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Comparative Assessment of 5′ A/T-Rich Overhang Sequences with Optimal and Sub-optimal Primers to Increase PCR Yields and Sensitivity

M. Arif · F. M. Ochoa-Corona

Abstract Efficient PCR amplifications require precisely designed and optimized oligonucleotide primers, components, and cycling conditions. Despite recent software development and reaction improvement, primer design can still be enhanced. The aims of this research are to understand (1) the effect on PCR efficiency and DNA yields of primer thermodynamics parameters, and (2) the incorporation of 5′ A/T-rich overhanging sequences (flaps) during primer design. Two primer sets, one optimal ($\Delta G = 0$) and one sub-optimal ($\Delta G = 0.9$), were designed using web interface software Primer3, BLASTn, and mFold to target a movement protein gene of *Tobacco mosaic virus*. The optimal primer set amplifies a product of 195 bp and supports higher PCR sensitivity and yields compared to the sub-optimal primer set, which amplifies a product of 192 bp. Greater fluorescence was obtained using optimal primers compared to that with sub-optimal primers. Primers designed with sub-optimal thermodynamics can be substantially improved by adding 5′ flaps. Results indicate that even if the performance of some primers can be improved substantially by 5′ flap addition, not all primers will be similarly improved. Optimal 5′ flap sequences are dependent on the primer sequences, and alter the primer's T_m value. The manipulation of this feature may enhance primer's efficiency to increase the PCR sensitivity and DNA yield.

Introduction

PCR became a central technique in molecular biology, microbiology, diagnostics, and forensics after its development during the 1980s [1, 2]. For the first time, PCR allowed the amplification of a targeted DNA sequence from a single, or a few, copies into thousands to millions of copies, streamlining the manipulation of sequences and understanding of molecular biology processes, but also speeding the enhancement of powerful detection methods to new unseen sensitivity levels [3–5]. The selection of primer sequences flanking the target sequence is relevant to the success of PCR. However, despite the progress made on software development for primer design in recent years, improvements in sensitivity, specificity, and yield are still needed for applications in microbial forensics, detection, and diagnostics procedures in general [6, 7].

The potential for the formation of two dimensional RNA secondary structures was recognized by Doty et al. [8], Fresco et al. [9], and Manthey [10], who discussed the role of ΔG in this phenomenon. mFold software was developed to predict DNA and RNA folding structures [11], it was applied later in the prediction of primer secondary structure at defined annealing temperatures. The core algorithm of mFold predicts ΔG, minimum free energy, and minimum free energy for the folding of any particular base pair [11]. No relationship was observed between ΔG and T_m values [10].

The 5′ A/T-rich flaps are short nucleotide sequences non-complementary to the target, positioned at the 5′ terminus of a primer as an overhanging or protruding extension of

M. Arif · F. M. Ochoa-Corona (✉)
National Institute for Microbial Forensics & Food and Agricultural Biosecurity, Department of Entomology and Plant Pathology, Oklahoma State University, 127 Noble Research Center, Stillwater, OK 74078, USA
e-mail: ochoaco@okstate.edu

M. Arif
e-mail: m.arif@okstate.edu

nucleotides. Use of primers having 5′ flaps increased qPCR fluorescent signals. This is useful particularly when working with sequences difficult to amplify, such as bisulfate-treated DNA or highly variable viral sequences [12, 13]. Furthermore, the incorporation of 5′ flaps enhanced the quality of sequencing [14]. Initial studies incorporated overhanging sequences in the 5′ termini of primer sequences. For example, when bacteriophage promoter sequences SP6 and T7 were added to the 5′ end of Potyvirus universal primers to facilitate sequencing [15], cloning, greater amplification, and specificity compared with standard primers lacking 5′ flaps. Thereafter, 5′ A/T-rich sequences were added to primers to improve primer sequences by adding restriction and universal detection sites [13]. The addition of an A/T-rich sequence at the 5′ termini of primers led to an increase in PCR yields. Afonina et al. [13] found that the optimal size for a 5′ flap was 12 bp, and adding longer or shorter 5′ flaps had no significant impact on qPCR fluorescence levels. There is no consensus on whether it is better to add 5′ flaps on both of the primers or on only one. Wei and Clover [12] reported optimum target amplification efficiency, using nepovirus primers targeting subgroups A and B, using a forward primer with a 5′ flap, and a 5′ flap-less reverse primer, but Afonina et al. [13] used flaps on both reverse and forward primers. Moreover, 5′ G/C-rich flaps were less useful than the A/T-rich flaps [13]. 5′ A/T-rich flaps have been used primarily by those developing new detection/diagnostics methods and by PCR users requiring sensitive detection levels. The terms 5′ non-complementary A/T-rich sequences, 5′ A/T-rich extensions, 5′ overhangs, 5′ flaps, and flaps have been used by others in describing this technology. The term 5′ flaps is used for the purpose of this article.

The use of 5′ flaps for improving PCR yields and sensitivity raises the question of whether oligonucleotide primer design can be bypassed using any priming sequences to which universal 5′ flaps are added. The hypothesis is that any target sequence of interest can be transformed into primers by adding 5′ flaps, regardless of the primer design parameters selected. The aim of this research is to compare the use of 5′ flaps with the application of optimal thermodynamics parameters during primer design. The efficiency of these two approaches as components of sensitive PCR-based detection methods widely used in molecular biology, detection and diagnostics, microbial forensics, forensic entomology, agriculture, and biosecurity, are assessed.

Materials and Methods

Primers Design and Analysis

Two primer sets were designed to amplify a conserved region within the movement protein gene of *Tobacco mosaic virus*

(TMV). TMV movement protein sequences were retrieved from the National Center for Biotechnology Information (NCBI, Bethesda, MD). All primers were designed using Primer3 [16], BLASTn [17], and mFold [11]. The minimum, optimal, and maximum values of selected Primer3 parameters used for selecting optimal primers were "primer T_m" 57, 60, and 60 °C, respectively, "primer GC%" 45, 50, and 55, respectively, the lowest possible value for "maximun self complementarity" and zero for "maximum 3′ self complementarity". The "maximun poly-X" was kept at 3. Other parameters were kept as default. For selecting the sub-optimal primers, the values for "maximun self complementarity" and "maximum 3′ self complemetarity" were raised stepwise until sub-optimal primers were obtained. Of the two pairs of primers, one was optimal ($\Delta G = 0$) and the second was sub-optimal ($\Delta G = 0.9$) at the selected T_ms of 61 and 54 °C, respectively.

The optimal (TMV-2F/2R) and sub-optimal (TMV-3F/3R) sets amplify two products of almost identical size 195 and 192 bp, minimizing differences in cumulative fluorescence, band intensity, and PCR yield. Primers were synthesized by Integrated DNA Technologies, Inc. (IDT, Inc., Coralville, IA) and Sigma-Aldrich Corp. (The Woodlands, TX). To compare the sensitivity and PCR yields using primers with and without 5′ flaps, each primer was synthesized with and without a 12 nucleotide reference 5′ A/T-rich flap (5′-AATAAATCATAA-3′) [13]. Primer sequences and thermodynamic values are listed in Table 1. Table 2 shown thermodynamic features of other newly designed 5′ flap sequences from this study.

Infected Plant Material and Nucleic Acid Isolation

Seedlings of *Nicotiana tabacum* (tobacco) were inoculated mechanically with TMV under controlled conditions [18]. Symptoms induced by TMV were evident 7 days after inoculation and symptomatic leaves were used for isolating total RNA using an RNeasy Plant Mini Kit (QIAGEN, Valencia, CA).

PCR Amplification

One-Step End-Point RT-PCR

Assays were carried out in 20 µl reactions containing 10 µl reaction mix buffer (Invitrogen, Carlsbad, CA), 0.5 µl RNAseOut (40 U/µl) (Invitrogen), 1 µl of each forward and reverse primer (5 µM), 0.4 µl SS III RT/Platinum *Taq* mix (Invitrogen), 0.5 µl BSA (Promega, Madison, WI), 2 µl of RNA template, and 4.6 µl nuclease free water (Ambion, Austin, TX). The cycling parameters were 30 min at 50 °C (cDNA synthesis), initial denaturation at 94 °C for 2 min followed by 40 cycles at 94 °C for 30 s,

Table 1 Thermodynamic parameters, secondary structures and product size of optimal and sub-optimal primer sequences with and without 5′ flaps

Primers code	Primer sequences (5′–3′)	T_m[a]	GC%	ΔG[b]	Optimal energy[c] (ΔG)	Number of secondary structures[d]	Product size (bp)
TMV 2F	GTGGACAAAAGGATGGAAAGA	58	43	0.0	1.1	1	195
TMV 2R	CAGAGAAAGCGGACAGAAACC	60	52	0.0	2.1	1	
TMV 2F_ref.flap	**AATAAATCATAA**GTGGACAAAAGGATGGAAAGA	66	30	0.9	1.0–1.9	6	195 + 12 +
TMV 2R_ref.flap	**AATAAATCATAA**CAGAGAAAGCGGACAGAAACC	67	36	1.0	1.1–2.1	3	12 = 219
TMV 3F	TCTAGTTGTTAAAGGAAAAG	46	30	0.9	1.8–2.7	18	192
TMV 3R	TATCCACTATCAATAAGCTTA	46	29	0.9	1.0–1.9	6	
TMV 3F_ref.flap	**AATAAATCATAA**TCTAGTTGTTAAAGGAAAAG	58	22	1.0	0.9–1.9	9	192 + 12 +
TMV 3R_ref.flap	**AATAAATCATAA**TATCCACTATCAATAAGCTTA	58	21	1.0	0.5–1.5	7	12 = 216

5′ flap sequence is bold [13]

[a] Oligo's melting temperature

[b] ΔG in plot file

[c] Optimal energy of secondary structures (kcal/mol) at 61 and 54 °C with optimal and sub-optimal primer sets, respectively

[d] Number of secondary structures calculated by mFold

61 °C (TMV-2F/2R) or 54 °C (TMV-3F/3R) for 15 s, 72 °C for 30 s, and 7 min final extension at 72 °C.

qPCR

SYBR Green qPCR assays were performed using plasmid TOPO-TA (Invitrogen) DNAs carrying each of the diagnostic target TMV regions as template for primers TMV-2F/2R (optimal) and TMV-3F/3R (sub-optimal). The plasmid DNA concentration was 1.0 ng/reaction. PCRs for both primer sets were carried out in 20 µl of total volume containing 10 µl of Platinum SYBR Green qPCR Super-Mix-UDG (Invitrogen), 0.8 µl of each forward and reverse primer (5 µM), 0.4 µl of BSA (10 mg/ml), 2 µl plasmid DNA (0.05 and 0.5 ng/µl), and 7.0 µl of nuclease free water. Each reaction was performed in three replicates. The cycling parameters were: two holds at 50 °C for 2 min and at 95 °C for 2 min followed by 40 cycles at 95 °C for 30 s, 61 °C (TMV-2F/2R) or 54 °C (TMV-3F/3R) for 30 s, 72 °C for 30 s. PCR assays were carried out in a Rotor-Gene 6000 (Corbett Research, Sydney, Australia).

RT-PCR Yield Quantification

One-step RT-PCR was performed in three replicates. Primer sets were TMV-2F/2R and TMV-3F/3R with and without a 5′ flaps (Table 1). Total RNA was used at 100 ng/reaction. Each amplicon was eluted from an agarose gel using Quantum Prep Freeze 'N Squeeze Spin Columns (Bio-Rad, Hercules, CA) and the product yield was determined twice using a NanoDrop spectrophotometer (Thermo Fisher Scientific Inc., Worcester, MA). The

variability and standard deviation among replicates were calculated.

Gradient RT-PCR, Sensitivity Assays, and Cloning

Gradient RT-PCRs

Optimal and sub-optimal primer sets, with and without a 5′ flap, were tested in a range of annealing temperature values from 48 to 66 °C. The concentration of template RNA for all gradient RT-PCRs was 100 ng/reaction. After amplification, 20 µl of PCR product from each reaction were visualized in a 1.5 % agarose gel electrophoresis in 1× Tris acetate EDTA (TAE) buffer.

Sensitivity Assays

The sensitivities of both optimal and sub-optimal primers, with and without 5′ flaps, were determined in ten-fold serial dilution assays using total RNA starting from 500 ng to 50 fg per reaction. The concentration of total RNA was determined by NanoDrop.

Cloning

The one-step RT-PCR products obtained using primers TMV-3F (sub-optimal) and TMV-2R (optimal) were purified from excised agarose band using Quantum Prep Freeze 'N Squeeze Spin Columns and cloned using a TOPO-TA Cloning Kit (Invitrogen). Plasmid DNA carrying the target sequences for both primer sets, TMV-3F/3R and TMV-2F/2R, was isolated using a PureLink™ Quick

Table 2 ΔG and optimal energy of secondary structures of optimal and sub-optimal primer sequences with and without 5′ flaps

Flap code	5′ Flap sequence (5′–3′)	With optimal primer 2F			With optimal primer 2R			With sub-optimal primer 3F			With sub-optimal primer 3R			Ct value with TMV-3F/R
		ΔG^a	ΔG^b	Sctr^c	ΔG^a	ΔG^b	Sctr^c	ΔG^a	ΔG^b	Sctr^c	ΔG^a	ΔG^b	Sctr^c	
WF1	AAAAATATTTT	0.0	−1.0	2	0.8	0 to 0.8	3	0.9	−0.8 to 0.1	3	0.7	−0.6 to 0.1	2	17.75
WF2	AAATTTTTTTT	0.7	−0.1 to −0.8	3	0.9	0.6 to 1.5	5	0.6	−0.2 to −0.8	3	0.7	0.3 to 1.0	3	16.57
WF3	AAAATTATTTT	0.0	−1.9	1	0.6	0.3 to 0.9	3	0.9	−0.8 to 0.1	3	0.8	−0.1 to 0.7	2	16.06
WF4	AAATATTTTTT	0.9	0.1 to −0.8	3	1.0	0.7 to 1.7	5	0.6	−0.2 to −0.8	2	1.0	0.6 to 1.6	8	16.14
WF5	AAAATTTTTTT	0.0	−2.0	1	0.9	−0.1 to 0.8	3	0.9	−0.8 to 0.1	3	0.7	−0.6 to 0.1	1	17.23
WF6	AAAAATTTTTT	0.9	0.1 to −0.8	4	0.9	0 to 0.9	4	0.9	−0.8 to 0.1	3	0.7	−0.6 to 0.1	1	16.51
WF7	AAATATATTTT	0.9	−0.1 to −1.0	2	1.0	0.7 to 1.7	3	0.5	−0.3 to −0.8	2	0.7	−0.6 to 0.1	1	17.28
WF8	AAATTTATTTT	0.9	−0.1 to −1.0	2	1.0	0.7 to 1.7	4	0.5	−0.3 to −0.8	2	1.0	0.5 to 1.5	8	16.44
WF9	AAAAAAAAAAA	0.0	1.5	1	0.0	2.1	1	1.0	0 to 1.0	2	0.5	0.5 to 1.0	2	16.71
WF10	TTTTTTTTTTT	0.8	0 to −0.8	3	1.0	0.7 to 1.7	4	0.8	−0.8 to 0	3	0.9	1.0 to 1.9	4	17.04
WF11	ATATATATATA	1.0	1.4 to 2.4	3	0.7	1.4 to 2.1	3	1.0	0.4 to 1.4	3	0.7	−0.2 to 0.5	2	17.36
WF12	TTTTTTTT	0.9	0.1 to −0.8	4	0.9	0.7 to 1.6	5	0.6	−0.2 to −0.8	6	0.9	1.0 to 1.9	8	16.91
ref.flap	AATAAATCATAA	–	–	–	–	–	–	1.0	0.9 to 1.9	9	1.0	0.5 to 1.5	7	17.66
–	Primers without flap	–	–	–	–	–	–	0.9	1.8 to 2.7	18	0.9	1.0 to 1.9	6	21.36

[a] ΔG in plot file

[b] Optimal energy of secondary structures (kcal/mol) at 61 and 54 °C with optimal and sub-optimal primer sets, respectively

[c] Number of secondary structures calculated by mFold

Plasmid Miniprep Kit (Invitrogen). The concentration of RNA and plasmid DNA were determined using a NanoDrop.

Results

Design of Optimal/Sub-Optimal Primer Pairs

and 5′ Flaps

Two primer sets: TMV-2F/2R, optimal, $\Delta G = 0$, amplifying a product of 195 bp and TMV-3F/3R, sub-optimal, $\Delta G = 0.9$, amplifying a product of 192 bp, were designed from sequences within the TMV movement protein gene (Fig. 1). The ΔG values were calculated using mFold (Table 1; Fig. 2). The optimal primer set, TMV-2F/2R, was predicted to have optimal folding (Fig. 2a, b), while the sub-optimal set, TMV-3F/3R, was predicted to have sub-optimal folding (Fig. 2c, d). Understanding the optimal folding of base pairs is the least likely secondary structure formation during reaction (Fig. 2).

Gradient RT-PCR of Optimal and Sub-Optimal Primers

To assess the range of annealing temperature values in which the optimal and sub-optimal primers, with and without 5′ flaps performed, each set was tested by gradient RT-PCR from 48 to 64.6 °C and 48 to 54 °C, respectively. The optimal primers amplified the targeted product within a range of 48–64.6 °C without the 5′ flap and 48–66 °C with the 5′ flap (Fig. 3a, b). The sub-optimal primer set, TMV-3F/3R, amplified the targeted product within a range of 48–54 °C (without 5′ flap) and 48–59.9 °C (with 5′ flap), respectively (Fig. 3c, d).

Thus, the addition of 5′ flaps to primers increased the range of annealing temperature performance for both optimal and sub-optimal primers. The optimal primer set had a larger range of annealing temperature performance with the 5′ flap, and an improvement in the annealing temperature range of amplification was induced in sub-optimal primers after 5′ flap addition.

RT-PCR Sensitivity Assays

The sensitivity of PCR using optimal and sub-optimal primers, with and without a 5′ flap, was assessed in one-step RT-PCR. A ten-fold serially diluted RNA, extracted from TMV-infected *Nicotiana* leaves, was used. The detection limit for the targeted TMV movement protein, using optimal primers, was 0.005 ng without 5′ flaps (Fig. 4a) and 0.0005 ng with 5′ flaps (Fig. 4b), while the detection limit using sub-optimal primers was 0.5 ng without flaps (Fig. 4c) and 0.005 ng with 5′ flaps (Fig. 4d). PCRs done using the optimal primer set, with 5′ flaps, were ten-fold more sensitive than without them. Sensitivity was 100-fold higher with sub-optimal primers when a 5′ flap was added, than when sub-optimal primers lacked 5′ flaps.

Quantification of PCR Yield

The yield of PCR products was ~85 % higher when amplified using optimal primers than when using sub-optimal primers (Fig. 5). However, the latter was ~20 % higher after the addition of 5′ flaps. PCR product concentration obtained using optimal primers, with or without 5′ flaps, were indistinguishable (Fig. 5). A high standard deviation resulted when optimal primers were used (Fig. 5).
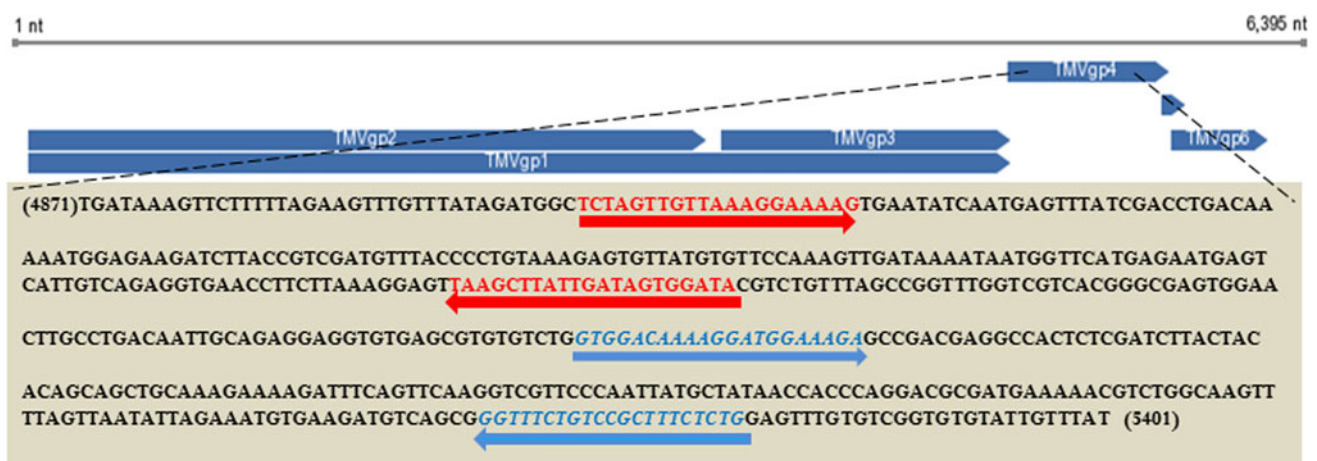

Fig. 1 Location of primer sets TMV-2F/2R (optimal; *blue and italics*) and TMV-3F/3R (sub-optimal; *red*) within the movement protein gene of *Tobacco mosaic virus* (Color figure online)
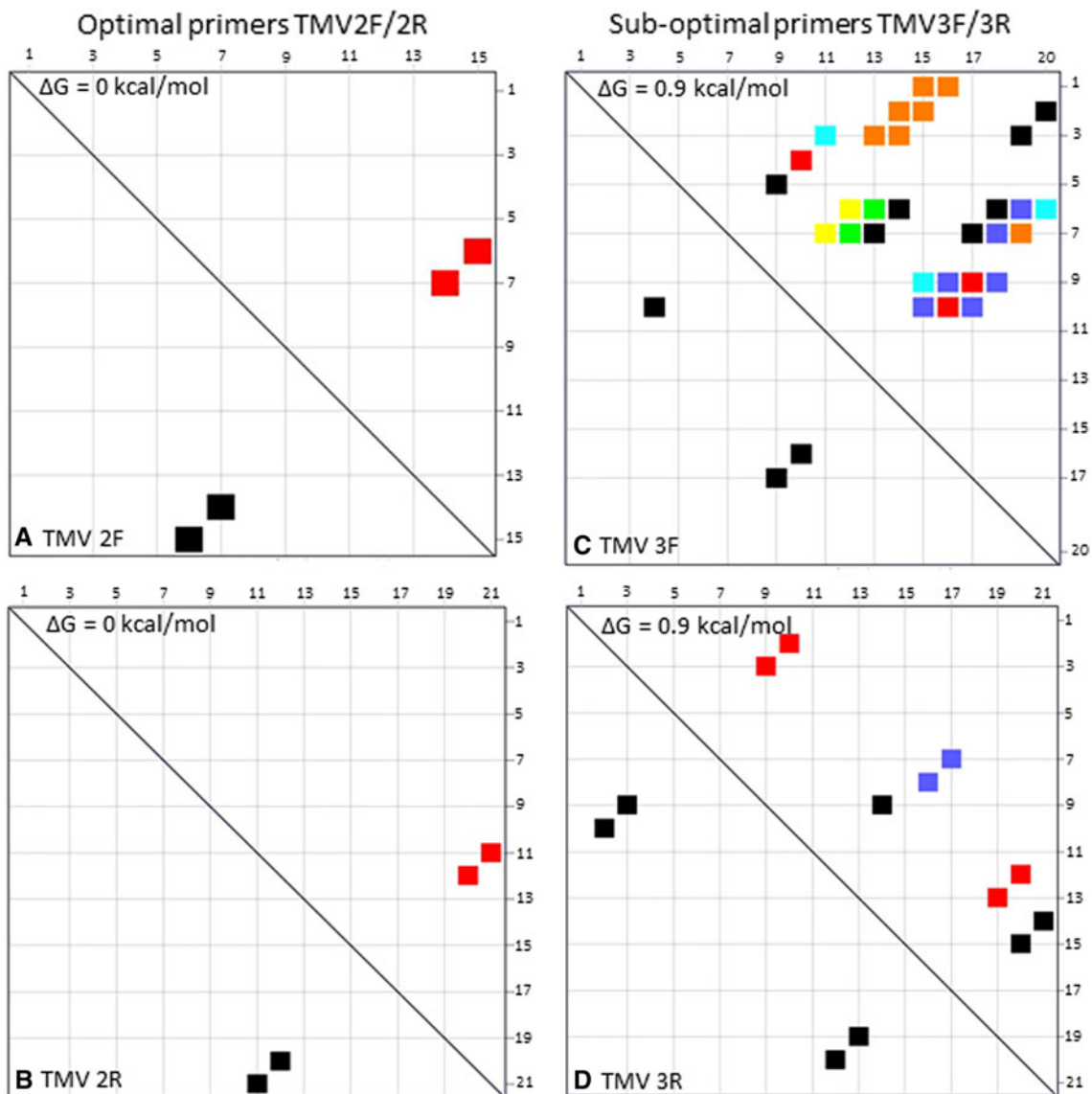
Fig. 2 Optimal energy values of each oligonucleotide primer calculated and plotted using mFold. The *black* and *red dots* represent all the optimal foldings. The *colored dots* represent the superposition of all possible sub-optimal foldings within $p\%$ deviation from the minimum free energy, the *red* and *green*, representing foldings within $p3\%$ and $p2\%$, respectively, and the *yellow dots* represent base pairs least likely to form. X and Y axis are the nucleotide position in primer sequence [11] (Color figure online)

Real-Time qPCR Assays

In general, use of primers with 5′ flaps resulted in low Ct (threshold cycle) values in SYBR Green qPCR. The Ct value obtained using the optimal primer set was 6.02 lower than that with the sub-optimal primers (Fig. 6a). Further, the use of sub-optimal primers with a 5′ flap yielded Ct values averaging 5.20, lower than those with sub-optimal primers lacking 5′ flap (Fig. 6b). No difference in fluorescence was detected whether the optimal primers were used with or without 5′ flaps (Fig. 6c). All standard deviations were calculated based on three Ct value replicates, and ranged from $0.09Ct$ to $0.17Ct$.

5′ Flap Comparison

The performance of primer sets having either the reference 5′ flap [13] or one of twelve other designed 5′ flaps (this article) were compared. Out of the twelve 5′ flaps, seven (WF2, WF3, WF4, WF6, WF8, WF9, and WF12) produced greater qPCR fluorescence than the reference 5′ flap [13] on sub-optimal primers. Use of primer sets with these seven 5′ flaps led to lower Ct values than using the primer set having the published 5′ flap (Table 2). For example, the effect of adding 5′ flap WF3 to sub-optimal primers on qPCR fluorescence is shown in Fig. 7.
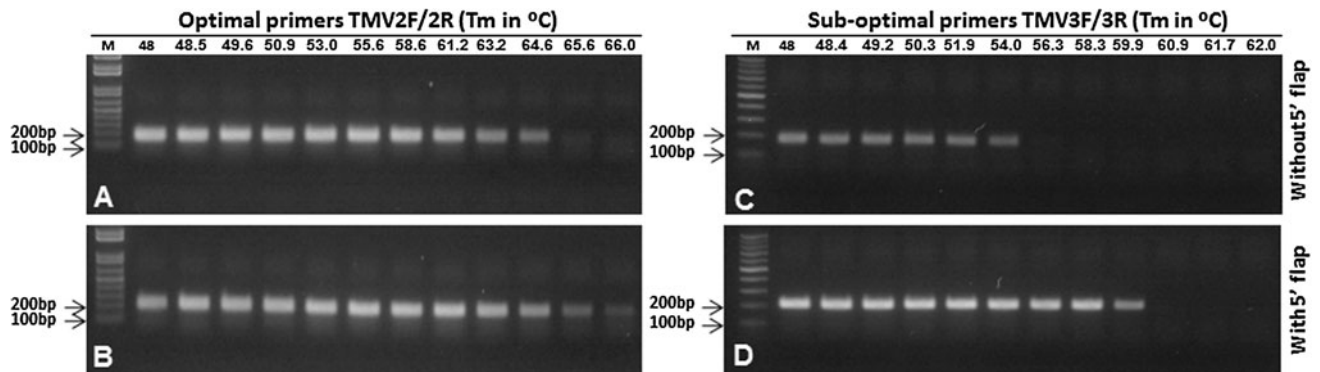
Fig. 3 TMV gradient one-step RT-PCR amplification. Annealing temperature ranged from 48 to 66 °C (**a**, **b**) and 48 to 62 °C (**c**, **d**). Optimal primer set TMV-2F/2R, **a** without and **b** with 5′ flap. Sub-optimal primer set TMV-3F/3R, **c** without and **d** with 5′ flap. *Lane M* 1 kb ladder
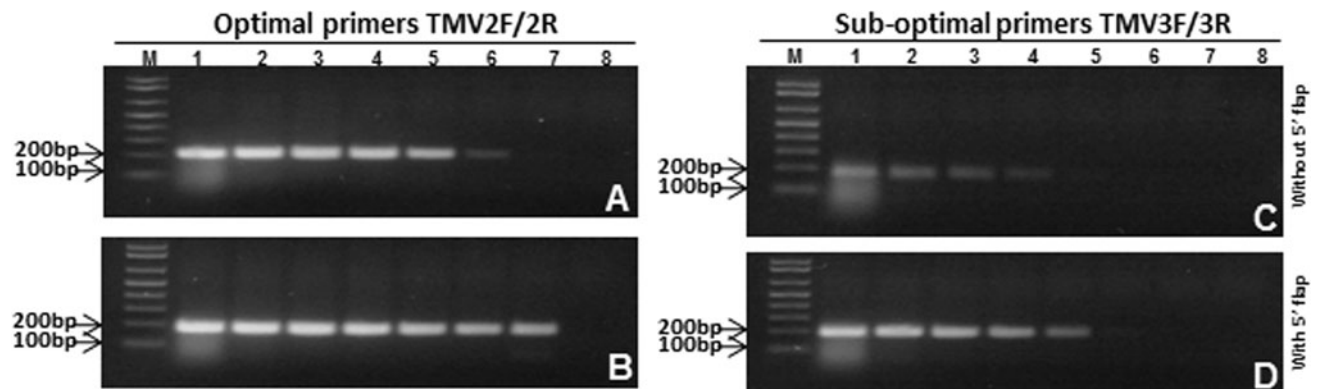

Fig. 4 Sensitivity assay of one-step RT-PCR. Sub-optimal primer set TMV-3F/3R, **a** without and **b** with 5′ AT-rich flap. Optimal primer set TMV-2F/2R, **c** without and **d** with 5′ AT-rich flap. *Lane M* 1 kb

ladder, *lanes 1–8* ten-fold serial dilutions starting from 500 ng/ reaction of RNA isolated from tobacco leaves infected with TMV
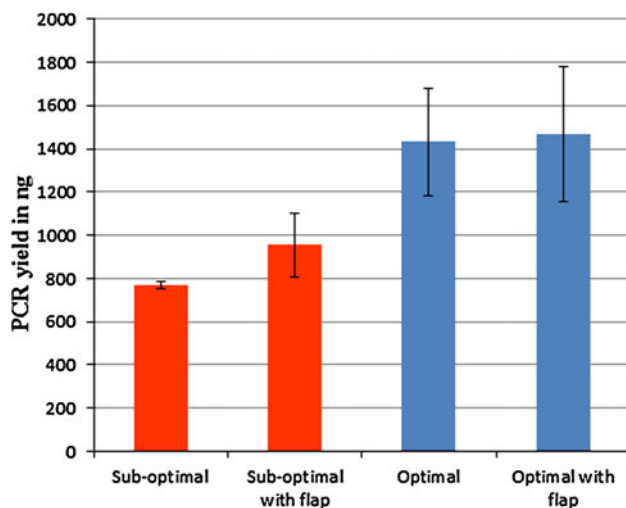

Fig. 5 NanoDrop reading of one-step RT-PCR DNA recovered from eluted band in three replicates

Discussion

We describe a comparative assessment of two primer sets, designed to be optimal and sub-optimal using the web

interface software pathway Primer3–BLASTn–mFold [19], known thermodynamic parameters, and 5′ flaps to understand reported improvement in PCR sensitivity and DNA yields. A segment of the TMV movement protein gene was chosen as a diagnostic target because TMV is easy to manipulate, abundant TMV genomic information is available [20], and TMV isolates are easy to obtain for use as reference controls.

Effect on Annealing Temperatures

Optimal primers with ΔG values and maximum 3′ complementarity equal to zero enable a robust PCR in which the DNA products are amplifiable in a broad range of annealing temperatures ranging from 48 to 64.6 °C without a 5′ primer flap (Fig. 3a) and from 48 to 66 °C with a 5′ flap (Fig. 3b). The addition of 5′ flaps to optimal and sub-optimal primers caused to broad the range of annealing temperature allows the primers to perform well among different operators, laboratories, and/or PCR machines, and favors assay repeatability and consistency. Adding 5′ flaps to sub-optimal primers also allows extension of the annealing temperature range (Fig. 3c, d).
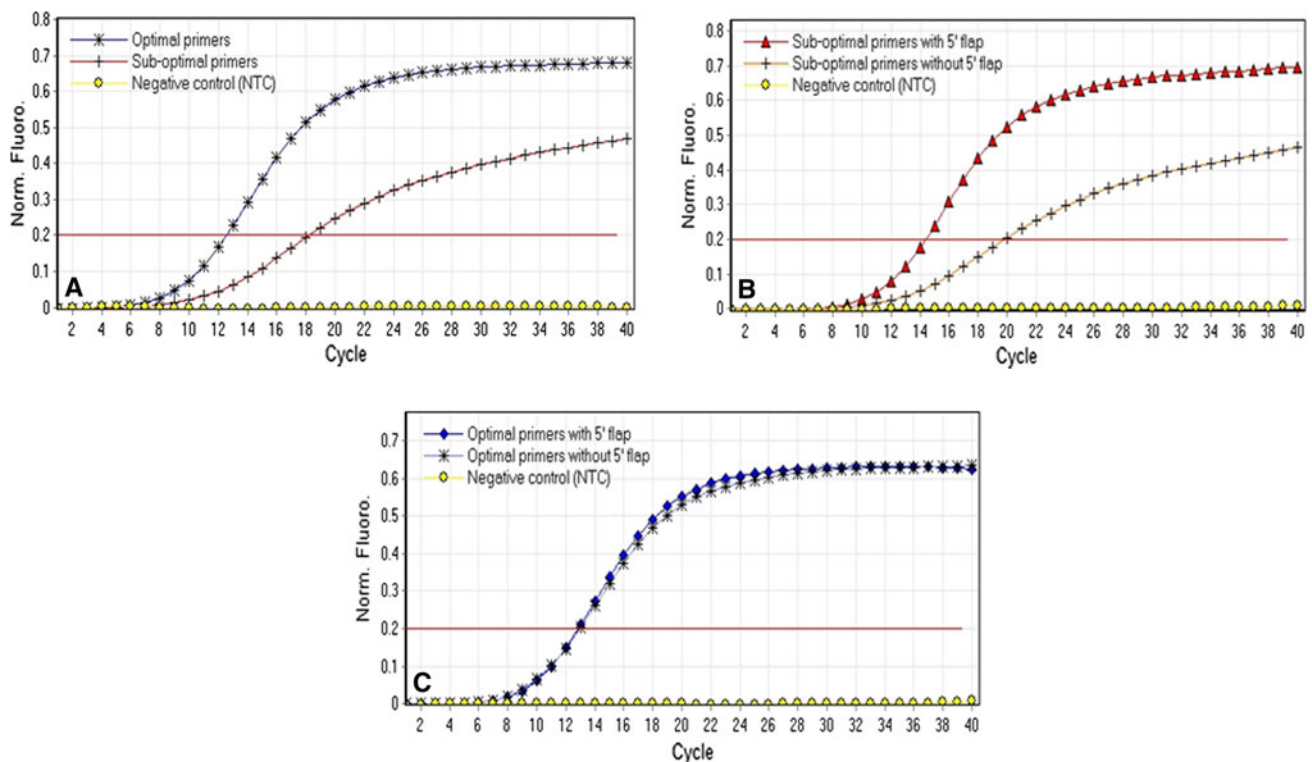
Fig. 6 Difference in fluorescence of SYBR Green qPCR amplification signal of optimal TMV-2F/2R and sub-optimal TMV-3F/3R with and without 5′ flaps. **a** Optimal versus sub-optimal primer, **b** sub-optimal primer with 5′ flap versus sub-optimal primer without flap, and **c** optimal primer with 5′ flap versus optimal primer without flap

Fig. 7 Difference in fluorescence of SYBR Green qPCR amplification signal of sub-optimal primer set with published ref.flap (5′-AATAAATCATAA-3′) [13] and new flap WF3 (5′-AAAATTATTTT-3′)
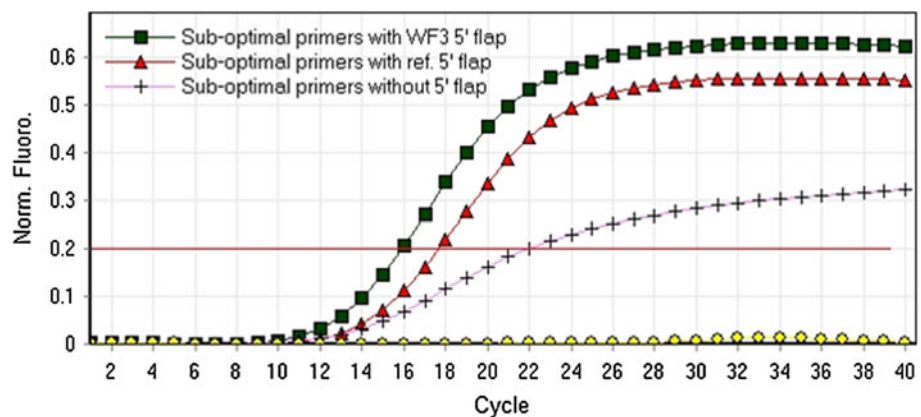
Effect on Sensitivity

The incorporation of 5′ flaps has the potential to increase the detection sensitivity of optimal primers up to tenfold (Fig. 4a, b) and that of sub-optimal primers up to 100-fold (Fig. 4c, d). Only one secondary structure was predicted in each optimal primer (Table 1), while up to 18 secondary structures were predicted to form in the sub-optimal primer TMV-3F. The 5′ reference flap incorporation in primer TMV-3F decreased the number of calculated secondary structures ranging from 18 to 9,

improving reaction efficiency (Table 1). Wei and Clover [12] reported optimum target amplification efficiency using only 5′ flap on the forward primer. The T_m values of the forward primer NepoA-F and reverse primer NepoA-R are 50.4 and 58.7 °C, respectively. The T_m difference (8.3 °C) between these two primers may render the PCR amplification less efficient. The addition of 5′ flap [13] on NepoA-F increases the T_m to 55.4 °C which decreases the T_m difference to 3.3 °C between these two primers and improves the PCR amplification efficiency.

Effect on DNA Yield

DNA yield was increased by about 90 % using optimal primers with 5′ flaps compared to those using sub-optimal primers without 5′ flaps (Fig. 5). DNA yield was 20–25 % higher when sub-optimal primers were 5′ flap-modified, but 5′ flap addition had no effect on yield using optimal primers with and without 5′ flap (Fig. 5). The effect of 5′ flap addition on PCR yields is related to multiple factors, i.e., the GC% of the primers decreased and the ΔG and the annealing temperature increased (Table 1). The addition of 5′ flaps on both forward and reverse primers enhances PCR yields because the product size increases; i.e., from 195 to 219 bp with optimal primers, which adds about 12 % to the yield. However, the % increase in PCR yield will vary depending on the length of the PCR product and the 5′ flaps. For example, using the optimal primers with 5′ flaps but doubling the product ranging from 195 to 390 bp without 5′ flap and 219 to 414 bp with 5′ flap, then the increment in PCR yield will be of about 6 %. Therefore, higher % of PCR yields is expected with primer sets with 5′ flaps that amplify smaller size product.

Effect on qPCR Sensitivity

A PCR product amplified using the TMV-3F and the TMV-2R primers containing both target sequences for optimal and sub-optimal primers, was inserted into a plasmid vector (pCR2.1-TOPO) so as to minimize other factors that can create variation in qPCR assays. From preliminary qPCR results (unpublished data), we estimated that $\sim 3Ct$ values are equivalent to a ten-fold difference in the concentration of the starting template. Therefore, having a Ct value difference of 6.02 between the optimal and sub-optimal primer sets would be equivalent to a 100-fold difference in sensitivity (Fig. 6a). The addition of 5′ flaps on to the sub-optimal but not optimal primers increased the sensitivity ~ 100-fold (Fig. 6b, c). Thus, incorporating 5′ flaps can improve the yields of PCRs performed using sub-optimal primers, but not those using optimal primers. Hence, 5′ flaps can be applied for improving assays, already in use or accredited, that were developed using sub-optimal primers. The approach is useful for diagnostic regions prone to form secondary structures and where primer design is challenging.

Effect of 5′ Flap Composition

The number of predicted secondary structures decreased from 18 to 2, and ΔG values decreased after addition of 5′ flap to sub-optimal primers (Table 2). These results show the performance of some primers can be improved substantially by the 5′ flap addition, however, not all primers will be similarly improved. Primers of which thermodynamics were optimized during primer design may not be enhanced by 5′ flaps (Table 1). In contrast, primers designed with sub-optimal thermodynamics can be substantially improved by adding 5′ flaps. However, there is currently no universal 5′ flap (Tables 1, 2); each primer may require a different customized 5′ flaps. Optimal 5′ flap sequences are dependent on the primer sequences, and may alter the primer's T_m value. The manipulation of this feature may render the primers compatible with multiplex PCR [21].

Incorporating 5′ primer flaps can be useful to improve existing PCR-based assays that are standardized for use within rigid standard operational procedures (SOPs) in forensics, biological detection, and/or diagnostic assays. Questions remain regarding the broad use of 5′ primer flaps. For example, can we design better custom 5′ flaps? What is the effect of combining different 5′ flaps in a single set of primers?

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