

# LightCycler™ -Primer Set

Ready-to-use amplification primer mix for RT-PCR using the LightCycler™ Instrument

## Human eNOS (nitric oxide synthase 3)

Kit for 96 reactions

Lot # 260704 Exp.26.07.2005

**Note:** After Thawing keep on ice!

Store the kit at -20°C

1.Kit Contents				
<b>caution</b>	After Thawing keep on ice!		<b>Sample material</b>	
<b>Kit contents</b>	<b>Vial</b>	<b>Label</b>	<b>Sample Preparation</b>	
	1	eNOS Primer mix <b>Yellow cap</b>		cDNA reverse transcribed from human RNA  Reliable and reproducible results are achieved with 1µg total RNA isolated with the HighPure total RNA Isolation Kit (Roche) reverse transcribed with the 1 <sup>st</sup> Strand cDNA Synthesis Kit (AMV) (Roche). <b>! The resulting cDNA has to be diluted to a final volume of 200-500 µl with PCR-grade water</b>
	2	Standard <b>Red cap</b>		
	3	Standard Stabilizer <b>Green cap</b>		
	4	Control cDNA <b>Blue cap</b>		
5	H <sub>2</sub> O, sterile, PCR grade <b>White cap</b>			
<b>Content and use</b>			<b>Application</b>	
		<ul style="list-style-type: none"> <li>• 200 µl ready-to-use primer mix for target specific amplification using the LightCycler™ FastStart Master Sybr Green I contains optimal MgCl<sub>2</sub> concentration and amplification primer pair</li> <li>• 60 µl amplification standard for approximately 23000 copies/µl of eNOS cDNA</li> <li>• 300 µl Solution for dilution of standard</li> <li>• 50 µl contains a cDNA mix from several human hematopoietic cell lines</li> <li>• 1 ml to adjust the final reaction volume</li> </ul>	Quantitative evaluation of gene expression in human cells and tissue	
<b>Additional equipment and reagents required</b>	1 <sup>st</sup> Strand cDNA Synthesis Kit for RT-PCR (Roche Cat. # 1 483 188) LightCycler™ FastStart Master SybrGreen I (Roche Cat. # 3 003 230) LightCycler™ Instrument (Roche Cat. # 2 011 468) LightCycler™ Primer Set Housekeeping genes (Search GmbH)		<b>Assay time</b>	
			Set up the PCR amplification <b>15 min</b> LightCycler™ PCR run <b>50 min</b>	
			<b>Number of tests</b>	
			The Kit is designed for 96 Reactions	
<b>2. Introduction</b>			<b>Quality Control</b>	
The LightCycler™-Primer Set allows to perform quantitative RT-PCR using the LightCycler™ instrument. An optimized primer pair has been selected for specific amplification of targets. The amplicon is detected by fluorescence using the double-stranded DNA binding dye Sybr®Green I.			The LightCycler™-Primer Set is tested using the LightCycler™ FastStart Master Sybr®Green I according to the protocol described below.	
			<b>Kit storage/stability</b>	
			The unopened kit is stable at -20°C 12 month from date of manufacture	
			<b>Specificity</b>	
			The LightCycler™-Primer Set "eNOS" is specific for the sequence of human eNOS and does not detect genomic eNOS specific sequences if used as directed.	

### 3. Procedure

<b>Introduction</b>	A fragment of the human eNOS cDNA sequence is amplified and monitored with the dsDNA specific Sybr <sup>®</sup> Green I dye						
<b>Additional reagents required</b>	LightCycler <sup>™</sup> FastStart Master Sybr <sup>®</sup> Green I (Cat.# 3 003 230)						
<b>Thawing the solutions</b>	Thaw the following reagents, mix gently, and store on ice:  <table border="0"> <tr> <td style="border-bottom: 1px solid black;">From the ...</td> <td style="border-bottom: 1px solid black;">Thaw the...</td> </tr> <tr> <td>LightCycler<sup>™</sup> FastStart Master Sybr<sup>®</sup>Green I</td> <td>vial <b>1a/b</b></td> </tr> <tr> <td>LightCycler<sup>™</sup> Primer Set</td> <td>all tubes</td> </tr> </table> <p><b>It is recommended to define the experimental protocol before preparing the solutions</b></p>	From the ...	Thaw the...	LightCycler <sup>™</sup> FastStart Master Sybr <sup>®</sup> Green I	vial <b>1a/b</b>	LightCycler <sup>™</sup> Primer Set	all tubes
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<b>Experimental Protocol</b>	The described protocol consists of four programs. <ul style="list-style-type: none"> <li>• Program 1: Denaturation of the template and activation of the polymerase</li> <li>• Program 2: Amplification of the target</li> <li>• Program 3: Melting curve analysis for product control</li> <li>• Program 4: Cooling the rotor and thermal chamber</li> </ul>						

### Denaturation

Parameter	Value
Cycles	1
Type	Regular
Temp. Targets	Segment 1
Target Temperature	95
Incubation time (h:min:s)	10:00
Temp. Transition Rate (°C/s)	20
Secondary Target Temp.	0
Step Size	0
Step Delay	0
Aquisition Mode	None

### Amplification

Parameter	Value		
Cycles	35		
Type	Quantification		
Temp. Targets	Seg.1	Seg.2	Seg.3
Target Temperature	95	68	72
Incubation time (h:min:s)	10	10	16
Temp. Transition Rate (°C/s)	20	20	20
Secondary Target Temp.	0	58	0
Step Size	0	0.5	0
Step Delay	0	1	0
Aquisition Mode	None	None	Single
Gains	F1 = 5		

### Melting Curve Analysis

Parameter	Value		
Cycles	1		
Type	Melting Curve		
Temp. Targets	Seg.1	Seg. 2	Seg.3
Target Temperature	95	58	95
Incubation time (h:min:s)	0	10	0
Temp. Transition Rate (°C/s)	20	20	0.1
Secondary Target Temp.	0	0	0
Step Size	0	0	0
Step Delay	0	0	0
Aquisition Mode	None	None	Cont.

### Cooling

Parameter	Value
Cycles	1
Type	Regular
Temp. Targets	Segment 1
Target Temperature	40
Incubation time (h:min:s)	30
Temp. Transition Rate (°C/s)	20
Secondary Target Temp.	0
Step Size	0
Step Delay	0
Aquisition Mode	None

<b>Preparation of the master mix</b>	Depending on the total number of reactions place LightCycler™ capillaries in pre-cooled centrifuge adaptors. It is recommended to use electronic pipettors with high quality tips (low volume retention). Prepare a master mix by multiplying the amount in the “Volume” column by the number of reactions to be analyzed, plus five additional reactions (Standard).
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Step	Action										
<b>1</b>	Prepare a fresh dilution series of the standard using the standard stabilizer solution 1:10 = 2300 copies/ $\mu$ l 1:100 = 230 copies/ $\mu$ l 1:1000 = 23 copies/ $\mu$ l										
<b>2</b>	In a 1.5 ml light protected reaction tube on ice, add the following components in the order mentioned below:										
	<table border="1"> <thead> <tr> <th>Component</th> <th>Vol.</th> </tr> </thead> <tbody> <tr> <td>H<sub>2</sub>O (white cap)</td> <td>6 <math>\mu</math>l</td> </tr> <tr> <td>LightCycler™ Primer Set (yellow cap)</td> <td>2 <math>\mu</math>l</td> </tr> <tr> <td>LightCycler™ FastStart DNA Master Sybr®Green I (premixed)</td> <td>2 <math>\mu</math>l</td> </tr> <tr> <td><b>Total Volume</b></td> <td><b>10 <math>\mu</math>l</b></td> </tr> </tbody> </table>	Component	Vol.	H <sub>2</sub> O (white cap)	6 $\mu$ l	LightCycler™ Primer Set (yellow cap)	2 $\mu$ l	LightCycler™ FastStart DNA Master Sybr®Green I (premixed)	2 $\mu$ l	<b>Total Volume</b>	<b>10 <math>\mu</math>l</b>
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<b>Total Volume</b>	<b>10 <math>\mu</math>l</b>										
<b>3</b>	<ul style="list-style-type: none"> <li>Pipet <b>10 <math>\mu</math>l</b> PCR mix into the pre-cooled LightCycler™ capillary</li> <li>Add <b>10 <math>\mu</math>l</b> of cDNA template</li> </ul>										
<b>4</b>	<ul style="list-style-type: none"> <li>Pipet <b>10 <math>\mu</math>l</b> of PCR mix into 4 pre-cooled LightCycler™ capillaries</li> <li>Add <b>10 <math>\mu</math>l</b> of undiluted and of the freshly diluted standards into each capillary</li> </ul>										
<b>5</b>	Seal each capillary with a stopper and place the adaptors, containing the capillary, into a benchtop microcentrifuge. Centrifuge at 2000 rpm for 30 s.										
<b>6</b>	Place capillaries in the rotor of the LightCycler™ Instrument.										
<b>7</b>	Cycle the samples as described above										

Typical results	
<b>Introduction</b>	The analysis of the obtained data is divided into two parts: <ul style="list-style-type: none"> <li>Part 1: Use of the quantification program, followed by</li> <li>Part 2: Specificity control of the amplification reaction by using the melting curve program</li> </ul>

<b>Quantification program</b>	The attached amplification curves in the QC sheet were obtained by performing the described procedure with the enclosed standards and control cDNA. The fluorescence values versus cycle number are displayed. The enclosed control cDNA contains approximately 20 copies per $\mu$ l of eNOS specific cDNA
<b>Melting curve program</b>	Assess the specificity of the amplified PCR product by performing a melting curve analysis. The resulting melting curves allow discrimination between specific and unspecific product The attached melting curves in the QC sheet display the amplification of the control cDNA. As a control for the specificity, 5ng of human genomic DNA was amplified in this experiment.



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