To Probe or Not to Probe

Comparison of qPCR Assays Using the LightCycler® 2.0 Instrument and either SYBR Green I Intercalation or the Universal ProbeLibrary as Detection Format

Onno Bakker

*Onno Bakker**
Molecular & Cellular Endocrinology Laboratory, Academic Medical Centre, Amsterdam, the Netherlands
**Corresponding author: o.bakker@amc.uva.nl*

In our efforts to measure the expression levels of the thyroid hormone receptor ß2 in small areas of mouse brain, we encountered primer-dimer problems when using SYBR Green I assays. With the Universal ProbeLibrary and the LightCycler® 2.0 Instrument we were able to solve the primer-dimer problem, increase PCR efficiency, and continue our investigations.

Table 1: LightCycler® Instrument programming.

PCR setup	Pre-incubation	Amplification		Melting curve
Old Primers	15 min at 95° C	45 cycles:	5 s at 95°C (20°C/s)	0 s at 95°C (20°C/s)
			10 s at 55°C (20°C/s)	0 s at 55°C (20°C/s)
			15 s at 72°C (20°C/s)	0 s at 95°C (0.1°C/s)
			(single acquisition)	continuous measurement
				60 s at 40°C (20°C/s)
UPL Primers	10 min at 95°C	55 cycles:	10 s at 95°C (20°C/s)	
			20 s at 50°C (20°C/s)	
			5 s at 72°C (20°C/s)	
			(single acquisition)	
		cooling:	30 s at 40°C (20°C/s)	

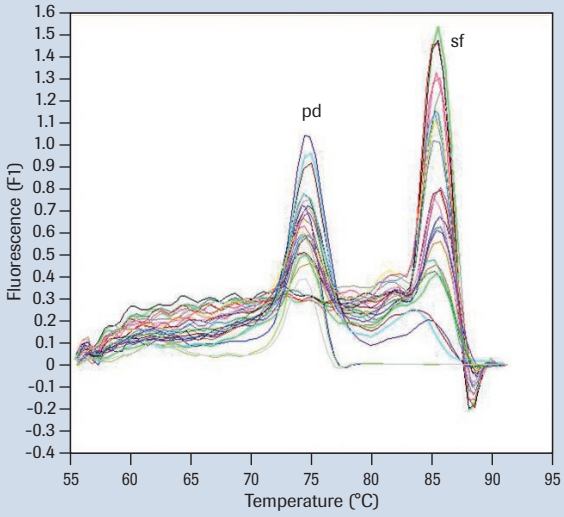

Figure 1: Old primers. The melting curve pattern shows a large portion of primer-dimer (pd) compared to the specific Tbr2 fragment (sf) when the PCR is performed using the "old" primers.

Introduction

In our laboratory, we study the actions of thyroid hormone from many different angles and in many different tissues. Thyroid hormone production is regulated by two peptide hormones: thyrotropin releasing hormone (TRH) and thyroid stimulating hormone (TSH or thyrotropin). TRH is expressed in the paraventricular nucleus (PVN) of the brain, which in turn induces the synthesis of TSH or thyrotropin in the pituitary gland. TSH then activates the thyroid to produce the thyroid hormone. The levels of thyroid hormone are regulated by a negative feedback system. When the hormone levels get too high, this is signalled by thyroid hormone receptors (TR, of which there are at least 5 isoforms) in the PVN and the pituitary gland. As a result, TRH and TSH production decrease, eventually decreasing TH production. One of our research interests is to understand this negative feedback mechanism under conditions of illness, like infections or diabetes. It is known that one of the isoforms of the TR, the TRß2, plays a role in this process. TRß2 is expressed in the PVN and the pituitary gland. We set up a PCR assay for this isoform using the LightCycler® 2.0 Instrument and SYBR Green I as detection format and often encountered the frustrating problem of primer-dimers. This problem appears to be related to the low level of expression of the TRß2 variant. We decided to overcome this problem by using the new Universal ProbeLibrary.

Materials and Methods

Preparation of mRNA and cDNA

After being excised, mouse pituitaries and the brain area that contains the paraventricular nucleus tissues were snap frozen in liquid nitrogen, and then kept at -80°C. About 100 mg (less in the case of the PVN) frozen tissue was placed in a tube with MagNA Lyser Green Beads. 100 µl lysis buffer was added, and the tube with the mixture was placed into the MagNA Lyser Instrument, and centrifuged at 6,500 *rpm* for 90 seconds. The lysed material was then transferred into a MagNA Pure LC Sample Cartridge and mRNA was purified using the MagNA Pure

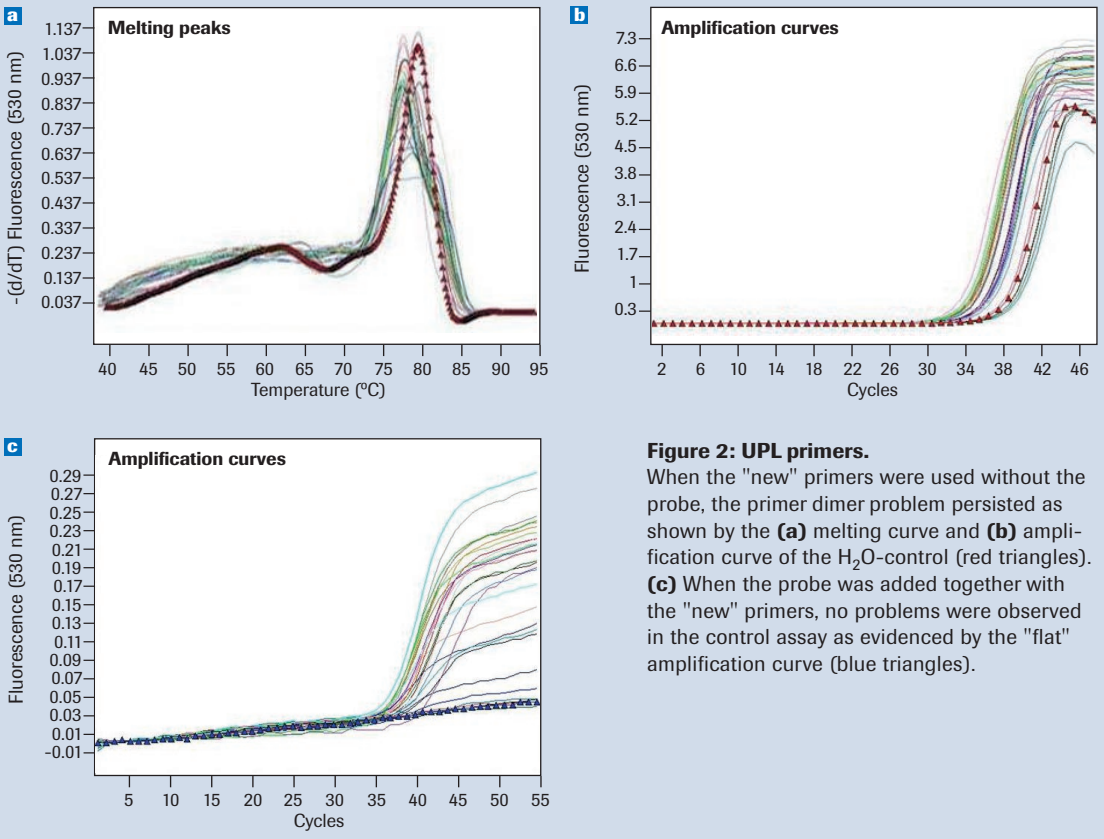

Figure 2: UPL primers.
When the "new" primers were used without the probe, the primer dimer problem persisted as shown by the **(a)** melting curve and **(b)** amplification curve of the H_2O-control (red triangles). **(c)** When the probe was added together with the "new" primers, no problems were observed in the control assay as evidenced by the "flat" amplification curve (blue triangles).

LC mRNA Isolation Kit II (Tissue). RNA concentration was measured after the purification using a NanoDrop, and equal microgram amounts were used for cDNA synthesis. cDNA was prepared in a two-step method using the Transcriptor First Strand cDNA Synthesis Kit and random primers.

RT-PCR

We used primers that we named the "old" primers (generated using a conventional primer design program) and the "new" primers (generated using the UPL Assay Design Center). Old primer sequences were forward: 5'-GTGAATCAGCCTTATACCTG-3' and reverse: 5'-ACAGGTGATGCAGCGATAGT-3' (255-bp fragment). New primer sequences were forward: 5'-CCACCGCACTCACAAAAA-3' and reverse: 5'-TTCAAGGAACGTGATGCAAT-3' (88-bp fragment). cDNA was amplified in a 20-µl reaction using the LightCycler 2.0® Instrument and the LightCycler® FastStart DNA Master, SYBR Green I when using the "old" primers and the "new" primers without the selected Universal ProbeLibrary (UPL) probe and the FastStart TaqMan® Probe Master when using the "new" primers in combination with the UPL probe #106 (formerly mouse #82). The instrument was programmed as indicated in Table 1.

Results

Old primers

As can be clearly seen in Figure 1, the "old" primers gave rise to primer-dimers (pd). The amounts of pd were so high that even the samples that had a specific fragment (sf) could not be used. This problem occurs because TRb2 is expressed at low levels. We have tried many methods to eliminate the primer-dimers, but all to no avail.

New primers and UPL

The "new" primers were designed using the Assay Design Center of the UPL website, www.universalprobelibrary.com. To make sure we did not miss a primer set that could work in the SYBR Green I assay, we performed the PCR without the UPL probe. As can be seen in Figure 2a and b this primer set caused serious problems (red triangles). When we then added the probe to the mixture, we obtained an assay devoid of primer-dimers (Figure 2c, blue triangles). With the old assay, none of the samples could be used in our calculations. Using the UPL probe, all samples could be used.

Conclusions

We attempted to determine whether the UPL could solve the primer-dimer problem in the TRß2 assay. The results show that using the UPL, the primer-dimer problem dis-

appeared. Another advantage of using the UPL is that the assay exhibits an increase in assay efficiency. The difference in efficiency between assays or individual samples can result in a significant difference in the final result (see www.gene-quantification.info). Therefore, we use the following rule of thumb: If the efficiency of an individual sample differs by more than 0.05 from the median efficiency of the assay in question, the sample is not taken into account for the calculations (a full discussion of this is unfortunately outside the scope of this paper). For example, in a SYBR Green I assay, which measures 5'-deiodinase in mouse liver, we had to remove one quarter of our samples. However, in an asssay employing a UPL probe with the same samples we were able to include all samples. This is solely the result of improved efficiency. ■

Order INFO

Product	Pack Size	Cat. No.
Universal ProbeLibrary Probe # 106	2 x 250 reactions	04 692 250 001
Universal ProbeLibrary Set, Mouse	1 set (90 probes for 250 reactions)	04 683 641 001
MagNA Lyser Instrument	(110 V)	03 358 968 001
	(220 V)	03 358 976 001
MagNA Lyser Green Beads	100 tubes	03 358 941 001
MagNA Pure LC Instrument	1 instrument	12 236 931 001
MagNA Pure LC mRNA Isolation Kit II (Tissue)	1 kit (192 isolations)	03 172 627 001
LightCycler® 2.0 Instrument	1 instrument	03 531 414 001
Transcriptor First Strand cDNA Synthesis Kit	1 kit (50 reactions)	04 379 012 001
LightCycler® FastStart DNA Master SYBR Green I	96 reactions	03 003 230 001
	480 reactions	12 239 264 001
FastStart TaqMan® Probe Master	100 reactions	04 673 409 001
	500 reactions	04 673 417 001
	2,000 reactions	04 673 433 001
LightCycler® TaqMan® Master	96 reactions	04 535 286 001
	480 reactions	04 735 536 001