LightCycler™ -Primer Set

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

Human KDR (VEGF receptor 2)

Kit for 96 reactions

Lot # 100703 Exp.10.07.2004

Note: After Thawing keep on ice!

Store the kit at –20°C

1.Kit Contents

caution — After Thawing keep on ice!

Kit contents

Vial	Label	Content and use
1	**KDR** 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 13000 copies/µl of KDR 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 material — cDNA reverse transcribed from human RNA

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 "KDR" is specific for the sequence of human KDR and does not detect genomic KDR specific sequences if used as directed.

3. Procedure	
Introduction	A fragment of the human KDR 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 Master Sybr®Green I vial **1a/b** 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 = 1300 copies/μl 1:100 = 130 copies/μl 1:1000 = 13 copies/μl
2	In a 1.5 ml light protected reaction tube on ice, add the following components in the order mentioned below:
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

Component	**Vol.**
H_2O (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**

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 7 copies per μl of KDR 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.

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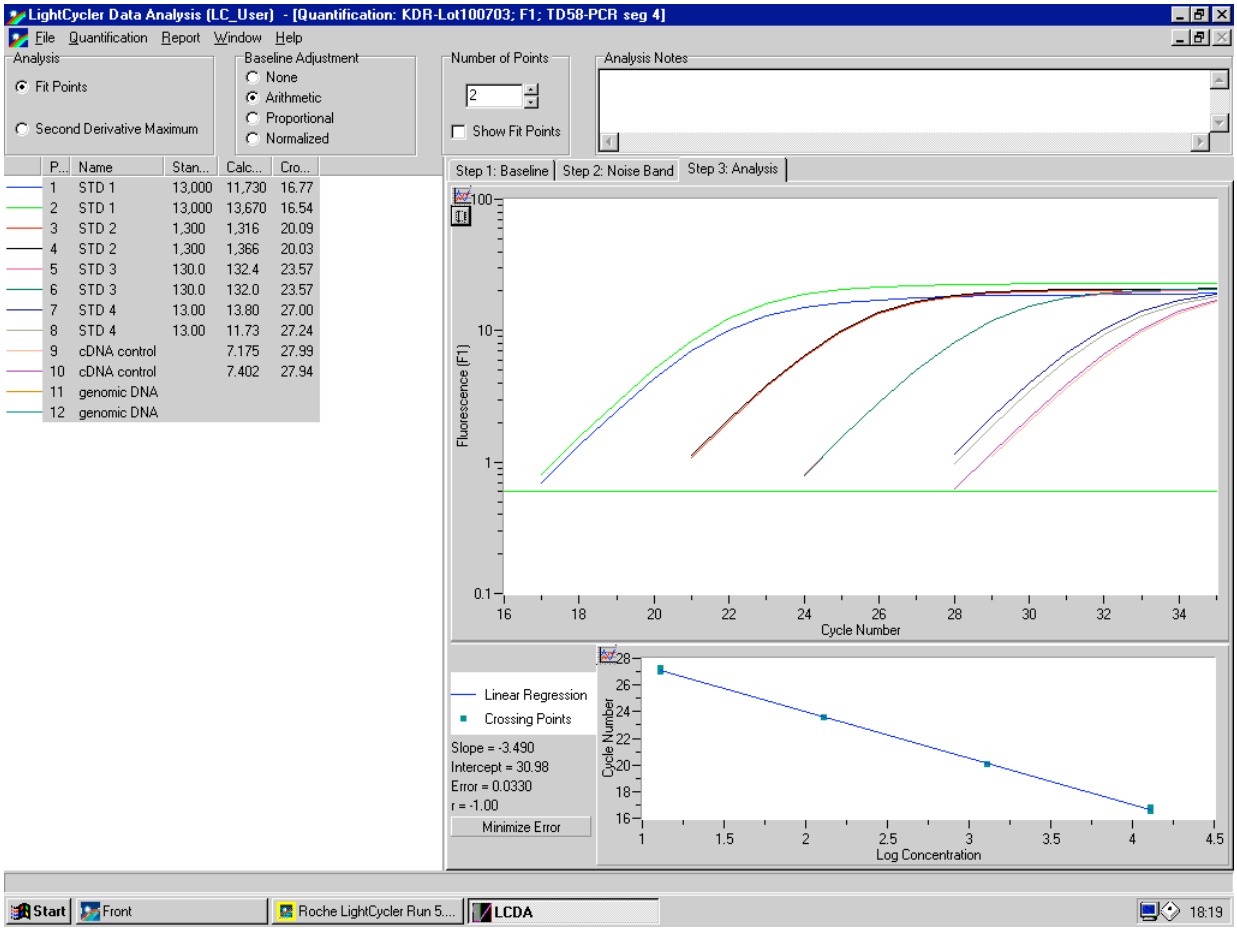
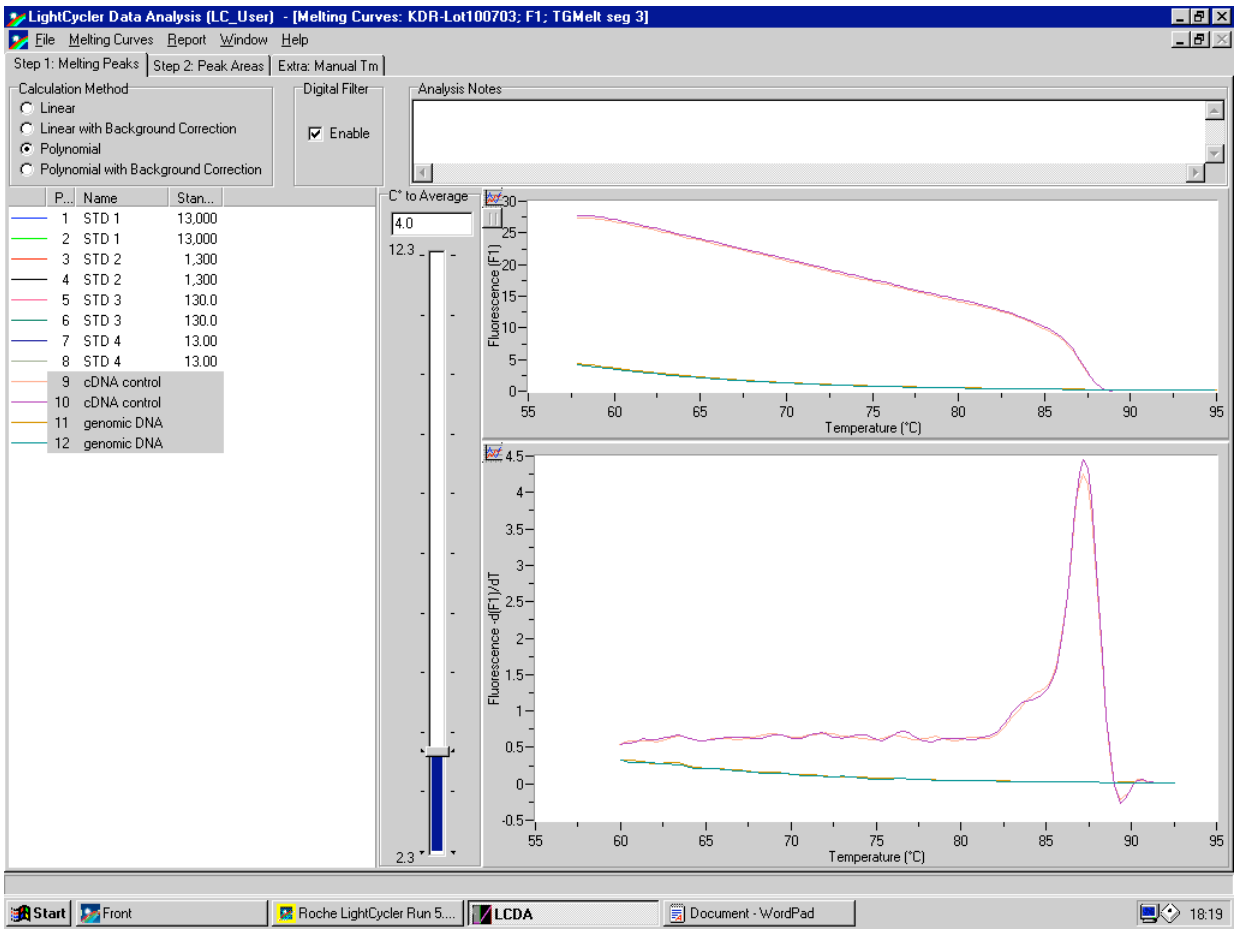

LightCycler Data Analysis (LC_User) - [Quantification: KDR-Lot100703; F1; TD58-PCR seg 4]
File Quantification Report Window Help
Analysis
Fit Points
Second Derivative Maximum
Baseline Adjustment
None
Arithmetic
Proportional
Normalized
Number of Points
2
Show Fit Points
Analysis Notes
P... Name Stan... Calc... Cro...
1 STD 1 13,000 11,730 16.77
2 STD 1 13,000 13,670 16.54
3 STD 2 1,300 1,316 20.09
4 STD 2 1,300 1,366 20.03
5 STD 3 130.0 132.4 23.57
6 STD 3 130.0 132.0 23.57
7 STD 4 13.00 13.80 27.00
8 STD 4 13.00 11.73 27.24
9 cDNA control 7.175 27.99
10 cDNA control 7.402 27.94
11 genomic DNA
12 genomic DNA
Step 1: Baseline
Step 2: Noise Band
Step 3: Analysis
Fluorescence (F1)
Cycle Number
Linear Regression
Crossing Points
Slope = -3.490
Intercept = 30.98
Error = 0.0330
r = -1.00
Minimize Error
Cycle Number
Log Concentration
Start
Front
Roche LightCycler Run 5....
LCDA
18:19


LightCycler Data Analysis (LC_User) - [Melting Curves: KDR-Lot100703; F1; TGMelt seg 3]
File Melting Curves Report Window Help
Step 1: Melting Peaks
Step 2: Peak Areas
Extra: Manual Tm
Calculation Method
Linear
Linear with Background Correction
Polynomial
Polynomial with Background Correction
Digital Filter
Enable
Analysis Notes
P... Name Stan...
1 STD 1 13,000
2 STD 1 13,000
3 STD 2 1,300
4 STD 2 1,300
5 STD 3 130.0
6 STD 3 130.0
7 STD 4 13.00
8 STD 4 13.00
9 cDNA control
10 cDNA control
11 genomic DNA
12 genomic DNA
C° to Average
4.0
12.3
2.3
Fluorescence (F1)
Temperature (°C)
Fluorescence -d(F1)/dT
Temperature (°C)
Start
Front
Roche LightCycler Run 5....
LCDA
Document - WordPad
18:19