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One-step RNA pathogen detection with reverse transcriptase activity of a mutated thermostable *Thermus aquaticus* DNA polymerase

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We describe the cloning and characterization of a mutated thermostable DNA polymerase from *Thermus aquaticus* (Taq) that exhibits an increased reverse transcriptase activity and is therefore designated for one-step PCR pathogen detection using established real-time detection methods. We demonstrate that this Taq polymerase mutant (Taq M1) has similar PCR sensitivity and nuclease activity as the respective Taq wild-type DNA polymerase. In addition, and in marked contrast to the wild-type, Taq M1 exhibits a significantly increased reverse transcriptase activity especially at high temperatures (>60°C). RNA generally hosts highly stable secondary structure motifs, such as hairpins and G-quadruplexes, which complicate, or in the worst case obviate, reverse transcription (RT). Thus, RT at high temperatures is desired to weaken or melt secondary structure motifs. To demonstrate the ability of Taq M1 for RNA detection of pathogens, we performed TaqMan probe-based diagnostics of Dobrava viruses by one-step RT-PCR. We found similar detection sensitivities compared to commercially available RT-PCR systems without further optimization of reaction parameters, thus making this enzyme highly suitable for any PCR probe-based RNA detection method.

Keywords: DNA polymerase · G-Quadruplex · RNA pathogen detection · RT-PCR · RNA secondary structures

1 Introduction

Emerging viral pathogens such as arthropod-borne flavi- and alphaviruses or rodent-borne hantavirus [1] or the recently occurring Influenza A virus subtype H1N1 [2] are a constant threat to global public health [3]. To monitor and detect their appearance and circulation, reliable pathogen detection methods are necessary. Apart from several antibody-based assays [4] – like the hemagglutination inhibition test (HI), enzyme immunoassay (EIA), and virus neutralization tests (VN) – nucleic acid detection assays (NA), such as the polymerase chain reaction (PCR), are among the most reliable detection techniques used for pathogen detection [5]. For PCR, a DNA polymerase needs specific primers (short DNA fragments) with sequences complementary to a target DNA region. During repeated cycles of heating and cooling new DNA is generated and is itself used as a template for replication. Due to the enzymatic replication under consumption of the primers and deoxynucleotide triphosphates (dNTPs), in theory the selected DNA sequence framed by the primers is exponentially amplified. Heat-stable DNA polymerases are employed in almost every PCR application, and re-

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Abbreviations: **Ct**, threshold-crossing points; **dNTPs**, deoxynucleotide triphosphates; **KlenTaq**, N-terminal-shortened form of DNA polymerase from *Thermus aquaticus*; **RT**, reverse transcription; **Taq M1**, mutated DNA polymerase from *Thermus aquaticus*; **Tth**, *Thermus thermophilus*; **wt**, wild type

main active during the thermal cycling steps necessary to physically separate the two strands of the DNA double helix (usually at high temperatures ~95°C). Nowadays, real-time PCR methods using unspecific fluorescent dyes, *e.g.*, SYBRgreen I, or specific probes, *e.g.*, TaqMan probes [6–9], report the amount of amplified DNA in real time and have significantly shortened conventional PCR methods. Consequently, they are the method of choice for detection and quantification of DNA and RNA targets such as retroviruses and viral pathogens [9]. In routine molecular diagnostics, probe-based real-time PCR systems are the state of the art since they are highly sensitive and include a specificity control.

Two enzymes are needed to detect RNA by a reverse transcription (RT)-PCR. In a first crucial step for RT-PCR, the RNA target is reverse transcribed into the complementary DNA strand. This is performed by a non-thermostable RNA-dependent DNA polymerase (reverse transcriptase) followed by real-time amplification of the transcribed target by a thermostable DNA polymerase [9]. In real-time RT-PCR, fluorescent probes are used to increase the level of specificity and to avoid detection of nonspecific side products [6, 7, 10]. The fluorescent probe hybridizes to a sequence in between the flanking primer sequences of the PCR target. In a TaqMan probe a fluorophor and a quencher molecule are covalently attached to the 5′ and 3′ end of the probe, allowing for Förster resonance energy transfer (FRET) to occur between both dye molecules, resulting in suppressed fluorescence of the fluorophor dye. During the PCR extension steps, a DNA polymerase, which harbors an active nuclease domain, degrades the DNA stretch of fluorescence probe that is annealed to the target strand. The fluorophor molecule is cleaved from the probe and released from close proximity to the quencher molecule, resulting in increased fluorescence. Thus, the generated fluorescence signal is directly proportional to the amplified target molecules after each cycle. The most critical step in this method is the conversion from the RNA target into DNA. This RT is prone to failure because RNA often hosts highly stable secondary structure motifs, such as hairpins and G-quadruplexes, that complicate or even prevent RT [11]. Thus, thermostable reverse transcriptases, which are able to work at higher temperatures, are urgently needed to increase reliability and sensitivity of RNA pathogen detection systems. It is known that some DNA polymerases for example from *Thermus aquaticus* exhibit a low intrinsic RT activity that is too inefficient for a fast and reliable RT-PCR-based RNA detection [12]. Myers and Gelfand [11] reported a DNA polymerase from *Thermus thermophilus* (Tth) that exhibits increased RT activity exclusively in the presence of Mn^{2+} ions, but unfortunately for many biotechnological applications, like pathogen detection or gene expression analysis, employment of Mn^{2+} is inappropriate [9].

We previously evolved an N-terminal-shortened form of a DNA polymerase from *Thermus aquaticus* (KlenTaq) with increased reverse transcriptase activity [13]. To investigate whether the full-length Taq DNA polymerase mutant (henceforth called Taq M1), which includes the nuclease domain, could be applicable for real-time one-step detection of pathogenic DNA using TaqMan probes, we fused the nuclease domain to the mutant KlenTaq. We demonstrate here the successful generation of the nuclease activity under conservation of the previously evolved reverse transcriptase activity. Our results show that Taq M1 has similar PCR sensitivity and nuclease activity to that of the respective wild-type (wt) Taq DNA polymerase. However, it also exhibits reverse transcriptase ability. In addition, we demonstrate the usefulness of Taq M1 for fast and reliable RNA pathogen detection in a case study for the detection of RNA from Dobrava virus, and its advantages in RT-PCR using RNA targets that form stable secondary structure motifs.

2 Materials and methods

2.1 Reagents and Instruments

Oligonucleotides were purchased from Purimex or Metabion, Germany. High Pure PCR Cleanup Micro Kit, High Pure Plasmid Isolation Kit, and RNA from Bacteriophage MS2 and Titan One Tube RT-PCR Kit were from Roche. RNeasy Mini Kit and QIAquick Gel Extraction Kit were purchased from Qiagen. PageRuler unstained Protein Ladder, DNaseI, RiboLock™ RNase Inhibitor and Rapid DNA Ligation Kit were purchased from Fermentas. Real-time PCR was performed in an iCycler or Chromo4 instrument from Bio-Rad. SYBRgreen I was purchased from Molecular Probes. Denaturing PAGE was analyzed with a Molecular Imager Fx from Bio-Rad. Phusion DNA polymerase, Antarctic Phosphatase, Low MW ladder, EcoRV, and BsmBI were purchased from New England Biolabs.

2.2 Cloning, protein expression and purification of Taq M1 polymerase

Respective plasmids (pASK-IBA37plus) harboring KlenTaq M1 and Taq wt gene were isolated from

the respective *E. coli* cultures using the High Pure Plasmid Isolation Kit. The KlenTaq M1 polymerase gene [14] was amplified using Phusion DNA polymerase with the forward primer (5′-GAT CTA CGT CTC CGC CCT GGA GGA GGC CC-3′) and reverse primer (5′-CAG GTC AAG CTT AGT TAG ATA TCA CTC C-3′). Taq nuclease domain DNA [15] including the whole pASK-IBA37plus plasmid sequence was amplified using Phusion DNA polymerase with forward primer (5′-GCC AAG GAG TGA TAT CTA ACT AAG CT-3′) and reverse primer (5′-ATG ATC CGT CTC AGG GCC TTG GGG CTT TCC AGA A-3′). Both amplificates were purified on a 0.8% agarose gel and isolated using the QIAquick Gel Extraction Kit. Isolated DNA was digested using *Eco*RV and *Bsm*BI, and purified using the High Pure PCR Cleanup Micro Kit. The Taq nuclease domain amplificate was dephosphorylated using Antarctic Phosphatase and ligated with the KlenTaq amplificate using the Rapid DNA Ligation Kit and transformed into electro competent *E. coli* XL10 gold cells. Clones were picked from agar plates and separately grown overnight in LB medium (100 µg/mL carbenicillin). The integrity of the whole mutant clone was proved by sequencing of the respective purified plasmid (GATC Biotech AG, Germany) using the sequencing primers p1–p5 (p1: 5′-GAG TTA TTT TAC CAC TCC CT-3′, p2: 5′-CCT GGC TTT GGG AAA AG-3′, p3: 5′-CCC GAG CCT TAT AAA GC-3′, p4: 5′-CGT AAG GGA TGG CTA GCT CC-3′, p5: 5′-CGC AGT AGC GGT AAA CG-3′). Enzyme purification and concentration determination was conducted as previously described [13, 16].

2.3 Assay of nuclease activity

Reaction mixtures (60 µL) contained 50 mM Tris-HCl (pH 9.2), 16 mM $(NH_4)_2SO_4$, 0.1% Tween 20, 2.5 mM $MgCl_2$, 50 nM of each dNTP, 150 nM substrate DNA (22 nt, 5′-[^{32}P]CCC CCC CCC CTC ATA CGT ACA C-3′), and 225 nM template DNA (5′-GTG TAC GTA TGA TCA TGC AGG TAG CCG ATG AAC TGG TCG AAA GAC CAG TTC ATC GGC TAC CTG CAT GAT-3′). After an initial denaturation and annealing step (95°C for 5 min, 0.5°C/s cooling down to 4°C), the reaction mixture was heated to 30°C and the reaction was started by addition of DNA polymerase (50 nM final concentration). Aliquots (5 µL) were taken at various time periods up to 60 min and reaction was stopped by addition of gel loading buffer (80% formamide, 20 mM EDTA). Product mixtures were analyzed by 12% denaturing PAGE and quantified using a Phosphorimager.

2.4 Real-time PCR, template dilution series

Reaction mixtures (20 µL) contained 50 mM Tris-HCl (pH 9.2), 16 mM $(NH_4)_2SO_4$, 0.1% Tween 20, 2.5 mM $MgCl_2$, 250 µM of each dNTP, tenfold dilution series of template RNA from bacteriophage MS2 (10 nM–10 fM) or DNA template MS2 [1 nM–10 fM, 100 nt, 5′-d(ATC GCT CGA GAA CGC AAG TTC TTC AGC GAA AAG CAC GAC AGT GGT CGC TAC ATA GCG TGG TTC CAT ACT GGA GGT GAA ATC ACC GAC AGC ATG AAG TCC G)-3′], 200 nM of each primer [5′-d(ATC GCT CGA GAA CGC AAG TT)-3′ forward, 5′-d(CGG ACT TCA TGC TGT CGG TG)-3′ reverse], 0.6x SYBRgreen I, 10 nM Taq DNA polymerase (wt or M1), and for the temperature dependence reactions (see below) 5 nM enzyme, respectively. After an initial RT cycle (95°C for 30 s, 55°C for 35 s and 65°C for 30 min), the product was amplified by 30 PCR cycles (95°C for 30 s, 55°C for 35 s and 72°C for 40 s), and analyzed by melting curve measurement from 30° to 94°C. For DNA templates, the PCR was performed without the RT step. Temperature dependence of reverse transcriptase activity was tested by applying a temperature gradient (from 60° to 72°C for 15 min) during the RT cycle and subsequent amplification as described above.

2.5 Primer extension assay with an RNA template

Reaction mixtures (20 µL) contained 50 mM Tris-HCl (pH 9.2), 16 mM $(NH_4)_2SO_4$, 0.1% Tween 20, 2.5 mM $MgCl_2$, 10 nM Taq DNA polymerase [wt or M1, 150 nM DNA primer (20 nt, 5′-[^{32}P]d(CGT TGG TCC TGA AGG AGG AT)-3′], and 225 nM template RNA (5′-AAA UCA ACC UAU CCU CCU UCA GGA CCA ACG-3′). After an initial denaturation and annealing step (95°C for 2 min, 0.5°C/s cooling to 40°C for 30 s), a temperature gradient (from 60° to 72°C, in detail: 60.1, 60.3, 61.2, 62.5, 63.9, 65.3, 66.7, 68.1, 69.5, 70.8, 71.7, 72.0°C) was applied and the reaction was started by addition of 100 nM dNTPs. After 10 min of incubation the reactions were stopped by addition of gel loading buffer (80% formamide, 20 mM EDTA). Product mixtures were separated by 12% denaturing PAGE and visualized using a Phosphorimager.

2.6 Real time RT-PCR conditions

Real time RT-PCR for Dobrava virus was performed as described [17] using the LightCycler® 480 RNA Master Hydrolysis Probes kit containing an aptamer blocked Tth (RT and DNA polymerase). Taq M1 was tested using the Tris-HCl-$(NH_4)_2SO_4$ buffer described above (pH 9.2) or a bicine buffer

(50 mM Bicine, pH 8.2, 115 mM KOAc, 2.5 mM $MgCl_2$, 8% glycerol). Primer concentrations were 500 nM for the primers and 200 nM for the probe, and the following temperature profile of RT 63°C for 5 min, activation 95°C for 1 min, 45 cycles of two-step PCR 95°C for 5 s and 60°C for 1 min was used for both enzymes. A transcribed quantitative RNA standard was used for sensitivity testing. All tests with the quantitative RNA standard were done in triplicates.

2.7 Real-time RT-PCR using an RNA template that forms stable secondary structure

psiCHECK2 (Promega) plasmids containing either a G-quadruplex structure 5′-d(GGG TGG GTG GGT GGG TGG GTG GG)-3′ or a similar control sequence 5′-d(GTG TGT GTG TGT GGG)-3′ were kindly provided by Prof. Hartig, University of Konstanz [18]. Using primer with a 5′-overhang containing the T7 promoter sequence 5′-d(TAA TAC GAC TCA CTA TAG GGC TTG TCG AGA CAG AGA AGA CTC TTG C)-3′ and the reverse primer 5′-d(CGA TGT GAG GCA CGA CGT GCC TCC)-3′, a 406- or 398-bp DNA fragment was generated. RNA templates with the G-quadruplex sequence 5′-(GGG UGG GUG GGU GGG UGG GUG GG)-3′ or the control sequence 5′-(GUG UGU GUG UGU GGG)-3′ were gained by *in vitro* T7 transcription. Remaining DNA was digested with DNaseI and the RNA purified with the Qiagen RNeasy kit. RiboLock™ RNase inhibitor (40 U) were added to the RNA templates. Reaction mixtures (20 µL) for Taq M1 contained 50 mM Tris-HCl (pH 9.2), 16 mM $(NH_4)_2SO_4$, 0.1% Tween 20, 2.5 mM $MgCl_2$, 1 mM KCl, 250 µM of each dNTP, 0.5 nM RNA template, 200 nM of each primer [5′-d(GGT GTC CAC TCC CAG TTC AAT TAC AG)-3′ forward, 5′-d(GCG TTT GCG TTG CTC GGG GTC GTA CAC C)-3′ reverse)], 0.6x SYBRgreen I, and 20 nM Taq M1 DNA polymerase. Experiments with Roche Titan one Kit were performed with the same template, primer, dNTP, SYBRgreen I and KCl concentrations in the supplied buffer. According to the manual DTT and enzyme mix were added. In the initial RT step a temperature gradient (from 55° to 70°C for 15 min) was applied. The product (133/141 bp) was amplified in 30 PCR cycles (94°C for 2 min, 55°C for 30 s and 68°C for 45 s) and analyzed by melting curve measurement from 30° to 94°C.

3 Results and discussion

Previously, we discovered a thermostable DNA polymerase (KlenTaq M1) with RT-PCR activity

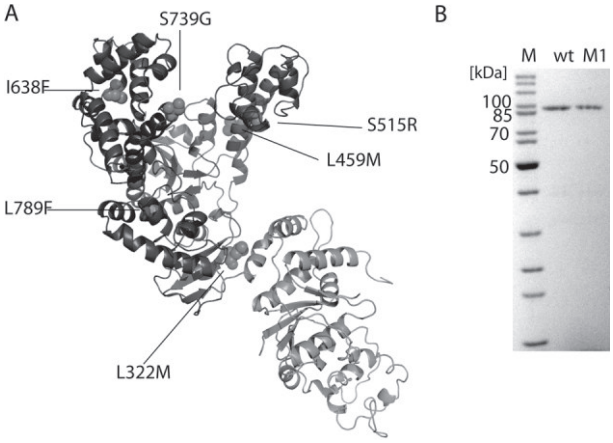

Figure 1. (A) Taq M1 mutations mapped on a ribbon representation of Taq DNA polymerase (PDB code 1TAQ, [19]). KlenTaq domain (dark grey) and nuclease domain (light grey) of Taq M1 are depicted. (B) SDS-PAGE gel of purified Taq DNA polymerases.

[13] by directed enzyme evolution. An overview of the mutations in the KlenTaq domain (dark grey) is shown in Fig. 1A on a ribbon representation of the crystal structure [19]. Here, using the scaffold of KlenTaq M1, we constructed a full-length Taq DNA polymerase (Taq M1) with the respective amino acid mutations of the KlenTaq M1. Taq M1 was overexpressed in *E. coli* cells and purified by Ni-NTA affinity chromatography followed by a gel filtration (see Fig. 1B).

First, we tested whether the mutations of KlenTaq M1 domain influenced the activity of the added

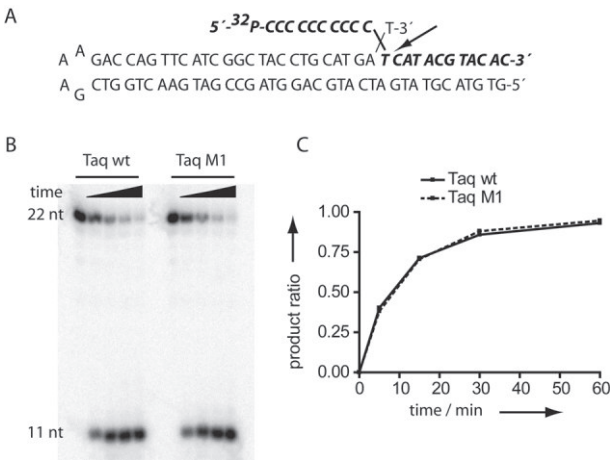

Figure 2. Nuclease activity. (A) Hairpin structure of template and 22-nt substrate (bold). The arrow indicates the expected cleavage position based on reported studies on *E. coli* DNA polymerase I and Taq DNA polymerase [15]. (B) Reaction products separated by denaturing PAGE. (C) Product formation (quantified ratio of product to the sum of product and substrate) after certain time periods (0, 5, 15, 30, 60 min).

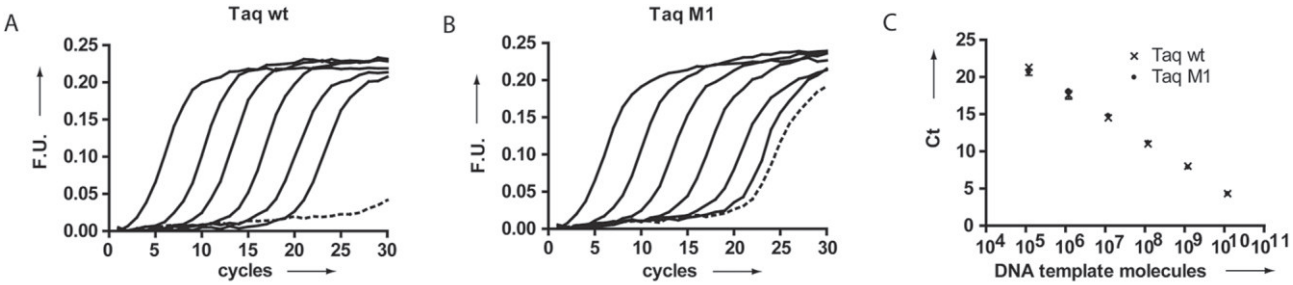

Figure 3. PCR activity test of Taq wt compared to Taq M1. (A, B) Real-time PCR curves of a template dilutions series using Taq wt (A) and Taq M1 (B) including a negative control without template (dashed line). Generally, all reactions were performed in triplicates. (C) Ct values *vs*. detected DNA template molecules.

N-terminal-attached nuclease domain. We used a stable DNA hairpin structure to which a radioactive labeled cleavage substrate anneals at the complementary site (Fig. 2A). This structure harbors a displaced 5′ end and a frayed 3′ primer terminus, and has been shown to be the preferred substrate for cleavage by the nucleases of Taq DNA polymerase and *E. coli* DNA polymerase I [15]. Figures 2B and C show the time-dependent cleavage of the 22-nt substrate resulting in the cleaved shorter product. Taq M1 exhibits nuclease activity similar to that of the wt Taq DNA polymerase (Taq wt). Thus, it appears that the mutations in the polymerase domain have little if any effect on the nuclease activity.

Next, we investigated the PCR activity of Taq M1 compared to Taq wt (Fig. 3). For this, we amplified a 100-nt DNA template, which was diluted tenfold stepwise from 1 nM to 10 fM concentration of template. The resulting real-time PCR amplification curves using SYBRgreen I were measured and are shown in Fig. 3. By comparing the threshold-crossing points (Ct) between Taq wt and Taq M1, we found very similar Ct values and thus the same PCR sensitivity as for Taq wt (Fig. 3C).

To investigate the ability of Taq M1 for RT in comparison with Taq wt, we first conducted primer extension reactions. A 20-nt 5′-[^{32}P]phosphate-labeled DNA primer strand was annealed to its complementary site on a 30-nt RNA template strand. As control we used the respective DNA template. We further conducted the reactions at different temperatures (60–72°C) to find an optimal RT temperature. After 10 min of incubation we analyzed the reaction products by denaturing PAGE. The control reaction in the presence of the respective DNA template yielded, with both employed enzymes, the expected 31-nt full-length product (Fig. 4; C, control reaction). Both enzymes add an additional nucleotide in a non-templated manner as previously observed for 3′-5′-exonuclease-deficient DNA polymerases [20–22]. In contrast, in reactions employing the RNA template, the wt enzyme extended the primer by seven nucleotides and only at temperatures below 65°C. Surprisingly, at higher reaction temperatures, no extension products were visible at all. Using the same reaction conditions, the mutant M1 reverse-transcribed the RNA template significantly more efficiently, and produced the full-length product. Interestingly, the RT efficiency was significantly reduced at temperatures higher than 70°C. Furthermore, by comparing the RT activities between the Taq M1 with the previously evolved KlenTaq M1, we observed a higher RT activity of the Taq variant, which may be due to an increased processivity, which is known for Taq DNA polymerases compared to their nuclease-lacking variants (data not shown) [23].

Next, we performed real-time RT-PCR experiments employing the 3569-nt RNA genome from bacteriophage MS2. The RNA template was diluted stepwise from 10 nM to 10 fM. In chosen set-up, a 100-nt RNA target sequence had to be first reverse-transcribed within 30 min and subsequently amplified according to a standard one-step RT-PCR pro-

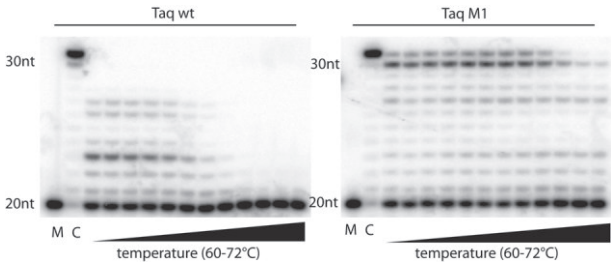

Figure 4. RT primer extension of Taq M1 compared to Taq wt under equal reaction conditions. M, Marker, reaction mix without enzyme; C, control reaction with the corresponding DNA template. Incubation (10 min, 10 nM enzyme concentration) was carried out at different temperatures ranging from 60° to 72°C (from left to right: 60.1, 60.3, 61.2, 62.5, 63.9, 65.3, 66.7, 68.1, 69.5, 70.8, 71.7, 72.0°C).

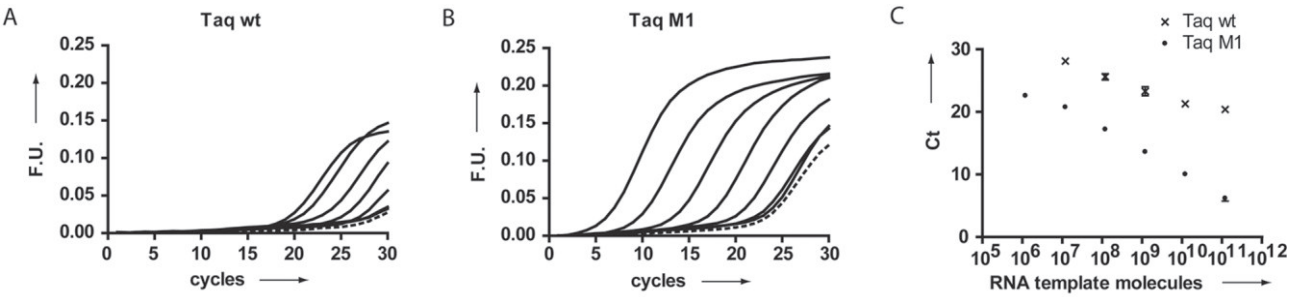

Figure 5. Real-time RT-PCR activity test of Taq wt compared to Taq M1. (A, B) Real-time RT-PCR curves of a tenfold template dilutions series with Taq wt (A) and Taq M1 (B) including a negative control without template (dashed line). Generally, reactions were performed in triplicates. (C) Ct values *vs.* number of RNA template molecules.

tocol (Fig. 5). Interestingly, we found that only the mutated Taq M1 was able to efficiently process the RNA target. Taq wt, in contrast, showed only low PCR activities and amplified the reverse-transcribed RNA target resulting in Ct value differences of more than ~10 cycles depending on the RNA template concentration. These results corroborate previous findings of a low intrinsic RT activity of the Taq DNA polymerase [11, 12]. Inspired by the finding of the temperature dependence of RT reaction (see Fig. 4), we conducted real-time RT-PCR experiments at different temperatures (60–72°C) during the RT step (see Fig. 6) and found a clear temperature dependence of the RT, which is in good agreement to the previously conducted RNA primer extensions (see Fig. 4). The RT optimum reaction temperature with the lowest Ct value was between 63° and 68°C. The efficiency drastically drops when the RT temperature was below 63°C or higher than 70°C.

After having obtained these promising results, we next validated these findings in the detection of pathogenic RNA obtained from natural sources in a case study. To test the performance of the newly generated TaqM1 enzyme in an established virus real-time RT-PCR TaqMan assay, the enzyme was adapted for use in an assay for the detection of Dobrava virus [17]. The real-time RT-PCR assay for Dobrava virus has an analytical sensitivity of 10^2 molecules when using the Roche Kit containing an aptamer blocked Tth DNA polymerase. We found that the TaqM1 enzyme did not perform well in the real-time RT-PCR assay using a Tris-HCl $(NH_4)_2SO_4$-based buffer (pH 9.2) employed in the previous real-time PCR and the extension assays. Better results were obtained with an analytical sensitivity of 10^3 molecules in a less basic 50 mM bicine buffer (pH 8.2). Comparison of the efficiencies of the Dobrava assays ($E = 10^{(-1/slope)} - 1$) of 0.56 and 0.61 for the Tth-based kit and TaqM1 in bicine buffer, however, clearly indicate that the novel TaqM1 enzyme shows a real-time RT-PCR performance comparative to the aptamer-blocked Tth DNA polymerase (Fig. 7).

Finally, we set out to investigate the ability of Taq M1 to reverse-transcribe RNA targets that form stable secondary structures [18]. We chose a well-described RNA sequence that forms a stable G-quadruplex motif, and conducted real-time RT-PCR experiments in comparison with a commercially available kit (Titan One Tube RT-PCR System, Roche) that contains an enzyme mix compris-

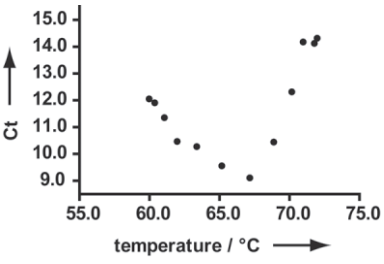

Figure 6. Temperature dependence of Taq M1 reverse transcriptase activity. Resulting Ct values of subsequent amplification *vs.* applied RT temperature. RT reaction (15 min incubation, 5 nM enzyme concentration) was carried out at different temperatures (60–72°C).

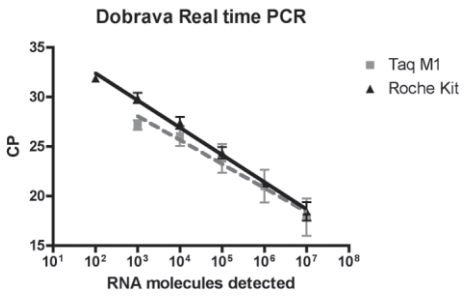

Figure 7. Dobrava virus detection by one-step real-time RT-PCR. Crossing points (CP) are plotted against RNA molecules detected. Each regression line was calculated from a triplicate data set.

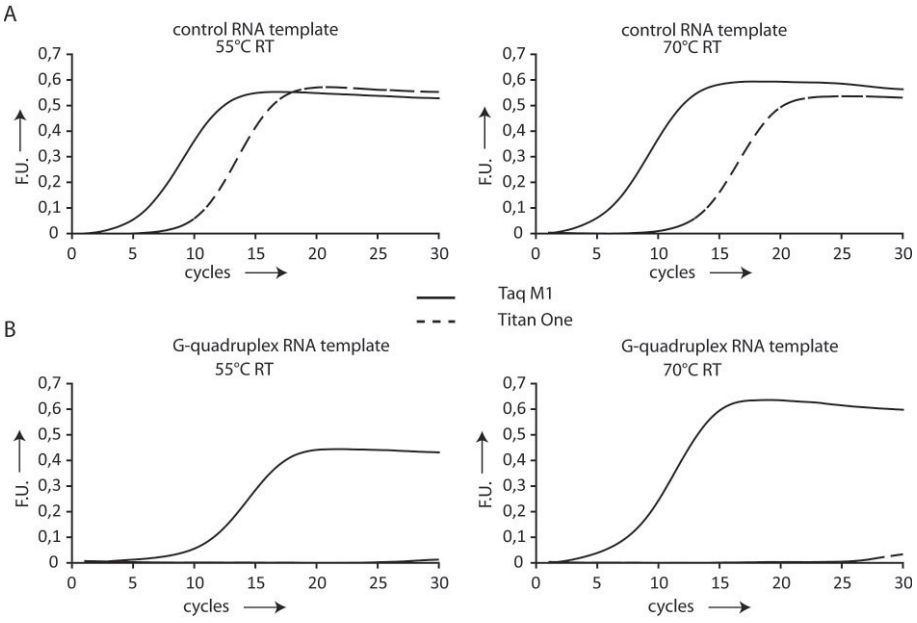

Figure 8. Real-time RT-PCR using a RNA template that forms secondary structure (G-quadruplex motif) (B) compared to a quadruplex-free control template (A). Amplification curves are deriving using Taq M1 (solid line) or a commercial kit with an RT step at 55°C or 70°C for 15 min, respectively.

ing AMV reverse transcriptase and a thermostable DNA polymerase blend (see Fig. 8). Both set-ups showed a strong amplification signal at the standard RT temperature (55°C), using an RNA template unable to form a G-quadruplex motif as a positive control (see Fig. 8A, left side). When applying increased RT temperatures (70°C) a similar PCR curve was obtained for Taq M1, whereas the C_t value of the commercial kit increased from 7 to more than 10, indicating an inactivation of the thermosensitive AMV RT. Using the G-quadruplex-forming RNA target, the commercial system was not able to amplify at the standard RT temperature (here 55°C) or at 70°C. In contrast, Taq M1 showed amplification at both temperatures (see Fig. 8B). These experiments clearly demonstrate the benefits from being able to perform RT at higher temperatures using Taq M1. Thus, this novel enzyme has a high potential for the detection of secondary structure-prone RNA molecules found in RNA viruses or tm-RNA in bacteria [24, 25].

4 Concluding remarks

We successfully combined a nuclease domain to a previously described N-terminal-shortened mutated Taq DNA polymerase [12] that has significantly increased reverse transcriptase activity without significantly compromising polymerase and nuclease function of the resulting chimera Taq M1. We have shown that Taq M1 has similar PCR activity as the Taq wt enzyme. Furthermore, the mutations in the polymerase domain have little effect on the activity of the attached nuclease domain. We have demonstrated that Taq M1 can be used for RT of RNA targets at high temperatures (~60–70°C). The nuclease domain of Taq M1 renders this enzyme highly suitable for any probe-based detection methods, as demonstrated here by the detection of RNA pathogens from natural sources. Noteworthy, without laborious optimization of parameters, detection sensitivities comparable to those of commercially available one-step RT-PCR systems, which are usually based on enzyme blends, were found. We think that the system might be further enhanced by optimizing reaction buffer composition, reaction conditions like pH and reagent concentrations. Further advancements of RNA detection by one-step RT-PCR might be feasible, in particular of complex RNA targets with highly stable secondary structure motifs for which RT at high temperatures is urgently needed. The scaffold of Taq M1 could serve as the basis for further progress along these lines employing directed enzyme evolution [26–30].

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The authors have declared no conflict of interest.

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