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Research paper

Amplification of nonspecific products in quantitative polymerase chain reactions (qPCR)

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ABSTRACT

Quantitative PCR allows the precise measurement of DNA concentrations and is generally considered to be straightforward and trouble free. However, a survey with 93 validated assays for genes in the Wnt-pathway showed that the amplification of nonspecific products occurs frequently and is unrelated to C_q or PCR efficiency values. Titration experiments showed that the occurrence of low and high melting temperature artifacts was shown to be determined by annealing temperature, primer concentration and cDNA input. To explore the range of input variations that occur in the normal use of the Cre assay these conditions were mimicked in a complete two-way design of template −plasmid DNA- and non-template −mouse cDNA- concentrations. These experiments showed that the frequency of the amplification of the correct product and the artifact, as well as the valid quantification of the correct product, depended on the concentration of the non-template cDNA. This finding questions the interpretation of dilution series in which template as well as non-template concentrations are simultaneously decreasing. Repetition of this cDNA concentration experiment with other templates revealed that exact reproduction qPCR experiments was affected by the time it takes to complete the pipetting of a qPCR plate. Long bench times were observed to lead to significantly more artifacts. However, the measurement of artifact-associated fluorescence can be avoided by inclusion of a small heating step after the elongation phase in the amplification protocol. Taken together, this trouble-shooting journey showed that reliability and reproducibility of qPCR experiments not only depends on standardization and reporting of the biochemistry and technical aspects but also on hitherto neglected factors as sample dilution and waiting times in the laboratory work flow.

1. Introduction

Quantitative PCR, qPCR or RT-qPCR, allows the sensitive, rapid and precise measurement of DNA or RNA targets. The method is used for e.g diagnostic purposes, gene expression studies for a restricted panel of genes in fundamental and translational research and validation of transcriptome analyses. In general, qPCR is considered to be straightforward and trouble free. As a consequence, most papers do not report the optimization and validation carried out to determine the specificity and sensitivity of an assay [1] although the MIQE guidelines specifically ask for such proof [2]. Optimization of qPCR is performed to avoid off-target products and artifacts. This optimization is commonly carried out with negative and positive control samples; the loss of specific product in a dilution series of the positive sample is then considered to indicate the lower detection limit of the assay [3]. For reactions with high C_q values, it is generally recommended to check the identity of the amplified product with melting curve, size and/or sequence analysis. Unnoticed amplification of a nonspecific product (artifacts), results in false positive results and underlines the emphasis of the MIQE guidelines on assay specificity.

Amplification artifacts can be shorter or longer than the intended amplicon. Short artifacts are most often primer dimers, which are mainly due to homology between the primers sequences [4]. Long artifacts comprise off-target products which contain additional sequences that do not or only partially overlap with the targeted sequence [5]. Although some papers describe how to prevent amplification of non-specific products [6–8], hardly any paper deals with the conditions leading to these off-target products.

The replacement of a 1-step RT-qPCR protocol by a 2-step protocol (cDNA synthesis followed by separate PCR reactions) reduces the occurrence of primer-dimers in the no-template control [9]. The 2-step protocol allows a better control of the temperature during the phase of

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the cycling protocol in which primers anneal to the available DNA strands and each other [10]. Hot-start PCR prevents primer extension due to interactions between reaction components at low temperature prior to the first denaturation step of the cycling protocol [6,7,11] and is therefore considered to reduce artifact formation. When these extended primers share homology with a sequence elsewhere in the genome, a phenomenon dubbed "jumping" can occur [12]. In such a within-genome recombination a completely new product is amplified [12,13]. The frequency of jumping is suggested to depend on the ratio of template to non-template DNA rather than on the absolute concentration of the template [14].

In this study we describe a series of trouble-shooting experiments that was done to get to grip on the conditions in which PCR artifacts occur in reactions with validated assays on the sample type for which they were designed. These analyses showed that the balance between primer, template and non-template concentrations determines whether the correct product and/or an artifact will be amplified. Qualitatively, low template concentrations increase artifact frequency whereas, quantitatively, low non-template concentrations lead to deviating C_q values and thus to incorrect quantification. Moreover, the occurrence of artifacts was shown to be highly dependent on the time it took to complete the pipetting of the qPCR plate despite implementation of a hot-start procedure. Measurement of the fluorescence at a temperature above the Tm of the primer-dimers was shown to give the expected results despite the formation of artifacts. These observations show that low-input gene expression experiments of a complicated design have to be carefully optimized, standardized, performed and evaluated, especially when 'not expressed' or 'not present' are among the possible outcomes.

2. Material and methods

2.1. Primer design

The PCR primers for genes in the Wnt pathway were designed using Primer-Blast (NCBI). The following criteria were taken into account when designing the primer pairs: (1) each primer should be 19–22 bp in length, (2) the annealing T_m should be 60 ± 1 °C, (3) the difference between the T_m of the two primers in a pair should be ≤ 1 °C, (4) the primers should have limited similarity with other sequences in the genome and especially not in the last 4 bases at the 3′ end of the primer [15] and (5) the amplicon should be between 70 and 150 bp in length. In addition, we aimed at primers that are exon–exon spanning, or that the amplicon is spanning an intron of at least 500 bp. However, this latter criterion could not be met for several targets, because many genes of the Wnt signaling pathway are without intron. Next the primers were analyzed using Oligoanalyzer v3.1 (IDT) with as analysis parameters: oligo-concentration: 1 μM, 50 mM Na^+, 2 mM Mg^+ and 0.25 mM dNTP. We aimed at primer pairs for which the homo-dimer and hetero-dimer strength is ΔG≤ −9 kcal/mol. Moreover, we checked that there are no extendable 3'ends in these home and hetero-dimers. Finally, we aimed at a T_m ≤ 55 °C of the primer-dimers.

The target specificity of the designed primer pairs was tested in qPCR reactions with cDNA of complete chicken embryos as positive control tissue. As negative controls we used minus-RT and no-tissue control (NTC) reactions. A reaction with genomic DNA served to determine cDNA specificity. After the PCR reaction the amplification curve was inspected with respect to background and plateau level (ratio ≥ 10), as well as the PCR efficiency (> 1.7; Fig. 1C). After the qPCR run, we performed melting curve analysis, gel electrophoresis and sequencing to verify that the expected target was amplified. If this verification failed an alternative primer pair was designed and tested. The primer pairs that were used in the reported analysis are shown in Supplemental Table 1.

MS2 primers were obtained both from literature [16] or designed (Supplemental Table 2) using Allele ID (PREMIER Biosoft). Primer pairs were evaluated *in silico* and using dilution series, gradient PCRs and checkerboard titrations.

The primers used to amplify the 341 bp Cre amplicon were; forward primer: GGTTCGCAAGAACCTGATGGACAT and reverse primer: CTAGAGCCTGTTTTGCACGTTCA [17].

2.2. Experiments with chicken embryos

Fertilized chicken eggs were obtained from a local hatchery (Drost BV, Nieuw Loosdrecht, the Netherlands) and incubated for three days at 38 °C. The embryonic tissue around the developing heart was divided into five regions. Samples were collected in TriReagent® (Life Technologies) and total RNA was immediately isolated; RNA purity and quantity were determined using the Nanodrop. Per RT-reaction 300–1000 ng of total RNA was converted into cDNA using anchored oligo-dT primers (T_{14}VV) and Superscript II reverse transcriptase (Life Technologies). Each 10 μL qPCR reaction contained an amount of cDNA equivalent to 5 ng of total RNA (5 RNA equivalents), *LightCycler® 480 SYBR Green I Master mix* (Roche), and 1 μM of the forward and reverse primer. The qPCR was run in a *LightCycler® 480 Multiwell Plate 384* cycler (Roche): 5 min 95 °C followed by 45 cycles 10 s 95 °C, 20 s 60 °C and 20 s 72 °C, and a melting curve analysis to verify the amplification product. Negative controls were (i) a no-tissue control (NTC; water) and (ii) a RT-reaction without enzyme. As positive control 5 RNA equivalents of cDNA prepared from entire chicken embryos was used. The expression level of 93 genes related to Wnt signaling and two reference genes was measured in triplicate per tissue.

2.3. Experiment with MS2 virus

The enterobacteriophage MS2 (DSM No 13767; strain PC-V3463; Genbank NC_001417) and *Escherichia coli* host cells (DSMZ 5695) were purchased (Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures, Braunschweig). A stock of MS2 was prepared of 4.8×10^{10} PFU per mL. The MS2 genome was isolated using the NucliSENS miniMAG kit (bioMérieux; Benelux, Zaltbommel, Netherlands) and after treatment with DNase converted into cDNA using the QuantiTect Reverse Transcription Kit (Qiagen). The cDNA was used undiluted (approx. 2×10^6 PFU) or diluted in 10-fold steps down to 10^{-10} and analyzed in 20 μL reactions using iQ™ Supermix (Biorad) or SsoFast™ EvaGreen® Supermix (Biorad) and primers at various concentrations in checkerboard designs. The qPCR was run in a BioRad CFX96™touch Real-time PCR detection system: 10 min 95 °C followed by 50 cycles of 15 s 95 °C and 1 min 60 °C, and a melting curve analysis to verify the amplification of the correct product. Each PCR variable was tested in one or more runs to reach at least triplicate measurements

2.4. Template/non-template checkerboard experiments

These experiments were carried out to evaluate the effect of the relative concentration of template versus non-template DNA in the reactions. A plasmid (pDNA) containing the sequence of *Cre* recombinase enzyme [17] was used as template. cDNA from hearts of newborn mice served as non-template DNA. RNA and cDNA preparation and PCR reaction were as described in the above chicken experiment. The input of the reactions consisted of a complete checkerboard design with all combinations of template (10-times dilution series from 15.10^3 down to 1.5 and 0 copies of pDNA) and non-template (5-times dilution series from 10 down to 0.0032 and 0 ng RNA equivalents of cDNA) concentrations. Note that this two-way design encompasses the range of variations in cDNA and target concentrations that occurs in a (dilution) series of different tissues for which the Cre-assay was designed. The experiment was replicated in 2 PCR runs and carried out with 2 primer concentrations (1 μM and 0.1 μM) with four technical replicate reactions per combination of inputs.