GENE QUANTIFICATION EUROPE CONFERENCE

COMPARING DIFFERENT RT-PCR APPROACHES TO MEASURE SPECIFIC CELLULAR TRANSCRIPTS

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Introduction:

Several techniques are currently available to measure changes in gene expression. Because of its high sensitivity, reverse-transcription with following polymerase-chain-reaction (RT-PCR) is being increasingly used to quantify physiologically relevant changes in gene expression. The quantification technique of choice depends on the target sequence, the expected range of the mRNA amount, the degree of accuracy required, and whether quantification needs to be relative or absolute. Herein three different RT-PCR quantification methods are described and compared:

- external standardised RT-PCR with quantification on ethidium bromide stained gels in combination with densitometry;
- external standardised RT-PCR with online-detection using LightCycler CybrGreen technology. SybrGreen binds to newly synthesised DNA giving a fluorescence signal once per cycle that is proportional to the DNA concentration;
- internal standardised competitive RT-PCR measured after HPLC separation and UV detection; A reference cRNA standard mutant is co-amplified in the same reaction tube with the native mRNA sequence of interest.

Quantification methodologies:

Quantification of RT-PCR products in **ethidium bromide stained gels** could be performed using external cRNA standards. Within a limited range of linear detection (4 - 400 pg HAS cRNA; r = 0.984) a 5-fold difference between two distinct cDNA samples could be detected (fig. 1). When comparing to a LightCycler quantification an increase of the reliability and sensitivity was observed (1 - 1000 pg HAS cRNA; r = 0.996) to later technique (fig. 2).

LightCycler online-quantification with an external cRNA standard curve offers high sensitivity (280 ag IGF-1 cRNA = 1600 molecules), low intra-assay variation (11.8%; fig. 3) and test linearity over a wide range of IGF-1 cDNA template input (280 ag - 28 ng; r = 0.992).

In **internal standardised competitive RT-PCR** identical PCR efficiencies of native mRNA and competitive standard cRNA were confirmed. The validated IGF-1 assay has a detection limit of 1600 IGF-1 cRNA molecules/reaction, offers low intra-assay variation (7.4%) and linearity is given between 140 - 840 ng total-RNA input (r = 0.997). The comparison of RT-PCR results of individual samples quantified by LightCycler vs. competitive RT-PCR (fig. 4) showed high similarity (r = 0.908; n = 30).

Conclusions:

Absolute quantification of specific transcripts still is a high sophisticated approach. Here we could show that three different RT-PCR techniques possess the following qualities:

- ethidium bromide gels are easiest to perform, but with lowest reliability of produced data;
- internal standardised RT-PCR is a very time consuming and laborious technique yielding the most precise results;
- online detection during RT-PCR, e.g. using LightCycler, combines the ease and necessary exactness to be able to produce most reliable as well as quick results.

All three techniques are found to be suitable to detect transcriptional changes depending on the aims and equipment of the researcher.

Fig.1: Quantification in ethidium bromide-stained agarose gel

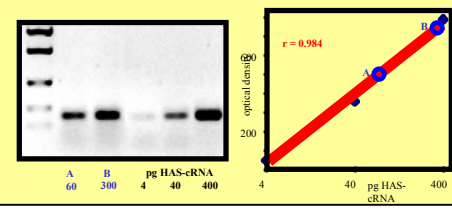

Fig.2: Online-quantification using LightCycler

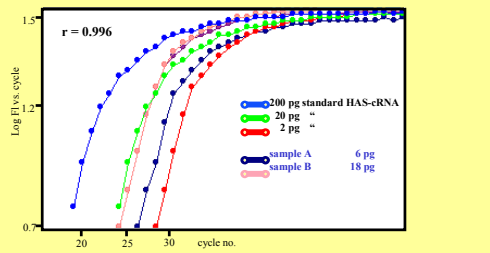

Fig. 3: LightCycler PCR intra-assay variation

- comparison of 3 LightCycler standard curves;
- 500-500.000 IGF-1 plasmid-DNA start molecules; one charge LC premix;
- regression, Pearson correlation coefficient (r) and 99% confidence interval;
- coefficient of variation (CV) of LC-PCR at indicated molecule input;

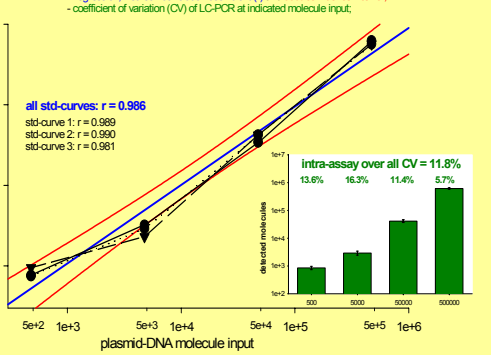

Fig.4: Comparison of two quantitative PCRs

- internal standardised competitive IGF-1 RT-PCR (abscissa)
- external standardised LightCycler Online-PCR (ordinate)
- calculated on gramme tissue basis of several bovine tissues