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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Please refer to our web-site at http://biochem.roche.com/prod_inf/biochemi/no3_2000/pfaffl.htm for the complete version of this article including experimental procedures, materials employed, and important references. The complete article will also be published soon in: Rapid Cycle Real-time PCR, Methods and Applications, Springer Press, Heidelberg.

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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. In addition, locally expressed IGF-1 is an important growth regulator acting in an auto- and paracrine manner.

To investigate local tissue-specific expression, even in tissues with low abundancies, a very sensitive method that allows for reliable quantification of IGF-1 mRNA is required. 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 protection-assay or northern hybridisation, respectively. 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. 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:

- Internally standardised competitive RT-PCR measured by HPLC separation and UV detection or high resolution gel electrophoresis followed by densitometric analysis: In 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.

- Externally standardised RT-PCR with online detection using LightCycler SYBR Green I technology: 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 must be equal to the analyte for accurate quantification.

- Externally standardised RT-PCR with online detection using specific LightCycler hybridisation probes: 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.

Results

Confirmation of primer specifity

Figure 1 shows specific LightCycler PCR products from a calibration curve of the synthetic template and of different species: cattle, sheep, pig, and primate *(Callithrix jacchus),* 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 1). Unspecific products and primer-dimers have melting temperatures lower than 82°C (Figure 2).

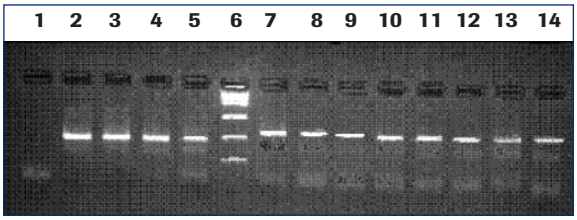

Figure 1: IGF-1 specific LightCycler PCR products after 50 cycles: *Lane 1, negative control; lane 2-5, calibration curve with synthetic 184 bp product (2 x10^8 to 2 x10^5 start copies of RNA); lane 6, 100 bp ladder; lane 7-14, native IGF-1 240 bp products: 2 cattle (Bos taurus), 2 sheep (Ovis aries), 2 pig (Sus scrofa), and 2 primates (Callithrix jacchus).*

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

Table 1: *Melting temperatures of IGF-1 products*

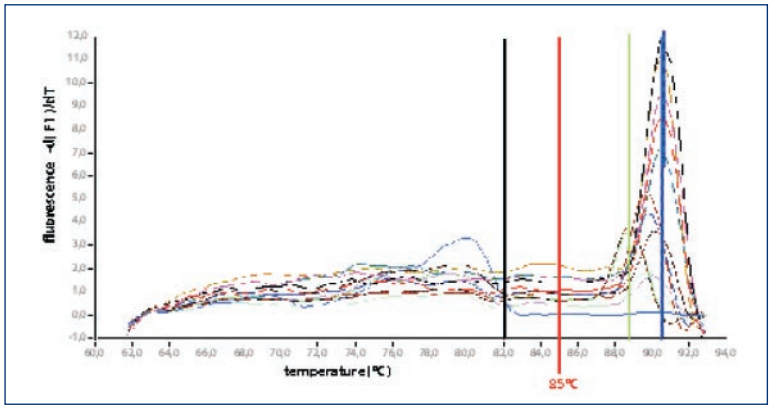

Figure 2: Melting curves of IGF-1 LightCycler products from multiple species. *Melting temperatures of IGF-1 products are between 88.7°C (Callithrix jacchus) and 90.5°C (Bos taurus) and for primer-dimers are lower than 82°C. The fourth segment during the amplification program melts the unspecific LightCycler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

Advantage of a high temperature fluorescence aquisition during amplification

The fourth segment during the amplification program melts non specific LightCycler PCR products at 85°C to eliminate the non specific fluorescence signal and ensures accurate quantification of the desired IGF-1 products (Figure 2). High temperature quantification keeps the fluorescence of the no template control to 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) (Figure 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) (Figure 3a).

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 curves, and for 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. The relationship between the initial amount of target present (A) and the amount of DNA produced after n PCR cycles(Yn) can be expressed as Yn=A x (1+E)n, where E is the amplification efficiency of one PCR step. Figure 4 shows the log fluorescence versus cycle number during the exponential phase (cycles 23–28), inter phase (cycles 29–32) and plateau phase (cycles 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 2) during the exponential phase were nearly identical, with high reproducibility. In the interphase, efficiencies were variable; 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 ng (16 RNA molecules) to 28 ng (1.6 x 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 x 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 x 10^5 molecules/capillary.

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 interassay variation in four experiments on 4 days using four different master premixes (Table 3).

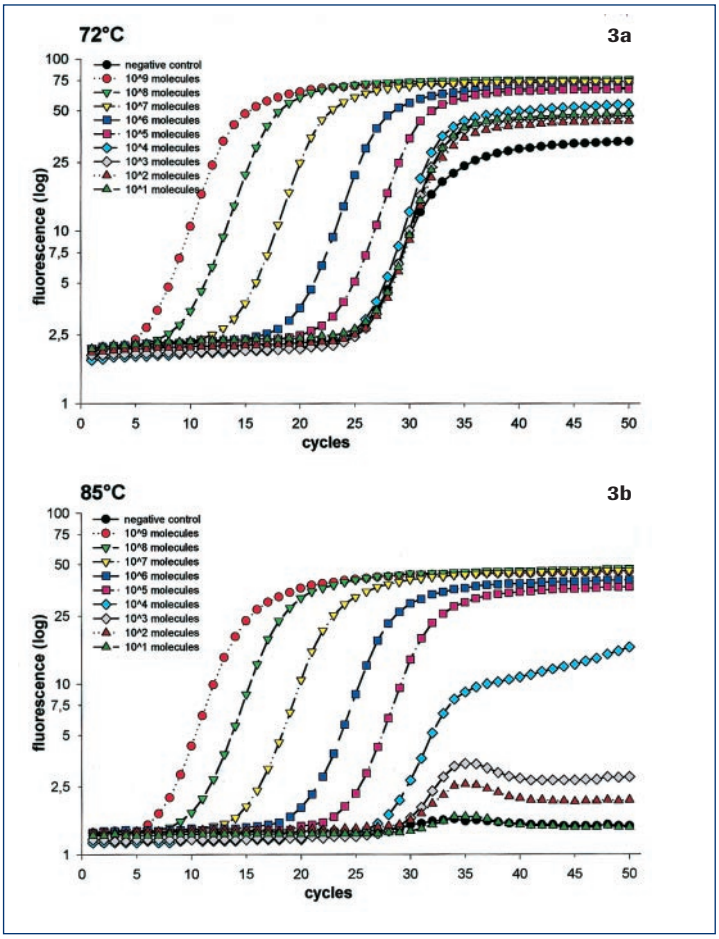

Figure 3: Effect of aquisition at different temperature levels: *the effects of SYBR Green I acquisition at 72°C in the third segment (a) and 85°C in the fourth segment (b) from 10^1 to 10^9 RNA start molecules with one negative water control. Both online quantifications were done in one LightCycler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

Conclusions

Primer design is essential for successful online LightCycler PCR quantification. 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.

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%. Reasons for this efficiency 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 minute 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.

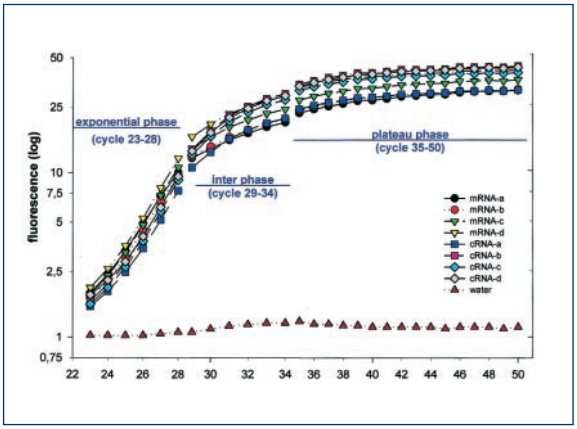

Figure 4: Logarithmic fluorescence plot versus cycle number *resulting from the amplification of liver RNA samples (5 ng) containing approximately 6.06 x10^5 native IGF-1 mRNA molecules (n=4) or recombinant 6 x 10^5 IGF-1 RNA (n=4) and a water control containing no IGF-1 templates (n=1). Amplification 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.*

Template	Exponential phase (cycles 23–28)	Interphase (cycles 29–34)	Plateau phase (cycles 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 RNA	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)

Table 2: *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).*

cDNA template molecules	Intra-assay variation (n=3)	Inter-assay variation (n=4)
500	13.6%	47.1%
5000	16.3%	22.5%
50,000	11.4%	31.9%
500,000	5.7%	11.3%
	Intra-assay overall CV=11.8%	Inter-assay overall CV=28.2%

Table 3: *Intra-assay and inter-assay variation of LightCycler IGF-1 PCR using 500–500,000 IGF-1 cDNA template molecules*

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, such as 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.

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.

Acknowledgements

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Order INFO

Product	Cat. No.	Pack Size
LightCycler Instrument	2 011 468	1 piece
LightCycler Capillaries	1 909 339	1 set
LightCycler-DNA Master SYBR Green I	2 015 102	1 kit (96 reactions)