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Note

A novel and rapid method for synthesizing positive controls and standards for quantitative PCR

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Abstract

We developed and tested a method to produce DNA standards and controls for quantitative PCR by designing and performing partial hybridization of long oligonucleotides before double stranded DNA fragments were synthesized and subsequently amplified by conventional PCR. This approach does not require any natural DNA template. Applications include the production of standards, which cannot be easily produced from DNA extracted from bacteria or plants.

Keywords: Quantitative PCR; DNA shuffling; Real-time PCR; Positive controls; Standards; *recA*; *Dehalococcoides*

PCR and quantitative (real-time) PCR (qPCR) have become increasingly useful methods in microbiology for detecting and quantifying target nucleic acids in both environmental (Cupples, in press) and clinical samples (Smith et al., 2006). However, any PCR amplification requires the use of appropriate positive controls to verify the efficiency of different parameters (temperature, primer efficiency, specificity of the reaction), and for the generation of standard curves in qPCR. The incorporation of suitable standards is compromised when organisms containing the target gene are difficult to culture (because of slow growth or unknown growth conditions). Producing positive controls is even sometimes impossible when targeted sequences come from metagenomic databases containing non cultivable and/or unknown organisms. Other sequences, like transgenes associated with genetically modified organisms, are known but not readily available (Hernandez et al., 2003) for DNA extraction. In addition, the detection and quantification of target genes from pathogenic strains often requires a biosafety level two or biosafety level three laboratory if only for the growth of the control culture.

Our objective was to develop a method in which these DNA sequence standards could be produced artificially without natural DNA template. This was done by using the sequence of the targeted gene to design three long (95 bp) oligonucleotide primers that overlap one another at complementary regions that are at least 25 bp in length and encompass the 200 bp qPCR target region as shown in Fig. 1. In addition, these primers were designed with the use of Primer Select (DNASTAR, Inc., Madison, WI) such that they should not form homo- and heterodimers when the change in Gibbs free energy (ΔG) is less than -10 kcal/mole (ΔG calculated for a salt concentration of 50 nM at 25 °C). These values correspond to the default parameters in the software. Each of the primer pairs (or sets) has similar melting temperatures, and an additional assay indicated that they would not form dimers at a difference of more than 10 °C. No design constraints were applied for primer hairpins.

Construction of the synthetic positive controls included an initial PCR-like cycle in the presence of each of the three long oligonucleotide primers but without the addition of any other DNA template (Fig. 1). Hybridization of the primer complementary regions led the polymerase to synthesize double strands of DNA complementary to the whole targeted DNA region. In a

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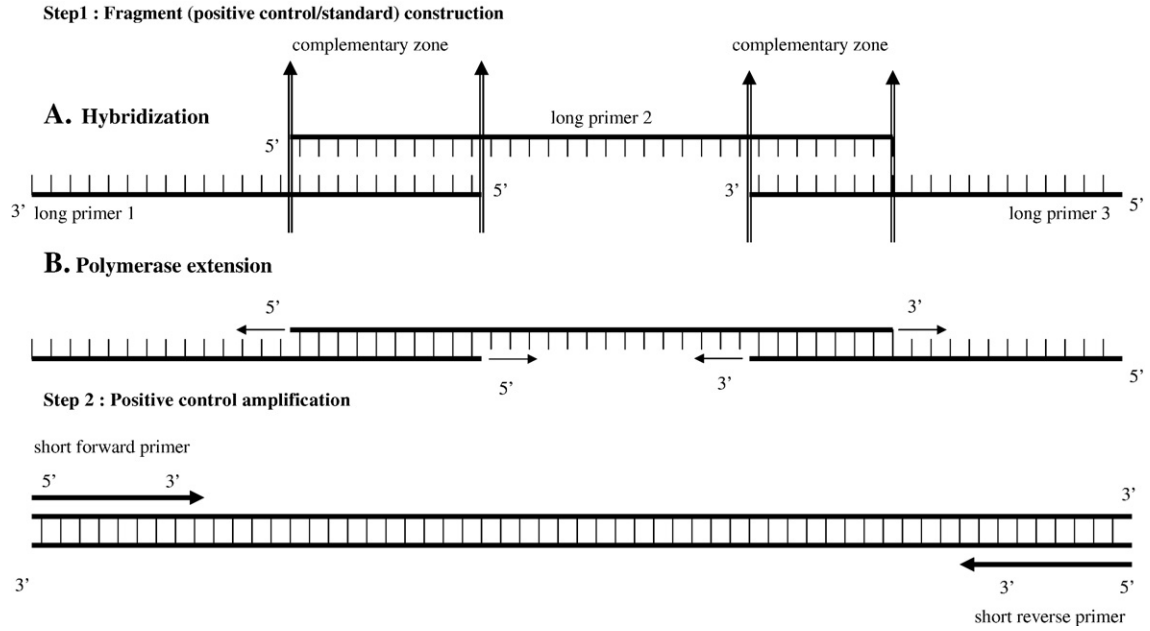

Fig. 1. Experimental steps in the synthesis of positive controls/standards. Step 1A: The three long oligonucleotides hybridize on each other's complementary sequence, which was bioinformatically designed. Step 1B: After hybridization, the polymerization without supplementary primers begins. Step 2: The last step of the positive control synthesis consists of classic PCR cycles with the supplementary short primers.

second step, a classical conventional PCR reaction was performed to amplify the synthetic sequence using the first reaction products as DNA template (Fig. 1).

The initial PCR-like cycle was carried out in the presence of 50 ng of each of the three long oligonucleotide primers, 1X Titanium Taq PCR Buffer, 0.2 mM deoxynucleoside triphosphates, 1 μL Titanium Taq DNA polymerase (Clontech-Takara Bio Europe, Saint-Germain-en-Laye, France) and 1 μL of T4 gene 32 protein (Roche Diagnostics S.A.S., Meylan, France). The PCR amplification was performed as a single-tube reaction in a Thermal Cycler (Biometra T1 Labgene scientific instrument, Archamps, France) programmed as follows: 6 min at 96 °C, followed by 35 cycles of 95 °C for 1 min; 53 °C for 30 s; and 68 °C for 40 s, with a final extension at 68 °C for 6 min. The second reaction, a classical conventional PCR reaction, was performed to amplify the synthetic sequence using 50 ng (quantified with the Qubit system (Invitrogen, Cergy Pontoise, France)) of the first reaction product as DNA template. This second PCR reaction was carried out in the presence of 1X Titanium Taq PCR Buffer, 0.2 mM deoxynucleoside triphosphates, 1 μL Titanium Taq DNA polymerase (Clontech-Takara Bio Europe, Saint-Germain-en-Laye, France), 1 μL of T4 gene 32 protein (per 50 μL reaction) (Roche Diagnostics S.A.S., Meylan, France), and 0.5 μM of forward and reverse primers. These primers are described as "short" forward and reverse primers in Table 1 and Fig. 1.

PCR products resulting from the second reaction were then cloned for sequencing using a TOPO TA cloning kit with a TOPO 4 vector and TOP10 chemically competent cells (Invitrogen, Cergy Pontoise, France) following the manufacturer's instructions. The resulting clones were sequenced by Cogenics (Meylan, France) to validate the control construction. After sequence verification, the clones containing the positive controls were extracted using a Nucleospin Extract II kit (Macharey Nagel, Hoerdt, France) following the manufacturer's instructions and the resulting DNA was tested as template in qPCR protocols.

To confirm our approach, two different PCR methods were used to test for the presence and effectiveness of the positive controls/standards that we synthesized for four different genetic elements. First, we used reverse transcription quantitative PCR (RT-qPCR) to test the positive controls/standards that we synthesized for the bacterial *recA* gene of *Dehalococcoides strain 195*, the *tceA* gene of *Dehalococcoides ethenogenes* sp. and the *Pseudomonas putida* toluene dioxygenase gene: *todC*. In addition, we used a classic qPCR assay to test the positive control/standard that we synthesized for the junction between the chloroplast transit peptide (*CTP*) and 5-enolpyruvylshikimate-3-phosphate synthase (*EPSPS*), which confers herbicide resistance in Roundup Ready (RR) corn (Monsanto, St. Louis, MO).

For the *recA*, *tceA* and *todC* positive controls/standards, the RT-qPCR reaction mixture yielded a final solution comprised of 1X Quantitech SYBR Green PCR mix (10 μL) (Quantitech SYBR Green RT-PCR kit, Qiagen, Courtaboeuf, France), 0.5 μM of each primer, 0.2 μL of Quantitech Reverse Transcriptase and 2.5 mM $MgCl_2$ in a final volume of 20 μL. The RT-qPCR conditions for each gene were as follows: 50 °C for 20 min for reverse transcription; 95 °C for 15 min for deactivation of