LightCycler™ -Primer Set

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

Human RANK-L (receptor activator of NFκB ligand)

Kit for 96 reactions

Lot # 100403 Exp.10.04.2004

Note: After Thawing keep on ice!

Store the kit at –20˚C

1.Kit Contents					
caution	After Thawing keep on ice!			**Sample material**	cDNA reverse transcribed from human RNA

Kit contents

Vial	Label	Content and use
1	**RANK-L Primer mix Yellow cap**	**200 µl** ready-to-use primer mix for target specific amplification using the LightCycler™ FastStart Master Sybr Green I contains optimal MgCl$_2$ concentration and amplification primer pair
2	Standard **Red cap**	60 µl amplification standard for approximately 18000 copies/µl of RANK-L cDNA
3	Standard Stabilizer **Green cap**	300 µl Solution for dilution of standard
4	Control cDNA **Blue cap**	50 µl contains a cDNA mix from several human hematopoietic cell lines
5	H2O, sterile, PCR grade **White cap**	1 ml to adjust the final reaction volume

Additional equipment and reagents required

1st 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)

2. Introduction

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.

Sample Preparation

Reliable and reproducible results are achieved with 1µg total RNA isolated with the HighPure total RNA Isolation Kit (Roche) reverse transcribed with the 1st Strand cDNA Synthesis Kit (AMV) (Roche). **The resulting cDNA has to be diluted to a final volume of 200-500 µl with PCR-grade water**

Application

Quantitative evaluation of gene expression in human cells and tissue

Assay time

Set up the PCR amplification **15 min**
LightCycler™ PCR run **50 min**

Number of tests

The Kit is designed for 96 Reactions

Quality Control

The LightCycler™-Primer Set is tested using the LightCycler™ FastStart Master Sybr®Green I according to the protocol described below.

Kit storage/ stability

The unopened kit is stable at –20˚C 12 month from date of manufacture

Specificity

The LightCycler™-Primer Set "RANK-L" is specific for the sequence of human RANK-L and does not detect genomic RANK-L specific sequences if used as directed.

3. Procedure	
Introduction	A fragment of the human RANK-L cDNA sequence is amplified and monitored with the dsDNA specific Sybr®Green I dye
Additional reagents required	LightCycler™ FastStart Master Sybr®Green I (Cat.# 3 003 230)
Thawing the solutions	Thaw the following reagents, mix gently, and store on ice: From the ... Thaw the... LightCycler™ FastStart vial **1a/b** Master Sybr®Green I LightCycler™ Primer Set all tubes **It is recommended to define the experimental protocol before preparing the solutions**

Experimental Protocol	The described protocol consists of four programs.
	    Program 1: Denaturation of the template and activation of the polymerase
	    Program 2: Amplification of the target
	    Program 3: Melting curve analysis for product control
	    Program 4: Cooling the rotor and thermal chamber

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

Preparation of the master mix	Depending on the total number of reactions place LightCycler™ capillaries in precooled 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).

Step	Action
1	Prepare a fresh dilution series of the standard using the standard stabilizer solution 1:10 = 1800 copies/μl 1:100 = 180 copies/μl 1:1000 = 18 copies/μl
2	In a 1.5 ml light protected reaction tube on ice, add the following components in the order mentioned below:

Component	Vol.
H$_2$O (white cap)	6 μl
LightCycler™ Primer Set (yellow cap)	**2 μl**
LightCycler™ FastStart DNA Master Sybr$^®$Green I (premixed)	2 μl
Total Volume	**10 μl**
3	Pipet **10 μl** PCR mix into the precooled LightCycler™ capillary Add **10 μl** of cDNA template
4	Pipet **10 μl** of PCR mix into 4 precooled LightCycler™ capillaries Add **10 μl** of undiluted and of the freshly diluted standards into each capillary
5	Seal each capillary with a stopper and place the adaptors, containing the capillary, into a benchtop microcentrifuge. Centrifuge at 2000 rpm for 30 s.
6	Place capillaries in the rotor of the LightCycler™ Instrument.
7	Cycle the samples as described above

Typical results	

Introduction	The analysis of the obtained data is divided into two parts:
	    Part 1: Use of the quantification program, followed by
	    Part 2: Specificity control of the amplification reaction by using the melting curve program

Quantification program	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 16 copies per μl of RANK-L specific cDNA
Melting curve program	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.