8. Sample data - typical results

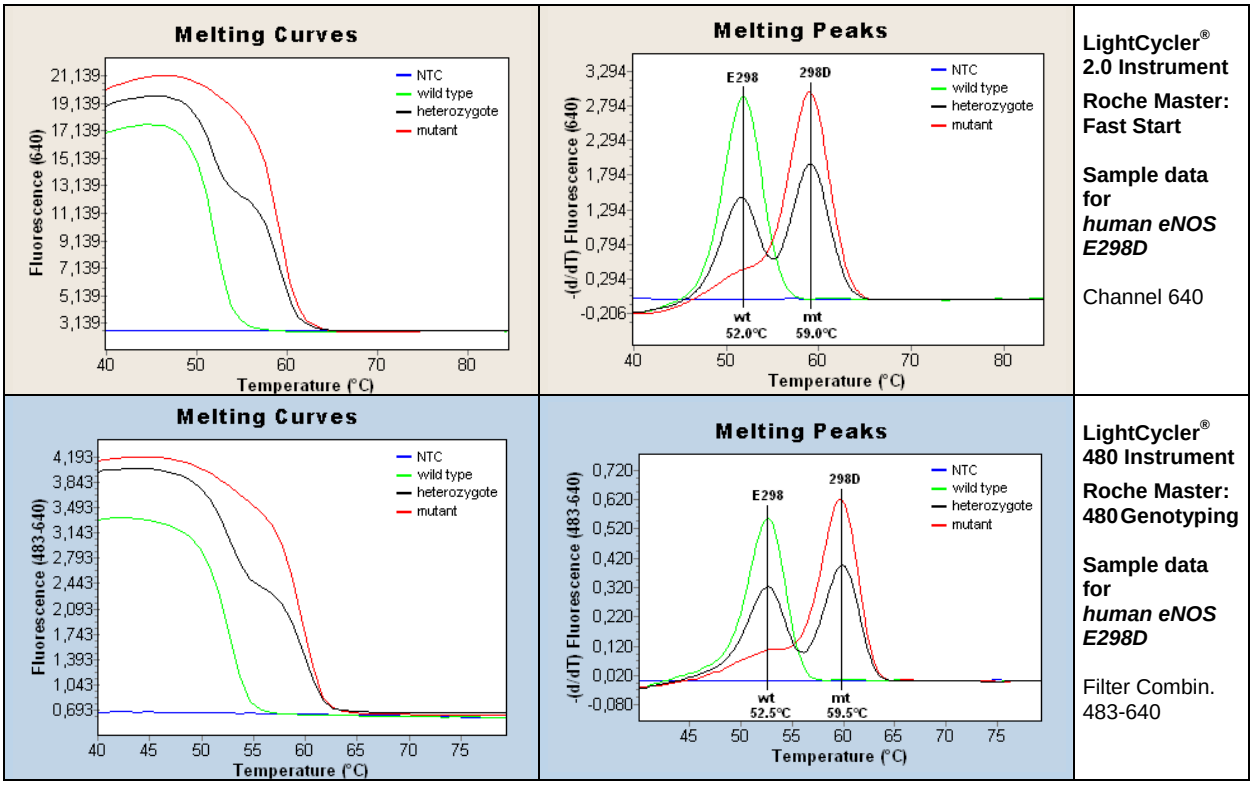

Fig.1. Sample data for the *human eNOS E298D* detection system.

Upper panels: Data from LightCycler® 2.0 Instrument. Left panel channel 640 melting curves for *human eNOS E298D*. Right panel channel 640 melting peaks for *human eNOS E298D*. Wildtype (wt) corresponds with *human eNOS 298E/E*, heterozygote (hetero) corresponds with *human eNOS 298E/D* and mutant (mt) corresponds with *human eNOS 298D/D*.

Lower panels: Data from LightCycler® 480 Instrument (384 well format). Left panel filter combination 483-640 melting curves for *human eNOS E298D*. Right panel channel 640 melting peaks for *human eNOS E298D*. Wildtype (wt) corresponds with *human eNOS 298E/E*, heterozygote (hetero) corresponds with *human eNOS 298E/D* and mutant (mt) corresponds with *human eNOS 298D/D*.

9. Interpretation of data

Genotype:	wild type homozygote *eNOS 298E/E*	heterozygote *eNOS 298D/E*	mutant homozygote *eNOS 298D/D*
Number of melting peaks (color)	1 (green)	2 (black)	1 (red)
Melting temperature of peaks	52.0℃	52.0℃ and 59.0 ℃	59.0℃
Temperature difference between peaks	---	7.0℃	---
Phenotype	---	---	under discussion

Tab. 3. Typical analysis results (LightCycler® 2.0 Instrument, Roche Master: Fast Start)

***Notes**:* The values of the respective melting temperatures (T_M) may vary ±2.5℃ between different experiments. Th e ΔT between the melting peaks for heterozygote genotypes may vary ±1.5℃. Samples with deviating melting curves sh ould be subject to further investigations; sequence analysis can be provided by TIB MOLBIOL Berlin (contact service@tib-molbiol.de).

These reagents were developed and manufactured by TIB MOLBIOL GmbH, Berlin, Germany.
LightCycler® hybridization probes produced under license from Roche Diagnostics GmbH.

LightMix® Kit *human eNOS E298D*

Cat.-No. 40-0380-16 Version 06/2011: double amount of control DNA per vial

Kit with reagents for the detection of the *human eNOS E298D* polymorphism using the LightCycler® 1.x / 2.0 / 480 Instruments.

Lyophilized mix of primers and probes (6 tubes with 16 rxns each) for a total of 96 reactions with a final volume of 20 µl each. **Store protected from light at room temperature (18-25°C), do NOT freeze!**

Additional reagents required

Roche Diagnostics:

LightCycler® FastStart DNA Master HybProbe	Cat.-No. 03 003 248 001
LightCycler® 480 Genotyping Master (LightCycler® 480 Instrument only)	Cat.-No. 04 707 524 001
High Pure PCR Template Preparation Kit	Cat.-No. 11 796 828 001

1. Introduction

Nitric oxide (NO), produced by the nitric oxide synthase (eNOS or NOS3) in the endothelial cells, regulates vasomotor tone and blood flow through its activation of the endothelium-derived relaxing factor EDRF. Yoshimura et al.[1] described the G894T variant located in exon 7 of the eNOS gene, causing the E298D amino acid exchange. E298D has been related to Alzheimer late onset as well as to hypertension, but due to contradictory results the role of the E298D is still subject to ongoing debate. More recently Berger et al.[2] reported that E298D is significantly associated with ischemic stroke. Fatini et al. published a LightCycler assay for the analysis of this polymorphism[3].

[1]The missense Glu298Asp variant of the endothelial nitric oxide synthase gene is strongly associated with placental abruption. Yoshimura et al., Hum. Genet. **108**:181-183 (2001).

[2]The glu298asp polymorphism in the nitric oxide synthase 3 gene is associated with the risk of ischemic stroke in two large independent case-control studies. Berger K et al. Hum. Genet. **121**:169-178 (2007)

[3]Endothelial Nitric Oxide Synthase –786T>C, but Not 894G>T and 4a4b, Polymorphism Influences Plasma Homocysteine Concentrations in Persons with Normal Vitamin Status Fatini C et al. Clin Chem **51**:1159-1164 (2005)

The LightMix® Kit *human eNOS E298D* provides a fast, easy and accurate system to identify the genotype of this target in a nucleic acid extract.

This LightMix® Kit is tested with the Roche Diagnostics 'LightCycler® FastStart DNA Master HybProbe' in the LightCycler® 1.x / 2.0 / 480 (96 and 384 well formats tested) Instruments and with the Roche Diagnostics 'LightCycler® 480 Genotyping Master' in the LightCycler® 480 Instrument (96 and 384 well formats tested).

2. Description

A 217 bp fragment of the *human eNOS* gene is amplified with specific primers. The resulting PCR fragment is analyzed with hybridization probes labeled with LightCycler® Red 640 (detected in channel 640). The genotype is identified by running a melting curve with specific melting points (Tm). The wildtype *human eNOS E298D* DNA exhibits a Tm of 52.0°C in channel 640. The mu tant *human eNOS E298D* exhibits a Tm of 59.0°C in channel 640.

The supplied control DNA allows for the accurate comparison with unknown samples.

For use in LightCycler® 1.x Instruments with software version 3.5.3 read channel F2 instead of channel 640, channel F3 instead of channel 705 and channel F1 instead of channel 530 for detection. We recommend upgrading LightCycler® 1.x Instruments to software version 4.1.

For use in LightCycler® 480 Instruments use filter combination 483-640 instead of channel 640.

3. Set contents

6 Vials with red caps containing premixed lyophilized primers and probes for 16 PCR reactions each of *human eNOS E298D*
3 Vials with colorless caps containing control DNA (*human eNOS E298D* mt, wt, hetero), 10^5 target equivalents per reaction

4. Programming

The protocol consists of four program steps

- 1: Denaturation: sample denaturation and enzyme activation
- 2: Cycling: PCR-amplification of the target DNA
- 3: Melting: melting curve analysis for identification of the PCR product derived from the target DNA
- 4: Cooling: cooling the instrument

	Program Step:	Denaturation	Cycling			Melting			Cooling
	Parameter								
	Analysis Mode	None	Quantification mode			Melting Curves mode			None
	Cycles	1	45			1			1
Settings	Target [℃]	95	95	60	72	95	40	85	40
LC 1.x/2.0	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 [℃/s]	20	20	20	20	20	20	0.2	20
	Acquisition Mode	None	None	Single	None	None	None	Continuous	None
	Analysis Mode	None	Quantification mode			Melting Curves mode			None
	Cycles	1	45			1			1
Settings	Target [℃]	95	95	60	72	95	40	80	40
LC 480	Hold [hh:mm:ss]	00:10:00	00:00:05	00:00:10	00:00:15	00:00:30	00:01:00	-	00:00:30
	Ramp Rate [℃/s] **96**	4.4	4.4	2.2	4.4	4.4	1.5	-	1.5
	Ramp Rate [℃/s] **384**	4.6	4.6	2.4	4.6	4.6	2.0	-	2.0
	Acquisition Mode	None	None	Single	None	None	None	Continuous	None
	Acquisitions [per ℃]	-	-	-	-	-	-	**1**	-

5. Data analysis

Perform data analysis, as described in the LightCycler® Instrument operator's manual.

View *human eNOS E298D* data in channel 640 "Tm Calling" Analysis mode (LightCycler® 2.0/480 Instruments) or Melting Curves mode (LightCycler® 1.x Instrument). The negative control (NTC) must show no signal.

6. Product characteristics

PCR results are obtained within 1 hour.

Sensitivity
These reagents detect 10 ng of *human* genomic DNA.

Measuring range
The measuring range of the assay is 10 ng to 100 ng of human genomic DNA.

Storage and Stability
- Lyophilized reagents are stable for at least 3 months after shipment if stored protected from light at room temperature (18-25℃).
- **Do not freeze** lyophilized reagents.
- Dissolved reagents are stable for at least 5 days if stored protected from light and refrigerated (4℃).

7. Experimental protocol

The following procedure was developed for use with the LightCycler® 1.x / 2.0 / 480 Instruments. Start programming before preparing the solutions. See the LightCycler® Instrument operator's manual for details.

Sample material: Use aqueous nucleic acid preparations (e.g. Roche Diagnostics 'High Pure PCR Template Preparation Kit').

Negative control: Always run at least one negative control - replace the template DNA with water.

Positive control: Run a positive control - replace the template DNA with the provided control DNA.

7.1. Preparation of parameter-specific reagents (16 reactions):

One reagent vial with a **red** cap contains all primers and probes to run 16 LightCycler® reactions for *human eNOS E298D.*

Add 66 µl PCR-grade water to each reagent vial, mix the solution (vortex) and spin down.

►Use 4 µl **reagent** for a 20 µl PCR reaction.

This solution is stable at least five days when stored refrigerated at 4°C. Avoid prolonged exposure to light.

7.2. Preparation of the control DNA

Add 80 µl PCR-grade water to each vial (1.6 x 10^6 target molecules) with a colorless cap. Mix the target DNA by pipetting the solution up and down 10 times (final concentration 10^5 target molecules in 5 µl).

Note: Control DNA can be dissolved up to 160 µl to achieve 32 control reactions.

►Use 5 µl control DNA for a 20 µl PCR reaction.

This solution is stable at least five days when stored refrigerated at 4°C, for long term storage freeze at -20°C. Avoid repeated freezing thawing cycles. For the heterozygote control DNA provided with the kit please note that the relative amounts of wild type DNA and mutant DNA may change during time.
Please note that opening these vials may cause contaminations of the work-space (aerosol).

7.3. Preparation of the LightCycler® reaction mix

In a cooled reaction tube, prepare the reaction mix by multiplying each volume for a single reaction by the number of reactions to be cycled plus one additional reaction.

For use with the Roche FastStart Master		**For use with the Roche 480 Genotyping Master**
Single reaction	**Component**	**Single reaction**
7.4 µl	water, PCR-grade (colorless cap, provided with the Roche Master kit)	7.0 µl
1.6 µl	Mg^{2+} solution 25 mM (blue cap, provided with the Roche FastStart kit)	-- µl
4.0 µl	**reagent** mix (parameter specific reagents containing primers and probes see **7.1.**)	4.0 µl
2.0 µl	Roche Master (red/yellow cap, for preparation see Roche manual)	4.0 µl
15.0 µl	**Volume of reaction mix**	**15.0 µl**

Mix gently, spin down and transfer 15 µl each of the reaction mix to a LightCycler® capillary (LightCycler® 1.x / 2.0 Instrument) or to a multiwell plate (LightCycler® 480 Instrument).

Add 5 µl of sample or control DNA to each capillary or well for a final reaction volume of **20 µl.**

Start run.