Improved Performance of the LightCycler® FastStart DNA Master^{PLUS} SYBR Green I Kit in Highly Sensitive Transcript Quantification

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Introduction

Real-time reverse transcription-PCR (RT-PCR) is considered the method of choice for any experiments requiring sensitive, specific, and reproducible quantification of mRNA molecules of interest. It is an essential tool for basic research, molecular medicine, and biotechnology.

The successful application of real-time RT-PCR and accurate quantitative measurement of transcription depends on careful experimental design and a clear understanding of methodological problems [1]. For example, the efficiency of a PCR is affected by cDNA synthesis, primer annealing, and the sequence and size of corresponding amplicons.

Neuropeptide Y (NPY) is a powerful stimulant of food intake. mRNA expression and pharmacological studies as well as feeding observations suggest that the NPY Y5 receptor is the primary mediator of NPY-induced feeding [2]. It is therefore of scientific interest to quantify *npy5r* transcripts encoding the NPY receptor Y5 (postulated as "feeding" receptor) in hypothalamic tissue of pigs. *Hsd3b* encodes a key enzyme of steroid biosynthesis, 3 β-hydroxysteroid dehydrogenase (3 β-HSD). The gene is expressed in steroidogenic organs such as ovary and placenta [3], and the level of its expression is an indicator for steroid production and thus a marker for growth and differentiation of ovarian granulosa cells [4, 5].

In this article, we describe the comparative application of two sequence-independent kits for cDNA quantification, the LightCycler® FastStart DNA Master SYBR Green I Kit and the LightCycler® FastStart DNA Master^{PLUS} SYBR Green I Kit. We compared the performance of the kits by quantifying *npy5r* transcripts in cDNA samples of porcine

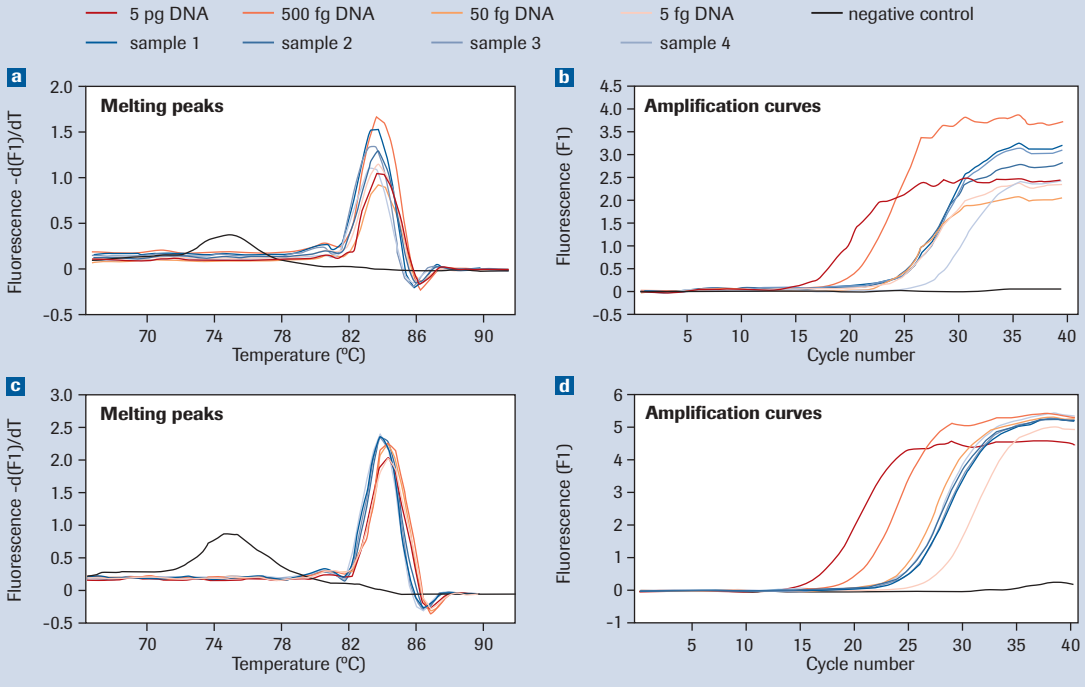

Figure 1: Quantification of *npy5r* transcripts in porcine hypothalamus. LightCycler® PCR was performed using (*a* and *b*) LightCycler® FastStart DNA Master SYBR Green I Kit or (*c* and *d*) LightCycler® FastStart DNA Master^{PLUS} SYBR Green I Kit. Identical standards (plasmid DNA) and samples were used.

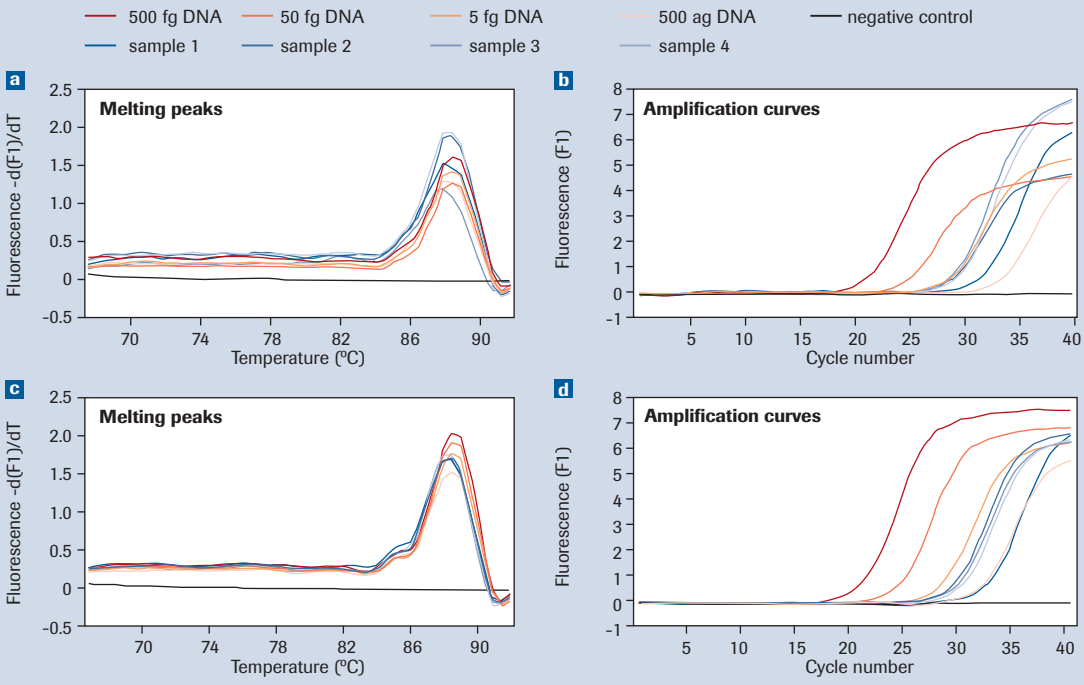

Figure 2: Quantification of *hsd3b* transcripts in porcine granulosa cells. LightCycler® PCR was performed using (*a* and *b*) LightCycler® FastStart DNA Master SYBR Green I Kit or (*c* and *d*) LightCycler® FastStart DNA Master^{PLUS} SYBR Green I Kit. Identical standards (plasmid DNA) and samples were used.

hypothalamus and *hsd3b* transcripts in cDNA samples of porcine ovarian granulosa cells using the LightCycler® System.

Materials and Methods

RNA isolation

Ovaries and hypothalami derived from domestic pigs kept under standardized housing conditions were collected immediately after slaughtering. Granulosa cells were aspirated from medium to large ovarian follicles. RNA isolation was performed using commercial kits.

Reverse transcription

For cDNA synthesis 2 µg total RNA from different hypothalami and 0.15 µg total RNA from granulosa cells were reversely transcribed in a 25 µl reaction volume with 500 nM of reverse primers (*hsd3b*: 5´-CTATGCTGCTGGTGTGGATGAAG-3´ or *npy5r*: 5´-GGAGAGCAAATGGCAAGG-3´). The synthesized cDNA samples were cleaned with the High Pure PCR Product Purification Kit and eluted in 50 µl elution buffer.

Table 1: Amplification efficiency of samples quantified using either LightCycler® FastStart DNA Master SYBR Green I Kit or LightCycler® FastStart DNA Master SYBR Green^{PLUS} I Kit.

	Amplification efficiency*		Error of standard curve	
	npy5r	*hsd3b*	*npy5r*	*hsd3b*
LightCycler® FastStart DNA Master SYBR Green I Kit	1.90	1.81	0.09	0.14
LightCycler® FastStart DNA Master^{PLUS} SYBR Green I Kit	1.92	1.88	0.02	0.06

**amplification efficiency = $10^{-1/slope\ of\ standard\ curve}$*

PCR using the LightCycler® Instrument

Primers for real-time PCR were designed according to published sequences of the pig *hsd3b* (EMBL/GenBank AF232699, 5´-AGGGTTTCTGGGTCAGAGGATC-3´ and 5´-CGTTGACCACGTCGATGATAGAG-3´) and *npy5r* genes (EMBL/GenBank AF106083, 5´-TGGTTTCATGGGGAATCTC-3´ and 5´-ACTGTCGCAATCAGGAAGTAG-3´). For comparative performance analysis, 0.3 µl of the purified cDNA samples were amplified with both the LightCycler® FastStart DNA Master SYBR Green I Kit and the LightCycler® FastStart DNA Master^{PLUS} SYBR Green I Kit in 10 µl total reaction volume each. Amplification and quantification of generated products were performed in a LightCycler® Instrument under the following cycling conditions: Pre-incubation at 95°C for 10 minutes, followed by 40 cycles denaturation at 95°C for 15 seconds, annealing at 60°C (*hsd3b*) or 52°C (*npy5r*) for 10 seconds, extension at 72°C for 10 seconds, and single-point fluorescence acquisition at 83°C (*hsd3b*) or 82°C (*npy5r*) for 6 seconds in order to avoid quantification of primer artifacts.

The melting peaks of all PCR products were routinely determined by melting-curve analysis, and their molecular sizes were monitored by agarose gel electrophoresis analysis (data not shown) in order to ascertain that only the expected products had been generated.

To generate external standards, the RT-PCR products of *hsd3b* and *npy5r* were cloned into the pGEM-T plasmid vector (Promega). Routinely, dilutions of the standards covering five orders of magnitude (5 pg to 500 ag plasmid DNA) were co-amplified during each run. Fluorescence signals, which were recorded on-line during amplification, were subsequently analyzed using the "Second Derivative Maximum" method of the LightCycler® Software Version 3.

Results and Discussion

To compare the application of two different sequence-independent kits for transcript quantification, the LightCycler® FastStart DNA Master SYBR Green I Kit and the LightCycler® FastStart DNA Master^{PLUS} SYBR Green I Kit were used to quantify *npy5r* cDNA in samples of porcine hypothalamus and *hsd3b* cDNA in porcine granulosa cell samples.

Both kits were suitable to amplify *npy5r* and *hsd3b* transcripts during real-time PCR. In *npy5r*- and *hsd3b*-specific assays, the amplification curves generated with the new LightCycler® FastStart DNA Master^{PLUS} SYBR Green I Kit were even more reproducible than those generated with the LightCycler® FastStart DNA Master SYBR Green I Kit (Figures 1 and 2). The melting-peak curves derived from assays using the new kit showed uniform peak levels (Figures 1 and 2). The efficiency of real-time PCR as calculated from the amplification of standard plasmids was higher using the new kit. At the same time, the reactions showed lower mean errors of the standard curve in case of both *npy5r* and *hsd3b* assays (Table 1).

Conclusion

The results of the study indicate that the LightCycler® FastStart DNA Master^{PLUS} SYBR Green I Kit provides efficient, robust, and reproducible amplification conditions and is less susceptible to end-product inhibition. Therefore, we strongly recommend the use of this kit for highly sensitive transcript quantification. ■

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