

3. Procedure

Introduction	A fragment of the human TGF-beta1 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: <table border="1" style="width: 100%; border-collapse: collapse;"> <tr> <td style="width: 50%;"><u>From the ...</u></td> <td style="width: 50%;"><u>Thaw the...</u></td> </tr> <tr> <td>LightCycler[™] FastStart Master Sybr[®]Green I</td> <td>vial 1a/b</td> </tr> <tr> <td>LightCycler[™] Primer Set</td> <td>all tubes</td> </tr> </table> <p>It is recommended to define the experimental protocol before preparing the solutions</p>		<u>From the ...</u>	<u>Thaw the...</u>	LightCycler [™] FastStart Master Sybr [®] Green I	vial 1a/b	LightCycler [™] Primer Set	all tubes
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LightCycler [™] FastStart Master Sybr [®] Green I	vial 1a/b							
LightCycler [™] Primer Set	all tubes							
Experimental Protocol	<p>The described protocol consists of four programs.</p> <ul style="list-style-type: none"> • 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).
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Step	Action										
1	Prepare a fresh dilution series of the standard using the standard stabilizer solution 1:10 = 770 copies/ μ l 1:100 = 77 copies/ μ l 1:1000 = 7,7 copies/ μ l										
2	In a 1.5 ml light protected reaction tube on ice, add the following components in the order mentioned below: <table border="1" data-bbox="188 943 697 1137"> <thead> <tr> <th>Component</th> <th>Vol.</th> </tr> </thead> <tbody> <tr> <td>H₂O (white cap)</td> <td>6 μl</td> </tr> <tr> <td>LightCycler™ Primer Set (yellow cap)</td> <td>2 μl</td> </tr> <tr> <td>LightCycler™ FastStart DNA Master Sybr® Green I (premixed)</td> <td>2 μl</td> </tr> <tr> <td>Total Volume</td> <td>10 μl</td> </tr> </tbody> </table>	Component	Vol.	H ₂ O (white cap)	6 μ l	LightCycler™ Primer Set (yellow cap)	2 μ l	LightCycler™ FastStart DNA Master Sybr® Green I (premixed)	2 μ l	Total Volume	10 μl
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LightCycler™ FastStart DNA Master Sybr® Green I (premixed)	2 μ l										
Total Volume	10 μl										
3	<ul style="list-style-type: none"> Pipet 10 μl PCR mix into the precooled LightCycler™ capillary Add 10 μl of cDNA template 										
4	<ul style="list-style-type: none"> 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: <ul style="list-style-type: none"> 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 1200 copies per μ l of TGF-beta1 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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