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Development and Validation of an Externally Standardised Quantitative Insulin-Like Growth Factor-1 RT-PCR Using LightCycler SYBR Green I Technology

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Introduction

The cytokine insulin-like growth factor-1 (IGF-1) is considered to mediate anabolic growth hormone actions in various tissues and species. During postnatal growth, IGF-1 stimulates protein synthesis and improves glucose utilisation [7,3]. In addition, locally expressed IGF-1 is an important growth regulator acting in an auto- and paracrine manner [12]. To investigate local tissue-specific expression even in tissues with low abundancies, a very sensitive method is required which allows for reliable quantification of IGF-1 mRNA. Because of its high sensitivity, reverse-transcription with subsequent polymerase chain reaction (RT-PCR) is being increasingly used to quantify physiologically relevant changes in gene expression. RT-PCR has a detection limit 10–100 fold lower than other methods, e.g. protection-assay or northern-hybridisation, respectively [11]. The RT-ribonuclease PCR quantification technique of choice depends on the target sequence, the expected range of the mRNA amount present in the tissue, the degree of accuracy required, and whether quantification needs to be relative or absolute. Externally standardised RT-PCR with quantification on ethidium bromide stained gels followed by densitometry is widely used, but the degree of accuracy is limited and the quantification is more relative than absolute [10]. For an exact quantitative measurement of low abundant gene expression only a few PCR methods allow reliable mRNA quantification. At present the following RT-PCR methods are suitable for sensitive quantification:

1. Internally standardised competitive RT-PCR measured by HPLC separation and UV detection [9] or high resolution gel electrophoresis followed by densitometric analysis [8]: In a competitive RT-PCR, a reference RNA mutant is reverse transcribed and co-amplified in the same reaction tube with the native mRNA sequence of interest. Internally standardised RT-PCR is a very time-consuming and laborious technique. It is generally believed to yield the most precise results, because all parameters throughout RT-PCR act on both the analyte and reference mutant.

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2. Externally standardised RT-PCR with online-detection using LightCycler SYBR Green I technology [6, 13]: LightCycler PCR with SYBR Green I online detection produces reliable and rapid results. Because it uses an external standard curve, the amplification efficiencies for the calibration curve and the analyte must be equal for accurate quantification.

3. Externally standardised RT-PCR with online-detection using specific LightCycler hybridisation probes [14]: This detection format is based on fluorescence resonance energy transfer.

The development and validation of an IGF-1 mRNA RT-PCR assay on the LightCycler using SYBR Green I is described here.

Materials

Equipment
Eppendorf Mastercycler Gradient Thermal Cycler (Eppendorf)
ImageMaster (Pharmacia Biotech)
LightCycler Instrument (Roche Diagnostics)
LightCycler Capillaries (Roche Diagnostics)
LightCycler Software Version 3.39 (Roche Diagnostics)
Mac DNASISPro version 3.5 Primer design software (Hitachi)
SIGMA PLOT for Windows Version 4.01 (SPSS)

Reagents
RNA-Clean (AGS)
Cloning vector pCRII (Invitrogen)
dNTPs (dATP, dGTP, dCTP, and dTTP) (MBI Fermentas)
Guanidinium thiocyanate (ICN)
LightCycler-DNA Master SYBR Green I (Roche Diagnostics)
NuSieve agarose (FMC BioProducts)
Primers (Pharmacia Biotech)
Random Hexamer Primers (Pharmacia Biotech)
Rnasin RNase inhibitor (MBI Fermentas)
SP 6 polymerase (MBI Fermentas)
Superscript IIPlus RNase H⁻ Reverse Trancriptase (Gibco Life Technologies)
Taq DNA Polymerase (Roche Diagnostics)

Procedures

Total RNA Extraction
A total of 0.5 g frozen tissue was homogenised in 4 M guanidinium thiocyanate buffer according to Chirgwin et al. [2] to destroy RNase activity. In the following steps, the RNA-Clean protocol with phenol/chloroform extraction for total RNA was used. In order to quantify the amount of RNA extracted, the optical density was determined at three different dilutions at 260 nm corrected by the 320 nm background absorption. RNA integrity was electrophoretically verified by ethidium bromide staining and by a 260/280 nm absorption ratio >1.75.

The primers used for the production of recombinant IGF-1 RNA and for quantitative LightCycler RT-PCR were derived from the bovine IGF-1 sequence [5] (EMBL Ac. no. X15726). They were designed to produce a 240 bp amplification product spanning two RNA splicing sites in a highly conserved region (exon 3–4) [7] of the IGF-1 sequence coding for the mature IGF-1 protein. Primer design and optimisation was done with the Mac DNASIS primer design software with >94% homology between cattle, sheep, pig, water buffalo, primates, mouse and human. Primer melting temperatures (T_m in °C) were calculated with the %GC method. Primers were additionally designed as multi-species primers with >94% homology between: cattle, sheep, pig, water buffalo, primates, mouse and human (Table 1).

Primer Design

Table 1. Oligonucleotides

IGF-1 (EMBL Accession # X15726)					
	Position	Exon	Length	GC (%)	T_m (°C)
TCGCATCTCTTCTATCTGGCCCTGT	88	3	25	52.0	68.9
GCAGTACATCTCCAGCCTCCTCAGA	327 R	4	25	56.0	69.0
Bovine RT-PCR product:	88–327	3–4	240		

A recombinant IGF-1 RNA was designed for validation of the LightCycler reaction and to determine test sensitivity and quantification range. An additional primer [9] was used to generate an internal deletion of 56 bases within the IGF-1 DNA target for length differentiation on gel electrophoresis. The truncated PCR product was cloned into pCRII and recombinant IGF-1 RNA was transcribed using SP 6 polymerase.

Construction of a Recombinant IGF-1 RNA Mutant

The conditions for the RT-PCR were optimized on a gradient cycler for the annealing temperature, Taq DNA polymerase, primer, $MgCl_2$ and dNTP concentrations. RT-PCR amplification products were separated on a 4% high resolution NuSieve agarose gel and analysed with the Image Master system. Both RT and LightCycler PCR master mixes were assembled with a minimal pipetting volume of 2 µl, to minimise pipetting errors and to improve homogeneity between all reaction capillaries.

Optimization of the RT-PCR

The RT was performed in 40 µl at the following total amounts and concentrations: 1 µg total RNA, 50 mM Tris, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT and 300 µM each dNTP (dATP, dGTP, dCTP, and dTTP) 10 µM random hexamers, 100 U of Superscript II Plus RNase H- reverse transcriptase and 12.5 U of Rnasin RNase inhibitor. The RNA, salts, DTT and dNTPs were first denatured for 5 min at 65°C in an Eppendorf Mastercycler Gradient, followed by the addition of random hexamers, reverse transcriptase and RNase inhibitor. RT was done at 42°C for 60 min and terminated by heating for 1 min at 99°C.

Reverse-Transcription

LightCycler PCR Mastermix

For each LightCycler reactions a mastermix of the following components was prepared to the indicated final concentration:

	Volume (µl)	Final
LightCycler-DNA Master SYBR Green I	2.0	1×
$MgCl_2$ (25 mM)	2.4	4.0 mM
Primers (20 µM)	0.2 + 0.2	0.2 µM
H_2O (PCR grade)	13.2	–
Total volume	18.0	

To 18 µl of LightCycler mastermix in the LightCycler glass capillaries, a maximum of 10 ng cDNA in a 2-µl volume was added as PCR template. The capillaries were closed, centrifuged in a micro-centrifuge using the LightCycler centrifuge adapters and placed into the LightCycler rotor.

LightCycler Programs: Four-Segment IGF-1 Amplification Program and Melting Curve Analysis

The following LightCycler protocol was used for IGF-1 online detection using SYBR Green I, including a four-segment LightCycler PCR amplification program and melting curve analysis:

- Denaturation at 95°C for 30 s
- Amplification

Parameter	Value			
Cycles	50			
Type	Quantification			
	Segment 1	Segment 2	Segment 3	Segment 4
Target temperature (°C)	95	62	72	85
Incubation time (s)	1	10	20	3
Temperature transition rate (°C/s)	20	20	20	20
Acquisition mode	None	None	None	Single
Gain	F1 = 5			

● Melting Curve Analysis

Parameter	Value		
Cycles	1		
Type	Melting Curve analysis		
	Segment 1	Segment 2	Segment 3
Target temperature (°C)	95	60	95
Incubation time (s)	10	10	0
Temperature transition rate (°C/s)	20	20	0.10
Acquisition mode	None	None	Cont.
Gain	F1 = 5		

For exact length verification LightCycler PCR products were separated by gel electrophoresis. Amplified IGF-1 LightCycler PCR products were removed from the glass capillaries by reverse centrifugation into 1.5 ml reaction tubes. Samples were diluted in agarose gel loading buffer and loaded onto 4% high resolution NuSieve agarose gels in 1× TAE buffer. Gel analysis was performed with the Image Master.

Gel Electrophoresis of PCR Product

Results

Figure 1 shows specific LightCycler PCR products from a calibration curve of the synthetic template and of different species: cattle, sheep, pig [8] and primate (callithrix jacchus) [4], after 50 cycles. Specificity of the desired IGF-1 products was documented with melting curve analysis (LightCycler Software 3.39). The melting temperatures of the products are species dependent (Table 2). Unspecific products and primer-dimers have melting temperatures lower than 82°C (Fig. 2).

Confirmation of Primer Specificity

Table 2. Melting temperatures of IGF-1 products

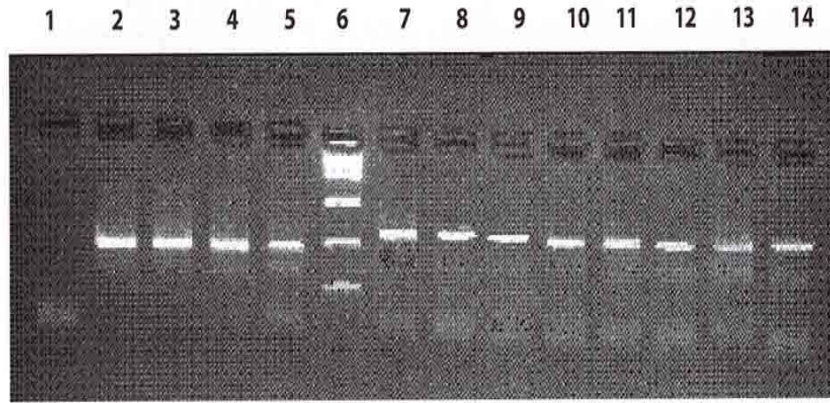| Species	Observed melting temperature
Cattle (bos taurus)	90.5°C
Sheep (ovis aries)	89.9°C
Pig (sus scrofa)	89.7°C
Primate (callithrix jacchus)	88.7°C

Fig. 1. IGF-1 specific LightCycler PCR products after 50 cycles: *Lane 1*, negative control; *lane 2–5*, calibration curve with synthetic 184 bp product ($2*10^8$ to $2*10^5$ start copies of RNA); *lane 6*, 100 bp ladder; *lane 7–14*: native IGF-1 240 bp products: 2 cattle (bos taurus), 2 sheep (ovis aries), 2 pig (sus scrofa) and 2 primates (callithrix jacchus)
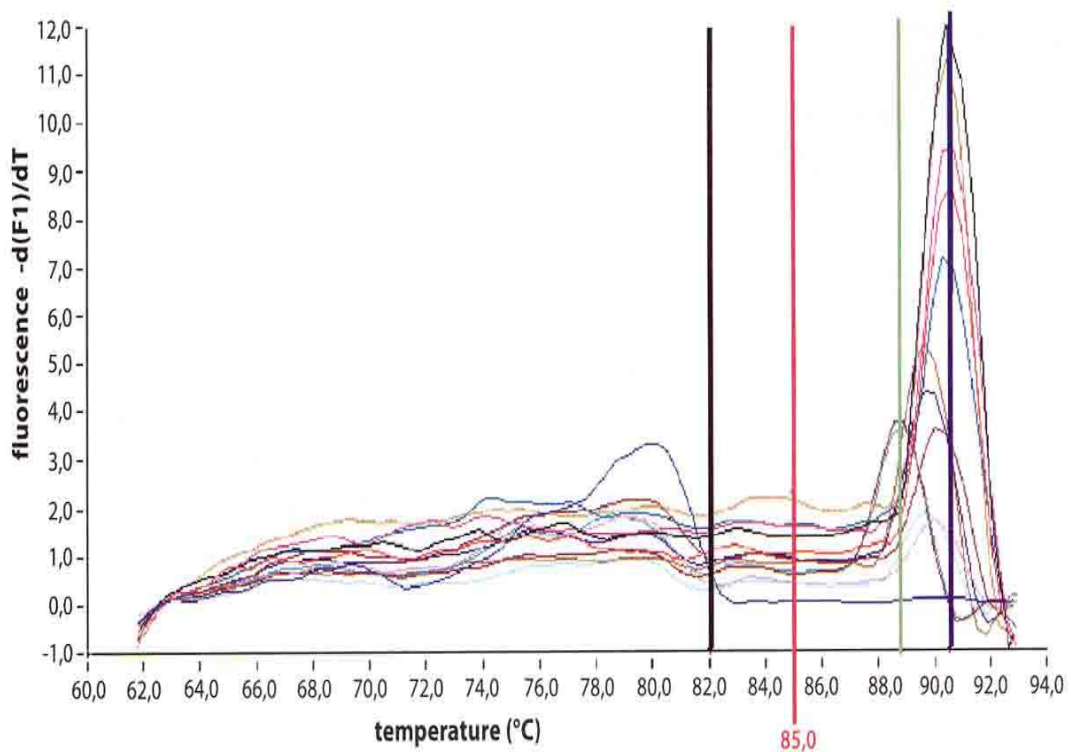

Fig. 2. Melting curves of IGF-1 LightCycler products from multiple species. Melting temperatures of IGF-1 products are between 88.7°C (callithrix jacchus) and 90.5°C (bos taurus) and for primer-dimers are lower than 82°C. The fourth segment during the amplification program melts the unspecific LightCycler PCR products at 85°C and eliminates any non-specific fluorescence signals

Advantage of a High Temperature Fluorescence Acquisition During Amplification

The fourth segment during the amplification program melts unspecific LightCycler PCR products at 85°C to eliminate the non-specific fluorescence signal and ensures accurate quantification of the desired IGF-1 products (Fig. 2). High temperature quantification keeps the fluorescence of the no template control around

1 unit, while the specific IGF-1 signal rises up to 40–50 fluorescence units. SYBR Green I determination at 85°C results in reliable and sensitive IGF-1 quantification with high linearity (correlation coefficient $r=0.99$) over seven orders of magnitude (10^2 to 10^9 RNA starting molecules) (Fig. 3b). In contrast, a conventional determination at 72°C results in a truncated quantification range ($r=0.99$) over only four orders of magnitude (10^5 to 10^9 RNA starting molecules) (Fig. 3a).
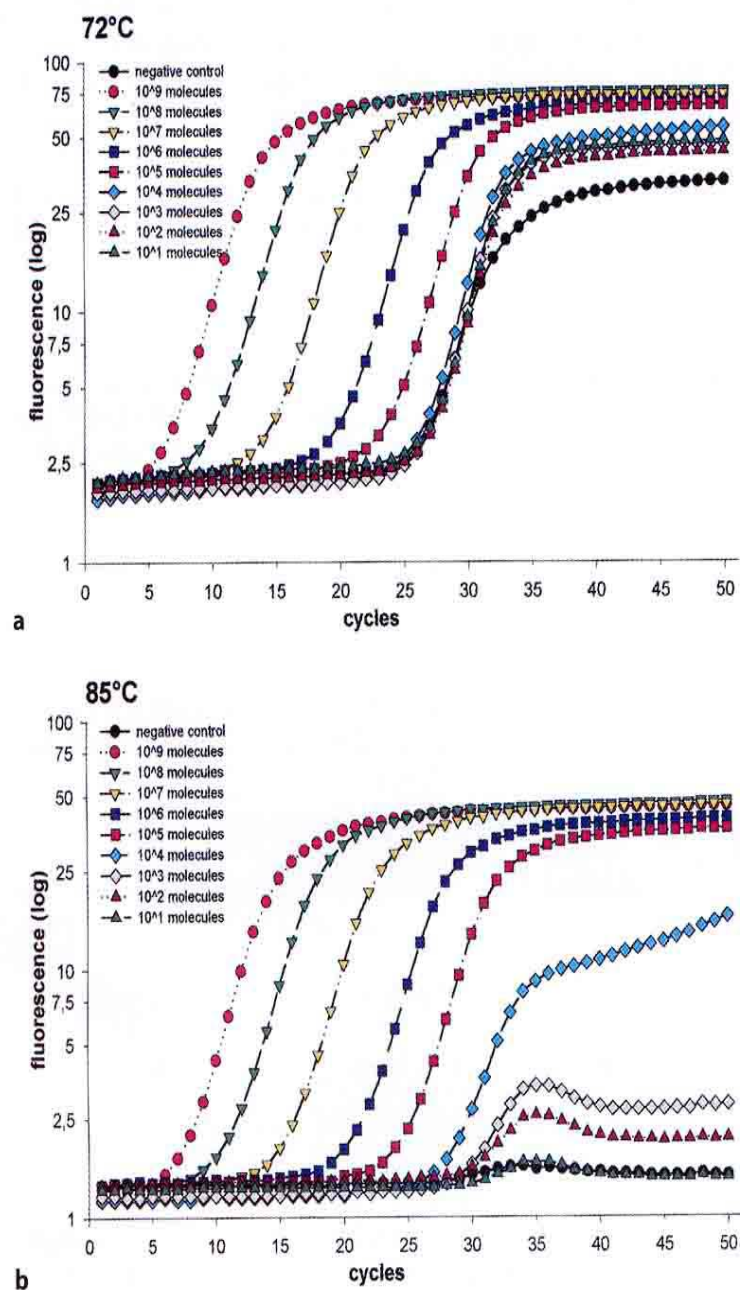

a

b

Fig. 3. The effect of SYBR Green I acquisition at 72°C in the third segment (**a**) and 85°C in the fourth segment (**b**) from 10^1 to 10^9 RNA start molecules with one negative water control. Both online quantifications were done in one LightCycler experiment with the same capillaries. Data analysis and plotting were performed with SIGMA PLOT software

Amplification Efficiencies of Recombinant IGF-1 RNA and Native IGF-1 mRNA

For reliable quantification, the amplification efficiency during PCR must be equal for recombinant RNA used in the calibration curve and native mRNA present in the investigated sample RNA. The recombinant 184 base RNA and native 240 base mRNA have previously been shown to have almost identical amplification efficiencies (E) of 66.2% and 64.7%, respectively, during the exponential phase [9]. The relationship between the initial amount A of target present and the amount Y_n of DNA produced after n PCR cycles can be expressed as $Y_n=A*(1+E)^n$, where E is the amplification efficiency of one PCR step [1]. Figure 4 shows the log fluorescence versus cycle number during the exponential phase (cycle 23–28), inter phase (cycle 29–32) and plateau phase (cycle 35–50). In order to compare the amplification efficiencies of both targets linear regressions were calculated for the exponential-, inter- and plateau phases, using SIGMA PLOT software. The resulting efficiencies (Table 3) during the exponential phase were nearly identical with high reproducibility. In the inter phase, efficiencies were variable and approached zero during the plateau phase. Negative controls containing water and no template showed no amplification products.

Sensitivity and Linearity

The sensitivity of LightCycler PCR was evaluated using different starting amounts of IGF-1 recombinant RNA from 2.8 ag (16 RNA molecules) to 28 ng ($1.6*10^{11}$ RNA molecules). The minimal detectable amount of IGF-1 RNA using SYBR Green I was 16 RNA molecules/capillary, with satisfactory test linearity ($r=0.985$) demonstrated from 1600 to $1.6*10^{11}$ RNA molecules/capillary. Using IGF-1 cDNA as template 50 molecules could be detected with high test linearity ($r=0.982$) in a range of 50 to $5*10^5$ molecules/capillary.

Table 3. LightCycler PCR efficiencies (in %; mean ± std. dev.) of native IGF-1 mRNA in 5 ng liver RNA ($n=4$), of recombinant IGF-1 RNA ($n=4$) and of a negative water control ($n=1$); ($r=$Pearson correlation coefficient)

| Template	Exponential phase (cycle 23–28)	Interphase (cycle 29–34)	Plateau phase (cycle 35–50)
Native IGF-1 mRNA	42.81±1.34 ($r=0.997$)	11.35±0.56 ($r=0.987$)	1.71±0.21 ($r=0.952$)
Recombinant IGF-1 RNA4	40.75±1.86 ($r=0.992$)	16.16±0.93 ($r=0.974$)	1.49±0.06 ($r=0.933$)
Water (no template)	0.85 ($r=0.831$)	2.59 ($r=0.952$)	(−0.43) ($r=0.789$)

Intra-assay and Inter-assay Variation

To confirm the reproducibility of LightCycler PCR even with low template copies (500 to 500,000 cDNA molecules), intra-assay variation was determined in three repeats in one LightCycler run and inter-assay variation in four experiments on 4 days using four different Master premixes (Table 4).

Table 4. Intra-assay and inter-assay variation of LightCycler IGF-1 PCR using 500–500,000 IGF-1 cDNA template molecules

cDNA template molecules	Intra-assay variation (n=3)		Inter-assay variation (n=4)	
500	13.6%	Intra-assay overall CV=11.8%	47.1%	Inter-assay overall CV=28.2%
5000	16.3%		22.5%	
50,000	11.4%		31.9%	
500,000	5.7%		11.3%	
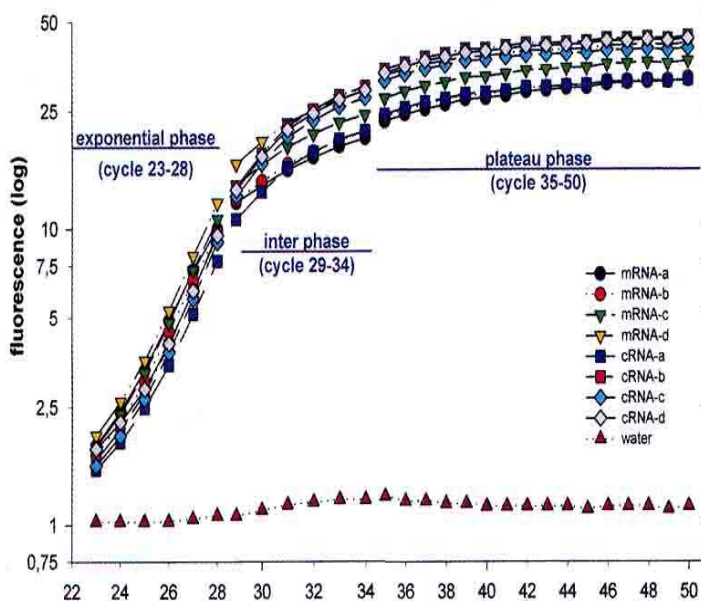
Fig. 4. Logarithmic fluorescence plot versus cycle number resulting from the amplification of liver RNA samples (5 ng) containing approximately 6.06 * 10^5 native IGF-1 mRNA molecules (n=4) or recombinant 6 * 10^5 IGF-1 RNA (n=4) and a water control containing no IGF-1 templates (n=1). Amplification efficiencies for the IGF-1 mRNA and recombinant RNA templates were recorded during the exponential phase (cycle 23–28), inter phase (cycle 29–32) and plateau phase (cycle 35–50) and calculated from fluorescence raw data

Comments

Primer design is essential for successful online LightCycler PCR quantification. Even one mismatch within the primers can result in missing PCR products or reduced specificity (data not shown). Because of its high ramping rates, LightCycler PCR offers highly stringent reaction conditions for PCR.

Optimal Primer Design and Influence on LightCycler PCR

The high stringency in LightCycler PCR results in lower PCR efficiencies. Prior work with conventional thermal cyclers gave efficiencies of 64.7% and 66.2% for IGF-1 mRNA and recombinant RNA, respectively, whereas amplification on the LightCycler resulted in lower values of 40.8% and 42.8% [9]. Reasons for this effi-

Amplification Efficiencies in the LightCycler

ciency drop may include the higher ramping rates, and the short annealing and elongation times of LightCycler PCR. The product length determines the required elongation step duration, which is limited by the polymerase extension rate (~1000 bp per min elongation time). Efficiency is usually high with a product size of around 200–400 bp and a longer elongation step duration may enhance reaction efficiency. The starting amount of reverse transcribed total-RNA should not exceed 20 ng/capillary. Higher concentrations inhibit PCR.

Rare Transcript IGF-1 Quantification

The use of RT followed by LightCycler PCR is a simple and sensitive method of detecting low amounts of mRNA molecules and offers important insights into the local expression of transcripts present in low abundance. The reliability of the assay depends on the condition of identical amplification efficiencies for both the wild-type mRNA and the recombinant RNA. As demonstrated herein, amplification efficiencies were nearly identical. The sensitivity, linearity and reproducibility of the LightCycler PCR assay allows for the absolute and accurate quantification of IGF-1 mRNA molecules even in tissues or cells with low abundancies or when very small amounts of RNA are available. The variability of the IGF-1 test rises as the number of starting template molecules decrease. We have used this IGF-1 mRNA quantification system to compare the IGF-1 expression rates in bovine tissues (bos taurus) [EMBL Ac. no. X15726]. The method can also be used in other species like sheep (ovis aries) (EMBL Ac. no. M30653), pig (sus scrofa) (EMBL Ac. no. X17492) and primates (callithrix jacchus) (EMBL Ac. no. Z49055) with sufficiently high homologies of the amplified IGF-1 fragment.

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