Quantification of steroid hormone receptor (AR, ERα, ERβ, PR) mRNA expression by LightCycler real-time RT-PCR

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INTRODUCTION

Steroid hormones, like estrogens, androgens and gestagens, play an important role in the cell and tissue differentiation as well as in the regulation of metabolic processes. The steroid hormone effect in the target cell is mediated by its cytoplasmic receptor which binds the steroid with high affinity and high selectivity. As a consequence of the steroid-hormone-receptor interaction the transcription of specific genes is being activated and led to an increased synthesis of specific proteins in the target cell.

Following receptor types of the steroid receptor family were investigated:

- Androgen receptor (**AR**)
- Estrogen receptor alpha (**ERα**) and Estrogen receptor beta (**ERβ**)
- Progesterone or Gestagene receptor (**PR**)

Beside the physiological function the presence of steroid receptors is well documented in patho-physiological disorders like hormone dependent cancer in the following tissues:

- In prostate: AR in prostatic cancer
- In mammary gland: ER α in breast cancer (ER β ??)
- In uterus: ER α in endometrial cancer (ER β ??)
- In gastrointestinal tract: ERα and PR in gastric cancer and ER β in colon cancer

The aim of this study was the development of multi-species RT-PCR expression systems for the steroid receptor family on the LightCycler system. To quantify these possible transcripts also in low abundant tissues, sensitive and reliable real-time RT-PCR quantification methods were developed and validated on the LightCycler.

MATERIAL & METHODS

Primer pairs were designed to produce an amplification product spanning two RNA-splicing sites in the region of the receptor ligand binding domain. Primer design and annealing optimisation was done with DKFZ primer design software. Primer sequences were designed as multi-species primers, according to the available EMBL sequences, which fit to the following species with high precision: human *(Homo sapiens)*, cattle *(Bos taurus)*, sheep *(Ovis aries)*, pig *(Sus scrofa)*, mouse *(Mus musculus)* and rat *(Rattus norvegicus)* and produced commercially (MWG Biotech).

Conditions for RT-PCRs were optimised in a gradient cycler (Biometra) with regard to Taq DNA Polymerase (Roche), PCR water, pH, primers (MWG), $MgCl_2$ concentrations and various annealing temperatures. Results were transferred on the LightCycler RT-PCR protocol. For LightCycler reactions a master-mix 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 1.0 µl LightCycler DNA Master SYBR Green I (1x). 9 µl of LightCycler master-mix was filled in the LightCycler glass capillaries and up to 25 ng reverse transcribed total RNA in 1 µl was added as PCR template.

To improve SYBR Green I quantification a new 4th segment with an high temperature fluorescence acquisition point was included to the amplification cycle program (Table 1). High temperature fluorescence acquisition melts the unspecific PCR products, eliminates the non-specific fluorescence signal derived from primer dimers and ensures an accurate quantification of only the desired steroid receptor products.

For all quantitative assays an external standard curve was used based on a single stranded DNA (ssDNA) molecule calculation. Therefore RT-PCR products from *Bos taurus* were cloned in pCR4.0 (Invitrogen) vector, linearised by a unique restriction digest and dilutions of each recombinant plasmid from single copies ssDNA up to 10^{10} ssDNA molecules were used as DNA standard.

Assay sensitivities, reproducibility, reliabilities and multi-species applications were shown in figure 1 and table 2.

Table 1: Specific LightCycler cycle conditions.

No.	Segment	Duration (sec)	AR	ERα	ERβ	PR
1st	denaturation	1	95°C	95°C	95°C	95°C
2nd	annealing	10	60°C	64°C	64°C	65°C
3rd	elongation	20	72°C	72°C	72°C	72°C
4th	fluorescence acquisition	3	83°C	82°C	87°C	81°C

Figure 1: ERα intra-assay and inter-assay variations at different ssDNA molecules input.

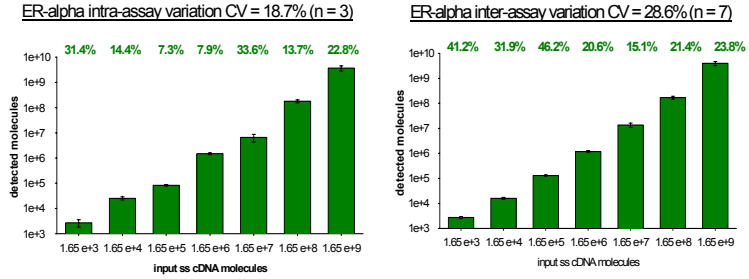

Table 2: Characterization and validation of multi-species steroid receptor RT-PCR.

	AR	ERα	ERβ	PR
product length	172 bp	234 bp	262 bp	227 bp
detection limit	12 molecules	2 molecules	10 molecules	14 molecules
quantification limit	120 molecules	165 molecules	106 molecules	760 molecules
quantification range (test linearity)	120 - $1.20*10^{10}$ molecules (r = 0.998)	165 - $1.65*10^{9}$ molecules (r = 0.995)	106 - $1.06*10^{10}$ molecules (r = 0.996)	760 – $7.60*10^{9}$ molecules (r = 0.998)
PCR efficiency	90.7%	81.2%	81.3%	93.9%
intra-assay variation	31.2% (n = 3)	18.7% (n = 4)	17.6% (n = 4)	5.7% (n = 4)
inter-assay variation	24.3% (n = 7)	28.6% (n = 4)	29.7% (n = 4)	25.7% (n = 4)
Species specific T_{melt} (°C)				
Homo sapiens	85.4 84.4	86.0 85.0	[87.9] 89.0	83.5 [82.9]
Rattus norvegicus	85.0		[89.9]	83.9
Callithrix jacchus (primate)	85.5	85.3	90.1	83.8
Bos taurus		85.4	90.5	83.1
Ovis aries	84.5	86.0	90.2	83.5
Sus scrofa				

FIRST EXPERIMENT

In a first experiment 15 different tissue ERα/ERβ expression profiles were quantified via real-time RT-PCR. ERα and ERβ real-time RT-PCR were product specific and in all tissues both transcripts were found in different expression ratios. High ERα /ERβ ratios were examined in some muscles, liver, udder and uterus; except in kidney and jejunum the ERα /ERβ ratios were <1. To make the individual tissue expression pattern evident all ER α and ERβ expression rates were compared and shown with bi-directional error bars (Figure 2).

Our expression results indicate the existence of two ER subtypes in various bovine tissues, their different expression pattern and co-expression. These different expression patterns of ERα and ERβ could be regarded as support for the hypothesis that the ER subtype proteins may have different biological functions, especially in kidney and the jejunum where ERβ expression ratio is vice versa in comparison to the other investigated tissues.

The localisation and dominant expression of ERβ in both kidney regions and in the jejunum leads to the hypothesis that ERβ plays a dominant role in these tissues. ERα was already detected earlier in the bovine gastrointestinal tract, but ERβ might be the major actor in the absorptive processes and in pathological processes like gastrointestinal cancer. However, any notations on direct physiological or pathological effects of estrogens on gastrointestinal tissues and kidney remain speculative.

DISCUSSION & CONCLUSION

In view of the data provided for sensitivity, linearity and reproducibility, the RT-PCR assays developed herein allows for the absolute and accurate quantification of low abundant steroid receptors mRNA molecules in various tissues and species.

In future more detailed studies of steroid receptors expression must be performed in all cell types to continue investigations of the steroid receptor regulation. In order to develop better cancer therapies, it is essential to understand the nature of gene expression via steroid receptor activation and its regulative function in physiological and patho-physiological processes.

Furthermore, the activity of endocrine disruptors known to interfere with the regulation of endogen steroid hormones, will be monitored using this quantitative real-time approach.

Figure 2: ERα and ERβ expression cluster in 15 bovine tissues.