LightMix® Kit *PAI-1 4G/5G*

Cat.-No. 40-0099-32

Kit with reagents for the detection of the *PAI 4G/5G* polymorphism using the Roche Diagnostics LightCycler® 1.x / 2.0 / 480 / Cobas® Z480 Instruments.

Lyophilized mix of primers and probes (3 tubes with 32 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!**

Note: A CE-IVD marked diagnostic-use LightMix Kit PAI-1 is available order no. 40-0099-64

1. Introduction

The *human Plasminogen Activator Inhibitor-1 (PAI-1)* is one of the strongest inhibitors of fibrinolytic activities. Due to an induction of the transcription, the homocygot *4G/4G* genotype is associated with higher levels of *PAI-1* and cholesterol in plasma. This finding may explain the involvement of the *PAI-1 4G/5G* polymorphism in the clustering of atherothrombotic risk factors, and why people with the *4G/4G* genotype are at increased risk for myocardial infarction.

The LightMix® Kit human *PAI 4G/5G* 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 well format) Instruments and with the Roche Diagnostics 'LightCycler® Genotyping Master' in the LightCycler® 480 Instrument 96 and 384 well format.

2. Description

A 345 bp fragment of the human *PAI-1* gene is amplified with specific primers. The resulting PCR fragments are 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 human *PAI-1 4G* DNA exhibits a Tm of 54.5°C in channel 640 and the human *PAI-1 5G* DNA exhibits a Tm of 62.0°C in the same channel.

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 with LightCycler® 480 systsm use filter comb. 483-640 (480), 498-640 (480 II) or 498-645 (Z).

3. Set contents

3	Vials with red cap containing lyophilized primers and probes for 32 PCR reactions *PAI-1 4G/5G*
1	Vial with colorless cap containing control DNA (*PAI-1 4G/4G mt*), 10^5 target equivalents per rxn
1	Vial with colorless cap containing control DNA (*PAI-1 5G/5G wt*), 10^5 target equivalents per rxn
1	Vial with colorless cap with control DNA (*4G/5G heterozygote*), 10^5 target equivalents per rxn

4. Additional Reagents and items required

	Roche Diagnostics
LightCycler® FastStart DNA Master HybProbe	Cat.-No. 03 003 248 001
or ONLY for LightCycler® 480 Instruments LightCycler® 480 Genotyping Master	Cat.-No. 04 707 524 001
High Pure PCR Template Preparation Kit	Cat.-No. 11 796 828 001

5. Product characteristics

PCR results are obtained within 1 hour.

Sensitivity
These reagents detect 1 ng of genomic DNA using the Roche 'LightCycler® FastStart DNA Master HybProbe' with the LightCycler 1.x / 2.0 / 480 Instruments. The detection limit is 10 ng of genomic DNA if using the Roche 'LightCycler® 480 Genotyping Master' with the LightCycler 480 Instrument.

Measuring range
The measuring range of the assay is 1 ng to 100 ng of genomic DNA.

Storage and Stability
- Lyophilized reagents are stable for at least 6 months after shipment when stored protected from light at room temperature (18-25°C). See expiry date on the product label.
- **Do not freeze** lyophilized reagents.
- Dissolved reagents are stable for at least 10 days when stored protected from light and refrigerated (4°C).

6. Material Safety Data

According to OSHA 29CFR1910.1200, Australia [NOHSC:1005, 1008 (1999)] and the EU 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.

7. 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
Settings LC 1.x/ LC 2.0	**Parameter**								
	Analysis Mode	None	Quantification mode			Melting Curves mode			None
	Cycles	1	40			1			1
	Target [°C]	95	95	60	72	95	40	85	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
	Acquisition Mode	None	None	Single	None	None	None	Continuos	None
Settings LC 480	Analysis Mode	None	Quantification mode			Melting Curves mode			None
	Cycles	1	40			1			1
	Target [°C]	95	95	60	72	95	40	85	40
	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		1.5
	Ramp Rate [°C/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 °C]	-	-	-	-	-	-	**3***	-

Table 1

*** Use Mono Color HybProbes detection format**

8. Data analysis

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

View 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.

9. 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 Instrument operator's manual for details.

Sample material: Use aqueous nucleic acid preparations (e.g. 'High Pure PCR Template Prep. 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.

9.1. Preparation of parameter-specific reagents (32 reactions):

One reagent vial with a **red** cap contains all primers and probes to run 32 reactions for *PAI-1 4G/5G*

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

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

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

9.2. 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 free ze at -20°C, avoid repeated freezing thawing cycles. For the heterozygote control DNA provided with the kit please note that the relative amounts of *PAI-1 4G* DNA and *PAI-1 5G* DNA may change during time.
Please note that opening of these vials may cause contaminations of the work-space (aerosol).

9.3. 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 LC1.x/LC2.0 LC480-96 well format		**For use with the Roche 480 Genotyping Master LC480 96 and 384 well format**
Single reaction	**Component**	**Single reaction**
9.4 µl	water, PCR-grade (colorless cap, provided with the Roche Master kit)	9.0 µl
1.6 µl	Mg^{2+} solution 25 mM (blue cap, provided with the Roche FastStart kit)	--
2.0 µl	**reagent** mix (parameter specific reagents containing primers and probes, see **7.1.**)	2.0 µl
2.0 µl	Roche Master (red/yellow cap, for preparation see Roche manuals)	4.0 µl
15.0 µl	**Volume of reaction mix**	**15.0 µl**

Table 2

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.

10. Sample data - typical results

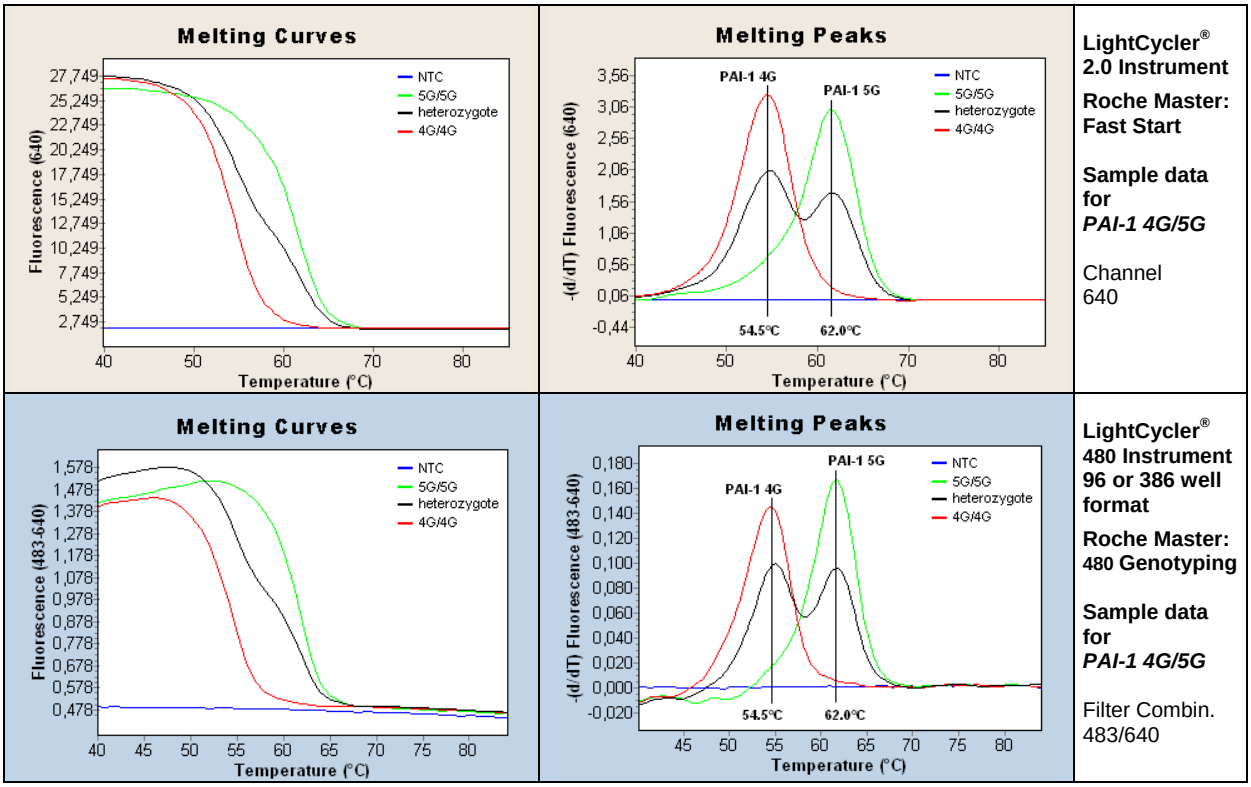

Fig.1. Sample data for the *PAI-1 4G/5G* detection system.

Upper panels: LightCycler® 2.0 Instrument. Left panel melting curves, right panel channel 640 melting peaks for *PAI-1 4G/5G*.
Lower panels: LightCycler® 480 Instrument. Left panel melting curves, right panel channel 640 melting peaks for *PAI-1 4G/5G*.

11. Interpretation of Data

Genotype:	homozygote *PAI-1 4G/4G*	heterozygote *PAI-1 4G/5G*	homozygote *PAI-1 5G/5G*
Number of melting peaks (color)	1 (red)	2 (black)	1 (green)
Melting temperature of peaks	54.5℃	54.5℃ and 62.0℃	62.0℃
Temperature difference between	---	7.5℃	---
Phenotype	higher plasma levels PAI-1	asymptomatic	asymptomatic

Table 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.
The ΔT between the melting peaks for heterozygote genotypes may vary ±1.5℃.
Samples with deviating melting curves should be subject to further investigations; sequence analysis can be provided by TIB MOLBIOL Berlin (contact service@tib-molbiol.de).

12. Version History

Notes in red mark events require to change procedures

V111027 Editorial changes
V130813 Z480 included, MSDS inlcluded, Version History included

Roche SAP order n° 05947219001

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