BOVINE GAPDH EXPRESSION USING REAL-TIME RT-PCR

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Summary. Gene expression analysis is increasingly important in biological research, while real-time reverse transcription PCR (RT-PCR) is becoming the method of choice for high-throughput and accurate expression profiling of selected genes. The aim of this study is measuring GAPDH expression levels for future researche as housekeeping gene for IGF-1. For compensating of variations in input RNA amounts and efficiency of reverse transcription, differnt endogenous housekeeping genes have been quantified, and results were normalized to these values. However, the normalization using housekeeping genes, in many cases of IGF-1 studies, was unsuccessful. We measured the GAPDH expression in different cattle tissues, blood, liver and skeletal muscle, with ready-to-use kits from Roche. For the experiments the real-time RT-PCR LightCycler technology was used. The GAPDH expression determination with SYBR Green I was performed with high linearity (R=1.0) and with small mean squared error (Error=0.098) over three orders of magnitude of molecules. The highest gene expression was observed in cattle muscle. The recommendation for changes in quantification protocol has been given. The most effective temperature for quantification is 84 - 85°C.

Abbreviations used: GAPDH - glyceraldehyde-3-phosphate-dehydrogenase, IGF-1 – insulin like growth factor 1, RT-PCR – reverse transcription polymerase chain reaction.

Keywords: glyceraldehyde-3-phosphate dehydrogenase, housekeeping gene, IGF-1, real-time RT-PCR, LightCycler.

GALVIJŲ GLYCERALDEHYDO-3-FOSFATO-DEHYDROGENAZĖS (GAPGDH) EKSPRESIJA ATVIRKŠTINĖS TRANSKRIPCIJOS (RT-PGR) METODU

Santrauka. Genų ekspresinės analizės metodai yra svarbūs biologijos mokslų tyrimams. Vienas dažniausiai taikomų metodų, pasižymintis dideliu našumu ir tikslumu atliekant genų ekspresiją, yra atvirkštinės transkriptazės polimerazės grandinės reakcija (RT-PGR). Šio tyrimo tikslas – atlikti glyceraldehydo-3-fosfato-dehydrogenazės (GAPDH) ekspresijos lygio matavimą su insulino augimo faktoriaus 1 (IGF -1) genu. Siekiant kompensuoti naudojamos RNR kiekio įvairavimą ir atvirkštinės transkriptazės efektyvumą, įvairūs reti genai buvo ištirti kiekybiškai, o rezultatai buvo sunorminti pagal gautas vertes. Tačiau norminimas naudojant šiuos genus daugeliu IGF-1 tyrimų atvejų buvo nesėkmingas. Buvo išmatuota GAPGDM ekspresija įvairiuose galvijų audiniuose, kraujyje, kepenyse ir skeleto raumenyse panaudojant „Roche“ firmos rinkinius. Eksperimentuojama buvo pagal RT-PCR LightCycler technologiją. GAPGDH ekspresijos nustatymas su SYBR Green I buvo atliktas ir gauti teigiami rezultatai (R=1,0; paklaida error = 0,098). Didžiausia genų ekspresija aptikta galvijų raumenyse. Straipsnyje pateiktos kiekybinio tyrimo protokolo pakeitimo rekomendacijos. Geno kiekybinei analizei efektyviausia temperatūra 84–85^{o}C.

Raktažodžiai: GAPDH-glyceraldehydo-3-fosfato-dehydrogenazė, IGF-1 –insulino augimo faktorius 1, RT-PCR - atvirkštinės transkriptazės polimerazės grandinės reakcija.

Introduction. Glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) tetramer of identical 36 kDa subunits is a key enzyme in glycolysis (Bustin, 2000). Apart from playing a key role in glycolysis, this ubiquitously expressed enzyme also displays other activities unrelated to its glycolytic function. The enzyme GAPDH is reported to be involved in the processes of DNA replication, DNA repair, nuclear RNA export, membrane fusion and microtubule bundling. The GAPDH gene is constitutively expressed at high levels in almost all tissues, such as rat and human muscle and heart. Research at Ambion has shown GAPDH to be expressed at relatively low levels in normal mouse liver, spleen, lung, embryo and hypothalamus and at relatively high levels in mouse brain, muscle, heart and kidney. The expression of GAPDH can be induced by several physiological factors in certain cell types. In adipocytes, insulin increases the levels of GAPDH mRNA through cis-acting sequences located within the GAPDH promoter. In endothelial cells, GAPDH is induced by hypoxia at the transcriptional level, this upregulation occuring to a much greater extent in epithelial cells than in other cell types (Tanner, 2004; Smith, 2002). Translocation of GAPDH into the nucleus is seen during its role in the early stages of apoptosis and oxidative stress, because of its high-level, constitutive expression, GAPDH is widely used as an endogenous control or housekeeping gene for quantitative RT-PCR analysis. However, the molecular mechanism that sustains high-level expression of this housekeeping enzyme is still unclear; GAPDH is almost always a tetramer and is localized in the cytoplasm of healthy cells (Bustin, 2000).

A lot of researchers have used GAPDH as a housekeeping gene for compensating variations in input of RNA amounts (Bustin, 2000; Hsio, 2001; Inderwies, 2003; Leuteneggera, 2000; Mao, 2001; Radoni, 2001; Scmittgen, 2000). Our main research focuses on finding a suitable housekeeping gene for IGF-1 expression. Recently, GAPDH has been widely used as a housekeeping gene. Similarly for IGF-1 study, the investigators assume that GAPDH would be good claimant to housekeeping gene. However, there are still no satisfactory results in bovine tissues. The present work is aimed to contribute to problems concerning suitability

of GAPDH use as housekeeping gene in bovine tissues. The question if GAPDH can be appropriate housekeeping gene for IGF-1 is of fundamental and practical interest. Insulin-like growth factor-I (IGF-I) is one of cytokines, a peptid that believe to play an important role in the regulation of cellular growth and differentiation (Pfaffl, 2001; Pfaffl 2002).

To gain insight about gene expression our research strategy involved using a real-time reverse transcription (RT) polymerase chain reaction (PCR) with only ready to use kits from Roche and was developed to detect and quantify the mRNA expression of this enzyme. In the research RT-PCR has was used with online-detection and LightCycler SYBR Green 1 technology. LightCycler PCR with SYBR Green 1 online detection produce reliable and rapid results (Bustin, 2000; Lutz, 2003; Pfaffl 2001; Rasmussen, 2001).

Materials and Methods.

Total mRNA extraction and cDNA synthesis.

The mRNA extraction was performed in bovine (*Bos taurus*) liver, blood and sceletal muscle. Tissue samples of 2 cows and 1 bull were taken immediately after slaughter (blood samples were taken from live animal before slautering), instantly frozen in dry ice and stored (for about 2 hours) at - 20°C until use. Samples were distrupted and homogenized using mortar and pestle. For mRNA isolation, 74-76 mg of homogenized sample was taken. mRNA was purified using the mRNA isolation kit (Roche Diagnostics Corporation, USA), based on a principle of purifying polyadenylated RNA species from sample homogenate non-polyadenylated RNA (rRNA and tRNA) by poly (A) tails. Synthesis of first strand complementary DNA (cDNA) was performed with First Strand cDNA Synthesis Kit for RT-PCR (AMV) (Roche Diagnostics). The mRNA template (10 µl of the mRNA sample or 10 µl of RNase-free water for negative control) was denaturated for 10 min at 65˚C in a termostated waterbath. During RNA denaturation cDNA master mix (total volume 30.0 µl) was prepared as follows: 6.4 µl H_2O PCR grade, 4.0 µl 10* RT-Buffer, 1.6 µl AMV Reverse Transcriptase, 4.0 µl deoxynucleotide mix, 8.0 µl 25 mM $MgCl_2$, 4.0 µl random primer $p(dN)_6$, 2.0 µl Rnase Inhibitor. The mix of cDNA and denaturated sample was treated according to the kit instructions. In order to quantify the synthesed cDNA, optical density of the cDNA work solution was determined at 260 nm. The stock solution was diluted into a work solution at the 1/100. Final concentration of reversely transcribed total cDNA was about 1.6 µg/ml.

Additionally, optical density of the OD_{260} nm/OD_{280} nm (nucleic acid/protein) absorption ratio was measured. Ratio OD_{260}/OD_{280} in water ranges from 1.4 to 1.8.

Oligonucleotide primers.

To sketch the oligonukleotide used in this work, the sequences of the concerning genes were required. The suitable genetic sequences were represented in (Mao, 2001). According to literature data, the GAPDH primers, for this experiment, were synthesed in TIB MOLBIOL (www.tib-molbiol.com). Primer information and the TIB reference number are listed in Table 1.

Table 1. **Sequence of PCR primers, position of the primers (f = forward; r = reverse), G/C content and TIB reference no of the used published nucleic acid sequences**

Primer	Sequence (5´→3`)	GC (%)	TIB reference no.
GAPDH f	CATTGACCTTCACTACATGGT	42.9	003120463
GAPDH r	ACCCTTCAAGTGAGCCCCAG	60.0	

Quantification by real-time PCR.

Polymerase chain reaction was performed with 40µl reversely transcribed total RNA (1.64 µg/ml) using LightCycler FastStart DNA Master SYBR Green I (Roche Diagnostics Inc.) in LightCycler Instrument (Roche Diagnostics Inc.). The procedure described by Lutz (Lutz, 2003) was taken for a basis. In our experiment the GAPDH expression was done with ready to use kits from Roche and some small changes in the protocol were made. In Master mix of the following reaction components was prepared: 11.8 µl of water, 2.4 µl of $MgCl_2$ (4 mM), 0.4 µl of forward primer (10 µM), 0.4 µl of reverse primer (10 µM), 0.8 µl bovine serum albumine (1 µg/ml) and 2.4 µl of LightCycler FastStart DNA Master SYBR Green I (Roche). An aliquot of 18 µl of LightCycler master mix was added to the LightCycler glass capillaries and 2 µl volume of PCR template was added. The capillaries were closed, centrifuged 5 s 700 g and placed into the LightCycler rotor. Subsequently, the LightCycler PCR reactions were performed by series of the target cDNA as follows:

– For all blood samples the 1/10 dilutions were prepared

– For all liver and sceletal muscle samples the undiluted, 1/10 and 1/100 templates were used

– Negative control

The PCR protocol by Lutz was modified: the pre-incubation time was used as recommended in LightCycler FastStart DNA Master SYBR Green I protocol – 10 min instead of 15 (table 2). The increase of the sensitivity of displaying default fluorescence during the run, at a single fluorescence acquisition point, was achieved by increasing the fluorimeter gains F1 value to the 10.

Calculations and statistics.

Expression mRNA was evaluated by amplification curve analysis of the LightCycler real-time RT-PCR. After incorporation into double stranded DNA (dsDNA), SYBR Green I (DNA binding dye) shows fluorescence emission and increases according to target amplification with cycle number. The exponential phase of the PCR becomes detectable when the fluorescence signal from accumulated PCR product is greater than the background fluorescence. LightCycler software version 3.5 was used to estimate the reproducibilities of the calibration curves (slopes and intercepts) and controls were estimated by use of the "second derivative maximum method", in which the software based on default values determines the log-

linear area (Rasmussen, 2001). Expression data of the GAPDH in different tissues was calculated according to the equation: amount = (RNA total concentration 6.023 x 10^{23} molecules) / (1930 bases x 340 daltons/base), where the 1930 is the average size of mRNA molecule (Steffen, 1999; Pfaffl, 2002). The average amount of RNA was 134 Mcopy per mg of fresh tissue.

Table 2. **Cycling conditions of glyceraldehyde-phosphate-dehydrogenase (GAPDH) in a four segment LightCycler real-time reverse transcription–polymerase chain reaction (RT–PCR). The amplication and quantication programme was repeated 55 times with a single fluorescence acquisition point at an elevated temperature**

Segment no.	Target Temperature (°C)	Incubation time (s)	Temperature transition rate (°C/s)	Second temperature (°C)	Step size (°C)	Acquisition mode
Denaturation, 1 cycle						
1	95	600	20	0	0	none
Quantification, 55 cycles						
1 (denat.)	95	15	20	0	0	None
2 (anneal.)	70	10	20	56	0.5	None
3 (elong.)	72	30	20	0	0	None
4 (quant.)	87	10	20	0	0	Single
Melting Curve Analysis						
1	95	0	20	0	0	None
2	65	15	20	0	0	None
3	95	0	0.1	0	0	Cont.
Cooling						
1	45	30	20	0	0	None

Results.

Quantification.

The quantification strategy is the principal marker in gene quantification. Generally two strategies can be performed in real-time RT-PCR. The levels of expressed genes may be measured by absolute or relative quantitative real-time RT-PCR. Absolute quantification relates the PCR signal to input copy number using a calibration curve, while relative quantification measures the relative change in mRNA expression levels (Rasmussen, 2001). The reliability of an absolute real-time RT-PCR assay depends on the condition of identical amplification efficiencies for both the native target and the calibration curve in RT reaction and in following kinetic PCR. The results of quantification of GAPDH mRNA are given in figure A, where the total fluorescence was measured at 87°C at the end of each cycle. The negative control contains no inpute cDNA.

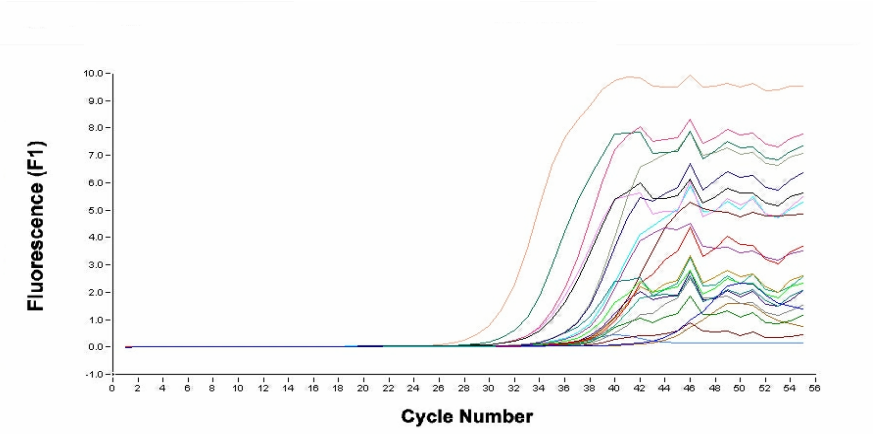
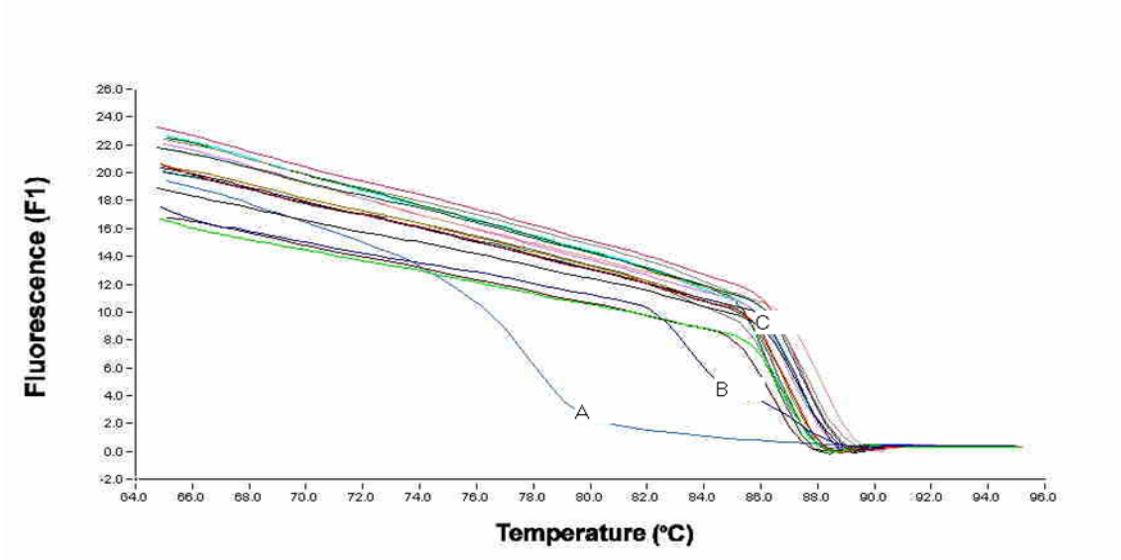


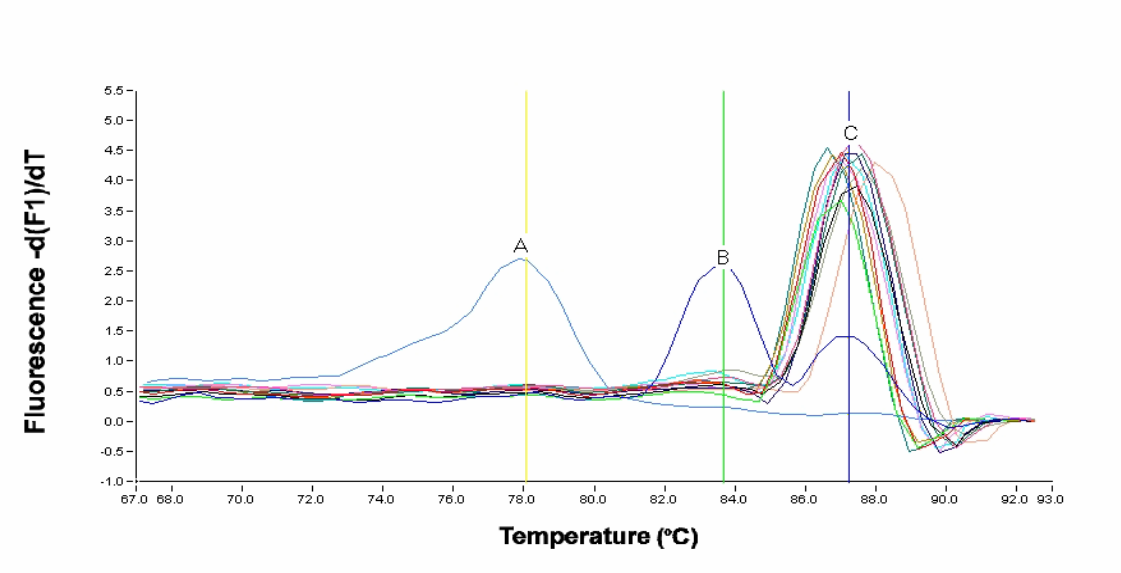
Figure 1. Fluorescence vs. the number of cycles for differing initial amounts of template copies. Data from an experiment by using a LightCycler to quantify copy number of GAPDH in bovine samples.

This figure shows the number of cycles (55 cycles) to be sufficient for product amplification, since a lot of samples had their crossing points about 35 cycle. But some others started to emerge from background in 26, 28, 30, 32, 34, and 36 and even in 40 cycles (5522 serum and 0757 muscle 1/100). On the other hand, Figure 2 (see panel a and b) shows melting curve analyses where the melting peaks are determined by plotting the negative derivate of fluorescence emitted by each sample during the increase of temperature by which PCR products were slowly denaturated. The melting of the DNA product is easily identified as a sharp peak centered at the Tm of the

product. Non-specific amplification products tend to melt at much lower temperatures and over a broader range. Fluorescence is measured continuously. The negative control sample contains only primer-dimers and had the Tm 77.9°C, while the GAPDH amplicon has the melting point Tm 87.2°C.



a



b

Figure 2. **Amplification of GAPDH dilution series of different bovine tissues: 2a shows melt curve analysis of GAPDH amplification reaction demonstranting the gradual reduction in fluorescence as temperature increases. The rapid fall off at 87.2°C (see C) indicates the presence of a specific product that melts at this temperature. The Tm of this product can be visualized more clearly as a peak in a first derivate plot (2b). In figures 2a and 2b, the A – primer dimers, B –unidentified product (in sample bull 5522 – EDTA blood) and C – product of interest (GAPDH)**

The area under overall melting peaks is related to the total amount of amplification products. The relative area under the melting peak approximately 87.2° C is related to the amount of specific PCR product.

Confirmation of primer and PCR-product specificity.

Specificity of the desired products in different bovine tissues total RNA was documented with melting curve analysis. The system uses a fluorescent dye (SYBR Green I) which binds specifically to double stranded DNA in order to detect the accumulation of PCR products and this therefore eliminates the need for electrophoresis for the detection of the specific amplicons. With the LightCycler, the accumulation of amplicons can be visualised in real time, and the specific product can be determined by its characteristic melting temperature (T_m). Derived mean melting temperatures of PCR products were the same for all samples, where can be the expression measured are listed in Figure 2**b**. Since SYBR green does not distinguish between one DNA-s, an important means of quality control is to check that all samples have a similar melting temperature. All PCR products for a particular primer pair should have the same melting temperature - unless there is contamination,

mispriming, primer-dimer artifacts, or some other problems.

Heterozygous detection with LightCycler System.

Melting curves are measures of the change in reporter fluorescence when we change the temperature of the reaction mix. In most cases, the reporter fluorescence depends on the reporter binding to double strand DNA. As such, melt curve analysis tells us whether the DNA is double stranded or not. Different DNAs melt at different points depending on the percentage of guanine and cytosine residues in the PCR product (the "%GC") and the length of the product. This allows us to infer the identity of PCR products without gel electrophoresis, but the strength of melt curve analysis lies in determining the stability of the generated PCR product (the "duplex"). In products, nucleotide mismatches can reduce the stability of the product ("heteroduplex") and as such reduce the melting temperature of the product (Bernard, 2001). These mutation detection protocols are usually designed with hybridization probes. However, in our experiment we observed analogous additional product in bull blood serum (5522) (Figure 3).

Some housekeeping genes (*e.g.* GAPDH, HPRT and β-Actin) are known to have pseudogenes that contain altered internal sequences but can be amplified by the same primer pair. Amplification of pseudogenes cannot be eliminated by higher stringency. However, redesigning the primers so they recognize a different priming region may help (Optimization strategy, 2000).

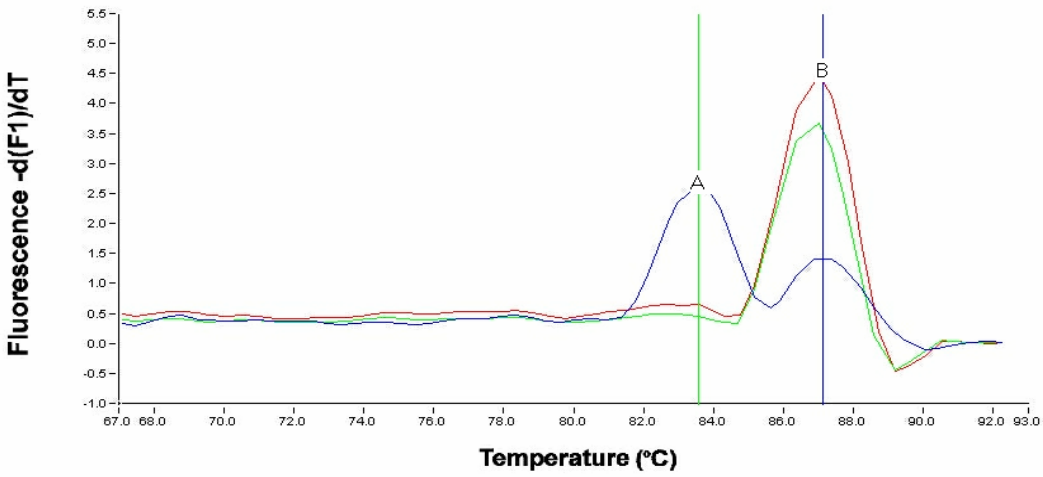


Figure 3. **Melting curves of GAPDH RT-PCR products in bovine EDTA blood. A sample from bull 5522 (A) exhibits a melting curve with two characteristic peaks. Other samples (B) show only one peak match the GAPDH amplicon**

Distribution of mRNA expression.

The highest gene expression was observed in cattle muscle. The standard curve is the linear regression line through the data points on a plot of crossing point (threshold cycle) versus logarithm of sample concentration. The value shown at the bottom of the standard curve in Figure 4**b** is derived from the calculated regression line:

Slope=-3.357 (=-1/logE), where E=1.99, efficiency of the reaction.

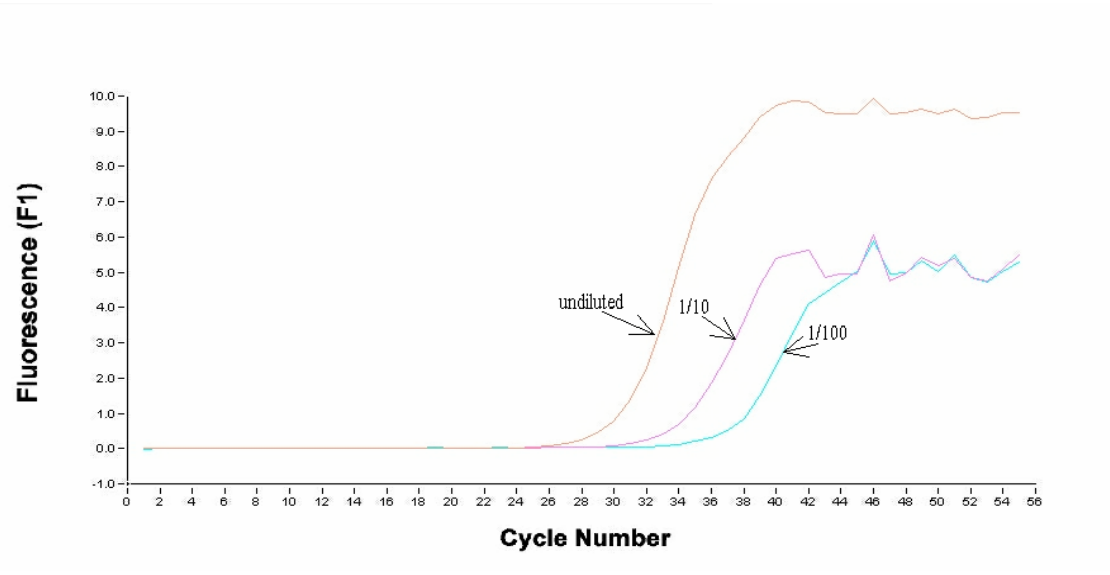
Y-Intercept=32.71 (=logNcp/loge; Ncp= 5.55×10^9, amount of PCR product at Cp

In theory this the slope should be around –3.3. Slopes lower than –4 are indicative of low PCR efficiency.

The GAPDH expression determination with SYBR Green I was performed with high linearity (R=1.0) and with small mean squared error (Error=0.098) over three orders of magnitude of molecules. Differences of GAPDH expression and/or stability in different tissues will be investigated by increased number of samples (animals).

Discussion.

The idea to analyze the expression of the GAPDH was born from some articles where have been published the need of the housekeeping gene for IGF-1 studying (Smith, 2002). Several researchers have been published data of GAPDH expression in humans (Glare, 2002; Hsio, 2001; Radoni 2004) or in bovine (Inderwies, 2003; Leuteneggera, 2000; Mao, 2001; Schmittgen, 2000; Smith, 2002) tissues for different purposes. They have amplified the interested gene and the GAPDH, as a housekeeping gene, together. There are a lot of companies, which provide the kits for selection of the optimal housekeeping gene. One of them, the LightCycler-h-Housekeeping Gene Selection Set, is specifically developed for PCR in glass capillaries using the LightCycler Instrument. But the product contains a mixture of primers and Hybridization Probes specific for detection of the mRNA for β2-microglobulin (β2M), glucose-6-phosphate dehydrogenase (G6PDH), 5-amino-levulinate synthase (ALAS), hypoxanthin-phosphoribosyl transferase (HPRT) and porphobilinogen deaminase (PBGD). In addition, *in vitro* transcribed RNAs - encoding fragments of the respective housekeeping gene - are provided as positive controls (Selection of Housekeeping genes, 2002). Of course it is an easy way to find suitable housekeeping gene for IGF-1, but unfortunately these products are made for human: all primers and hybridisation probes contained each by housekeeping gene are for human.



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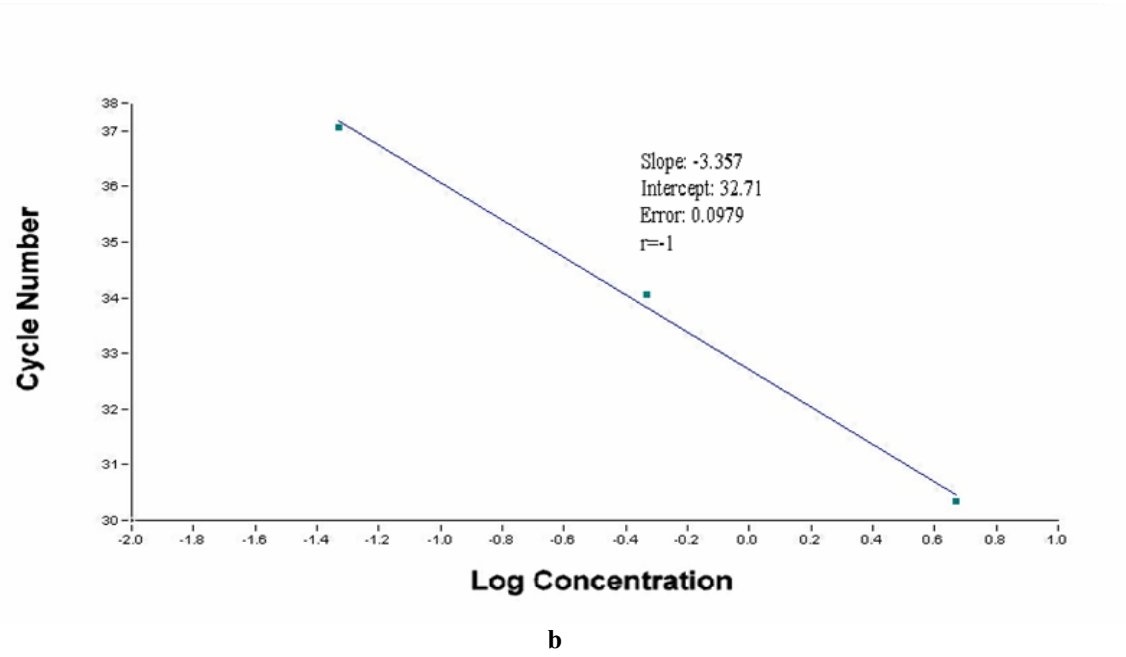

b

Figure 4. **Expression of GAPDH mRNA in skeletal muscle. Figure 4a shows raw fluorescence data for dilutions of a stock solution of GAPDH cDNA. Panel b shows a linear standard curve in three magnitudes of copies of GAPDH**

For our research we used the primer sequences (Mao, 2001) and protocol (Lutz, 2003) for bovine tissues customized for our study. To characterize the measurement range, the LightCycler PCR reactions were performed in series of the sample dilutions: blood samples were prepared as a 1/10 dilutions, muscle samples were for liver and sceletal undiluted, 1/10 and 1/100 templates were used. We tested only the ready to use kits from Roche regarding their usefulness for the GAPDH expression in bovine tissues with LightCycler instrument. All kits, what were used in experiment were easy to handle and gave us the satisfactories results. Correspondingly the LightCycler provides fast measuring and data analyses. In skeletal muscle GAPDH mRNA was highly expressed (E=1,9856). In contrast, the expression in other tissues was less. Our results show that all bovine tissues had clearly measured expression of GAPDH. Higher total RNA concentration (189 Mcopy/mg tissue comparing with the average number 134±31 Mcopy/mg tissue) gave the higher expression in all dilutions of this sample. In other words increase concentration of total RNA in tissue, is usually supported by higher level of mRNA GAPDH expression in that tissue. The received data give us a theme for reflection for the answer to a question interesting us: can GAPDH be appropriate housekeeping gene for IGF-1. This is of fundamental and practical interest. As to conditions: in several recent articles the issue of conditions of measuring the quality of mRNA or cDNA has been covered rather unsatisfactorily, whereas some researchers dilute sample, before measuring the quality, in water, some in buffer (Rolfs, 1992; Скоупс, 1985). Our results affirm the use usefulness of Bovine Serum Albumine (BSA) additives, as a detergent, in mastermix of PCR reaction. BSA

stabilizates DNA-polymerase and also it can interfere with formation of secondary structures (Steffen, 1999; Rolfs, 1992). The PCR protocol, taken from Lutz with small changes in denaturation step, fulfilled our aim. The melting curve analyse demonstrate that the primer-dimers Tm is on 78°C. Therefore the temperature of fluorescence measurement in quantification must be higher than 80°C (at what the SYBR Green will not show the excitation, because on this temperature there are present only single-stranded primers), but lower than temperature in which GAPDH amplicon will be denaturated. So, this confirms that the protocol, presented by Lutz (Lutz, 2003), is reasonable in GAPDH expression measurements, but the most effective temperature for quantification is in range 84-85°C, whereas at higher temperature we will decrease the sensitivity.

In conclusion, our results affirm the simple introduction and permormance of GAPDH gene expression studies in cattle by implementation of minor necessary changes in PCR protocol.

Acknowledgements.

We appreciate the support of Mr M.Roasto at Veterinary Faculty EAU, for kindly providing LightCycler equipment.

This work was supported by ESF Grant No 5734.

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