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Validities of mRNA quantification using recombinant RNA and recombinant DNA external calibration curves in real-time RT-PCR

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Abstract

Reverse transcription (RT) followed by polymerase chain reaction (PCR) is the technique of choice for analysing mRNA in extremely low abundance. Real-time RT-PCR using SYBR Green I detection combines the ease and necessary exactness to be able to produce reliable as well as rapid results. To obtain high accuracy and reliability in RT and real-time PCR a highly defined calibration curve is needed. We have developed, optimised and validated an Insulin-like growth factor-1 (IGF-1) RT-PCR in the LightCycler, based on either a recombinant IGF-1 RNA (recRNA) or a recombinant IGF-1 DNA (recDNA) calibration curve. Above that, the limits, accuracy and variation of these externally standardised quantification systems were determined and compared with a native RT-PCR from liver total RNA. For the evaluation and optimisation of cDNA synthesis rate of recRNA several RNA backgrounds were tested. We conclude that external calibration curve using recDNA is a better model for the quantification of mRNA than the recRNA calibration model. This model showed higher sensitivity, exhibit a larger quantification range, had a higher reproducibility, and is more stable than the recRNA calibration curve.

Introduction

Because of its high sensitivity, RT-PCR is increasingly used to quantify physiologically changes in gene expression. The RT-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 (Freeman *et al.* 1999). The 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 a fully quantitative measurement of low abundant gene expression only a few PCR methods are reliable. At present internally standardised, competitive RT-PCR (Becker-Andreé *et al.* 1989, Gilliland *et al.* 1990, Siebert & Larrick 1992), externally standardised, real-time RT-PCR using SYBR Green I technology (Morrison *et al.* 1998, Wittwer & Garling 1991), or externally standardised real-time RT-PCR using specific fluorescence dye labelled hybridisation probe(s) (Leutenegger *et al.* 1999, Loeffler *et al.* 2000, Winer *et al.* 1999), are suitable for sensitive quantification. Probe-based detection formats are based on fluorescence resonance energy transfer (FRET) (Wittwer *et al.* 1997) in the LightCycler system (Roche Diagnostics, Basel, Switzerland) or on reporter fluorescence primarily by Förster type energy transfer (Förster 1948, Lakowicz 1983) in the TaqMan system (PE Applied Biosystems, Foster City, USA). Only real-time RT-PCR with SYBR Green I technology combines the ease and necessary exactness to be able to produce reliable as well as rapid results. Because of its external calibration curve this method has to be highly validated and the identical LightCycler PCR amplification efficiencies for calibration curve and target mRNA must be confirmed. In general

two types of external calibration curves are possible. Type one based on a recombinant RNA (recRNA) standard target and the type two on a recombinant DNA (recDNA) target. Alternatively, only a purified RT-PCR product can be used as calibration material, but the long term stability and the reproducibility of the calibration is not very satisfactory (data not shown). In case of using the calibration recRNA model both, standard recRNA and unknown sample RNA, underwent parallel RT and real-time PCR conditions. If recDNA is used as calibration model, the standard samples only underwent the real-time PCR conditions and the amplification results may differ from the RT-PCR results in the unknown samples.

In this paper these two models are investigated in the LightCycler real-time PCR system, using a multi-species RT-PCR for IGF-1. IGF-1 is considered to mediate the anabolic growth hormone actions in various tissues and species. During postnatal growth, IGF-1 stimulates protein synthesis and improves glucose utilisation (Simmen 1991). Above that, locally expressed IGF-1 is an important growth regulator acting in an auto- and paracrine manner (Thissen *et al.* 1994).

The aim of this study was the comparison of two types of calibration models, leading back on the identical gene target, as well as the optimisation and validation of these models using SYBR Green I fluorescence technology. Results were compared with a native RT-PCR from liver total RNA. Derived limits and accuracy of this methodology are described and optimisation strategies for LightCycler PCR are discussed to achieve accurate, reproducible and reliable results in the unknown samples RNA.

Material and methods

Performed total RNA extraction

RNA extraction was performed in tissue, bacterial cell pellet (*Escherichia coli*) or insect cell pellet (*Spodoptera frugiperda*) as described earlier (Pfaffl *et al.* 1998b). RNA integrity was electrophoretically verified by ethidium bromide staining and by OD_{260}/OD_{280} nm absorption ratio higher than 1.85/1.

Multi-species IGF-1 primer design

The primers used for the production of IGF-1 recRNA and recDNA were derived from the bovine IGF-1 sequence (Fotsis *et al.* 1990) and designed to produce a 240 bp amplification product spanning two RNA-splicing sites in the highly conserved region of exon 3–4 (Simmen 1991) coding for the mature IGF-1 protein. Primer design and optimisation in regard to primer dimer, self priming formation and primer melting temperature was done with the Mac DNASIS primer design software (Hitachi Software, Yokohama, Japan) with >94% homology between: cattle (*Bos taurus*), water buffalo (*Bubalus arnee bubalis*), sheep (*Ovis aries*), pig (*Sus scrofa*), mouse (*Mus musculus*), and human (*Homo sapiens*). Following primers were used: Forward primer 5′-TCG CAT CTC TTC TAT CTG GCC CTG T-3′ Reverse primer 5′-GCA GTA CAT CTC CAG CCT CCT CAG A-3′.

Construction of recRNA mutant and recDNA

A recombinant IGF-1 RNA mutant was designed for recRNA calibration curve model. RecRNA standard material was already successfully used in an internal standardised competitive IGF-1 RT-PCR (Pfaffl *et al.* 1998b). In brief: a PCR technology with an additional primer was performed to generate an internal deletion of 56 bases within the 240 bp IGF-1 DNA target for length differentiation. The truncated PCR product (184 bp) was cloned in pCRII (Invitrogen, Leek, The Netherlands) and IGF-1 recRNA (184 bases) was transcribed using SP 6 polymerase (MBI Fermentas, Vilnius, Lithuania). RecDNA standard was derived by cloning the native 240 bp IGF-1 RT-PCR product in pCRII (Invitrogen).

Generation of RNA and DNA external calibration curves

RecRNA and linearised plasmid bearing the recDNA were gel purified and quantified by multiple ($n = 10$) optical measurements in various dilutions and concentrations. From the derived calculated molecular concentration [using the molecular mass of single stranded (ss) IGF-1 recRNA and double stranded (ds) IGF-1 recDNA] accurate calibration point aliquots from 10^{10} ss molecules μl^{-1} down to <10 ss molecules μl^{-1} were diluted for both models. Calibration curve dilution were frozen at $-80\,^{\circ}C$ with RNAase inhibitor (MBI Fermentas).

Reverse-transcription (RT) with different concentrations of RNA background

One μg total RNA from sample preparation or recRNA (10^1 to 10^9 ss recRNA start molecules) were reverse transcribed with 100 U of Super script II Plus RNase H^- Reverse Trancriptase (Gibco Life technologies, Gaithersburg, USA) using 100 μM random hexamer primers (Pharmacia Biotech) according to the manufactures instructions.

For the determination of cDNA synthesis efficiency during RT, various recRNA dilutions were balanced with insect total RNA (*Spodoptera frugiperda*) or bacterial total RNA (*Eschericha coli*). In each experiment 4×10^4 to 4×10^9 recRNA start molecules were reverse transcribed with various (none, 2, 10, 20 ng) prokaryotic or eukaryotic total RNA per capillary.

Optimisation of IGF-1 specific RT-PCR

Conditions for IGF-1 RT-PCR were optimised in a gradient cycler (Mastercycler Gradient; Eppendorf, Germany) with regard to Taq DNA polymerase (Roche), PCR water pH, IGF-1 forward and reverse primer (Pharmacia), $MgCl_2$(Roche), dNTPs (MBI Fermentas) concentrations and various annealing temperatures. Amplification products were separated on a 4% high resolution NuSieve agarose (FMC Bio Products, Rockland, USA) gel electrophoresis and analysed with the Image Master system (Pharmacia). Optimised results were transferred on the following LightCycler PCR protocol.

Multi-species IGF-1 real-time PCR

For LightCycler reactions a mastermix of the following reaction components was prepared to the indicated end-concentration: 6.4 μl water, 1.2 μl $MgCl_2$(4 mM), 0.2 μl Forward Primer (0.4 μM), 0.2 μl Reverse Primer (0.4 μM) and 1 μl LightCyler DNA Master SYBR Green I (Roche). 9 μl of LightCycler mastermix was filled in the LightCycler glass capillaries and 1 μl volume was added as PCR template. Capillaries were closed, centrifuged and placed into the LightCycler rotor. To improve SYBR Green I quantification a high temperature fluorescence measurement point at 85 °C was performed (Pfaffl 2000). It melts the unspecific PCR products below 85 °C, e.g., primer dimers, eliminates the non-specific fluorescence signal and ensures accurate quantification of the desired IGF-1 product. The following LightCycler protocol was used: denaturation program (95 °C for 30 s), a four-segment amplification and quantification program repeated 50 times (95 °C for 1 s; 62 °C for 10 s; 72 °C for 20 s; 85 °C for 3 s with a single fluorescence measurement), melting curve program (60 °C to 95 °C with a heating rate of 0.1 °Cs^{-1} and continuous fluorescence measurements) and finally a cooling program down to 40 °C.

Results

Confirmation of primer specificity

Specificity of the desired IGF-1 products was documented with high-resolution gel electrophoresis and additionally with LightCycler melting curve analysis. Derived melting temperature of IGF-1 RT-PCR products are species specific [88.7 (Primate: *Callithrix jacchus*), 89.7 °C (*Sus scrofa*), 89.9 °C (*Ovis aries*) and 90.5 °C (*Bos taurus*)]. IGF-1 primer-dimer formation(s)showed melting temperatures of about 80 °C.

Amplification efficiencies, sensitivity and linearity

Sensitivity of the LightCycler PCR was evaluated using different starting amounts of recRNA, recDNA or a native liver total RNA (Table 1). In the recRNA calibration model 16 recRNA molecules could be detected with no RNA background. The SYBR Green I determination at the elevated temperature 85 °C resulted in a reliable and sensitive IGF-1 quantification with high linearity (correlation coefficient $r = 0.99$) over seven orders of magnitude from 1.6×10^3 to 1.6×10^{10} recRNA start molecules (Pfaffl 2000). Using IGF-1 recDNA as template 6 molecules set the detection limit with high test linearity in the quantification range of 60 to 6×10^{10} recDNA molecules/capillary (Table 1). In a native liver total RNA sample 13 start molecules could be detected in 80 pg RNA, using the recDNA calibration curve. Real-time PCR efficiencies were calculated from the given slopes in LightCycler Software 3.01 (Roche) and showed efficiency rates per cycle in the recDNA calibration curve model of 1.93, liver sample PCR of 1.89 and recRNA calibration curve model of 1.77.

Intra-assay and inter-assay variation

To confirm precision and reproducibility of real-time PCR the intra-assay precision was determined in 4

Table 1. Characterisation of real-time IGF-1 LightCycler PCR using either recRNA or ss recDNA external calibration curve in comparison with the native liver total RNA. Intra- and inter-assay variation of calibration curve models are mean values ($n = 4$) determined in Table 2.

	IGF-1 recRNA calibration curve	IGF-1 recDNA calibration curve	Unknown IGF-1 mRNA
Start template	IGF-1 recRNA	IGF-1 recDNA	IGF-1 mRNA
Amplification efficiency	1.77	1.93	1.89
Detection limit	16 molecules	6 molecules	80 pg liver total RNA
Quantification limit	1600 molecules	60 molecules	500 pg liver total RNA
Quantification range (test linearity)	1600–1.6×10^{10} molecules ($r = 0.992$)	60–6×10^{10} molecules ($r = 0.996$)	500 pg–50 ng liver total RNA ($r = 0.933$)
Intra-assay variation	2.7% ($n = 4$)	0.7% ($n = 4$)	1.2% ($n = 4$)
Inter-assay variation	4.5% ($n = 4$)	2.6% ($n = 4$)	4.9% ($n = 4$)

repeats within one LightCycler run. Inter-assay variation was investigated in 4 different experiment runs in two days using 4 different premix cups within one batch of LightCycler DNA Master SYBR Green I kit (Roche). Determination of variation was done in both calibration curve models over a wide molecule range and for native mRNA quantification in 10 ng liver total RNA containing approximately 6×10^5 IGF-1 mRNA molecules. In the intra-assay experiments the variability rose as the number of starting template molecules decreased. In the inter-assay experiment it was vice versa (Table 2). Calculation of test precision and test variability is based on the variation of crossing points (CP) from the CP mean value. Figure 1 shows the fluorescence history and the influence of different recDNA IGF-1 start molecules on the CP. Four replicates of 6×10^6 down to 6×10^1 recDNA start molecules concentrations were shown. Analysis line was set to a fluorescence level of 2 where determination of CP was performed. Corresponding CP data of different starting concentrations are shown in Table 2. There is a linear relationship between the log of the start molecules and the corresponding CP during real-time PCR. The CP is defined as the point at which the fluorescence rises appreciably above the background fluorescence. From the given start molecules a linear calibration curve with a distinct coefficient of correlation (r) can be constructed by plotting the log of start molecules versus the CP.

cDNA synthesis efficiency of recRNA with bacterial and insect cell RNA background

To enhance the RT, as well as the real-time PCR efficiencies in a recRNA calibration model, different RNA background were tested during RT. The influence of rising concentrations of RNA background (none, 2, 10 and 20 ng/capillary) of bacterial or insect cell total RNA on cDNA synthesis efficiency are shown in Table 3. The investigated *Escherichia coli* and *Spodoptera frugiperda* RNA backgrounds were free of the IGF-1 mRNA target. Calibration curves with different background concentrations were only shifted parallel to each other with high linearity ($r > 0.993$) over the investigated molecule ranges. Efficiency data were normalised in comparison to no RT of the recDNA calibration curve (no RT = 100%) from 4×10^2 to 4×10^6 ss recDNA molecules, which was amplified in parallel in a single run. Prokaryotic and eukaryotic RNA background suppressed the cDNA synthesis rate extremely, up to >9.7 to >6.5 times respectively in comparison to no RT recDNA calibration curve. Further real-time PCR efficiency was suppressed in experiments with high bacterial or insect RNA background down to 1.32 or 1.43 respectively. In both experiments the best RT and real-time efficiencies were derived with low background concentrations for prokaryotic or eukaryotic RNA added.

Discussion

RT with followed by LightCycler PCR is a simple and sensitive method to detect low amounts of mRNA

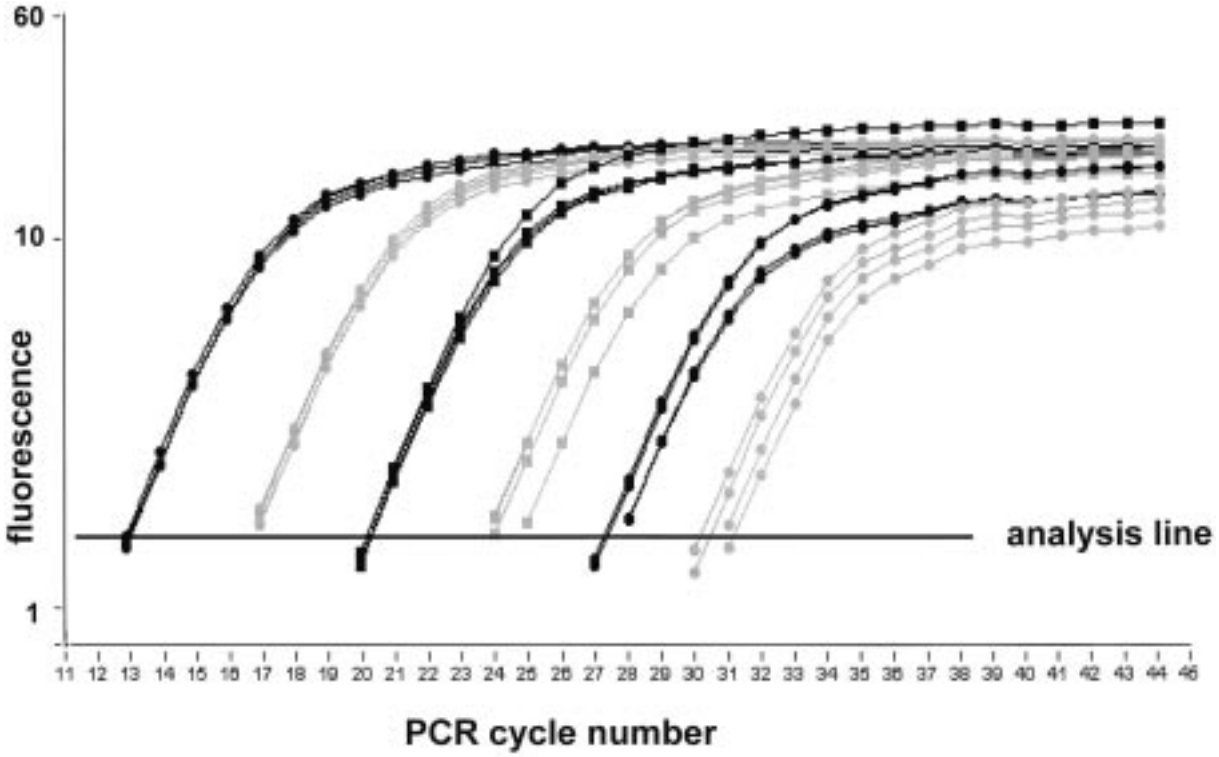

Fig. 1. Logarithmic fluorescence plot versus cycle number is shown, resulting from the determination of intra-assay variation in recDNA calibration curve model. Four replicates of different concentrations of recDNA start molecules were plotted [6×10^6 (●); 6×10^5 (●) ; 6×10^4 (■); 6×10^3 (■); 6×10^2 (●); 6×10^1 (■) start molecules]. Analysis line was set to a fluorescence level of 2 where measurement of crossing points (CP) was done. Corresponding CP mean data are summarized in Table 2 in column of intra-assay variation of recDNA.

molecules and offers important insights into the local expression of low abundant transcripts (Ferré 1992). We optimised and validated two types of calibration models for a real-time RT-PCR using the SYBR Green I technology in the LightCycler. As demonstrated, the amplification in both calibration curve types is linear over a wide range of input copies, with high sensitivity, precision and reproducibility. Even 16 molecules recRNA, 6 molecules recDNA or 13 molecules IGF-1 mRNA could be detected with the established quantification models.

An optimal primer fit is the most influencing determinant and essential for a successful real-time PCR quantification (McPherson *et al.* 1991). If the primers are not properly designed in regard to maximal annealing specificity, annealing temperature, primer dimer or self priming formation and buffer conditions, a successful real-time PCR is uncertain. Because of its high ramping rates, limited annealing and elongation time, the rapid cycle PCR in the LightCycler system offers stringent reaction conditions to all PCR components and leads to a primer sensitive and template specific PCR (Wittwer *et al.* 1997). Amplification of genomic DNA was avoided by primer pairs located on different exons, therefore a DNAse treatment of RNA samples is unnecessary.

High reproducibility and low test variability of maximal 6.8% could be derived in both calibration models as well in the native sample (Tables 1 and 2). An inverse relationship between template start molecules and reproducibility in intra-assay experiments and inter-assay experiment could be recognised. To characterise the PCR variation an average variation coefficient was calculated over the whole range of the calibration curve. In our opinion this reflects the real PCR variation.

The reliability of a real-time PCR assay depends on the condition of identical amplification efficiencies for both the native target and the calibration curve (Souazé *et al.* 1996). Using an external recRNA calibration curve model, both RNA targets should have as well the same attributes in terms of reverse tran-

Table 2 Intra-assay (test precision) and inter-assay variation (test variability) of two LightCycler calibration curve models using recRNA or recDNA as start template. Calculation of variation is based on the variation of crossing point (CP) and expressed as mean with coefficient of variation (CV). To characterise the mean RT-PCR variation, the average variation coefficient was calculated over the whole range of the calibration curve.

	Intra-assay variation ($n = 4$)		Inter-assay variation ($n = 4$)	
recRNA	Mean CP	CV	Mean CP	CV
2×10^8	13.32	1.7%	13.79	6.8%
2×10^7	16.88	1.4%	17.38	3.7%
2×10^6	20.59	1.3%	20.99	5.8%
2×10^5	22.97	2.8%	23.35	3.2%
2×10^4	23.29	6.6%	23.77	3.1%
Average		2.7%		4.5%
recDNA	Mean CP	CV	Mean CP	CV
6×10^6	13.75	0.5%	14.03	5.7%
6×10^5	17.45	0.6%	17.82	3.2%
6×10^4	20.93	0.4%	21.37	2.3%
6×10^3	24.57	0.5%	24.94	2.1%
6×10^2	28.26	1.0%	28.41	1.2%
6×10^1	31.39	1.4%	31.06	0.9%
Average		0.7%		2.6%

scription (Siebert & Larrick 1992). Mutant recRNA and native IGF-1 mRNA has already been validated in the competitive IGF-1 RT-PCR and showed identical amplification efficiencies, *E*, in each cycle of 1.66 and 1.65 respectively during exponential phase (Pfaffl *et al.* 1998b). As demonstrated herein, for the externally calibration curve model with recRNA an almost similar amplification efficiency of 1.77 could be confirmed. Higher amplification efficiencies of 1.93 were achieved with the recDNA calibration model. Double-stranded recDNA (linearised plasmid) is more stable than single stranded recRNA taking temperature degradation, RNAase activity, multiple freezing and thawing during long time storage into consideration. The quantification efficiency of the native liver sample real-time PCR (1.89) was between both calibration models and can be adapted more to the recDNA calibration curve model cycle conditions.

If a calibration curve with recRNA model is used to quantify native mRNA the different RT efficiency must be investigated. In calibration curves only these specific recRNA molecules are present during RT and the cDNA synthesis kinetis are not like those in native RNA (unknown sample) with a high percentage of ribosomal RNA (∼80%) and transfer RNA (10–15%). This missing RNA species can influence the cDNA synthesis rate and in consequence RT efficiency rises and calibration curves are then overestimated (Freeman *et al.* 1999, Zimmermann & Mannhalter 1996). To compensate for background effects and mimic a natural RNA distribution like in native total RNA, bacterial or insect cell line total RNA was inserted. Total RNA derived from these organisms was tested to be free of the desired IGF-1 mRNA template, to prevent a false positive signal. Alternatively commercially available RNA sources can be used as RNA background, e.g., poly-A RNA or transfer RNA, but they do not represent a native RNA distribution over all RNA subspecies. Depending on the amount of spiked RNA in comparison of no background higher RT efficiencies were demonstrated. Very high background concentrations had a more significant suppression effect on

Table 3 Influence of different concentrations [none, 2, 10, 20 ng RNA background per capillary] of prokaryotic bacterial RNA (*Escherichia coli*) or eukaryotic insect cell RNA (*Spodoptera frugiperda*) on RT synthesis efficiency using 4×10^4 to 4×10^9 ss recRNA molecules. Data were normalised in comparison to no RT (recDNA calibration curve = 100%) from 4×10^2 to 4×10^6 ss recDNA molecules, which were amplified in parallel within one run. [calculated RT efficiency (RTE), real-time PCR amplification efficiency (E), and Pearson correlation coefficient (r) of each calibration curve model].

Added RNA background	*Escherichia coli* total RNA	*Spodoptera frugiperda* total RNA
0 ng/capillary	RTE = 4.9%	RTE = 6.2%
	$E = 1.78$	$E = 2.01$
	$r = 0.997$	$r = 0.996$
2 ng/capillary	RTE = 10.3%	RTE = 15.5%
	$E = 1.75$	$E = 1.78$
	$r = 0.998$	$r = 0.994$
10 ng/capillary	RTE = 3.1%	RTE = 16.0%
	$E = 1.79$	$E = 1.57$
	$r = 0.998$	$r = 0.998$
20 ng/capillary	RTE = 1.4%	RTE = 13.4%
	$E = 1.32$	$E = 1.43$
	$r = 0.993$	$r = 0.996$
recDNA	No RT = 100%	No RT = 100%
	$E = 1.76$	$E = 1.81$
	$r = 0.982$	$r = 0.999$

cDNA synthesis rate as well as on later real-time PCR efficiency. But, this result suggest that a minimum of RNA background is generally needed for a better RT synthesis efficiency rate. On the other hand, especially low concentrations of recRNA should always be buffered with background or carrier RNA, otherwise the low amounts can be degraded by RNAses. A general statement can be made, that a cDNA synthesis rate is 16% or even lower compared with the same recDNA.

Conclusion

The sensitivity, linearity and reproducibility of the developed real-time PCR assays allows for the absolute and accurate quantification of IGF-1 mRNA molecules even in tissues with low abundancies down to a few molecules. Using a recDNA calibration curve model only the existing cDNA molecules derived out of RT can be quantified precisely. It is not possible to draw a conclusion on the existing mRNA molecules present in the native total RNA sample. Always the cDNA synthesis efficiency must be recognised. Using a recRNA calibration curve model the advantage is that both RNA templates underwent parallel a RT and real-time PCR. But in this approach the following real-time PCR efficiency is suppressed and yields in a sub-optimal real-time quantification. In our opinion the external calibration curve using recDNA is a better model for the quantification of mRNA than the recRNA calibration model – consideration of RT efficiency is needed. The model showed higher sensitivity, exhibit a larger quantification range, had a higher reproducibility, and is more stable than the recRNA. We have used this recDNA system to compare the IGF-1 expression rates in bovine (*Bos taurus*) [EMBL accession no. X15726] (Pfaffl *et al.* 1998b) and porcine tissues (*Sus scrofa*) [EMBL accession no. X17492] (Pfaffl *et al.* 1998a). It can also be used in other species like sheep (*Ovis aries*) [EMBL accession no. M30653] and primates (*Callithrix jacchus*) [EMBL accession no. Z49055] (Pfaffl 2000) with sufficiently high homologies of the amplified IGF-1 fragment.

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