The Universal ProbeLibrary – a Versatile Tool for Quantitative Expression Analysis in the Zebrafish

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The teleost zebrafish is a widely used model organism for the study of developmental and disease-related processes in vertebrates, including humans. Understanding these processes at a molecular and cellular level frequently requires the estimation of gene expression levels. Quantitative real-time PCR (qPCR) is an important tool towards this goal but has, until now, been relatively neglected in zebrafish. Here, we describe the comparison of the Universal ProbeLibrary (UPL)-based qPCR to a conventional SYBR Green I-based qPCR assay using embryonic zebrafish cDNA. We found that the Universal ProbeLibrary-based assay shows equal or better performance compared with the SYBR Green I assay.

Introduction

The Universal ProbeLibrary combined with the ProbeFinder Assay Design Software is an easy-to-use qPCR system that enables researchers to find the correct primer/probe combination and to set up a qPCR assay for the transcript of interest in a relatively short time. In the conventional SYBR Green-based qPCR, an intercalating dye (*e.g.*, SYBR Green I) is used to non-specifically label all PCR-amplified dsDNA. During this labeling process, unwanted products and primer-dimers are also labeled by the dye. In contrast to this, the Universal ProbeLibrary assay is a probe-based assay that relies on the sequence-specific detection of a desired PCR product by labeled probes [*e.g.*, fluorescein (FAM)-labeled hydrolysis probes]. The combination of gene-specific primers and the probe results in increased specificity and sensitivity.

The Universal ProbeLibrary is based on locked nucleic acid (LNA) probes; these are short (8–9 nucleotides) modified nucleic acid molecules that are able to bind to their target sites despite the short length, and can therefore be used as probes in real-time PCR or for *in situ* hybridization. They are designed as hydrolysis probes that are labeled with fluorescein (FAM) and can be used with standard PCR conditions and detection formats.

The short size of the LNA-probes enables the detection of transcripts with different splice forms at the same time (common assay) or distinction between several splice forms with a probe that binds to only one or the other transcript (differentiating assay).

Here, we show the evaluation of the Universal ProbeLibrary for zebrafish. Newly designed Universal ProbeLibrary assays were compared with custom-designed SYBR Green I assays. In addition, Universal ProbeLibrary assays were tested for three different housekeeping genes, to determine relative gene expression using multiple reference genes.

Materials and Methods

RNA isolation and reverse transcription

Total RNA was extracted from 50 zebrafish embryos at 24 hpf (hours post-fertilization) using a commercially available RNA isolation kit. First-strand cDNA synthesis was performed using the Transcriptor First Strand cDNA Synthesis Kit with 1 µg of total RNA and oligo(dT) primers.

Primer design

Primers for the Universal ProbeLibrary assays were designed using the ProbeFinder Software. Primers for the SYBR Green I assays were designed using VectorNTI (Invitrogen).

Quantitative PCR

For the Universal ProbeLibrary-based assays, the LightCycler® TaqMan® Master reaction mix was used. For the SYBR Green I-based assays, the LightCycler® FastStart DNA Master^{PLUS} SYBR Green I reaction mix was used. Both assay types were performed with a LightCycler® 1.2 Instrument.

Amplification conditions for the Universal ProbeLibrary assays were initial denaturation for 10 minutes at 94°C followed by 40–50 cycles of 10 seconds at 94°C, 20 seconds at 55°C, and 5 seconds at 72°C. For the SYBR Green I assays, we started with 10 minutes at 94°C followed by

Table 1: Universal ProbeLibrary and SYBR Green I assays.			
Transcript	**Universal ProbeLibrary Forward/reverse primer**	**Probe No. (UPL)**	**SYBR Green I Forward/reverse primer**
her5	GGAGCAAAAAGACATGAGAAGG/ TCTCAAGGTTTCTAGGCTTTGATT	63	TGAAAACATACACAAATCGCACTC/ CCTCATAGAGATGCACCTGAGTTT
pax2a	GGCAGCTACCCACCTCTA/ ATTTCCTGAAAAGTCGCTTCC	151	TGTGTCAGCAAAATTCTCGGA/ TTCCACAACTTTGGGCGTC
fgf8	GAAGATGGCGACGTTTGTG/ CCCTCCTGTTCATACAGATGTAAA	17	TTTACACAGCATGTGAGTGAGCA/ GGTTCGGCTGTAAAGCTGGTA
fgfr1	CTCTCAGGGGTCTCCGAATA/ GAGGTTTCCCGAGAACCAG	43	AAAGTAGAGCCGGCCGACT/ TCCACCTCGTCTTCTTCATCTG
canopy1	CCTCTTGTTTTCACGTAACGTCT/ GCCACAAGAACAAGGCAAAT	54	AGTCAAAGAAAGATGAAGCACTGTACT/ TTTTTTGGATCCGTCTGACTGA
her9	GAGCGAGAATCAACGAGAGC/ TCCAATTTAGAGTGTCTGGAGCTA	44	CCTTGGGCAGCTGAAGACTC/ GGTGCTTGACTGTCATCTCCAG
neurog1	GATTCTGCAAAACCTCAAGCA/ TCGGAGTATACGATCTCCATTGT	23	CCACTGTGCACGTCGTGAA/ CCAATGCGTCGTTAAGGTTG
pea3	CCAGCAAGTGCCTTATACTTTAGC/ TGCGTCCATGTATTTCCTTTT	147	TCCTGCAGCCACAAGCAA/ TAGATCCTCCTGCTCCTGCC
fgf4	GTGGAGAGGGGAGTTGTGAC/ TGCATTCGTTTGTGAACTGC	150	CCGGGGAAAAAAGAGATATCAG/ AAACCCAATGCCAACGTTG
hey2	CAGCGACATGGATGAAACC/ CATCTTATAAATGAACCATTGCTTTG	71	TCTTGAGCATTGGCTTCCG/ AACCAGACGGACACGGAGAG
wnt1	TGTCCTCTCTCACTGGCACA/ CGAGGATGCCACATTCACTA	77	CTCTGAAGGGGCCATTGAGT/ ACATTGTCGCTGCAGCCTC
GAD67	AAGGGAGAAGCTGCACAGG/ GTCTCCCTGTGGTTGGTAGC	157	ATGCGCTCTTCTCACCTGGT/ CAGACATGCCTTTGGTTTTGAC
elavl3	ACCCAGCTCTACCAGACAGC/ TGGTTATGGGGGAGAATCTG	131	GTCAGAAAGACATGGAGCAGTTG/ GAACCGAATGAAACCTACCCC
bactin1	TCACTCCCCTTGTTCACAATAA/ GGCAGCGATTTCCTCATC	76	CACACCGTGCCCATCTATGA/ AGGATCTTCATCAGGTAGTCTGTCAG
bactin2	AAGGCCAACAGGGAAAAGAT/ GTGGTACGACCAGAGGCATAC	56	CTGACTGACTACCTCATGAAGATCCT/ CCTTGATGTCACGGACAATTTC
gapdh	AACTTTGGTATTGAGGAGGCTCT/ TCTTCTGTGTGGCGGTGTAG	114	n.d.

40–50 cycles of 10 seconds at 94°C, 5 seconds at 55°C, and 5 seconds at 72°C. For all SYBR Green I assays a melting curve from 60°C to 95°C was recorded.

Results and Discussion

We used the ProbeFinder Software to design Universal ProbeLibrary assays for 15 different zebrafish transcripts expressed at embryonic stages. For the same subset of transcripts, a custom design of primer pairs for use with conventional SYBR Green I assays was developed.

The ProbeFinder Software in the online Assay Design Center (www.universalprobelibrary.com) proved to be very user friendly and successful in finding good assay designs using known accession numbers and keywords or gene names. The integration of databases for zebrafish genes is apparently of good quality and results are presented in a clear and well-designed manner.

Sequential 1:10 dilution series of the zebrafish cDNA were used to compare the performance of both assay types. For the Universal ProbeLibrary assays, 14 out of 15 assays showed satisfactory to excellent results, whereas one assay failed completely (*hey2*; Table 1). For the SYBR Green I assays, all assays worked well (Table 1). In the failed assay, no gel product was detected (not shown), showing that the failure was most likely due to unsuccessful primer design. With respect to linearity, the Universal ProbeLibrary assays showed an overall better performance than SYBR Green I assays.

In addition to comparing the two detection formats, we tried to find more reference genes which the zebrafish researcher could use for more accurate normalization when assessing relative gene expression. In most publications to date, zebrafish researchers used only one gene (mostly *bactin1*) for normalization. In general, the

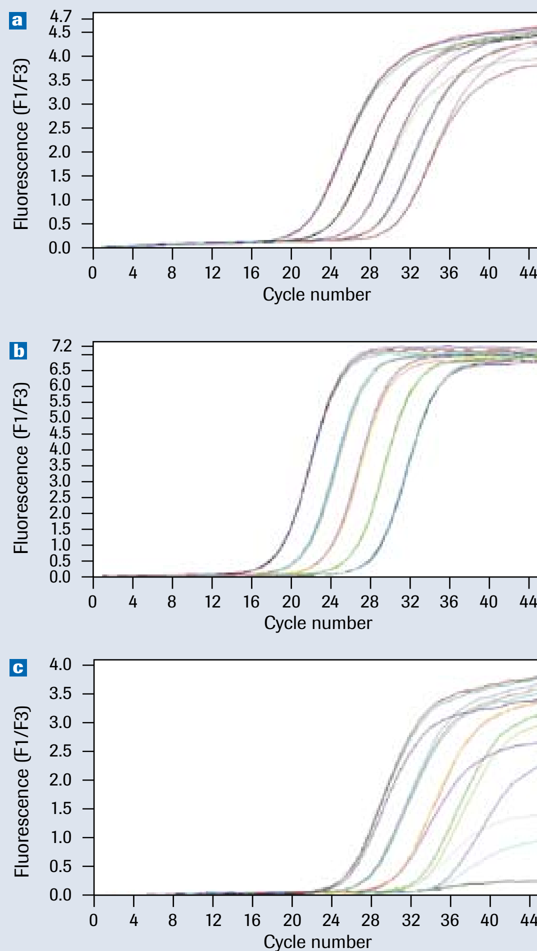

Figure 1: Three potential reference genes. Amplification curves of **(a)** *bactin1*, **(b)** *bactin2*, and **(c)** *gapdh* of five dilutions starting with a 1:5 dilution of cDNA from zebrafish embryos (24 hours post-fertilization).

use of multiple normalization genes is advantageous compared with the use of only one reference gene (www.gene-quantification.info and [1]). Since there are two *bactin* genes in zebrafish, *bactin2* and additionally *gapdh* were assayed. For all genes, the first selected Universal ProbeLibrary assay was successful (Figure 1). In Figure 1, a 1:5 dilution series for each of these three genes is shown.

Conclusions

In our hands the Universal ProbeLibrary-based real-time PCR using the LightCycler® Instrument and the ProbeFinder Software proved to be a very useful tool to determine accurate expression ratios in zebrafish embryos. Traditionally, *in situ* hybridization using DIG- or fluorescein-labeled RNA probes is the only method widely used in zebrafish to assess expression levels in embryos. This is not an ideal situation, since this method is not quantitative. The Universal ProbeLibrary-based real-time PCR now offers a reliable and time-efficient tool to overcome this problem by designing assays for any gene of interest in a very short time, and with an increased rate of success. Moreover, we established assays for three potential reference genes, paving the road for successful relative gene quantification in the zebrafish. ■

References

1. Vandesompele J *et al.* (2002) Genome Biol 3:RESEARCH0034

Product	Pack Size	Cat. No.
Universal ProbeLibrary Extension Set, Probes #91 to #165	1 set	04 869 877 001
Universal ProbeLibrary Set, Human	1 set	04 683 633 001
Transcriptor First-strand cDNA Synthesis Kit	1 kit (50 reactions)	04 379 012 001
LightCycler® TaqMan® Master	1 kit (96 reactions) 1 kit (480 reactions)	04 535 286 001 04 735 536 001
LightCycler® FastStart DNA Master^{PLUS} SYBR Green I	1 kit (96 reactions) 1 kit (480 reactions)	03 515 869 001 03 515 885 001