Enhanced PCR amplification incorporating 5’ primer flap sequences and free energy values near equilibrium

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INTRODUCTION

• The objective of this study is to enhance the sensitivity of PCR-based detection by combining primer design, optimal thermodynamics and 5’ AT-rich non-complementary sequences.
• Primers with AT-rich non-complementary sequences, i.e. 5'-AATAATCATAA, located at the 5’ terminal position were reported to increase PCR amplification yield (1).
• Primers with ∆G value zero circa perform well in broad gradients of melting temperature (Tm) (2).
• Primer quality can be qualitatively assessed using reference ∆G values and bioinformatics tools such as mFOLD and Primer3 during primer design (3).
• The polymerase chain reaction (PCR) is a cornerstone technique in molecular biology, with extended use and application in microbial forensics and agricultural biosecurity.

MATERIALS AND METHODS

1. Optimal and suboptimal primers were designed based on the sequence of the movement protein gene of Tobacco mosaic virus (TMV) and the rDNA subunit of Pythium cryptorregulare (Pc), using the Web interface software pathway Primer3-miFOLD-BLASTn (Fig. 1), (3).
2. Two pairs are optimal, ’TMV-2’ (∆G = 0; product size 195 bp) & Pc-1 (∆G = 0; product size 434 bp), and two other pairs are suboptimal ’TMV-3’ (∆G = -1; product size 192 bp) & ’Pc-2’ (∆G = -1; product size 454 bp).

3. The effect of combining free energy values (∆G) selected during primer design (Fig. 2) with and without the addition of 5’ AT-rich non-complementary nucleotides was measured by one step RT-PCR (for TMV: Fig. 3) and end point PCR (for Pc: not shown) of serially diluted RNA/DNA, and by quantifying the DNA yield using a Nanodrop® (C.D.300 nm) after elution of excised bands. The effect on DNA yields was also quantified by SYBR® Green real time RT-PCR assays.

RESULTS

Primers having optimal ∆G and 5’ AT-rich non-complementary nucleotides widened the range of effective annealing temperatures of PCR assays (Fig. 4), improving also sensitivity (Fig. 5), and PCR product yields (Tables 1 & Fig. 6).

M. Arif

Fig. 4. TMV-gradient one step RT-PCR amplification. Tm’s ranged from 40°C to 65°C (A & B) and 48°C to 67°C (C & D). TMV-2 optimal primer set, (A) without and (B) with 5’ AT-rich non-complementary nucleotides sequences. TMV-3 suboptimal primer set, (C) without and (D) with 5’ AT-rich non-complementary nucleotides sequences.

Fig. 5. Sensitivity assay of one step RT-PCR. TMV2 optimal primer set, (A) without and (B) with 5’ AT-rich non-complementary nucleotides sequences. TMV-3 suboptimal primer set, (C) without and (D) with 5’ AT-rich non-complementary nucleotides sequences. Lane M: 100 bp ladder; Lane 1-6: Tm serial dilutions of isolated TMV RNA starting from 500 ng/reaction.

Fig. 6. Real Time PCR amplification of optimal TMV-2 and suboptimal TMV-3 with and without 5’ AT-rich flaps.

CONCLUSIONS

1. Designing primers having ∆G = 0 and 5’ AT-rich non-complementary nucleotides increased DNA yields and the sensitivity of one step RT-PCR and PCR detection.
2. The addition of 5’ AT-rich non-complementary nucleotides to suboptimal primer assays has the potential to improve DNA yields.

LITERATURE CITED


LAB PROTOCOLS

1. PCR-RT amplification was carried out in 20 µl containing 10 µl 2X reaction mix, 0.5 µl RNaseOut, 2 µl of each forward and reverse primer (10X), 0.5 µl BSA (10µg/uI). 2 µl RNA template and nuclease free water 1.6 µl. Real time RT-PCR reactions were also in 20 µl volume containing 10 µl RT SYBR Green qPCR master mix, 6.4 µl nuclease free water, 0.8 µl of each forward and reverse primer (5 µm) and 2 µl of cDNA.
2. PCR cycling conditions were 95°C, 30 min. (cDNA synthesis), initial denaturation 94°C, 2 min. for 40 cycles at 94°C, 30 sec., annealing, 61°C (TMV-2) 30 sec. & 54°C (TMV-3), PCR reaction, 30 sec. at 72°C and 7 min. final extension. For real time PCR, 95°C, 10 min. initial denaturation 94°C, 2 min. for 40 cycles at 94°C, 30 sec., annealing, 54°C. Polymerization, 30 sec. at 72°C and 7 min. final extension.