LightCycler™ -Primer Set

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

Murine TNF-alpha

Kit for 96 reactions

Lot# 230701 Exp. 23.07.2002

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	**Murine TNF-alpha** 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 41000 copies/µl of TNF-alpha 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 a murine hematopoietic cell line and from murine spleens
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 reversely transcribed from murine RNA

Sample Preparation: Reliable and reproduceable results are achieved with 1µg total RNA isolated with the HighPure total RNA Isolation Kit (Roche) reversely 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 murine 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 "TNF-alpha" is specific for the sequence of murine TNF-alpha.

3. Procedure	
Introduction	A fragment of the murine TNF-alpha 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 = 4100 copies/μl 1:100 = 410 copies/μl 1:1000 = 41 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_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**

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 280 copies per μl of TNF-alpha 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 negative control, the template was replaced with PCR-grade water.

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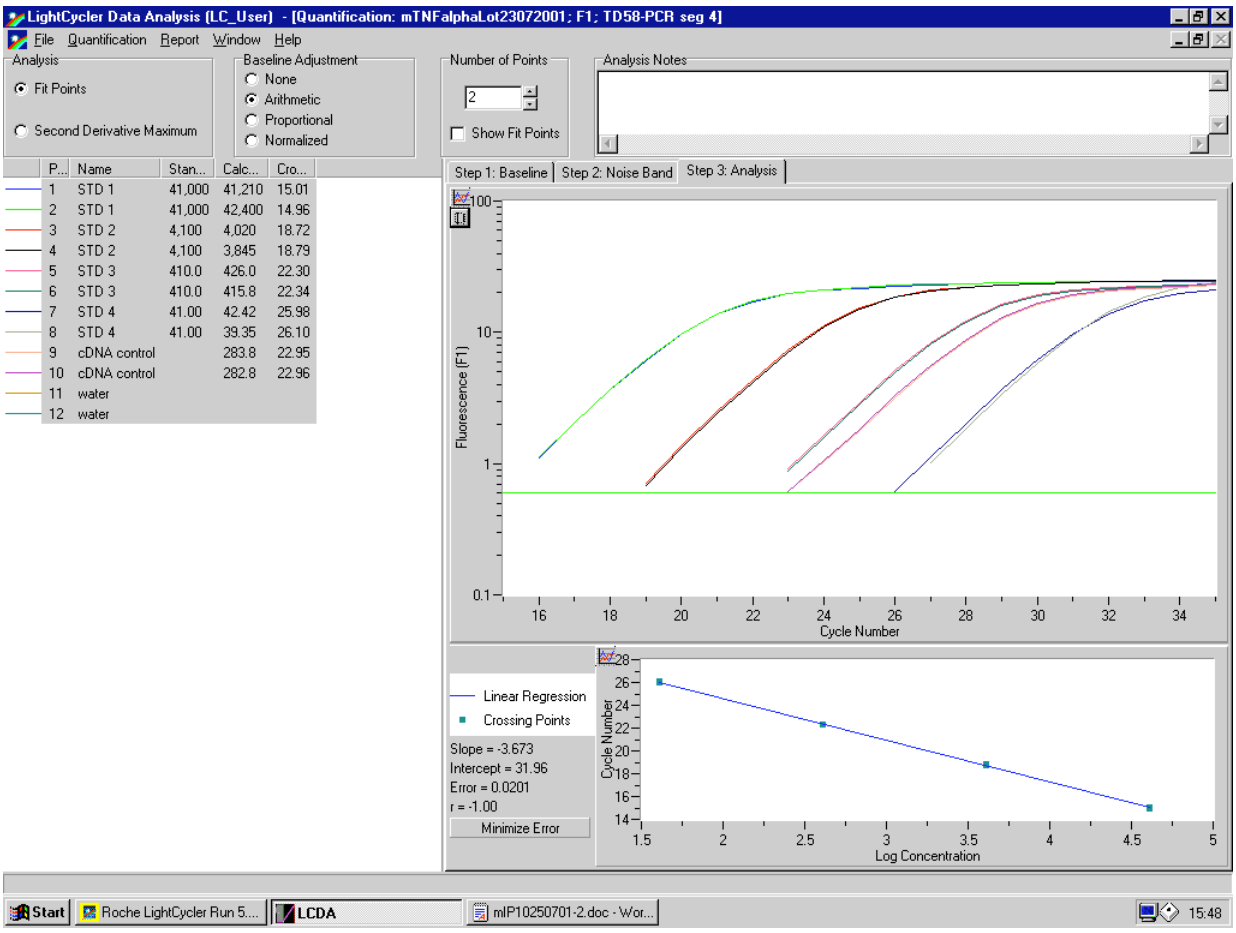
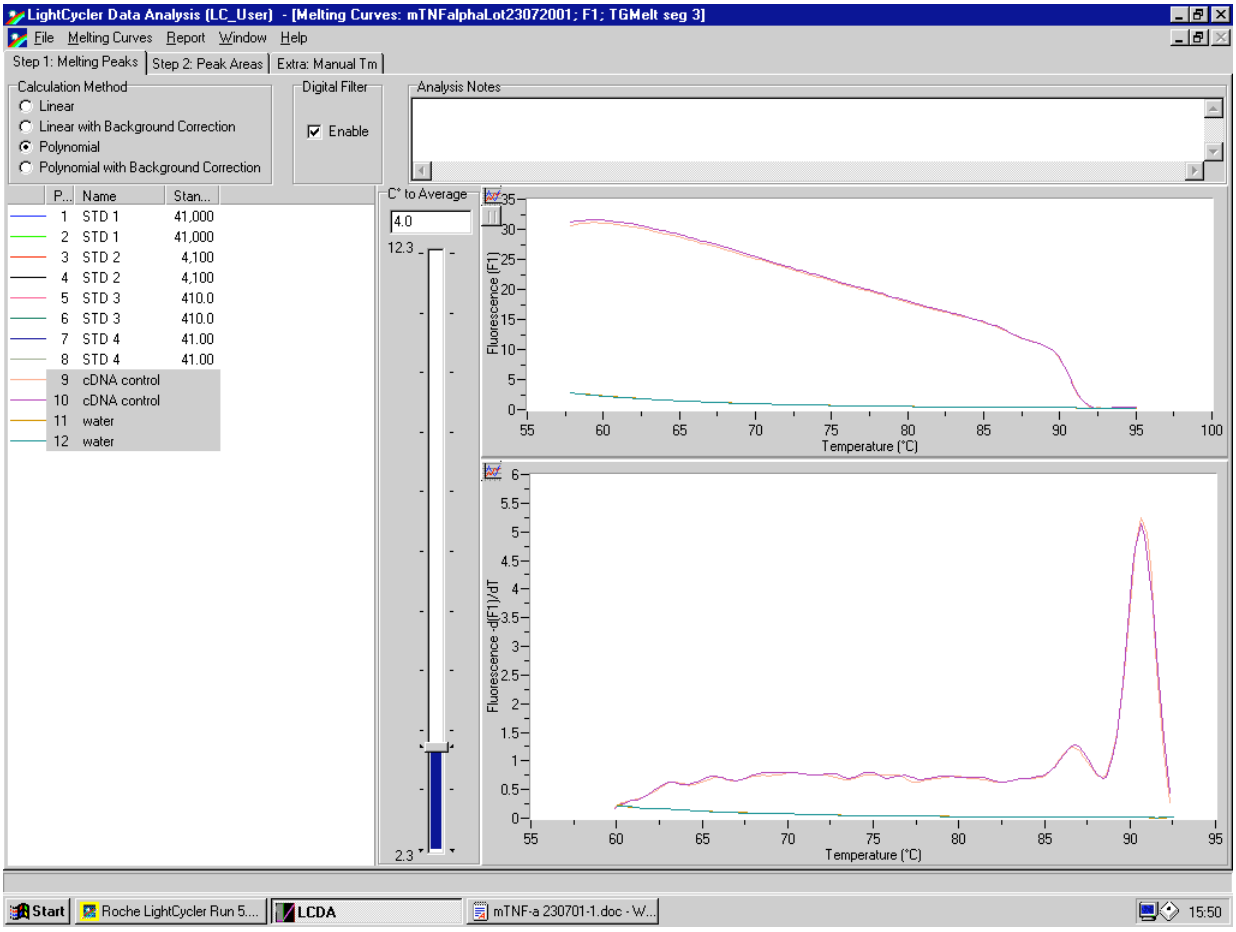

LightCycler Data Analysis (LC_User) - [Quantification: mTNFalphaLot23072001; F1; TD58-PCR seg 4]
File Quantification Report Window Help
Analysis
Fit Points
Second Derivative Maximum
Baseline Adjustment
None
Arithmetic
Proportional
Normalized
Number of Points
2
Show Fit Points
Analysis Notes
P... Name Stan... Calc... Cro...
1 STD 1 41,000 41,210 15.01
2 STD 1 41,000 42,400 14.96
3 STD 2 4,100 4,020 18.72
4 STD 2 4,100 3,845 18.79
5 STD 3 410.0 426.0 22.30
6 STD 3 410.0 415.8 22.34
7 STD 4 41.00 42.42 25.98
8 STD 4 41.00 39.35 26.10
9 cDNA control 283.8 22.95
10 cDNA control 282.8 22.96
11 water
12 water
Step 1: Baseline
Step 2: Noise Band
Step 3: Analysis
Fluorescence (F1)
Cycle Number
Linear Regression
Crossing Points
Slope = -3.673
Intercept = 31.96
Error = 0.0201
r = -1.00
Minimize Error
Cycle Number
Log Concentration
Start
Roche LightCycler Run 5....
LCDA
mIP10250701-2.doc - Wor...
15:48


LightCycler Data Analysis (LC_User) - [Melting Curves: mTNFalphaLot23072001; 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 41,000
2 STD 1 41,000
3 STD 2 4,100
4 STD 2 4,100
5 STD 3 410.0
6 STD 3 410.0
7 STD 4 41.00
8 STD 4 41.00
9 cDNA control
10 cDNA control
11 water
12 water
C° to Average
4.0
12.3
2.3
Fluorescence (F1)
Temperature (°C)
Fluorescence -d(F1)/dT
Temperature (°C)
Start
Roche LightCycler Run 5....
LCDA
mTNF-a 230701-1.doc - W...
15:50