Molecular and Cellular Probes 25 (2011) 60–64

Contents lists available at ScienceDirect

Molecular and Cellular Probes

journal homepage: www.elsevier.com/locate/ymcpr

Effect of template on generating a standard curve for absolute quantification of an RNA virus by real-time reverse transcriptase-polymerase chain reaction

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ARTICLE INFO

Article history:
Received 26 October 2010
Accepted 23 December 2010
Available online 28 December 2010

Keywords:
Real-time PCR
Absolute quantification
IPNV

ABSTRACT

The effect of different templates on generating standard curves that are needed for the absolute quantification of an RNA virus by real-time reverse transcriptase-PCR (RT-PCR) was evaluated. We used infectious pancreatic necrosis virus (IPNV), a major viral pathogen of wild and cultured salmon, as a RNA virus example for the study. A dilution series of four different templates representing the IPNV protease gene (two *in vitro* transcribed RNAs of 100 bases and 500 bases in length, a plasmid DNA and a DNA oligo) were used as template to produce standard curves to quantify IPNV load in rainbow trout. The slope, the goodness of fit (r^2), and the efficiency (e) of PCR were statistically equivalent irrespective of the nature of template used in the PCR. Using a factorial ANOVA, no significant difference in IPNV copy number was observed using the four different standard curves for absolute quantification of IPNV in experimentally-challenged rainbow trout. However, when IPNV transcript abundance was less than 100 copies per reaction and when the template size was bigger than the amplicon size amplification was more variable. The data suggests that the size of the template used to generate standard curve should be very similar to the size of the amplicon. A synthetic DNA oligo template would be optimal for this purpose as it can be custom made and only requires the sequence information for its synthesis. However, if the standard curve is generated with template copy number in excess of 100 copies per reaction, the nature of the template has no effect on the standard curve, and therefore, the cheaper template would be the preferred choice of template over the other more expensive options.

1. Introduction

Detection and quantification of RNA viruses by real-time reverse transcriptase-polymerase chain reaction (RT-PCR) is a highly sensitive, high throughput and widely used method [3,6,7]. In real-time RT-PCR, the viral transcripts are quantified using either relative or absolute quantification methods. In the relative quantification method, the transcript level is routinely measured with respect to an internal control gene that is presumed to be constitutively expressed in the cell, using a comparative threshold cycle (C_T) method [6,9]. In absolute quantification, the viral load is measured as copy number per cell or copy number per total RNA following a transformation of the data using a standard curve (Chen et al., 2005). A standard curve is generated by performing an RT-PCR using a dilution series of template DNA made from either *in vitro* transcribed RNA, plasmid DNA or DNA oligo. The current literature suggests that there are advantages and disadvantages to using different types of templates for the construction of a standard curve to be used for absolute transcript quantification. The advantage of using *in vitro* transcribed RNA as template is that it involves cDNA synthesis and therefore takes into account the efficiency of the reverse transcription reaction. The disadvantage is that it involves *in vitro* RNA and cDNA synthesis steps, which are time consuming and expensive. An advantage of using plasmid DNA as template is that it is relatively cheap and fairly easy to generate, however, plasmid DNA does not account for the cDNA synthesis step, a requirement for the amplification of a viral RNA transcript. The advantage of using DNA oligo is that it can be custom synthesized, and only requires the sequence information. However, DNA oligo of 75-to 100 bases that represents an average size of an amplicon is relatively expensive to synthesize and, like the plasmid DNA, it does not involve any cDNA synthesis.

In this study, we compared the strengths of four separate templates commonly used to generate standard curves in real-time

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doi:10.1016/j.mcp.2010.12.002

RT-PCR studies for their abilities to accurately quantify viral transcripts. For this study, our model was infectious pancreatic necrosis virus (IPNV), a major viral pathogen infecting rainbow trout, salmon and other salmonids [8]. IPNV is a bi-segmented double-stranded RNA containing virus belonging to the family *Birnaviridae*, genus *Aquabirnavirus* [8]. Recently, we developed a real-time RT-PCR method using SYBR Green chemistry to detect and quantify IPNV load in rainbow trout following invasive and non-invasive tissue sampling [1]. In the present study, to accurately measure the IPNV transcript level in rainbow trout, standard curves were generated using four different templates representing an IPNV protease gene: *in-vitro* transcribed RNA of 2 different sizes, 100 and 500 bases, a ~4.0 kb plasmid DNA containing a 84 bp amplicon representing the IPNV protease gene, and an 84 bases DNA oligo. After performing the real-time RT-PCR, the IPNV load in rainbow trout was calculated by extrapolating the C_T values to each of the four standard curves. Our data showed that PCR efficiencies were equivalent, irrespective of template type, and thus no significant difference was observed in IPNV copy number calculations when using the four different templates for the quantification of IPNV viral load in experimentally-challenged fish.

2. Materials and methods

2.1. Virus challenge, RNA isolation and cDNA synthesis

The experimental challenge of rainbow trout (*Onorhynchus mykiss*, mean weight, ~3 g) with IPNV *via* the waterborne route has been described earlier [1]. Briefly, a group of 36 fish were infected with IPNV in a volume of water that was 10× the total weight of the fish in grams for 1 h with aeration (~10^5 $TCID_{50}$/mL of IPNV). Six fish were collected at each of days 1, 3, 7, 14, 21, and 28 post-challenge. In addition, six fish were sacrificed prior to the IPNV immersion challenge, which served as healthy control fish. Spleen tissues were collected from each fish and stored in TRI reagent (Molecular Research Center, MRC, Cincinnati, Ohio) at −75 °C until RNA isolation was performed.

Total RNA was isolated from spleen tissue samples using TRI reagent and following the manufacturer's protocol (MRC, Cincinnati, Ohio). The purified RNA samples were treated with DNase I (Ambion, Inc., Austin, TX) before assessing the RNA quality by running the samples on a 1% formaldehyde agarose gel. The RNA samples were quantified using a BIO-RAD SmartSpec™ 3000 spectrophotometer (Hercules, CA). The cDNA synthesis was carried out in a 40 μL reaction volume containing 1 μg DNase I treated total RNA, 1X RT-PCR buffer, 1 mM dNTPs, 0.75 μM oligo dT, 4 U of RNase inhibitor, and 5 U of MultiScribe reverse transcriptase (Applied Biosystems, Foster City, CA) at 42 °C for 1 h. The cDNA was diluted to 1:10 using DNase and RNase-free molecular biology grade water and 2 μL of the diluted cDNA was taken for each reaction in the real-time RT-PCR assay.

2.2. SYBR® Green real-time RT-PCR assay

The primers and the optimized reaction conditions for SYBR® Green real-time RT-PCR were the same as described earlier [1]. IPNV primers were designed based on the non-structural protease (NS) gene present in Segment A of the IPNV genome (GenBank accession number NC_001915), and the primers for the internal control genes, rainbow trout β-actin and elongation factor 1-α (EF-1-α), were based on the published sequence (GenBank accession numbers AF157514 and AF498320, respectively; Table 1). The real-time RT-PCR amplifications were performed in a Bio-Rad iCycler iQ™ (Bio-Rad Laboratories, Inc., Richmond, CA). The SYBR® Green real-time RT-PCRs were carried out in a 25 μL reaction volume containing 12.5 μL of 2X SYBR® Green Supermix (iQ SYBR® Green Supermix), 300 nM each of forward and reverse primers, and 2 μL of the 1:10 diluted cDNA. The amplifications were carried out in a 96 well plate and each sample had 3 replicates. The thermal profile used for the SYBR® Green real-time RT-PCR was 95 °C for 10 min, followed by 40 cycles of 95 °C for 10 s, and 60 °C for 1 min. After the run, the melting curve of each amplicon was examined to determine the specificity of the amplification.

For each of the four different templates used in real-time PCR, five independent standard curves were run and the mean cycle threshold (C_T) value at each 10-fold dilution of five independent run was taken for the final standard curve. The slope and y-intercept were obtained using linear regression.

2.3. Plasmid standard curve

The real-time RT-PCR amplicon (84 bp) from spleen tissue of an IPNV infected fish was run in a 2% agarose gel, and gel-purified using QIAQuick gel-extraction kit (Qiagen, Valencia, CA). The gel-purified amplicon was re-amplified by PCR using the primers 1916F and a nested 1999R-T_n (5′ TTTTTTTTTTTTTTTTCCAG CG3′). The PCR amplified product was run in a 2% agarose gel, gel-purified using the QIAQuick gel-extraction kit (Qiagen, Valencia, CA), cloned into pCR2.1-TOPO vector (3931 bp size plasmid, Invitrogen, Carlsbad, CA), and sequence verified. Thus the size of the recombinant plasmid DNA containing a $(T)_{16}$ tailed 84 bp amplicon was 4031 bp. The standard curve was generated using a dilution series of 2.3×10^5 to approximately 2 copies (10 pg to 0.01 fg) of plasmid DNA and SYBR® Green real-time PCR.

2.4. In-vitro transcribed RNA standard curves

In order to generating a 100 bp *in vitro* transcribed RNA, the plasmid DNA of a clone representing the real-time RT-PCR amplicon was digested with the restriction enzyme *Bam*H1, run in a 1% agarose gel, and the digested plasmid was gel-purified. The gel-purified, plasmid DNA was used as template for *in vitro*

Table 1
The nucleotide sequence of the primers used for the amplification of IPNV, and the rainbow trout internal control genes, β-actin and, EF-1α.

Gene	Primer name	Primer sequence (5′-3′)	%GC	T_m[a]	Amplicon size (bp)
IPNV	1916F	AGGAGATGACATGTGCTACACCG	52	60	84
	1999R	CCAGCGAATATTTTCTCCACCA	45	60	
	1720F	TTCCAAAGCCAACAAAGGGAGTC	48	65	513
	2217R	TTTTTTTTTTTTTTTTATGTGTTTGCCACCATCTCG	28	72	
β-actin	1301F	CCCAAACCCAGCTTCTCAGTCT	55	64	113
	1413R	TGCTTCACCGTTCCAGTTGTG	52	64	
EF-1α	136F	TGATCTACAAGTGCGGAGGCA	52	64	101
	236R	CAGCACCCAGGCATACTTGAA	52	63	

[a] Melting temperature at 50 mM Na^+.