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The Effect of Primer-Template Mismatches on the Detection and Quantification of Nucleic Acids Using the 5′ Nuclease Assay

Ralph Stadhouders, Suzan D. Pas, Jeer Anber, Jolanda Voermans, Ted H.M. Mes, and Martin Schutten

From the Department of Virology, Erasmus University Medical Centre, Rotterdam, The Netherlands

Real-time polymerase chain reaction (PCR) is the current method of choice for detection and quantification of nucleic acids, especially for molecular diagnostics. Complementarity between primers and template is often crucial for PCR applications, as mismatches can severely reduce priming efficiency. However, little quantitative data on the effect of these mismatches is available. We quantitatively investigated the effects of primer-template mismatches within the 3′-end primer region on real-time PCR using the 5′-nuclease assay. Our results show that single mismatches instigate a broad variety of effects, ranging from minor (<1.5 cycle threshold, eg, A–C, C–A, T–G, G–T) to severe impact (>7.0 cycle threshold, eg, A–A, G–A, A–G, C–C) on PCR amplification. A clear relationship between specific mismatch types, position, and impact was found, which remained consistent for DNA versus RNA amplifications and *Taq*/Moloney murine leukemia virus versus r*Tth* based amplifications. The overall size of the impact among the various master mixes used differed substantially (up to sevenfold), and for certain master mixes a reverse or forward primer-specific impact was observed, emphasizing the importance of the experimental conditions used. Taken together these data suggest that mismatch impact follows a consistent pattern and enabled us to formulate several guidelines for predicting primer-template mismatch behavior when using specific 5-nuclease assay master mixes. Our study provides novel insight into mismatch behavior and should allow for more optimized development of real-time PCR assays involving primer-template mismatches. ***(J Mol Diagn 2010, 12:109–117; DOI: 10.2353/jmoldx.2010.090035)***

During the past decade, real-time polymerase chain reaction (PCR) has established itself as an essential technique for reliable detection and quantification of nucleic acids.[1–3] The result is a widespread application of real-time PCR assays in both research[1–4] and diagnostic[4–6] laboratories. Vital to the specificity, sensitivity, and efficiency of real-time PCR are the primers. The most important primer characteristics contributing to a successful amplification are primer-template association and dissociation kinetics, possible secondary structures, and primer-template complementarity (Watson-Crick base-pairing).[7,8] Full complementarity between primer and template sequences is generally considered crucial for the specific amplification of a nucleic acid sequence, but can be difficult to achieve, in particular for applications depending on highly heterogenic nucleic acid input for amplification (eg, diagnostic assays for influenza virus and human immunodeficiency virus). Conserved regions are often too small to accommodate a typical real-time PCR assay (50 to 150 bp), exhibit inferior G-C contents or are prone to the formation of secondary structures. Primer-template mismatches can therefore be unavoidable.

Unfortunately, mismatches between primers and template are known to affect both the stability of the primer-template duplex and the efficiency with which the polymerase extends the primer,[7–13] potentially leading to biased results or even PCR failure.[14,15] Even apparently small effects on nucleic acid quantification (0.5 to 1.0 log underestimation of initial copy number) can have serious consequences, as illustrated by studies on the relation between viral load and disease prognosis in HIV-1.[16]

The detrimental effects of primer-template mismatches can however also prove beneficial. They provide a discriminative force that can be used for PCR assays opting to distinguish between different nucleic acids (eg, single nucleotide polymorphism detection, allele-specific PCR), which have become important tools for modern molecular diagnostics.[4]

Every mismatch, irrespective of its location within the primer sequence, will result in a decreased thermal stability of the primer-template duplex, thus potentially affecting PCR specificity. However, mismatches located in the 3′ end region (defined as the last 5 nucleotides of the 3′ end region) of a primer have significantly larger effects on priming efficiency than more 5′ located mis-

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Address reprint requests to Martin Schutten, Ph.D., Department of Virology, Room L355, Erasmus MC - Centre location, Department of Virology, Room L355, 's-Gravendijkwal 230, 3015 CE Rotterdam, The Netherlands. E-mail: m.schutten@erasmusmc.nl.

matches,[9,11,13–15] since 3′ end mismatches can disrupt the nearby polymerase active site.[17,18]

Strategies to alter mismatch impact, eg, degenerate/modified bases or extensive adaptation of PCR conditions, can prove helpful in specific situations, but these strategies often require a lot of time-consuming optimization and can result in unwanted side effects (eg, increased primer-dimer formation). Quantitative data on the effects of 3′ end mismatches is necessary to improve knowledge and reliable prediction of mismatch behavior, which is beneficial for the development and optimization of real-time PCR assays involving mismatches.

Several studies on the effects of 3′ end primer-template mismatches have been published.[9,10,19–22] However, only few systematically examined the behavior of 3′ end primer-template mismatches (including the relationship between these effects and the position of the mismatch) using modern quantitative methods. In this study, we comprehensively investigate the effects of 3′ end primer-template mismatches using different commercially available 5′-nuclease assay master mixes. Diagnostic laboratories often employ such optimized premixed reagents, which are generally directly used with few adaptations. Our approach therefore provides a relevant system for quantification of mismatch impact on diagnostic real-time PCR assays. Our experiments resulted in a large quantitative dataset from which different aspects of mismatch effects on PCR amplification were further analyzed, ultimately leading to the formulation of a set of general guidelines for improved prediction of primer-template mismatch impact.

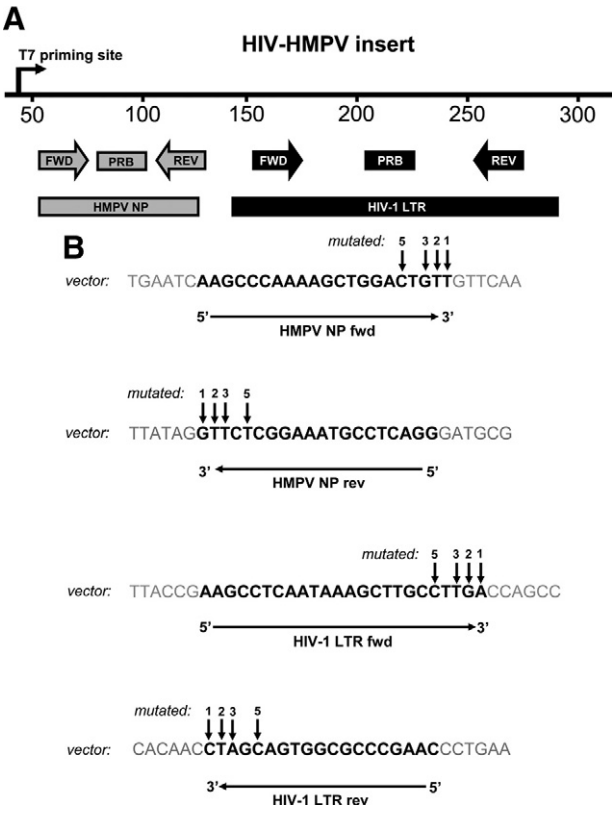

Figure 1. Oligonucleotides used to study the effects of 3′ end primer-template mismatches on nucleic acid detection and quantification with real-time Taqman PCR. Panel (**A**) represents a linear map of the HIV-HMPV insert, in which the location of both viral DNA sequences and corresponding primers and probes are depicted. Panel (**B**) shows the nucleotide sequences of the primers (black) as located in the vector. **Arrows** indicate the positions on the HIV-hMPVpGA4 vector that have been subjected to site-directed mutagenesis. fwd = forward primer, rev = reverse primer, PRB = probe.

Materials and Methods

Vector Construction

A pGA4 vector containing a 148 bp region from the HIV-1 5′ long terminal repeat (LTR) and a 75 bp region from the *nucleoprotein* (NP) gene in human metapneumovirus (hMPV) was constructed (the HIV-hMPVpGA4 model vector). Two real-time Taqman PCR assays were defined on the insert: i) a previously described and commonly used HIV-1 LTR assay[23] and ii) an in-house developed hMPV NP assay. The primers and probes (synthesized by Eurogentec, Seraing, Belgium) used for mismatch analysis, together with their location within the HIV-HMPV insert, are depicted in Figure 1A–B.

Mutagenesis and Generation of PCR Templates

Site-directed mutagenesis (QuikChange XL Site-Directed Mutagenesis Kit from Stratagene, La Jolla, CA) was used to generate 48 single bp mutations in the HIV-HMPV insert at 4 positions: the 3′ terminal base (position 1), the penultimate base (position 2), and the third (position 3), and fifth base (position 5) from the 3′ primer terminus (Figure 1B, indicated in the vector sequence by arrows). Mutations were individually introduced in only one primer, resulting in constructs containing a single mismatch in one of the four primer sequences. This strategy allowed the utilization of the non-mutated PCR as a control reaction for calculating mismatch effects. Additionally, three constructs with multiple mutations in the last 3 bases were generated (all located in the hMPV NP forward primer, including the 3′ terminal base).

Mutations were verified by colony PCR using M13 (−20) primers (forward: 5′-TGTAAAACGACGGCCAGT-3′, reverse: 5′-GGTCATAGCTGTTTCCTG-3′) and subsequent sequencing using the BigDye Terminator v.3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) with the previously mentioned M13 (−20) primers. Sequencing reactions were performed on a 3100 Genetic Analyzer (Applied Biosystems).

For real-time Taqman RT-PCR assays, sense RNA was transcribed from the HIV-hMPVpGA4 vector and its mutated derivatives using the T7 RiboMAX Express Large Scale RNA production System kit (Promega, Madison, WI) according to the manufacturer's instructions. RNA synthesis reactions were subsequently incubated at 37°C for 15 minutes in the presence of 2 units of TURBO DNase (Applied Biosystems) to remove any DNA contaminations. DNA removal was validated by testing $\log_{10}$ serial dilutions of RNA using the 2× Taqman Universal PCR Mastermix, as described below.