# Primers with 5′ flaps improve real-time PCR

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*Primers that contain portions noncomplementary to the target region are usually used to add to the PCR product a utility sequence such as a restriction site or a universal probe binding site. We have demonstrated that primers with short 5′ AT-rich overhangs increase real-time PCR fluorescent signal. The improvement is particularly significant for difficult to amplify templates, such as highly variable viral sequences or bisulfite-treated DNA.*

Primers that contain portions noncomplementary to the target (often called overhang, flap, or tail) are usually used to add to the PCR product a utility sequence such as a restriction site (1) or a universal detection site (2,3). Stem-loop primers make PCR amplification of short templates such as microRNA possible (4). Flap primers are used to reduce the number of sequencing errors in short PCR products (5). We have noticed that primers with short 5′ AT-rich flaps increase real-time PCR fluorescent signal, and this improvement is particularly significant for sequences that are difficult to amplify, such as bisulfite-treated DNA or highly variable viral sequences. The real-time PCR experimental data presented here (see Table 1 for oligonucleotide sequences) was generated by 5′ minor groove binder (MGB) fluorescent hybridization probes (6), although we have observed similar effects for other platforms such as TaqMan probes (BioSearch Technologies, Novato, CA, USA) (data not shown). An example of the typical benefits of primers containing 5′ AT-rich flaps is shown in Figure 1, in which they are used to amplify sodium bisulfite-treated DNA. The bisulfite treatment leads to a conversion of all unmethylated cytosines to uracils, leaving methylated cytosines unchanged (7), which constrains primer design. Human genomic DNA was treated with sodium bisulfite, and a portion of the differentially methylated region of the H19 gene was amplified using primers with or without 12-mer 5′ AT-rich flaps (Table 1). Fluorescent signal is increased, and PCR yield is higher when bisulfite-treated DNA is amplified with flap primers and fluorescence is generated by a hybridization probe (Figure 1A). This increase in signal intensity is also observed when fluorescence is generated by a free intercalating dye such as SYBR Green (Figure 1B), and the bands on ethidium bromide-stained agarose gel are also more pronounced (Figure 1C).

We have determined that a 12-bp 5′ AT-rich flap sequence is optimal. Addition of flap sequences longer than 12 bp did not show any significant improvement, while shorter sequences had a lesser impact on the fluorescent signal (see Supplementary Table S1, available online at www.BioTechniques.com). The addition of a flap to either one or the other primer provides a boost to the fluorescent signal, but is not as beneficial as having both primers with flaps. 5′ GC-rich flaps overall proved not as useful as 5′ AT-rich flaps, because they are more prone to form stable secondary structures and negatively impact the PCR (data not shown). The positive effect of the 12-bp 5′ AT-rich flap is more pronounced for shorter primers (Supplementary Table S2). To study the effect of flap addition to primers of decreasing length on cycle threshold ($C_T$) and

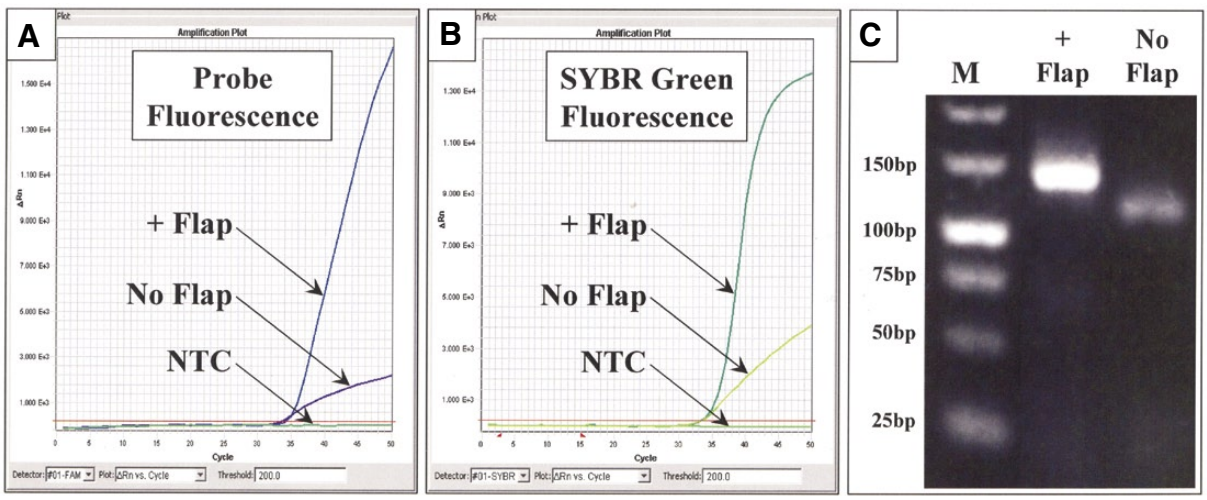


**Figure 1. Primers with 5′ flaps improve real-time PCR.** Bisulfite-treated genomic DNA with 50% methylated H19 differentially methylated region (DMR) was amplified with flap and non-flap primers using (A) JumpStart Taq ReadyMix and (B) SYBR Green JumpStart Taq ReadyMix (both from Sigma-Aldrich, St. Louis, MO, USA) using the following cycling parameters: 2 min at 95°C, followed by 50 cycles of 15 s at 95°C and 1 min at 60°C. (C) The JumpStart Taq ReadyMix PCR products were run on a 4% agarose gel.

**Table 1. Primers and Probes Used for Real-Time PCR**

| Primer or Probe | Sequence[a] | Final Concentration |
|---|---|---|
| | **VZV Reagents** | |
| VZV-forward primer | 5′-**AATAAATCATAA**GAACAATCACGAACCG-3′ | 0.25 μM |
| VZV-reverse primer | 5′-**AATAAATCATAA**CGGGCATGCTTACGGACGT-3′ | 1.0 μM |
| VZV-probe | 5′-MGB-FAM-CAGGACGTCAGATCACGT-NFQ-3′ | 0.2 μM |
| VZV DNA | VZV, strain Webster; ATCC VR-916 | $10^5$ copies/rxn |
| | **Enterovirus Reagents** | |
| ENV-forward primer | 5′-**AATAAATCATAA**GAAGAGYCZ*ATTGAGCTA-3′ | 1.0 μM |
| ENV-reverse primer | 5′-**AATAAATCATAA**GGA*TTRGCCGCA*TTC-3′ | 1.0 μM |
| ENV-probe | 5′-MGB-FAM-TCCGGCCCCTGAATGC-NFQ-3′ | 0.2 μM |
| ENV RNA | Coxsackievirus isolate A6; ATCC VR-165 | |
| | **H19 Differentially Methylated Region Reagents** | |
| DMR-forward primer[b] | 5′-**AATAAATCATAA**TTGG A*TGGTACGGA*ATTGG-3′ | 1.0 μM |
| DMR-reverse primer | 5′-**AATAAATCATAA**TCCACGAACGAACCC-3′ | 0.25[b] μM |
| DMR-probe | 5′-MGB-FAM-ACGA*ACT*CGA*ACT*ATAAT-NFQ-3′ | 0.2 μM |
| Bisulfite-treated DNA template | Human genomic DNA (DMR 50% methylated) was a generous gift of Alain Carrié [Institut Fédératif de Recherche des NeuroSciences (IFRNS), France] | 1 ng/rxn |

[a]Bolded sequences are noncomplementary flaps. Primers without bold type lack flaps.
[b]Raised to 1 μM for SYBR Green.
*Indicates modified base with increased stability.
VZV, varicella-zoster virus; MGB, minor groove binder; NFQ, nonfluorescent quencher; ATCC, American Type Culture Collection, Manassas, VA, USA; rxn, reaction; ENV, enterovirus; DMR, differentially methylated region

fluorescence, we chose a primer pair optimized for the cycling conditions recommended by the PCR Master Mix manufacturer (8) and designed several shorter primer pairs. As expected, shorter primers without flaps had higher $C_T$ values and lower fluorescence gain. The addition of a 12-bp 5′ AT-rich flap nullified the difference in performance. Fourteen- to fifteen-mer primers with these flaps had approximately the same $C_T$ value and fluorescence as the original 22- to 25-mer primers without flaps (Supplementary Table S2). This is useful information if primer design is constrained to short stretches of sequence for any reason.

Figure 2 demonstrates the effect of 5′ flap primers in real-time PCR with varicella-zoster virus (VZV) DNA template and one-step reverse transcription PCR (RT-PCR) with enterovirus RNA template. The region chosen for the VZV design [open reading frame (ORF) 38 gene] is conservative and does not impose any restrictions on primer design. An increase in total fluorescence gain of approximately 50% due to 5′ primer flaps (Figure 2A), and no significant shift in $C_T$ is typical for such unconstrained primer designs. In contrast to VZV, the sequences of the enterovirus genus (Taxonomy ID 12059; www.ncbi.nlm.nih.gov/Taxonomy) are highly variable. Therefore, to enable detection of all known isolates, the primer design is constrained to a short conservative region in the 5′ untranslated region (UTR). The primers also include degenerate and modified bases to account for unavoidable single nucleotide polymorphisms (SNPs) and to boost primer stability (9). As a result,

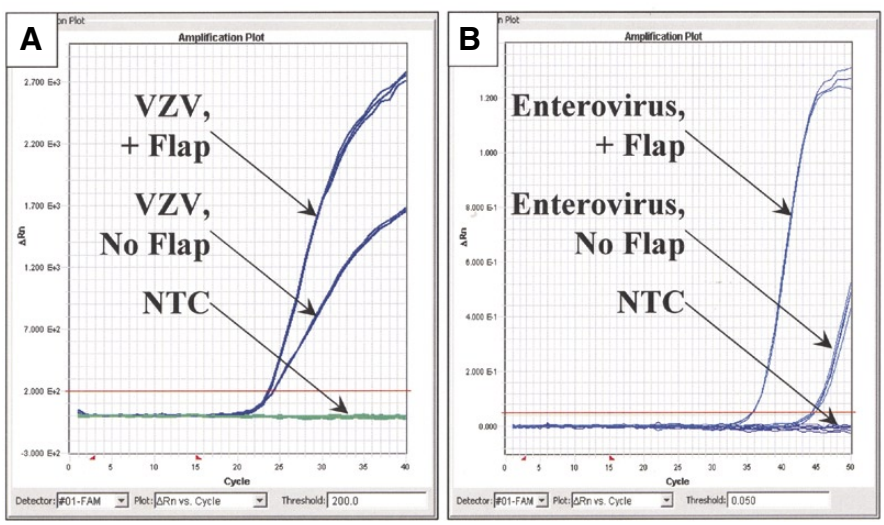


**Figure 2. Real-time PCR amplification of viral targets with 5′ flap primers.** (A) Varicella-zoster virus (VZV) DNA template was amplified with the LightCycler FastStart DNA Master HybProbe master mixture (Roche Applied Science, Indianapolis, IN, USA) using the following cycling parameters: 10 min at 95°C, followed by 40 cycles of 5 s at 95°C, 20 s at 56°C, and 20 s at 76°C. (B) Enterovirus RNA (coxsackievirus A6) template was amplified by one-step reverse transcription PCR (RT-PCR) with the QuantiTect Probe RT-PCR master mixture (Qiagen, Venlo, The Netherlands) using the following cycling parameters: 30 min at 50°C (RT), 15 min at 95°C, followed by 50 cycles of 15 s at 95°C, 30 s at 56°C, and 30 s at 76°C. In both cases detection was done during the annealing stage of PCR.

the PCR primers perform suboptimally, and PCR efficiency is compromised. When the flap is added to the suboptimal (non-flap) enterovirus primers, there is a significant increase in performance for both fluorescence and $C_T$ values (Figure 2B).

It is not clear why PCR primers containing the 5′ AT-rich flaps are better, but the benefits are obvious. This innovation could be of benefit to every real-time PCR laboratory, especially when sequence choice for primer design is constrained.

## COMPETING INTERESTS STATEMENT

*The authors declare no competing interests.*

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