# Real-time RT-PCR quantification of insulin-like growth factor (IGF)-1, IGF-1 receptor, IGF-2, IGF-2 receptor, insulin receptor, growth hormone receptor, IGF-binding proteins 1, 2 and 3 in the bovine species

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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 highly accurate and reliable results in a real-time RT-PCR a highly defined calibration curve is needed. We designed and developed nine different calibration curves, based on recombinant DNA plasmid standards and established them on a constant real-time PCR platform for the following factors: growth hormone receptor (GHR), insulin-like growth factor (IGF)-1, IGF-1 receptor (IGF-1R), IGF-2, IGF-2 receptor (IGF-2R), insulin receptor (INSR), and IGF-binding proteins (IGF-BP) 1, 2 and 3. Developed assays were applied in the LightCycler system on bovine ileum and liver total RNA and showed high specificity and sensitivity of quantification. All assays had a detection limit of under 35 recombinant DNA molecules present in the capillary. The SYBR

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Green I determination resulted in a reliable and accurate quantification with high test linearity (Pearson correlation coefficient $r > 0.99$) over seven orders of magnitude from $<10^2$ to $>10^8$ recombinant DNA start molecules and an assay variation of maximal 5.3%. Applicability of the method was shown by analysing mRNA levels in newborn calves: mRNA concentrations per gram tissue of mRNAs of IGF-1, IGF-1R, IGF-2, IGF-2R, GHR, INSR, and IGF-BP1, 2 and 3 were all different between in liver and ileum and the traits all exhibited individual differences. © 2002 Elsevier Science Inc. All rights reserved.

## 1. Introduction

Reverse transcription (RT) of mRNA into DNA, followed by polymerase chain reaction (PCR) using real-time PCR, is a simple and sensitive method not only to detect, but also to measure even minute amounts of mRNA molecules. This offers important insights into the local mRNA expression of low abundant transcripts in various tissues [1]. Thus, during the recent years, RT-PCR has become an increasingly useful tool for the mRNA quantification. Nowadays real-time RT-PCR is more and more used to quantify physiologically changes in gene expression. Because of its high ramping rates (heating and cooling rates), limited annealing and elongation time, the rapid-cycle PCR, such as in the LightCycler® (Roche Diagnostics, Mannheim, Germany) system, offers stringent reaction conditions to all PCR components and leads to a primer sensitive and template-specific RT-PCR [2,3]. Using external calibration curve, based on a recombinant DNA (recDNA) plasmid standard, the quantification methodology is sensitive, exact and highly reproducible for the reliable measurements of gene transcripts [4]. Real-time RT-PCR with external calibration curve using SYBR Green I® technology combines the ease and necessary exactness to be able to produce reliable and rapid results.

The available publications about mRNA quantification concerning the somatotropic axis in cattle are limited to different quantification methods: (1) semi-quantitative block RT-PCR with densitometric analysis of ethidium bromide stained gels [5–11]; (2) semi-quantitative RT-PCR with solid phase DNA assay technique [12,13]; and (3) fully quantitative competitive RT-PCR assays [14–16]. It has to be realised that the results of the different applied methodologies of the quantified factors are not directly comparable. However, to compare all factors among each other and give defined statements about the expression levels, a quantification system for all factors based on one constant PCR platform is essential.

Therefore, we developed and validated nine quantitative real-time RT-PCR assays for various elements involved in the action of the somatotropic axis, and established them on the Light-Cycler system: insulin-like growth factor (IGF)-1, IGF-1 receptor (IGF-1R), IGF-2, IGF-2 receptor (IGF-2R), insulin receptor (INSR), growth hormone receptor (GHR), IGF-binding protein (IGF-BP) 1, 2 and 3. Limits and accuracies of the developed assays are described to achieve precise, reliable and reproducible results in the unknown samples total RNA. Applicability of the method was shown by analysing samples obtained from calves immediately after birth.

## 2. Material and methods

### 2.1. Total RNA extraction

The RNA extraction was performed in bovine (*Bos taurus*) ileum and liver as described earlier [14,15]. The integrity of RNA was electrophoretically verified by ethidium bromide staining and by optical density (OD) absorption ratio $OD_{260\,nm}/OD_{280\,nm} > 1.9$.

### 2.2. Multiple species primer design

The primers used for the production of recDNA were derived either from bovine, ovine, human or mouse sequences. Primers were designed to produce an amplification product which spanned at least two exons in the highly conserved coding region (CDS) of the appropriate coding sequence of multiple species. Therefore, a multiple CDS alignment (clusteral alignment in HUSAR® software) of the available mRNAs was done at DKFZ (http://genome.dkfz-heidelberg.de/biounit/). Primer design and optimisation was done in the high homology regions of the multiple alignment with regard to primer dimer formation, self-priming formation and primer melting temperature (HUSAR® software at DKFZ). IGF-1 primer and IGF-1R primer sequences were taken from earlier publications [11,14]. Primer sequences, the position in the coding region and the expected real-time RT-PCR product length, and exon spanning region were summarised in Table 1. For IGF-1R no information about the intron/exon structure is available in the published sequence databases EMBL (http://www.ebi.ac.uk/) and GenBank (http://www.ncbi.nlm.nih.gov/Entrez/index.html).

### 2.3. Generation of recombinant DNA external calibration curves

The RT-PCR products of all investigated factors were cloned either in pCR2.1 [11,14] or in pCR4.0 vectors (Invitrogen, Leek, The Netherland). Recombinant plasmids were transformed in *Escherichia coli* Top F10 competent cells (Invitrogen) and grown on LB-medium. After plasmid midi preparation (Peqlab, Erlangen, Germany), plasmids were linearised with a unique cutting restriction enzyme digest (*Hin*dIII, *Not*I, *Pst*I or *Xba*I; delivered by Gibco Life Technologies, Gaithersburg, MD). Linear double stranded plasmids were quantified by multiple ($n = 10$) optical measurements in various dilutions and concentrations at $OD_{260\,nm}$. From the calculated molecular concentration, using the molecular mass of single stranded (ss) recDNA, accurate calibration point aliquots from $>10^9$ ss molecules/μL down to $<10$ ss molecules/μL were diluted. Derived calibration curves dilutions were aliquoted and frozen at $-20\,°C$.

### 2.4. Reverse-transcription

One microgram total RNA from the sample preparation was reverse transcribed with 100 U of Super script II Plus RNase H⁻ Reverse Transcriptase (Gibco Life Technologies) using 100 μM random hexamer primers (Pharmacia Biotech, Buckinghamshire, UK) according to the manufactures instructions.

Table 1
Forward (for) and reverse (rev) primer sequences (5′–>3′)[a], RT-PCR product length and exon spanning of the investigated factors[b]

| Primer | Sequence (5′–>3′) | Position and length | Exon spanning |
|---|---|---|---|
| IGF-1 for | TCG CAT CTC TTC TAT CTG GCC CTG T | bt 88–327 | 3–>4 |
| IGF-1 rev | GCA GTA CAT CTC CAG CCT CCT CAG A | 240 bp | |
| IGF-1R for | TTA AAA TGG CCA GAA CCT GAG | bt 367–680 | – |
| IGF-1R rev | ATT ATA ACC AAG CCT CCC AC | 314 bp | |
| IGF-2 for | GAC CGC GGC TTC TAC TTC AG | ov 139–343 | 3–>5 |
| IGF-2 rev | AAG AAC TTG CCC ACG GGG TAT | 205 bp | |
| IGF-2R for | TAC AAC TTC CGG TGG TAC ACC A | mm 2237–2380 | 18–>19 |
| IGF-2R rev | CAT GGC ATA CCA GTT TCC TCC A | 144 bp | |
| GHR for | CCA GTT TCC ATG GTT CTT AAT TAT | bt 816–953 | 8–>9 |
| GHR rev | TTC CTT TAA TCT TTG GAA CTG G | 138 bp | |
| INSR for | TCC TCA AGG AGC TGG AGG AGT | ov 2162–2324 | 10–>12 |
| INSR rev | GCT GCT GTC ACA TTC CCC A | 163 bp | |
| IGF-BP1 for | TCA AGA AGT GGA AGG AGC CCT | h 544–671 | 2–>3 |
| IGF-BP1 rev | AAT CCA TTC TTG TTG CAG TTT | 123 bp | |
| IGF-BP2 for | CAC CGG CAG ATG GGC AA | bt 604–739 | 2–>3 |
| IGF-BP2 rev | GAA GGC GCA TGG TGG AGA T | 136 bp | |
| IGF-BP3 for | ACA GAC ACC CAG AAC TTC TCC T C | bt 588–781 | 2–>4 |
| IGF-BP3 rev | GCT TCC TGC CCT TGG A | 194 bp | |

[a] Their position in the coding sequence of the indicated species (bt, *B. taurus*; ov, *O. aries*; mm, *M. musculus*; h, *H. sapiens*).

[b] Insulin-like growth factor (IGF)-1, IGF-1 receptor (IGF-1R), IGF-2, IGF-2 receptor (IGF-2R), insulin receptor (INSR), growth hormone receptor (GHR), IGF binding protein (IGF-BP) 1, 2 and 3. IGF-1 primer and IGF-1R primers sequences were taken from earlier publications [11,14].

## 2.5. Real-time RT-PCR

A master-mix of the following reaction components was prepared to the indicated end-concentration: 6.4 μL water, 1.2 μL MgCl₂ (4 mM), 0.2 μL forward primer (0.4 μM), 0.2 μL reverse primer (0.4 μM) and 1.0 μL LightCycler Fast Start DNA Master SYBR Green I[®] (Roche Diagnostics, Mannheim, Germany). Nine μL of master-mix was filled in the glass capillaries and 1 μL volume, containing 25 ng reverse transcribed total RNA, was added as PCR template. Capillaries were closed, centrifuged and placed into the rotor. To improve SYBR Green I quantification, a high temperature fluorescence measurement point was performed [17]. Temperature for the elevated fluorescence acquisition in the fourth segment are listed in Table 2. It melts the unspecific PCR products at the elevated temperature, e.g., primer dimers, eliminates the non-specific fluorescence signal and ensures an accurate quantification of the desired product.

The following general real-time PCR protocol was used: *denaturation program* (95°C for 10 min), a *four-segment amplification and quantification program* repeated 40 times (factor-specific amplification conditions with a single fluorescence measurement are summarised in Table 2), *melting curve program* (60–99°C with a heating rate of 0.1°C/s and continuous fluorescence measurements), and finally a *cooling program* down to 40°C.

Table 2
LightCycler real-time PCR cycling conditions of all nine assays

| Assay steps (segments) | Duration (s) | Temperature (°C) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | IGF-1 | IGF-1R | IGF-2 | IGF-2R | GHR | INSR | IGF-BP1 | IGF-BP2 | IGF-BP3 |
| I, Denaturation | 15 | 95 | 95 | 95 | 95 | 95 | 95 | 95 | 95 | 95 |
| II, Primer annealing | 10 | 62 | 63 | 62 | 62 | 58 | 62 | 58 | 58 | 58 |
| III, Elongation | 20 | 72 | 72 | 72 | 72 | 72 | 72 | 72 | 72 | 72 |
| IV, Fluorescence acquisition | 3 | 86 | 80 | 88 | 86 | 76 | 82 | 82 | 87 | 87 |

*Denaturation program* (95°C for 10 min), a *four segment amplification and quantification program* repeated 40 times with a single fluorescence measurement, *melting curve program* (60–995°C with a heating rate of 0.1°C/s and continuous fluorescence measurements) and finally a *cooling program* down to 40°C. For abbreviations: see Table 1.

## 2.6. First application of established assays

The developed assays were first applied in two biological matrix, in liver and ileum, of newborn calves ($n = 7$). Calves were born as singles after normal lengths of pregnancy (290 days) and were euthanised by i.v. barbiturate injection immediately after birth. Subsequently, liver and ileum were removed and shock-frozen in liquid nitrogen and then stored $-80°C$ until total RNA extraction.

## 2.7. Calculations and statistics

Results in liver and ileum of the different traits are given as numbers of molecules ($\pm$SEM) of mRNA per 25 ng cDNA (corrected or uncorrected for ubiquitin values), as numbers of molecules ($\pm$SEM) per mg fresh tissue, and as fg ($10^{-15}$ g) or pg ($10^{-12}$ g) per g fresh tissue (see Table 4). The amounts of mRNA of the different traits on a fg/mg or pg/mg of fresh tissue were calculated on the following basis: molecular weight of double stranded DNA (MW) = (number of base pairs) $\times$ (660 Da per base pairs). MW of the different traits were calculated according to the RT-PCR product length given in Table 1 as follows: IGF-1 = $15.8 \times 10^4$ g/mol, IGF-1R = $20.7 \times 10^4$ g/mol, IGF-2 = $13.5 \times 10^4$ g/mol, IGF-2R = $9.5 \times 10^4$ g/mol, GHR = $9.1 \times 10^4$ g/mol, INSR = $10.7 \times 10^4$ g/mol, IGF-BP1 = $8.1 \times 10^4$ g/mol, IGF-BP2 = $8.9 \times 10^4$ g/mol, and IGF-BP3 = $12.8 \times 10^4$ g/mol. The amounts present in 1 mg of fresh tissue (fg/mg or pg/mg) were calculated according to the equation: amount = (number of molecules/mg fresh tissue $\times$ MW)/$6.023 \times 10^{23}$ molecules (1 mol contains $6.023 \times 10^{23}$ molecules).

For statistical evaluations data were analysed by ANOVA using the General Linear Models (GLM) procedure of SAS program package release 6.11 (SAS Institute, Cary, NC, USA, 1995). Differences between tissue means were post-hoc localised by Bonferroni's $t$-test. Differences between groups shown in Table 4 were considered significant at $P < 0.05$, $P < 0.01$ or $P < 0.001$.
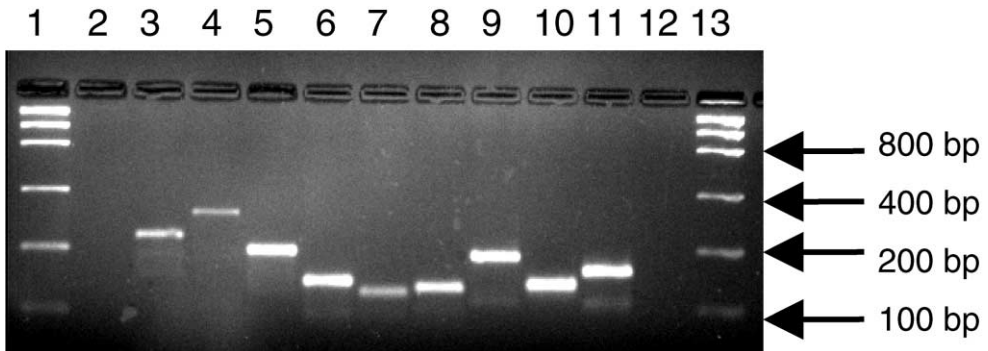


Fig. 1. High resolution 4% agarose gel electrophoresis of all real-time RT-PCR products derived from bovine liver total RNA. Lanes 1 and 13: length standard (2, 1.2 kbp, 800, 400, 200, 100 bp); lanes 3–11: IGF-1 at 240 bp; IGF-1R at 314 bp; IGF-2 at 205 bp; IGF-2R at 144 bp; IGF-BP1 at 123 bp; IGF-BP2 at 136 bp; IGF-BP3 at 194 bp; GHR at 138 bp; INSR at 163 bp.

## 3. Results

### 3.1. Confirmation of primer and RT-PCR product specificity

Specificity of the desired products in bovine liver and muscle total RNA were documented with high resolution gel electrophoresis (Fig. 1) and additionally with melting curve analysis. Derived melting temperature of RT-PCR products were product-specific between 78.9°C (bovine GHR) and 90.9°C (bovine IGF-BP2) and listed in Table 3. Real-time amplified RT-PCR products were sequenced (MWG Biotech, Ebersberg, Germany) and 100% homology to the bovine sequence could be confirmed. INSR and IGF-2R sequences were newly elucidated in *B. taurus* and published in the EMBL and GenBank (Ac. no. BTA320235 AJ320235; Ac. no. BTA320234 AJ320234).

### 3.2. Amplification efficiencies, sensitivity and linearity of the assays

The sensitivity of the LightCycler PCR was evaluated using different starting amounts of recDNA (Table 3). In all assays, the sensitivity of the quantification was very high, i.e., 35 recDNA molecules present in the capillary could be detected. The SYBR Green I determination at elevated temperatures resulted in a reliable and sensitive quantification with high test linearity (Pearson correlation coefficient $r > 0.99$) over seven orders of magnitude from $<10^2$ to $>10^8$ recDNA start molecules. Real-time PCR efficiencies were calculated from the given slopes in LightCycler Software 3.3 (Roche Diagnostics). The corresponding real-time PCR efficiency ($E$) of 1 cycle in the exponential phase was calculated according to the equation: $E = 10^{[-1/\text{slope}]}$ [18]. The maximal efficiency of PCR is $E = 2$ where every PCR product is replicated every cycle and the minimal value is $E = 1$, corresponding to no replication. Investigated transcripts showed high real-time PCR efficiency rates between 1.82 for IGF-BP3 and 1.98 for IGF-BP1 over the entire quantification range (Table 3).

### 3.3. Assay variation: precision and reproducibility

To confirm precision and reproducibility of real-time PCR, the variation was determined in three repeats within one LightCycler run over a wide molecule range ($<10^2$ up to $>10^8$ recDNA start molecules). In all RT-PCR assays, the variation was $<5.3\%$ (Table 3), showing high precision and reproducibility of the applied assays. Calculation of test variability is based on the variation of crossing points (CP) from the CP mean value. Analysis line was set to a fluorescence level of 2 where the determination of CP was performed. There a linear relationship between the log of the start molecules and the corresponding CP during real-time PCR is given. The CP is defined as the point at which the fluorescence rises appreciably above the background fluorescence [18].

### 3.4. Assay application in other species

We have designed these quantification assays to compare primarily the expression rates in bovine tissues (*B. taurus*) (Fig. 1). But these assays are also applicable and positive tested in

Table 3
PCR efficiency calculated according to the equation: $E = 10^{[-1/\text{slope}]}$ [18]

| | IGF-1 | IGF-1R | IGF-2 | IGF-2R | GHR | INSR | IGF-BP1 | IGF-BP2 | IGF-BP3 |
|---|---|---|---|---|---|---|---|---|---|
| PCR efficiency | 1.93 | 1.94 | 1.96 | 1.92 | 1.89 | 1.85 | 1.98 | 1.95 | 1.82 |
| Melting temperature (°C) | 90.5 | 87.2 | 90.6 | 89.6 | 78.9 | 87 | 85.2 | 90.9 | 88.3 |
| Detection limit [molecules] | 10 | 7 | 35 | 7 | 34 | 21 | 9 | 18 | 18 |
| Quantification range [molecules] | $10–9.2 \times 10^9$ ($r=1.00$) | $65–6.5 \times 10^{10}$ ($r=1.00$) | $35–3.5 \times 10^8$ ($r=1.00$) | $67–6.7 \times 10^{10}$ ($r=1.00$) | $34–3.3 \times 10^{10}$ ($r=1.00$) | $21–2.1 \times 10^8$ ($r=0.99$) | $9–8.4 \times 10^9$ ($r=1.00$) | $46–4.6 \times 10^9$ ($r=1.00$) | $180–1.8 \times 10^{10}$ ($r=0.99$) |
| Assay variation (%) | 0.7 | 4.7 | 1.5 | 3.6 | 5.3 | 1.7 | 4.0 | 1.5 | 4.6 |
| Species | B. taurus, O. aries, S. scrofa, H. sapiens, C. jacchus | B. taurus, O. aries, S. scrofa, H. sapiens, C. jacchus | B. taurus, O. aries, S. scrofa, H. sapiens, R. norvegicus | B. taurus, O. aries, H. sapiens, R. norvegicus, S. scrofa | B. taurus, O. aries, H. sapiens, S. scrofa | B. taurus, O. aries, S. scrofa, R. norvegicus | B. taurus, O. aries, S. scrofa | B. taurus, O. aries, H. sapiens, S. scrofa | B. taurus, O. aries, H. sapiens |

The detection limit, quantification range and test linearity (r, Pearson correlation coefficient) are given in molecules per capillary. Assay variation was determined in three repeats ($n = 3$) over the complete quantification range. Determination of variation is based on crossing point variation and was done in 25 ng reverse transcribed total RNA. For abbreviations: see Table 1.

Table 4
Number of mRNA molecules in ileum and liver of newborn calves

| Trait | Ileum | Liver | Differences between ileum and liver |
|---|---|---|---|
| IGF-1 | $41.3 \times 10^3 \pm 11.4 \times 10^{3a}$ | $227.8 \times 10^3 \pm 87.2 \times 10^{3a}$ | $P = 0.088^a$ |
| | $42.3 \times 10^3 \pm 12.4 \times 10^{3b}$ | $227.0 \times 10^3 \pm 86.3 \times 10^{3b}$ | $P = 0.055^b$ |
| | $3.82 \times 10^6 \pm 1.0 \times 10^{6c}$ | $27.3 \times 10^6 \pm 10.0 \times 10^{6c}$ | $P < 0.05^c$ |
| | $1.01 \pm 0.27 \, \text{pg}^d$ | $7.2 \pm 2.67 \, \text{pg}^d$ | $P < 0.05^d$ |
| IGF-1R | $16.0 \times 10^3 \pm 2.3 \times 10^{3a}$ | $19.2 \times 10^3 \pm 1.7 \times 10^{3a}$ | $P = 0.35^a$ |
| | $16.0 \times 10^3 \pm 2.3 \times 10^{3b}$ | $19.2 \times 10^3 \pm 1.8 \times 10^{3b}$ | $P = 0.31^b$ |
| | $1.5 \times 10^6 \pm 223.9 \times 10^{3c}$ | $2.3 \times 10^6 \pm 191.7 \times 10^{3c}$ | $P < 0.05^c$ |
| | $0.5 \pm 0.08 \, \text{pg}^d$ | $0.8 \pm 0.06 \, \text{pg}^d$ | $P < 0.05^d$ |
| IGF-2 | $569.8 \times 10^3 \pm 124.9 \times 10^{3a}$ | $17.9 \times 10^6 \pm 2.8 \times 10^{6a}$ | $P < 0.001^a$ |
| | $569.2 \times 10^3 \pm 120.8 \times 10^{3b}$ | $17.9 \times 10^6 \pm 2.8 \, 10^{6b}$ | $P < 0.001^b$ |
| | $54.4 \times 10^6 \pm 12.1 \times 10^{6c}$ | $2.2 \times 10^9 \pm 387.7 \times 10^{6c}$ | $P < 0.001^c$ |
| | $12.6 \pm 2.8 \, \text{pg}^d$ | $522.2 \pm 90.3 \, \text{pg}^d$ | $P < 0.001^d$ |
| IGF-2R | $612 \pm 65^a$ | $1.5 \times 10^3 \pm 69^a$ | $P < 0.001^a$ |
| | $614 \pm 66^b$ | $1.5 \times 10^3 \pm 70^b$ | $P < 0.001^b$ |
| | $60.0 \times 10^3 \pm 8.9 \times 10^{3c}$ | $181.1 \times 10^3 \pm 9.2 \times 10^{3c}$ | $P < 0.001^c$ |
| | $9.5 \pm 1.4 \, \text{fg}^d$ | $28.5 \pm 1.4 \, \text{fg}^d$ | $P < 0.001^d$ |
| GHR | $2.2 \times 10^3 \pm 795^a$ | $48.3 \times 10^3 \pm 4.6 \times 10^{3a}$ | $P < 0.001^a$ |
| | $2.3 \times 10^3 \pm 854^b$ | $48.4 \times 10^3 \pm 4.9 \times 10^{3b}$ | $P < 0.001^b$ |
| | $207.7 \times 10^3 \pm 73.4 \times 10^{3c}$ | $5.9 \times 10^6 \pm 632.9 \times 10^{3c}$ | $P < 0.001^c$ |
| | $30 \pm 10 \, \text{fg}^d$ | $900 \pm 90 \, \text{fg}^d$ | $P < 0.001^d$ |
| INSR | $77.2 \times 10^3 \pm 15.4 \times 10^{3a}$ | $336.2 \times 10^3 \pm 17.9 \times 10^{3a}$ | $P < 0.001^a$ |
| | $78.4 \times 10^3 \pm 17.0 \times 10^{3b}$ | $336.2 \times 10^3 \pm 17.9 \times 10^{3b}$ | $P < 0.001^b$ |
| | $7.0 \times 10^6 \pm 1.4 \times 10^{6c}$ | $41.2 \times 10^6 \pm 2.2 \times 10^{6c}$ | $P < 0.001^c$ |
| | $1.25 \pm 0.25 \, \text{pg}^d$ | $7.13 \pm 0.36 \, \text{pg}^d$ | $P < 0.001^d$ |
| IGF-BP1 | $1.3 \times 10^3 \pm 322^a$ | $1.6 \times 10^6 \pm 421.1 \times 10^{3a}$ | $P < 0.01^a$ |
| | $1.4 \times 10^3 \pm 323^b$ | $1.6 \times 10^6 \pm 418.7 \times 10^{3b}$ | $P < 0.01^b$ |
| | $126.8 \times 10^3 \pm 30.7 \times 10^{3c}$ | $208.6 \times 10^6 \pm 54.1 \times 10^{6c}$ | $P < 0.005^c$ |
| | $20 \pm 4 \, \text{fg}^d$ | $27.9 \pm 7.2 \, \text{pg}^d$ | $P < 0.005^d$ |
| IGF-BP2 | $186 \pm 81^a$ | $50.0 \times 10^3 \pm 10.7 \times 10^{3a}$ | $P < 0.005^a$ |
| | $192 \pm 87^b$ | $49.8 \times 10^3 \pm 10.4 \times 10^{3b}$ | $P < 0.001^b$ |
| | $17.6 \times 10^3 \pm 7.7 \times 10^{3c}$ | $6.2 \times 10^6 \pm 1.3 \times 10^{6c}$ | $P < 0.001^c$ |
| | $3.0 \pm 1.2 \, \text{fg}^d$ | $900 \pm 195 \, \text{fg}^d$ | $P < 0.001^d$ |
| IGF-BP3 | $6.9 \times 10^3 \pm 1.6 \times 10^{3a}$ | $42.1 \times 10^3 \pm 4.8 \times 10^{3a}$ | $P < 0.001^a$ |
| | $7.0 \times 10^3 \pm 1.7 \times 10^{3b}$ | $42.3 \times 10^3 \pm 4.9 \times 10^{3b}$ | $P < 0.001^b$ |
| | $647.3 \times 10^3 \pm 151.7 \times 10^{3c}$ | $5.2 \times 10^6 \pm 583.5 \times 10^{3c}$ | $P < 0.001^c$ |
| | $130 \pm 32 \, \text{fg}^d$ | $1130 \pm 130 \, \text{fg}^d$ | $P < 0.001^d$ |

[a] Number of mRNA molecules/25 ng cDNA (reverse transcribed total RNA).
[b] Number of mRNA molecules/25 ng cDNA, normalised for ubiquitin expression.
[c] Number of mRNA molecules/mg ileum and liver, normalised for ubiquitin expression.
[d] fg or pg of mRNA/mg ileum and liver, normalised for ubiquitin expression.

other species (Table 3) like, human (*Homo sapiens*), sheep (*Ovis aries*), pig (*Sus scrofa*), rat (*Rattus norvegicus*), and primates (*Callithrix jacchus*) with sufficiently high homologies of the amplified RT-PCR products.

### 3.5. Expression results of ileum and liver

Extracted total RNA concentrations of investigated tissues were significantly different: $2414 \pm 132$ ng/mg ileum versus $3079 \pm 92$ ng/mg liver ($P < 0.05$). To compare the quantified mRNA molecules of the established assays, Ubiquitin as a housekeeping gene was quantified as well. Ubiquitin normalisation of expression data were made based on percent difference between mean ubiquitin values (100%) in ileum and liver, respectively. The ubiquitin expression showed a minimal variation in all investigated tissues of 1.8% in liver ($n = 7$) and 4.6% in ileum ($n = 7$). All nine factors could be quantified in ileum and liver of newborn calves. Table 4 shows the quantified mRNA molecules (a) on the basis of 25 ng cDNA (25 ng reverse transcribed total RNA); (b) on the basis of 25 ng cDNA and normalised by the ubiquitin expression efficiency; (c) per mg extracted tissue normalised by the ubiquitin expression efficiency; and (d) as fg or pg per g tissue normalised by the ubiquitin expression efficiency.

## 4. Discussion

In this study, we have designed, optimised and validated nine assays of factors involved in the action of the somatotropic axis in real-time RT-PCR using the SYBR Green I technology with the LightCycler. The amplification of the PCR products was shown to be linear over a wide range of input copies, with high sensitivity, precision and reproducibility. As few as 35 molecules could be detected with the established quantification models. Amplification of genomic DNA was avoided by primer pairs located on different exons, therefore, a DNAse treatment of tissue total RNA samples was unnecessary. A great simplification for the determination at the mRNA level of the nine parameters involved in the somatotropic axis was achieved by use of only one cDNA for the determination of all parameters. This was done by a reverse transcription of total RNA extracted from the tissues using random hexamer primers.

High reproducibility and low test variability of $\leq 5.3\%$ could be derived. To characterise the RT-PCR variation at its best, an average variation coefficient was calculated over the whole range of the calibration curve. This reflects the realistic PCR variation over the complete quantification range [17]. An externally recDNA calibration curve mimics the real-time PCR better than other standard materials, like recombinant RNA or purified PCR products, and possesses an almost similar amplification efficiency as the native sample mRNA [4]. Double-stranded recDNA, derived from a linearised plasmid, is a stable and reliable standard material for calibration curves and will be not degraded over a long storage period [4]. Therefore, the test variability is minimised, the repeatability of is maximised, and the derived expression results are fully comparable over all applied quantification tests.

All factors could be quantified with the new established assays with high exactness and reproducibility. The quantified mRNA concentrations and expression levels are different between liver and ileum. It turned out, that IGF-2R is very low abundant in both tissues, whereas

expressions of GHR, IGF-BP1 and 2 are very low in ileum, but are medium or even highly expressed in liver. Extremely high mRNA concentrations could be measured for IGF-2 and IGF-BP1 in liver. Except for IGF and IGF-1R all factors showed significant different expression levels in ileum versus liver—either on 25 ng cDNA basis (raw and normalised data) or on mg tissue basis. For IGF and IGF-1R only significant expression levels could be determined on mg tissue basis ($P < 0.05$) and fg/pg mRNA/mg tissue ($P < 0.05$). Ligand concentration in comparison to their corresponding receptor in the IGF-1 and -2 systems showed extreme differences between the tissues. Ligand mRNAs were always higher expressed than receptors and showed ratios of ligand to receptor (IGF-1/IGF-1R) in liver (ratio = ~12) higher than in ileum (ratio = ~2.5). The IGF-2/IGF-2R ratio was more evident in both tissues and extremer in ileum (ratio = ~900) than in liver (ratio = ~125). Studies on the physiological relevance of these data with more tissues and under different physiological conditions are in progress.

The developed assays containing recDNA calibration curve, specific primers and cycling conditions can be applied as well on other real-time quantification systems: TaqMan® (PE Applied Biosystems, Foster City, CA, USA), RotoGene® (Corbett Research, Sydney, NSW, Australia), iCycler® Thermal Cycler (Bio-Rad, Hercules, CA, USA) and Multiplex Quantitative PCR System® (Stratagene, La Jolla, CA, USA). But the assay performances described herein are optimised to the demands of the LightCycler platform. For the above mentioned real-time PCR machines assay performances like sensitivity, linearity, reproducibility and PCR efficiency must be separately validated for each used platform. Performance variation can occur on the basis of different cycling techniques, fast-cycle versus conventional block-cycle technology, other fluorescence excitation techniques, laser versus light emitting diode excitation, and the applied fluorescence detection system, CCD camera (charge coupled device image sensor) versus photo-hybrid detection.

In conclusion, the sensitivity, linearity and reproducibility of the developed real-time PCR assays allows absolute and accurate quantification, down to a few molecules. In future we will use the established quantification systems to compare the expression rates in tissues of *B. taurus* and other species like *H. sapiens, O. aries, S. scrofa, R. norvegicus*, and *C. jacchus* to investigate physiological changes in gene expression.

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## References

[1] Ferré F. Quantitative or semi-quantitative PCR: reality versus myth. PCR Methods Applications 1992;2:1–9.
[2] Wittwer CT, Ririe KM, Andrew RV, David DA, Gundry RA, Balis UJ. The LightCycler: a microvolume multisample fluorimeter with rapid temperature control. Bio Techniques 1997;22:176–81.
[3] Morrison T, Weis JJ, Wittwer CT. Quantification of low-copy transcripts by continuous SYBR Green I monitoring during amplification. Bio Techniques 1998;24:954–62.

[4] Pfaffl MW, Hageleit M. Validities of mRNA quantification using recombinant RNA and recombinant DNA external calibration curves in real-time RT-PCR. Biotechnol Lett 2001;23:275–82.

[5] Knabel M, Kolle S, Sinowatz F. Expression of growth hormone receptor in the bovine mammary gland during prenatal development. Anat Embryol (Berlin) 1998;198:163–9.

[6] Kolle S, Sinowatz F, Boie G, Lincoln D. Developmental changes in the expression of the growth hormone receptor messenger ribonucleic acid and protein in the bovine ovary. Biol Reprod 1998;59:836–42.

[7] Schams D, Berisha B, Kosmann M, Einspanier R, Amselgruber WM. Possible role of growth hormone, IGFs, and IGF-binding proteins in the regulation of ovarian function in large farm animals. Domest Anim Endocrinol 1999;17:279–85.

[8] Armstrong DG, Gutierrez CG, Baxter G, Glazyrin AL, Mann GE, Woad KJ, Hogg CO, Webb RJ. Expression of mRNA encoding IGF-I, IGF-II and type 1 IGF receptor in bovine ovarian follicles. Endocrinology 2000;165:101–13.

[9] Izadyar F, Van Tol HT, Hage WG, Bevers MM. Preimplantation bovine embryos express mRNA of growth hormone receptor and respond to growth hormone addition during in vitro development. Mol Reprod Dev 2000;57:247–55.

[10] Sinowatz F, Schams D, Plath A, Kolle S. Expression and localization of growth factors during mammary gland development. Adv Exp Med Biol 2000;480:19–25.

[11] Plath-Gabler A, Gabler C, Sinowatz F, Berisha B, Schams D. The expression of the IGF family and GH receptor in the bovine mammary gland. Endocrinology 2001;168:39–48.

[12] Olney RC, Anhalt H, Neely EK, Wilson DM. A quantitative assay for IGF-I and IGF binding protein mRNAs: expression in malignant melanoma cells. Mol Cell Endocrinol 1995;110:213–23.

[13] Olney RC, Mougey EB. Expression of the components of the insulin-like growth factor axis across the growth-plate. Mol Cell Endocrinol 1999;156:63–71.

[14] Pfaffl MW, Meyer HHD, Sauerwein H. Quantification of the insulin like growth factor-1 (IGF-1) mRNA: development and validation of an internally standardised competitive reverse transcription-polymerase chain reaction. Exp Clin Endocrinol Diabetes 1998;106:502–12.

[15] Pfaffl MW, Schwarz F, Sauerwein H. Quantification of insulin-like growth factor-1 (IGF-1) mRNA: modulation of growth intensity by feeding results in inter- and intra-tissue-specific differences of IGF-1 mRNA expression in steers. Exp Clin Endocrinol Diabetes 1998;106:514–21.

[16] Cordano P, Hammon HM, Morel C, Zurbriggen A, Blum JW. mRNA of insulin-like growth factor (IGF) quantification and presence of IGF binding proteins, and receptors for growth hormone, IGF-I and insulin, determined by reverse transcribed polymerase chain reaction, in the liver of growing and mature male cattle. Domest Anim Endocrinol 2000;19:191–208.

[17] Pfaffl MW. Development and validation of an externally standardised quantitative insulin like growth factor-1 (IGF-1) RT-PCR using LightCycler SYBR® Green I technology. In: Meuer S, Wittwer C, Nakagawara K, editors. Rapid cycle real-time PCR, methods and applications. Heidelberg: Springer, 2001. p. 281–92.

[18] Rasmussen R. Quantification on the LightCycler. In: Meuer S, Wittwer C, Nakagawara K, editors. Rapid cycle real-time PCR, methods and applications. Heidelberg: Springer, 2001. p. 21–34.