every HFE LightCycler® run.
b) Ensure to program only 1 acquisition per second instead the default value 3; more acquisitions reduce the slope of the melting curve, increase experiment time and cause kit malfunction.
c) Number of cycles may be increased up to 50 cycles to increase the signals in channel 530.

5.4 LightCycler® Nano Instrument

For details see the LightCycler® Operator's Manual.

Color Compensation is not required!

Run Setting / Optical setting

Intercalating Dyes
Normal Quality

Profile

The temperature profile consists of four program steps (Tab.3):

1. **Denaturation** of sample and activation of the enzyme
2. **Cycling** PCR-amplification of the target DNA
3. **Denaturation** of amplified PCR product.
4. **Melting** Identification of PCR amplified DNA sequence

Step:	1	2			3	4	
Parameter							
Name	Hold	3 Step Amplification			Hold	Melting Stage Initial Stage	Melting Stage Final Stage
Cycles		45					
Temp [°C]	95	95	60	72	95	43	75
Ramp (°C/s)	5	5	4	5	5	4	0.2
Hold (s)	600	10	15	20	30	120	1
Acquire			√				

Tab. 3: Programming of LightCycler® Nano Instrument.

Note:

Store the program and the default values as **'Experiment file'** which can be loaded to start every HFE LightCycler® run.

6. Experimental Protocol

Program the Instrument before preparing the solutions (see 5. Programming and read the Operator's Manual for details).
The described performance of the assay can be guaranteed only when used with Roche Diagnostics LightCycler® systems or cobas z 480 Analyzer.

6.1 Sample Preparation

For preparation of genomic DNA use human peripheral blood (EDTA, citrate). The use of heparin is strongly discouraged since this anticoagulant might interfere with the PCR.
Perform nucleic acid purification using the High Pure PCR Template Preparation Kit or the MagNA Pure LC Instruments using the extraction kit appropriate to the MagNA Pure Instrument used (see 2. Additional Devices and Reagents) as described in the respective protocols.

In the depicted assays (7.5. Reading of the Results) the DNA was manually extracted from 200 µl of blood using the High Pure PCR Template Preparation Kit following the manufacturer's instructions; 100 µl of elution buffer were used for the final elution of the purified DNA from the column.

6.2 Reagents Preparation

6.2.1 Preparation of the LightCycler® FastStart DNA Master

1	Keep LightCycler® FastStart Enzyme **1a** cold.
2	Thaw the LightCycler® FastStart Reaction Mix **1b** by warming up the tube at 30°- 35°C for 3 - 5 minutes.
3	Quickly spin tubes to collect drops.
4	The solution must be free of particles.
5	Add 60 µl of **1b** to the vial **1a**.
6 ⚠	Mix the solution carefully with a pipette. Do not vortex! Avoid the production of bubbles.
7	Spin the tubes to collect drops.
8	Use reagent to prepare the Reaction Mix (6.3).
9	Store left over reagent at 4°C.

6.2.2 Preparation of Parameter-Specific Reagents (PSR)

►	Each **PSR** reagent tube is sufficient for 32 reactions.
1	Spin the premixed **PSR** tube at 10,000 RPM for 1 minute.
2	Check that the pellet is located at the bottom.
3	To each **PSR** tube add **66 µl** of PCR-grade **Water.**
4	Incubate for 20 sec at room temperature.
5	Vortex for 10 sec.
6	Spin the tubes to collect drops.

► Use 2 µl of **PSR** reagent for a 20 µl PCR reaction.

6.2.3 Preparation of Standards

►	Each **Standard** reagent tube is sufficient for 32 reactions.
1	Spin the three tubes at 10,000 RPM for 1 minute.
2	Check that the blue pellet is located at the bottom of the tube.
3	Dissolve pellet by adding 160 µl PCR-grade **Water**.
4	Incubate for 20 sec at room temperature.
5	Vortex for 10 sec.
6	Spin the tubes to collect drops.

► Use **5 µl** of each **Standard** for a 20 µl PCR reaction.
► All three **Standards** must be used in each run.
Please note: Opening the vials may cause contaminations of the work-space (aerosol).

6.2.4 Preparation of Genotyping Standards

The LightCycler® software 4.05 and later (capillary based instruments) and software 1.5 (Roche 480 Instruments) can be calibrated with reference standards to perform an automated genotyping of unknown clinical samples.

►	Genotyping Standards must be generated by mixing the Standards as follows:
	Prepare 3 clean tubes and label them as 5, 6 and 7
Tube 5	Mix 5 µl of **Standard 1** with 5 µl of **Standard 3**
	Genotype for p.C282Y and p.S65C
Tube 6	Mix 5 µl of **Standard 1** with 5 µl of **Standard 2**
	Genotype for p.C282Y and p.H63D
Tube 7	Mix 5 µl of **Standard 2** with 5 µl of **Standard 3**
	Genotype for 65C / 63D

► Use **5 µl** of Genotyping Standards (Tube 5-7) for a 20 µl PCR reaction
► All three Genotyping Standards must be used in the first run of the kit to calibrate the genotyping module.
Please note: Opening the vials may cause contaminations of the work-space (aerosol).

6.3 Preparation of the Reaction Mix

6.3.1 Preparation of 32 LightCycler® Reaction Mix

We recommend preparing 32 reactions to prevent storage of dissolved or activated reagents in varying volumes (6.2). See chapter 6.4 for storage and stability of dilute components. For preparation of the reaction mix for less samples, please go to step 6.3.2 “Reaction mix for single reaction”.

Prepare the reaction mix in the PSR reagent tube (cooled):

Components	**32 reactions**
To the **PSR** tube (red cap) already containing	66.0 µl
Add:	
H_2O, PCR-grade (colorless cap)	310.2 µl
Mg^{2+} solution 25 mM (blue cap)	52.8 µl
LightCycler® FastStart DNA Master HybProbe (red cap), see **6.2.1**	66.0 µl
⚠ Substitute of the “long neck cap” of the **PSR** tube with the red cap from FastStart	
Total Volume	**495.0 µl**

Tab. 4: Volumes of components for preparing 32 reaction mixture

6.3.2 Preparation of the Single LightCycler® Reaction Mix

Prepare the reaction mix by multiplying each volume (Tab. 5) by the number of biological samples to be analyzed plus five reactions (Negative Control, three **Standards**, one excess) and (optionally) three **Genotyping Standards**.

Prepare the reaction mix in a cooled vial:

Components	**Single reaction**
H_2O, PCR-grade (colorless cap)	9.4 µl
Mg^{2+} solution 25 mM (blue cap)	1.6 µl
PSR (red cap), see **6.2.2**	2.0 µl
LightCycler® FastStart DNA Master HybProbe (red cap), see **6.2.1**	2.0 µl
Volume of reaction mix	**15.0 µl**

Tab. 5: Volumes of components for preparing a single reaction mixture

Gently pipette up and down the reaction mix.
A high percentage of experimental failure is due
to a non homogeneous reaction mix!

6.3.3 Capillary / Well Loading Procedure

Each run must include one Negative Control (**NTC**) to demonstrate the absence of contaminations with genomic DNA or HFE PCR product and three Standards to identify run specific melting temperatures. Regulatory agencies or local laboratory rules might require including also the three Genotyping Standards.

Capillary / Well Loading Procedure

▶	Remember to include the controls when setting up the run.
1	Mix gently, spin down and check for the absence of air bubbles in the reaction mix vial.
2	Dispense **15 µl** per capillary/well of reaction mix
3	**Mandatory:** Add **5 µl** of PCR-grade **H_2O** as **Negative Control** (**NTC**) in position 1 (A1). Add **5 µl** of **Standard 1** in position 2 (A2). Add **5 µl** of **Standard 2** in position 3 (A3). Add **5 µl** of **Standard 3** in position 4 (A4). **Optional*:** Add **5 µl** of **Tube 5** Genotyping Standard in position 5 (A5). Add **5 µl** of **Tube 6** Genotyping Standard in position 6 (A6). Add **5 µl** of **Tube 7** Genotyping Standard in position 7 (A7).
4	Add **5 µl** of **Sample** in the remaining capillaries / wells.
5 ⚠	Close the capillary/plate and centrifuge. Check that no air bubbles are present.
6	Place the rotor/plate into the Instrument.
7	Capillary-based users only: input number of samples.
8	Start the run.
9	Input experiment’s name when instructed.
10	Save sample data in the samples’ window.

* See section **6.5** for the Sample loading and Genotyping Standards calibration.

6.4 Storage and Stability of Diluted Components

Reaction Mix
The complete reaction mix containing Parameter-Specific Reagents (**PSR**), LightCycler® FastStart DNA Master HybProbe and $MgCl_2$ can be stored refrigerated (4°C - 8°C) for 30 days.

Avoid prolonged exposure to light.

Parameter-Specific Reagents (PSR)
Once diluted, store PSR refrigerated at 4°C - 8°C for up to 30 days.

Avoid prolonged exposure to light.

LightCycler® FastStart DNA Master HybProbe
The combined FastStart DNA Master HybProbe master mix (1a+1b) can be stored refrigerated (4°C - 8°C) for 30 days.

Positive Controls
The Positive Controls, once dissolved, are stable for 30 days when stored refrigerated (4°C - 8°C).

Genotyping Standards
The **Genotyping Standards** are stable for 30 days (4°C - 8°C).

6.5 Loading of Controls and Genotyping Standards

Samples in positions 1 to 4 (plate: A1 to A4) must be filled in each run as described in the table below. Samples in positions 5 to 7 (plate: A5 to A7) are required for teaching of Genotyping Standards only in the first run of the kit.

Genotype results are based on melting temperatures.The use of the automated genotyping module present in the LightCycler® 2.0 and LightCycler® 480 software is optional.

Refer to the Instrument Operator’s Manual for details.

6.5.1 Capillary Based Instruments

In “Samples data - Capillary View”, input Sample Name as described in the second column.
Select “Analysis Type – Genotyping”. Select Channel 530 and 640 only!
From the pull down menu select “Sample Type” and copy the “Genotype” description.

Pos	Sample Name	Channel	Target Name	Sample Type	Genotype (Amino Acids)
1	**NTC**	530	Target 1	Negative Control	
		640	Target 4	Negative Control	
2	**Standard 1**	530	Target 1	Melting Standard	HFE 63H 65S Wild Type
		640	Target 4	Melting Standard	HFE 282Y Mutant
3	**Standard 2**	530	Target 1	Melting Standard	HFE 63D Mutant
		640	Target 4	Melting Standard	HFE 282C Wild Type
4	**Standard 3**	530	Target 1	Melting Standard	HFE 65C Mutant
		640	Target 4	Unknown	
5	**Tube 5**	530	Target 1	Melting Standard	HFE 65 S/C Heterozygous
		640	Target 4	Melting Standard	HFE 282 C/Y Heterozygous
6	**Tube 6**	530	Target 1	Melting Standard	HFE 63 H/D Heterozygous
		640	Target 4	Unknown	
7	**Tube 7**	530	Target 1	Melting Standard	HFE 65C / 63D Heterozygous
		640	Target 4	Unknown	

6.5.2 Roche 480 Instruments

In the “Sample Editor” window, in “Step1: Select Workflow” section, select “Melt Geno”. Input the description of **Standards** and **Genotyping Standards** as follows:

Pos	Filter combination	Sample Name	Melt Geno Sample Type	Melt Geno Genotype
A1	Fluos (465-510)	**NTC**	Negative Control	
A1	Red 640 (498-640)	**NTC**	Negative Control	
A2	Fluos (465-510)	**Standard 1**	Melting Standard	HFE 63H 65S Wild Type
A2	Red 640 (498-640)	**Standard 1**	Melting Standard	HFE 282Y Mutant
A3	Fluos (465-510)	**Standard 2**	Melting Standard	HFE 63D Mutant
A3	Red 640 (498-640)	**Standard 2**	Melting Standard	HFE 282C Wild Type
A4	Fluos (465-510)	**Standard 3**	Melting Standard	HFE 65C Mutant
A4	Red 640 (498-640)	**Standard 3**	Unknown	
A5	Fluos (465-510)	**Tube 5**	Melting Standard	HFE 65 S/C Heterozygous
A5	Red 640 (498-640)	**Tube 5**	Melting Standard	HFE 282 C/Y Heterozygous
A6	Fluos (465-510)	**Tube 6**	Melting Standard	HFE 63 H/D Heterozygous
A6	Red 640 (498-640)	**Tube 6**	Unknown	
A7	Fluos (465-510)	**Tube 7**	Melting Standard	HFE 65C / 63D Heterozygous
A7	Red 640 (498-640)	**Tube 7**	Unknown	

For the cobas z 480 Analyzer use channel 498-645 instead of 498-640.

6.5.3 LightCycler® Nano Instrument

Samples:

Input, as shown below, description of **Standards** and **Genotyping Standards** (optional) into the “Samples” window, **Name** and **Dye** into the “Target” window.

Samples:

Color	Name	Note
	NTC	
	Standard 1	
	Standard 2	
	Standard 3	
	Tube 5	
	Tube 6	
	Tube 7	

Target:

Color	Name	Dye	Reference
	channel 530	FAM	
	channel 640	Cy5	

Well as table

Pos	#	Note	Sample	Cy5	Type	FAM	Type
A1	1		**NTC**	channel 640	U	channel 530	U
A2	2		**Standard 1**	channel 640	U	channel 530	U
A3	3		**Standard 2**	channel 640	U	channel 530	U
A4	4		**Standard 3**	channel 640	U	channel 530	U
A5	5		**Tube 5**	channel 640	U	channel 530	U
A6	7		**Tube 6**	channel 640	U	channel 530	U
A7	7		**Tube 7**	channel 640	U	channel 530	U

7. Data Analysis and Interpretation

7.1 Limits and Interferences

The test is specific and enables to detect HFE gene variants at/near codons 63, 65 and 282. No interferences known.

7.2 Calibration

Calibrate as described in sections 6.2.4, 6.3.3, 6.5, 7.3.2 and 7.3.3.

7.3 Quality Control – Acceptance Criteria

In order to perform a reliable genotyping analysis, it is essential that Negative Control **NTC** and all three **Standards** are included in each run.

NOTE: The test is performed at an annealing temperature of 60ºC at which the probes will not bind well, yielding low or even no signals in 'quantification'. For this reason, the acceptance criteria are based only on the definition of the melting-curve patterns as described below.

7.3.1 Negative Control

NTC Negative Control (Mandatory - Position 1).
Melting-curve analysis of the Negative Control must provide a negative result: no assay-specific melting peaks (see 7.3.2) must be detected.
In case that the **NTC** should report one or more specific peaks (compare signal with sample results to avoid that the software enlarges background noise to window size suggesting the presence of melting peaks), a contamination or a pipetting error has occurred; the session is not valid and the procedure has to be repeated. If the problem sustains, change water and/or reagents and repeat. Feel free to ask for assistance at service@tib-molbiol.de.

In case a peak is detected (see paragraph 7.3.4), the software might incorrectly identify it as positive, making automatic genotyping impossible (SW 1.5 reports: "*Sample NTC in position A1 is a negative control not in the negative group"*).

In this case - to enable automatic genotyping – change the NTC sample from "Negative Control" to "Unknown" (see 6.5 Sample loading and calibration of Genotyping Standards); alternatively, results must be read from the melting temperatures (see 7.3.4 Samples and 7.6 Interpretation of the Results).

7.3.2 Standards (Positive Controls)

Melting-curve analysis should always show
HFE **Standard 1** (Mandatory - position 2).
one melting peak in channel 530
one melting peak in channel 640
HFE **Standard 2** (Mandatory - position 3).
one melting peak in channel 530
one melting peak in channel 640
HFE **Standard 3** (Mandatory - position 4).
one melting peak in channel 530
one melting peak in channel 640
The provided **Standards** mimic **homozygous** clinical samples (see **7.5**).
See **7.6 Interpretation of the Results** for expected melting temperatures.

7.3.3 Genotyping Standards DNA

Melting-curve analysis should always show:

Tube 5 Genotyping Standard (Optional - position 5).
two melting peaks in channel 530
two melting peaks in channel 640
Tube 6 Genotyping Standard (Optional - position 6).
two melting peaks in channel 530
two melting peaks in channel 640
Tube 7 Genotyping Standard (Optional - position 7).
two melting peaks in channel 530
one melting peak in channel 640
Genotyping Standards mimic **heterozygous** clinical samples.

See **7.6 Interpretation of the Results** for expected melting temperatures.

Before repeating a run consider common errors; check in particular the amplification profile, correct master-mix and $MgCl_2$ concentration used, and keep in mind that also inadequate storage of reagents may cause a failure of the device.

7.3.4 Samples

The result of the assay must always show one or two melting peaks for each channel. See 7.6 Interpretation of Results **expected melting temperatures.**

⚠ No more than two peaks per sample are expected in channel 530.
The melting peak profiles must be conformable to the acceptance criteria described in the present chapter and in **7.6 Interpretation of the Results** See also **7.7.2 Problematic profiles** and **7.7.3 Rare Variants**.

7.3.5 Abnormal Melting Curves

If an abnormal melting curve persists, it can be due to a defect in the product or can be caused by other variations (mutations) in the probe binding region; (see **7.7.3 Rare Variants**). In the latter case another method must be used for comparison / verification of the sequence.

Submit the PCR fragment for DNA sequencing to confirm the sequence or identify any unknown mutations. Report deviations to service@tib-molbiol.de Feel free to send deviant melting samples to the Berlin laboratories to confirm the obtained results and/or identify other mutations by DNA sequencing.

7.4 Saving External Genotyping Standards

⚠ (Not applicable for LC1.x software versions below 4.0 and for LightCycler® Nano Instrument).
After the genotyping analysis, if samples 1 to 7 comply with the acceptance criteria (see **7.3 Quality Control – Acceptance Criteria**), save the Genotyping Standards as follows and use External Standard in all successive runs.

7.4.1 Capillary Based Instruments

In the Melting Curve analysis - Genotyping window open the “Standard (Int)” menu and select “Save standards as External”.

7.4.2 Roche 480 Instruments

In the Melt Curve Genotyping analysis window open the “Standards (In-run)” menu and select “Save as ext.”

7.5 Reading the Results

Perform data analysis as described in the Instrument Operator’s Manuals.

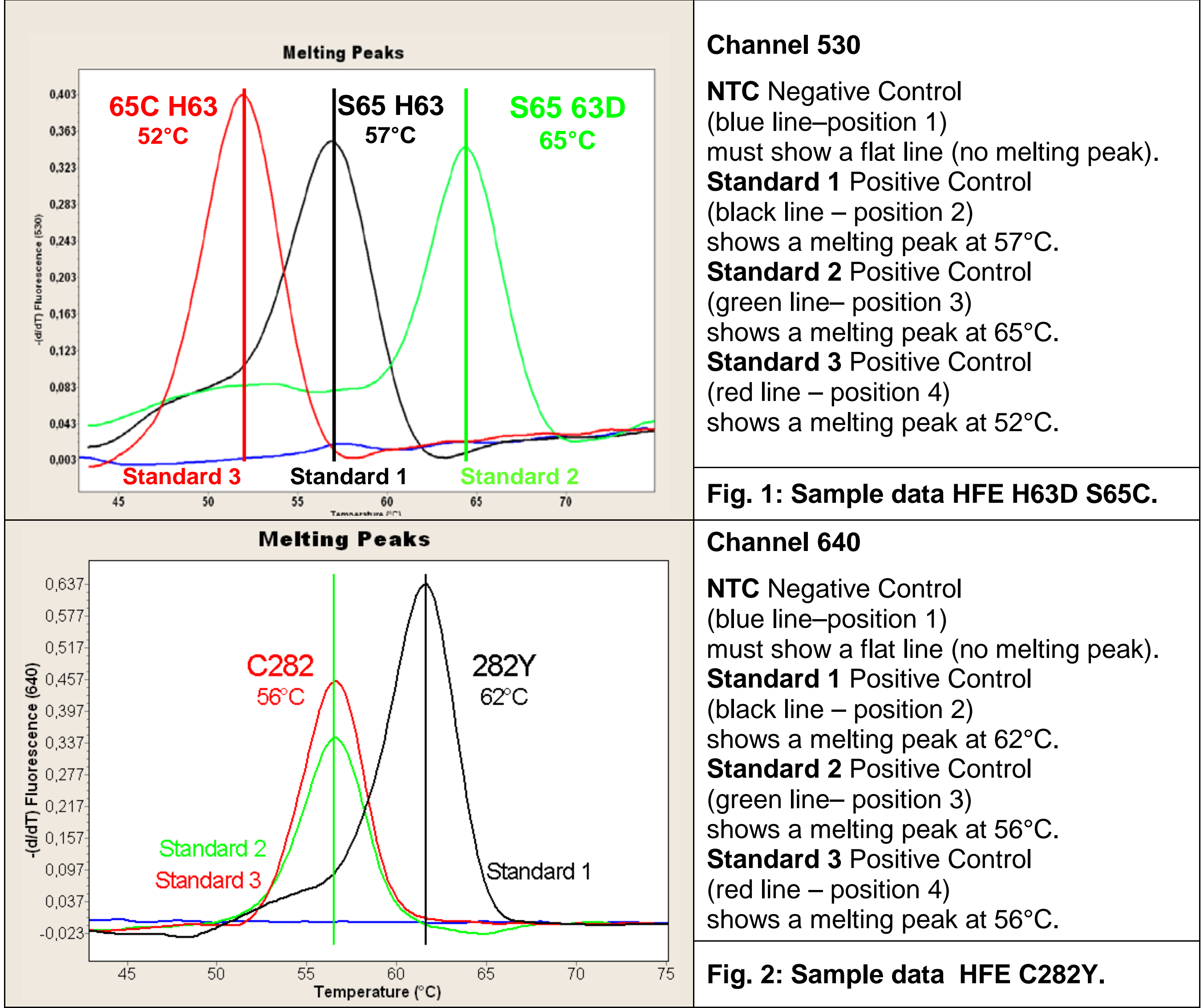
7.5.1 Melting Analysis: Capillary Based Instruments

The melting-curve peaks (Fig. 1 and 2) display homozygous genotypes.

⚠ Activate the Color Compensation to avoid wrong results.

View *HFE H63D S65C C282Y* data for Melting as follows:
LC 2.0 Instrument (or LC1.x with software versions 4.1):
View Melting data for HFE H63D S65C in channel 530 (Fig 3) and C282Y in channel 640 (Fig 4). Analysis Type “Melting Curve Analysis – Genotyping” mode.

LC1.x, software version 3.5.3
View Melting data for HFE H63D S65C in F1 (Fig 3) and C282Y in F2 (Fig 4). “Melting Curve” mode.



Channel 530

NTC Negative Control
(blue line–position 1)
must show a flat line (no melting peak).
Standard 1 Positive Control
(black line – position 2)
shows a melting peak at 57°C.
Standard 2 Positive Control
(green line– position 3)
shows a melting peak at 65°C.
Standard 3 Positive Control
(red line – position 4)
shows a melting peak at 52°C.

Fig. 1: Sample data HFE H63D S65C.

Channel 640

NTC Negative Control
(blue line–position 1)
must show a flat line (no melting peak).
Standard 1 Positive Control
(black line – position 2)
shows a melting peak at 62°C.
Standard 2 Positive Control
(green line– position 3)
shows a melting peak at 56°C.
Standard 3 Positive Control
(red line – position 4)
shows a melting peak at 56°C.

Fig. 2: Sample data HFE C282Y.

Note: The values of the melting temperatures may vary ±2.5°C between different experiments.
The ΔT between the melting peaks for heterozygous genotypes may vary ±1.5°C.
In case of variations see instructions: **7.3.5 Abnormal Melting Curves** and **7.7.3 Rare Variants**.
In case of low signals in channel 530 the number of cycles can be increased up to 50 cycles.

In case of automatic genotype module failure (score <0.6 or res<0.4), switch to manual reading (Tm calling) and compare with table 6 (**7.6. Interpretation**).

7.5.2 Melting Analysis: Roche 480 Instruments

The melting-curve peaks (Fig. 3 and 4) display homozygous genotypes.

⚠ Activate the Color Compensation to avoid wrong results.

View data for Melting as follows:

Roche 480 Instruments:
For use in LightCycler® 480 Instrument view HFE H63D S65C Melting data in channel 483-533 and HFE C282Y in channel 483-640, "Melt Curve Genotyping" mode.
For use in LightCycler® 480 II Instrument view HFE H63D S65C Melting data in channel 465-510 (Figure 5) and HFE C282Y in channel 498-640 (Figure 6) "Melt Curve Genotyping" mode.
For use in cobas z 480 Analyzer view HFE H63D S65C Melting data in channel 465-510 and HFE C282Y in channel 498-645. Analysis Type "Melt Curve Genotyping" mode.

Channel 465-510

NTC Negative Control
(blue line–position 1)
must show a flat line (no melting peak).
Standard 1 Positive Control
(black line – position 2)
shows a melting peak at 57°C.
Standard 2 Positive Control
(green line– position 3)
shows a melting peak at 65°C.
Standard 3 Positive Control
(red line – position 4)
shows a melting peak at 52°C.

Fig. 3: Melting sample data for HFE H63D S65C.

Channel 498 - 640

NTC Negative Control
(blue line–position 1)
must show a flat line (no melting peak).
Standard 1 Positive Control
(black line – position 2)
shows a melting peak at 62°C.
Standard 2 Positive Control
(green line– position 3)
shows a melting peak at 56°C.
Standard 3 Positive Control
(red line – position 4)
shows a melting peak at 56°C.

Fig. 4: Melting sample data for HFE C282Y.

Note: The values of the melting temperatures may vary ±2.5°C between different experiments.
The ΔT between the melting peaks for heterozygous genotypes may vary ±1.5°C.
In case of variations see instructions: **7.3.5 Abnormal Melting Curves.** and **7.7.3 Rare Variants**.
In case of low signals in channel 530 the number of cycles can be increased up to 50 cycles.

In case of automatic genotype module failure (score <0.6 or res<0.4), switch to manual identification of melting curve (Tm calling) and compare results with table 6 (**7.6. Interpretation of the Results**).

7.5.3 Melting Analysis: LightCycler® Nano Instrument

The melting-curve peaks (Fig. 5 and 6) display homozygous genotypes.

View data for Melting as follows:

Analysis

In window: **Select Analysis**

Select: Tm Calling

In window: **Setting**

Select: Use negative Derivative "Yes"

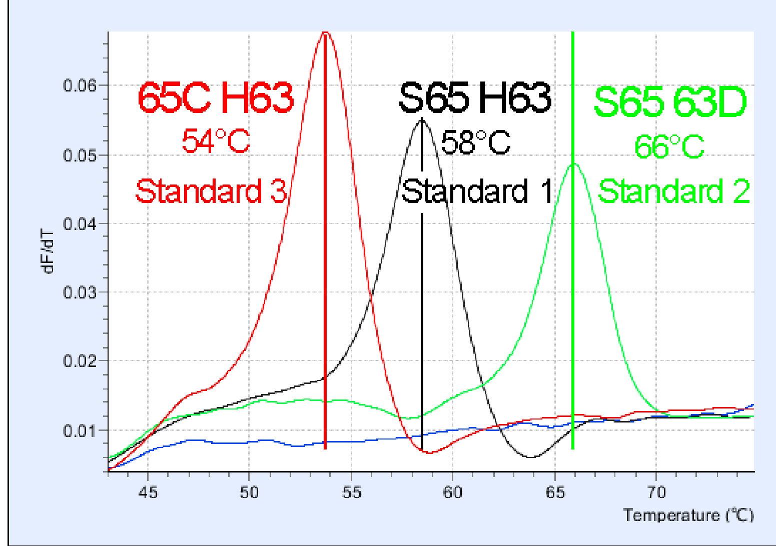
Select: Noise Reduction Range (°C) = 1

Select: Target: Channel 530 for HFE H63D S65C

Select: Target: Channel 640 for HFE C282Y

Melt Peaks

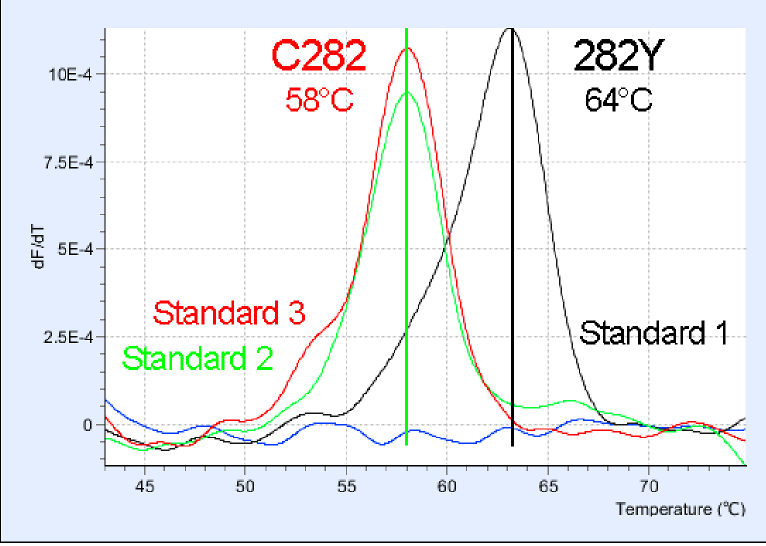
Manually compare the melting curve of each patient with the melting curve of Standards in both channels and use table 6 in chapter **7.6. Interpretation of the Results** for comparison.



Channel 530

NTC Negative Control
(blue line–position 1)
must show a flat line (no melting peak).
Standard 1 Positive Control
(black line – position 2)
shows a melting peak at 58°C.
Standard 2 Positive Control
(green line– position 3)
shows a melting peak at 66°C.
Standard 3 Positive Control
(red line – position 4)
shows a melting peak at 54°C.

Fig. 5: Melting sample data for HFE H63D S65C.



Channel 640

NTC Negative Control
(blue line–position 1)
must show a flat line (no melting peak).
Standard 1 Positive Control
(black line – position 2)
shows a melting peak at 64°C.
Standard 2 Positive Control
(green line– position 3)
shows a melting peak at 58°C.
Standard 3 Positive Control
(red line – position 4)
shows a melting peak at 58°C.

Fig. 6: Melting sample data for HFE C282Y.

Note: The values of the melting temperatures may vary ±2.5°C between different experiments.
The ΔT between the melting peaks for heterozygous genotypes may vary ±1.5°C.

In case of variations see instructions: **7.3.5 Abnormal Melting Curves** and **7.7.3 Rare Variants.**

7.6. Interpretation of the Results - Risk Alleles

1) Start to read results in channel 640 and identify all **high risk** individuals having the homozygous **282 Y/Y** mutation (single high-melting peak) [1].
   Note: *Homozygous 282Y/Y samples must be 63 His wild type; see channel 530).*
2) Still in channel 640 select all **282 H/Y** heterozygous individuals and analyze them in channel 530 to identify **63H/D** individuals having a **low risk** [2].
   Report remaining 282 H/Y heterozygous which are either **63 H/H** and **65 S/S** or **65S/C** as '**no risk**' (general population risk) [3,4].
   Note: *282 H/Y heterozygous samples can not be 63 D/D nor 65 C/C nor 63 D/65 C.*
3) Read channel 530 to select all individuals with the single high-melting peak which are homozygous **63 D/D** (**mild form**) [5]
   Note: *Homozygous 63 D/D samples are commonly wild type in channel 640. See ref. 11.*

Channel 530			Channel 640		Common Name	Disease
63H 65C	63H 65S	63D 65S	C282	282Y	HFE Genotype	Risk
530 Melting Peaks Temperature (°C)			640 Melting Peaks Temperature (°C)		**282Y homozygote** H63 S65 | H63 S65 282Y | 282Y	[1] High risk
-	57	-	-	62		
530 Melting Peaks Temperature (°C)			640 Melting Peaks Temperature (°C)		**H63D heterozygote** **C282Yheterozygote** Compound heterozygote H63 S65 | 63D S65 C282 | 282Y	[2] Low risk
-	57	65	56	62		
530 Melting Peaks Temperature (°C)			640 Melting Peaks Temperature (°C)		**S65C heterozygote** **C282Yheterozygote** Compound heterozygote H63 S65 | H63 65C C282 | 282Y	[3] Putative increased risk (disputed)
52	57	-	56	62		
530 Melting Peaks Temperature (°C)			640 Melting Peaks Temperature (°C)		**C282Y heterozygote** H63 S65 | H63 S65 C282 | 282Y	[4] General Population Risk
-	57	-	56	62		
530 Melting Peaks Temperature (°C)			640 Melting Peaks Temperature (°C)		**63D homozygote** 63D S65 | 63D S65 C282 | C282	[5] Mild Form
-	-	65	56	-		
ΔTm 5°C	ΔTm 8°C		ΔTm 6°C		**Tab. 6.1: HFE 282 and disease risk variants**	
ΔTm 13°C					See also 7.3.4 and 7.3.5	

7.6. Interpretation of the Results (continued)

4) Report the status of all remaining **282 C/C (wild type)** patients for their melting profile channel 530 as **'no risk'** (General population risk)[6-10].

   Note: 65 S/C heterozygous may have only a shoulder instead of a separated peak at the temperature for 63 H. The shape of the curves for all variants is significant if inspected by eye since it may be called wrong by the instrument software, irrespective if using the 'Genotyping' module or the 'Tm Calling' program.

Note: The values of the melting temperatures (Tm) may vary ±2.5°C between runs. The ΔT for melting peaks for heterozygous genotypes may vary ±1.5°C

In case that the peaks in channel 530 are low consider to run up to 50 cycles.

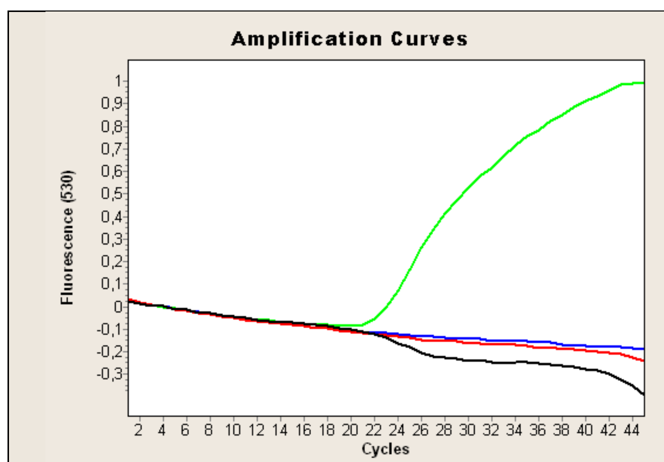
<table>
<tr><th colspan="3">Channel 530</th><th colspan="2">Channel 640</th><th>Common Name</th><th>Disease</th></tr>
<tr><th>63H
65C</th><th>63H
65S</th><th>63D
65S</th><th>C282</th><th>282Y</th><th>HFE Genotype</th><th>Risk</th></tr>
<tr><td colspan="3">Melting Peaks
530
Temperature (°C)</td><td colspan="2">Melting Peaks
640
Temperature (°C)</td><td rowspan="2">65C / 63D heterozygote
Compound heterozygote
H63 65C | 63D S65
C282 | C282</td><td rowspan="2">[6]
General Population Risk</td></tr>
<tr><td>52</td><td>-</td><td>65</td><td>56</td><td>-</td></tr>
<tr><td colspan="3">Melting Peaks
530
Temperature (°C)</td><td colspan="2">Melting Peaks
640
Temperature (°C)</td><td rowspan="2">H63D heterozygote
H63 S65 | 63D S65
C282 | C282</td><td rowspan="2">[7]
General Population Risk</td></tr>
<tr><td>-</td><td>57</td><td>65</td><td>56</td><td>-</td></tr>
<tr><td colspan="3">Melting Peaks
530
Temperature (°C)</td><td colspan="2">Melting Peaks
640
Temperature (°C)</td><td rowspan="2">65C homozygote
H63 65C | H63 65C
C282 | C282</td><td rowspan="2">[8]
General Population Risk</td></tr>
<tr><td>52</td><td>-</td><td>-</td><td>56</td><td>-</td></tr>
<tr><td colspan="3">Melting Peaks
530
Temperature (°C)</td><td colspan="2">Melting Peaks
640
Temperature (°C)</td><td rowspan="2">S65C heterozygote
H63 S65 | H63 65C
C282 | C282</td><td rowspan="2">[9]
General Population Risk</td></tr>
<tr><td>52</td><td>57</td><td>-</td><td>56</td><td>-</td></tr>
<tr><td colspan="3">Melting Peaks
530
Temperature (°C)</td><td colspan="2">Melting Peaks
640
Temperature (°C)</td><td rowspan="2">Wild Type
H63 S65 | H63 S65
C282 | C282</td><td rowspan="2">[10]
General Population Risk</td></tr>
<tr><td>-</td><td>57</td><td>-</td><td>56</td><td>-</td></tr>
<tr><td colspan="2">ΔTm 5°C</td><td>ΔTm 8°C</td><td colspan="2">ΔTm 6°C</td><td colspan="2">Tab. 6.2: C282 with common 63/65 variants</td></tr>
<tr><td colspan="3">ΔTm 13°C</td><td colspan="2"></td><td colspan="2">See also 7.3.4 and 7.3.5</td></tr>
</table>

7.7 Additional Information

7.7.1 Typical Data for Amplification

The amplification curves do not contain any analytical information (please see section **7.3 Quality Control – Acceptance Criteria**), but nevertheless, as an example, typical LightCycler® 2.0 curves are depicted below (Fig. 7 and 8).

View data for amplification as follows:
LC 2.0 Instrument (or LC1.x with software versions 4.1):
View HFE H63D S65C amplification in channel 530 and HFE C282Y amplification in channel 640, “Absolute Quantification” analysis mode.
Roche 480 Instruments:
For use in LightCycler® 480 Instrument, view HFE H63D S65C amplification in channel 483-533 and HFE C282Y in channel 483-640, “Abs Quant/2nd Derivative Max” mode.
For use in LightCycler® 480 II Instrument, view HFE H63D S65C amplification in channel 465-510 and HFE C282Y in channel 498-640 “Abs Quant/2nd Derivative Max” mode.
For use in cobas z 480 Analyzer view HFE H63D S65C amplification in channel 465-510 and HFE C282Y in channel 498-645.
LC Nano Instrument:
View HFE H63D S65C amplification in “Automatic Quantification” mode in channel 530 and HFE C282Y in channel 640.
LC1.x, software versions 3.5:
View HFE H63D S65C amplification in fluorescence channel F1 and HFE C282Y amplification in channel F2, “Quantification – Second Derivative Maximum” mode.

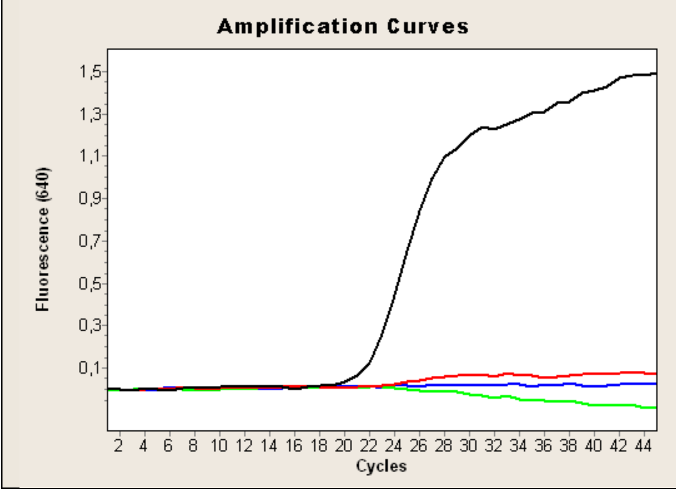


Data from channel 530
The **NTC** Negative Control (blue line) shows no amplification signal.

Standard 2 Positive Control (green line) shows an amplification signal typical for clinical samples that contain at least one 63D allele.

All other remaining standards and biological samples show no amplification signal.

Fig. 7: Amplification plot. Sample data for HFE H63D S65C



Data from channel 640
The **NTC** Negative Control (blue line) shows no amplification signal.

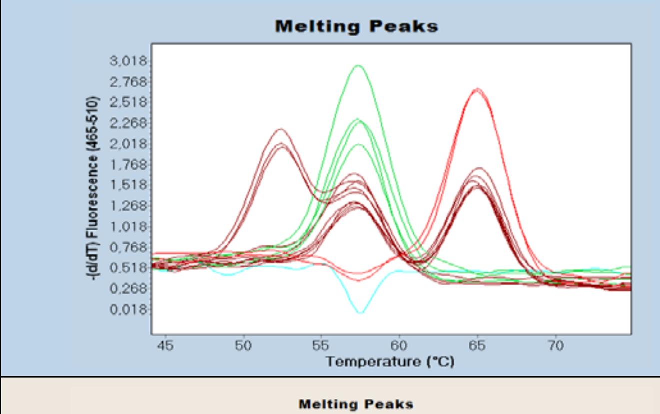
Standard 1 Positive Control (black line) shows an amplification signal typical for clinical samples that contain at least one 282Y allele.

All other remaining standards and biological samples show no amplification signal.

Fig. 8: Amplification plot. Sample data for HFE C282Y

7.7.2 Interpretation of Problematic Profiles

In the figures below, melting peak profiles from clinical samples are depicted.

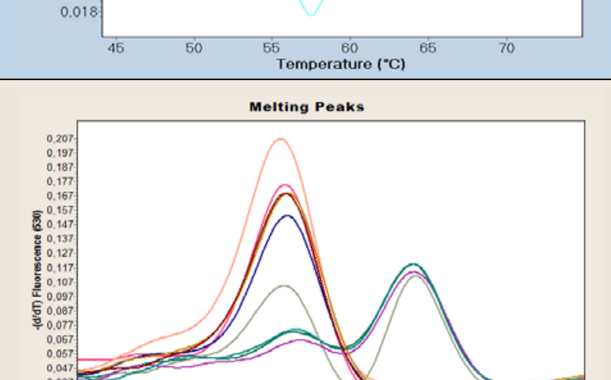


Instrument: LC480 II

Channel 465 - 510

NTC (light blue line): the negative control might show an abnormal negative peak, without affecting the automatic identification of other samples.

Fig. 9: Melting peaks HFE H63D S65C.



Instrument: LC2.0

Channel 530

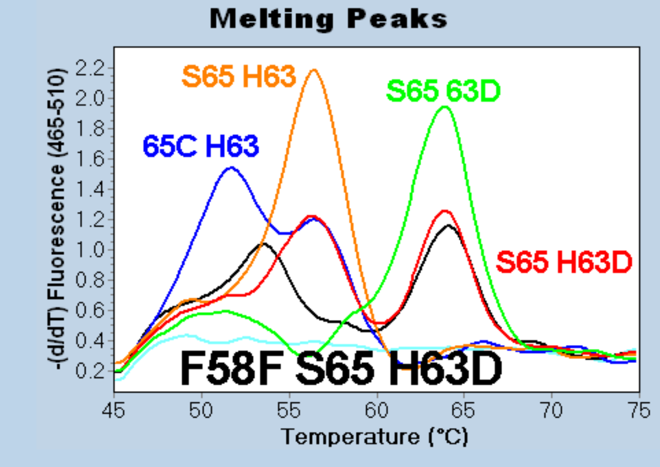
The shape of the curve can be influenced by the allele condition in the other channel without interfering with correct interpretation.

The green or violet and gray curves display two peaks (57°C and 65°C) and, despite the different shape of the curve, must be interpreted as heterozygous

Fig. 10 Melting peaks HFE H63D S65C.

7.7.3 Rare Variants

Other variants covered by the detection probe will yield a melting peak different from the full match, and usually with a shifted temperature compared to the common mutations. In the figures below we show some examples for rare variants observed by users of this device.



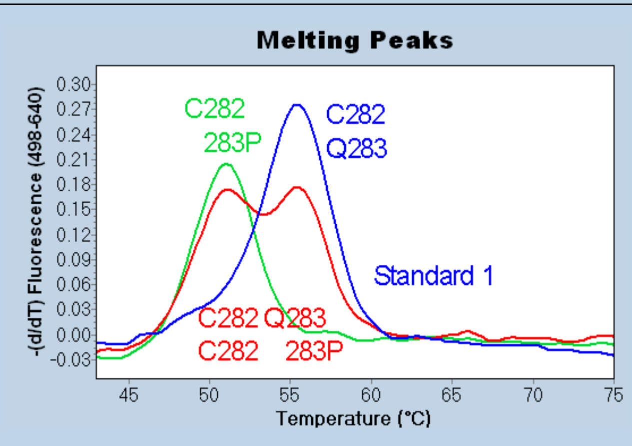
Instrument: LC480II

Exon 2: Channel 465 - 510

The silent mutation g.8658C>T p.**F58F** has been observed in 2012 for one patient with a melting peak at **54°C** - which is between S65 H63 (wild type) and 65C H63.
The patient was g.8658C>T heterozygous (black line, left peak) combined with 63D. [10]
At time of observation (2013) this polymorphism was not registered in dbSNP at NCBI.

No reference standard provided with the kit.

Fig. 11: Melting peaks for HFE F58F.



Instrument: LC480II

Exon 4: Channel 498 - 640

Variant p.**T281M** generates a Tm at **47°C** (not shown), published Biasiotto et al, 2008[12]
Variant p.**Q283P** generates a Tm at **51°C** (green), published by Ka et al. 2005 [9]
282C 283Q / 282C 283Q (wild type, blue) 56°C
282C 283Q / 282C 283P (red) 56°C / 51°C.
282C 283P / 282C 283P (green) 51°C

No reference standards provided with the kit.

Fig. 12: Melting peaks for HFE Q283P.

8. Troubleshooting

Instrument specific codes:	Capillary based instruments LightCycler® Nano	LightCycler® 480 instruments
Event	**Possible Reason**	**Solution**
No sample detected	No centrifugation	Centrifuge capillary
All negative PCRs	Incorrect selection of detection channel	Set correct channel before analyzing
	Incorrect amplification protocol	Check instrument program
Low 530 signals	The HFE 63/65 fragment is known to have a lower PCR efficiency	Run more PCR cycles. Up to 50 cycles have been evaluated/yield same results
Inconsistent base-line among various samples	Incorrect pipetting	Ensure homogeneity of mix quantity in each sample
	Non homogenous reaction mix	Pipette reaction mix up and down 10 x with a clean 200µl tip before distribution in reaction vessel
	Multiwell plate badly sealed	Ensure that multiwell plate is properly sealed
Baseline “Saw teeth like”	Bubble in well	Centrifuge plate before run
	Incorrect positioning of capillary in the carousel	Firmly press capillary in the carousel
No signal in the Positive Control	Error while setting the instrument	Check the position settings of the Positive Control
	Incorrect PSR / $MgCl_2$ concentration	Repeat assay
	Positive Control or standard degradation	Use a new aliquot of Positive Control or standard
Positive signal in Negative Control **NTC**	Error while setting the instrument	Check the position settings of the Negative Control
	Dispensing error	Comply with the work sheet when dispensing samples, Negative Controls, Positive Controls and standards
	Dispensing error	Always change tips among samples
	Dispensing error	Avoid spilling the contents of the sample test tube
	Contamination of the PCR- grade water.	Use a new aliquot of PCR- grade water
	Contamination of the reaction mix	Use new aliquots of reagents to prepare the reaction mix
	Contamination of the extraction/preparation area for amplification reactions	Clean surfaces and instruments with aqueous detergents, wash lab coats, replace test tubes and tips in use
	Contamination of the extraction/preparation area for amplification reactions	Add LightCycler® Uracil-DNA Glycosylase (Cat.-No.03 539 806 001) to the reaction mix according to instructions
No signal in samples	Low DNA amount	Check DNA concentration
	Sample inhibition	Dilute sample and repeat PCR, or repeat extraction and PCR, or add Positive Control and repeat
Melting curve outside the expected temperature range	With peaks Tm **concordant** with Positive Control: Incorrect reagent concentration	Manually assign results accordingly to Positive Control
	With peaks Tm **discordant** with Positive Control: Possible extraction inhibitor	Repeat assay diluting the DNA 1:3
	With peaks Tm **discordant** with Positive Control: Possible different mutation	Repeat assay by sequencing and report unexpected variant to service@tib-molbiol.de

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Classification / References

Reference	Classification
EDMA	16 01 01 05 00
CPV	33694000-1
EAN	4260159331073
Roche SAP No.	05945798001

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When the present kit is distributed through Roche Diagnostics or their local distributors, FastStart DNA Master HybProbe is supplied as a separate product:

Roche Diagnostics Cat.-No. 03 003 248 001 kit for 96 reactions
Roche Diagnostics Cat.-No. 12 239 272 001 kit for 480 reactions

Material Safety Data

According to OSHA 29CFR1910.1200, Commonwealth of Australia [NOHSC:1005, 1008 (1999)] and the European Union Directives 67/548/EC and 1999/45/EC any products which not contain more than 1% of a component classified as hazardous or classified as carcinogenic do not require a Material Safety Data Sheet (MSDS).

Product is not hazardous, not toxic, not IATA restricted. Product is not from human, animal or plant origin. Product contains synthetic oligonucleotide primers and probes.

Version History

Notes in red mark events that require to change laboratory procedures
Notes in blue mark improvements or change in composition

Version	Event	Date
V110620	Release Version	20-06-2011
V110815	Product information, color code vials, chapters, section 6 (protocol)	04-07-2011
V110822	Correction order of standards in section 6.3.3 and 6.5.1	22-08-2011
V110906	Correction in section 3.2 for genotypes/melting temperatures	06-09-2011
V120116	Former 'Positive Control' named 'Standard' Chapter 1.3 Productivity 7.6 Problematic Profiles, 7.7 Interpretation of results, new table 6. LightCycler® Nano included, Sample data Q283P (Fig12).	23-01-2012
V120209	Incubation times LightCycler® 480 corrected.	09-02-2012
V120504	One primer 282 substituted (640 improvement), from lot n° 1775xx Chapter 6: Melting starts from 43°C instead of 45°C	04-05-2012
V130503	Reverse primer 63/65 substituted (530 improvement) lot n° 2480xx Detailed specification for storage of PSR (section 6.4) Instructions for automated genotyping module failure (3.2) (7.3.1). Chapter 7.6 and 7.7 interchanged and improved Troubleshooting section low 530 signals / running more PCR cycles	27-06-2013
V130704	MagNa Pure 96 and MagNa Pure Compact included. Instructions for automated genotyping module failure (7.5.2)	04-07-2013
V131211	Reference (11) added - rare cases of 63D and 282Y on the same allele. Section 3.2 Signal levels of Standards and use for Automated Typing.	11-12-2013
V140626	Lot 2929xxxx: Adjustment of fluorescence signals to reduce negative peaks recorded in the 530 channel. No changes in Tm values.	26-06-2014
V150202	MagNa Pure 96 IVD included, HGVS gene nomenclature corrections	06-02-2015
V160101	Editorial changes. Section 1.3: 7.7.3 Tm for variant T281M mentioned	04-01-2016
V160626	Storage (1.1, 1.4). Editorial changes.	26-06-2016

Produced by:

TIB MOLBIOL Syntheselabor GmbH
Eresburgstrasse 22-23
12103 Berlin, Germany
www.tib-molbiol.com