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**A mutated thermostable *Thermus aquaticus* DNA polymerase with reverse transcriptase activity for one step RNA pathogen detection**

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**Research Article ((5072 words))****A mutated thermostable *Thermus aquaticus* DNA polymerase with reverse transcriptase activity for one step RNA pathogen detection**

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**Abbreviations:** **CP**, Crossing points; **Ct**, threshold-crossing points; **dNTPs**, deoxynucleotide triphosphates; ***E. coli***, *Escherichia coli*; **KlenTaq**, N-terminal shortened form of a DNA polymerase from *Thermus aquaticus*; **PAGE**, polyacrylamide gel electrophoresis; **PCR**, polymerase chain reaction; **RT**, reverse transcription; **Taq M1**, mutated DNA polymerase from *Thermus aquaticus*; **Tth** *Thermus thermophilus*.

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For Peer Review

**Abstract**

We describe the cloning and characterisation of a mutated thermostable DNA polymerase from *Thermus aquaticus* (Taq) which 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. Indeed, we found similar detection sensitivities compared to commercial available RT-PCR systems without further optimization of reaction parameters thus making this enzyme highly suitable for any PCR probe based RNA detection method.

## 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), the selected DNA sequence framed by the primers is exponentially amplified in theory. Almost in every PCR applications heat-stable DNA polymerases are employed which remain active during the thermal cycling steps necessary to physically separate the two strands of the DNA double helix (usually at high temperatures  $\sim 95^{\circ}\text{C}$ ). 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 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

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2  
3 complementary DNA strand. This is performed by a non-thermostable RNA-  
4  
5 dependent DNA polymerase (reverse transcriptase) followed by real-time  
6  
7 amplification of the transcribed target by a thermostable DNA polymerase [9]. In real  
8  
9 time RT-PCR, fluorescent probes are used to increase the level of specificity and to  
10  
11 avoid detection of non-specific side-products [6, 7, 10]: The fluorescent probe  
12  
13 hybridises to a sequence in-between the flanking primer sequences of the PCR  
14  
15 target. In a TaqMan probe fluorophor and a quencher molecule are covalently  
16  
17 attached to the 5' and 3' end of the probe allowing for Förster resonance energy  
18  
19 transfer (FRET) to occur between both dye molecules, resulting in suppressed  
20  
21 fluorescence of the fluorophor dye. During the PCR extension steps, a DNA  
22  
23 polymerase, which harbours an active nuclease domain, degrades the DNA stretch of  
24  
25 fluorescence probe that is annealed to the target strand. The fluorophor molecule is  
26  
27 cleaved from the probe and released from close proximity to the quencher molecule,  
28  
29 resulting in increased fluorescence. Thus, the generated fluorescence signal is  
30  
31 directly proportional to the amplified target molecules after each cycle. The most  
32  
33 critical step in this method is the conversion from the RNA target into DNA. This  
34  
35 reverse transcription is prone to failure, because RNA often hosts highly stable  
36  
37 secondary structure motifs such as hairpins and G-quadruplexes that complicate or  
38  
39 even prevent reverse transcription [11]. Thus, thermostable reverse transcriptases,  
40  
41 which are able to work at higher temperatures, are of urgent need to increase  
42  
43 reliability and sensitivity of RNA pathogen detection systems. It is known that some  
44  
45 DNA polymerases for example from *Thermus aquaticus* exhibit a low intrinsic RT  
46  
47 activity that is too inefficient for a fast and reliable RT-PCR based RNA detection [12].  
48  
49 Myers and Gelfand [11] reported a DNA polymerase from *Thermus thermophilus*  
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51 (Tth) that exhibits increased RT activity exclusively in the presence of  $Mn^{2+}$  ions, but  
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3 unfortunately for many biotechnological applications like pathogen detection or gene  
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5 expression analysis, employment of  $Mn^{2+}$  is inappropriate [9].  
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7

8 We previously evolved a N-terminal shortened form of a DNA polymerase from  
9  
10 *Thermus aquaticus* (KlenTaq) with increased reverse transcriptase activity [13]. In  
11  
12 order to investigate whether the full-length Taq DNA polymerase mutant (henceforth  
13  
14 called Taq M1) including the nuclease domain would be applicable in real time one  
15  
16 step detection of pathogenic DNA using TaqMan probes, we fused the nuclease  
17  
18 domain to the prior generated mutant KlenTaq. We demonstrate the successful  
19  
20 generation of the nuclease activity under conservation of the previously evolved  
21  
22 reverse transcriptase activity. Our results show that Taq M1 has similar PCR  
23  
24 sensitivity and nuclease activity as the respective wild-type Taq DNA polymerase  
25  
26 however, exhibits reverse transcriptase ability. In addition, we demonstrate the  
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28 usefulness of Taq M1 for fast and reliable RNA pathogen detection in a case study  
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30 for the detection of RNA from Dobrava virus and its advantages in RT-PCR using  
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32 RNA targets that form stable secondary structure motifs.  
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## 41 **2 Materials and methods**

### 42 **2.1 Reagents and Instruments**

43  
44 Oligonucleotides were purchased from Purimex or Metabion, Germany. High Pure  
45  
46 PCR Cleanup Micro Kit, High Pure Plasmid Isolation Kit, and RNA from  
47  
48 Bacteriophage MS2 were from Roche. RNeasy Mini Kit and QIAquick Gel Extraction  
49  
50 Kit were purchased from Qiagen. PageRuler unstained Protein Ladder, DNaseI,  
51  
52 RiboLock™ RNase Inhibitor and Rapid DNA Ligation Kit were purchased from  
53  
54 Fermentas. Real-time PCR was performed in iCycler or Chromo4 instrument from  
55  
56 BioRAD. SYBRgreenI was purchased from Molecular Probes. Denaturing PAGE was  
57  
58 analysed with a Molecular Imager Fx from BioRAD. Phusion DNA polymerase,  
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3 Antarctic Phosphatase, Low MW ladder, EcoRV, and BsmBI were purchased from  
4  
5 New England Biolabs.  
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## 10 **2.2 Cloning, Protein Expression and Purification of Taq M1 Polymerase**

11  
12 Respective plasmids (pASK-IBA37plus) harbouring KlenTaq M1 and Taq wild-type  
13  
14 gene were isolated from the respective *E. coli* cultures using the High Pure Plasmid  
15  
16 Isolation Kit. The KlenTaq M1 polymerase gene [14] was amplified by using Phusion  
17  
18 DNA polymerase with the forward primer (5'-GAT CTA CGT CTC CGC CCT GGA  
19  
20 GGA GGC CC-3') and reverse primer (5'-CAG GTC AAG CTT AGT TAG ATA TCA  
21  
22 CTC C-3'). Taq nuclease domain DNA [15] including the whole pASK-IBA37plus  
23  
24 plasmid sequence was amplified by using Phusion DNA polymerase with the forward  
25  
26 primer (5'-GCC AAG GAG TGA TAT CTA ACT AAG CT-3') and reverse primer (5'-  
27  
28 ATG ATC CGT CTC AGG GCC TTG GGG CTT TCC AGA A-3'). Both amplicates  
29  
30 were purified by 0.8% agarose gel and isolated using the QIAquick Gel Extraction Kit.  
31  
32 Isolated DNA was digested by EcoRV and BsmBI, purified using the High Pure PCR  
33  
34 Cleanup Micro Kit. The Taq nuclease domain amplicate was dephosphorylated  
35  
36 using Antarctic Phosphatase and ligated with the KlenTaq amplicate by using the  
37  
38 Rapid DNA Ligation Kit and transformed into electro competent *E. coli* XL10 gold  
39  
40 cells. Clones were picked from agar plates and separately grown overnight in LB  
41  
42 medium (100 µg/ml carbenicillin). Integrity of whole mutant clone was proved by  
43  
44 sequencing of the respective purified plasmid (GATC Biotech AG, Germany) using  
45  
46 the sequencing primers p1 - p5 (p1 5'-GAG TTA TTT TAC CAC TCC CT-3', p2 5'-  
47  
48 CCT GGC TTT GGG AAA AG-3', p3 5'-CCC GAG CCT TAT AAA GC-3', p4 5'-CGT  
49  
50 AAG GGA TGG CTA GCT CC-3', p5 5'-CGC AGT AGC GGT AAA CG-3'). Enzyme  
51  
52 purification and concentration determination was conducted as previously described  
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60 [13, 16].

### 2.3 Assay of nuclease activity

Reaction mixtures (60  $\mu$ l) contained 50 mM Tris·HCl (pH 9.2), 16 mM  $(\text{NH}_4)_2\text{SO}_4$ , 0.1% Tween 20, 2.5 mM  $\text{MgCl}_2$ , 50 nM of each dNTP, 150 nM substrate DNA (22 nt, 5'- $^{32}\text{P}$ ]-CCC CCC CCC CTC ATA CGT ACA C-3', 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). 5  $\mu$ l aliquots 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 analysed by 12 % denaturing PAGE and quantified using a Phosphorimager.

### 2.4 Real-time PCR, template dilution series

Reaction mixtures (20  $\mu$ l) contained 50 mM Tris-HCl (pH 9.2), 16 mM  $(\text{NH}_4)_2\text{SO}_4$ , 0.1% Tween 20, 2.5 mM  $\text{MgCl}_2$ , 250  $\mu$ M 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), 0.6 x SYBRgreenI, 10 nM Taq DNA polymerase wt / M1 and for the temperature dependence reactions (vide infra) 5 nM enzyme respectively. After an initial reverse transcription cycle (95°C for 30 sec, 55°C for 35 sec and 65°C for 30

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3 min) the product was amplified by 30 PCR cycles (95 °C for 30 sec, 55 °C for 35 sec  
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5 and 72 °C for 40 sec) and following melting curve measurement from 30-94 °C. In  
6  
7 case of DNA templates the PCR was performed without the RT step. Temperature  
8  
9 dependence of reverse transcriptase activity was tested by applying a temperature  
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11 gradient (from 60-72 °C for 15 min) during the reverse transcription cycle and  
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13 subsequent amplification as described above.  
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## 20 **2.5 Primer extension assay with an RNA template**

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22 Reaction mixtures (20 µl) contained 50 mM Tris·HCl (pH 9.2), 16 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>,  
23  
24 0.1% Tween 20, 2.5 mM MgCl<sub>2</sub>, 10 nM Taq DNA polymerase wt or M1, 150 nM DNA  
25  
26 primer (20 nt, 5'-[<sup>32</sup>P]-d(CGT TGG TCC TGA AGG AGG AT)-3'), 225 nM template  
27  
28 RNA (5'- AAA UCA ACC UAU CCU CCU UCA GGA CCA ACG-3'). After an initial  
29  
30 denaturation and annealing step (95 °C for 2 min, 0.5 °C/s cooling to 40 °C for 30 sec),  
31  
32 a temperature gradient (from 60–72 °C, in detail: 60.1, 60.3, 61.2, 62.5, 63.9, 65.3,  
33  
34 66.7, 68.1, 69.5, 70.8, 71.7, 72.0 °C) was applied and the reaction was started by  
35  
36 addition of 100 nM dNTPs. After 10 min of incubation the reactions were stopped by  
37  
38 addition of gel loading buffer (80% formamide, 20 mM EDTA). Product mixtures were  
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40 separated by 12% denaturing PAGE and visualised using a Phosphorimager.  
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## 48 **2.6 Real time RT-PCR conditions**

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50 Real time RT-PCR for Dobrava virus was performed as described [17] using the  
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52 LightCycler<sup>®</sup> 480 RNA Master Hydrolysis Probes kit containing an aptamer blocked  
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54 Tth (RT and DNA polymerase). Taq M1 was tested using the Tris·HCl-(NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>-  
55  
56 buffer described above (pH 9.2) or a bicine buffer (50 mM Bicine (pH 8.2), 115 mM  
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58 KOAc, 2.5 mM MgCl<sub>2</sub>, 8% glycerol). Primer concentrations were 500 nM for the  
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60 primers and 200 nM for the probe and the following temperature profile of RT 63 °C 5

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3 min, Activation 95°C, 1 min, 45 cycles of 2-step PCR 95°C, 5 sec and 60°C 1 min  
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5 was used for both enzymes. A transcribed quantitative RNA standard was used for  
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7 sensitivity testing. All tests with the quantitative RNA standard were done in  
8  
9 triplicates.  
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## 12 13 14 15 **2.7 Real time RT PCR using a RNA template that form stable secondary** 16 17 **structure**

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19 psiCHECK2 (Promega) plasmids containing either a G-quadruplex structure 5'-  
20 d(GGG TGG GTG GGT GGG TGG GTG GG)-3' or a similar control sequence 5'-  
21 d(GTG TGT GTG TGT GGG)-3' were kindly provided by Prof. Hartig, University of  
22  
23 Konstanz. [18] By using primer with a 5'-overhang containing the T7 promoter  
24  
25 sequence 5'-d(TAA TAC GAC TCA CTA TAG GGC TTG TCG AGA CAG AGA AGA  
26  
27 CTC TTG C)-3' and the reverse primer 5'-d(CGA TGT GAG GCA CGA CGT GCC  
28  
29 TCC)-3' a 406 respectively 398 base pair DNA fragment was generated. RNA  
30  
31 templates with the G-quadruplex sequence 5'-(GGG UGG GUG GGU GGG UGG  
32  
33 GUG GG)-3' or the control sequence 5'-(GUG UGU GUG UGU GGG)-3' were gained  
34  
35 by in vitro T7 transcription. Remaining DNA was digested with DNaseI and the RNA  
36  
37 purified with the Qiagen RNeasy kit. 40 units of RiboLock™ RNase Inhibitor were  
38  
39 added to the RNA templates. Reaction mixtures (20 µl) for Taq M1 contained 50 mM  
40  
41 Tris-HCl (pH 9.2), 16 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.1% Tween 20, 2.5 mM MgCl<sub>2</sub>, 1 mM KCl, 250  
42  
43 µM of each dNTP, 0.5 nM RNA-Template, 200 nM of each primer (5'-d(GGT GTC  
44  
45 CAC TCC CAG TTC AAT TAC AG)-3' forward, 5'-d(GCG TTT GCG TTG CTC GGG  
46  
47 GTC GTA CAC C)-3' reverse), 0.6 x SYBRgreenI, 20 nM Taq M1 DNA polymerase.  
48  
49 Experiments with Roche Titan one Kit were performed with the same template,  
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51 primer, dNTP, SYBRgreenI and KCl concentrations in the supplied buffer. According  
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53 to the manual DTT and enzyme mix were added. In the initial reverse transcription  
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3 step a temperature gradient (from 55-70°C for 15 min) was applied. The product  
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5 (133/141 base pairs) was amplified in 30 PCR cycles (94°C for 2min, 55°C for 30 sec  
6  
7 and 68°C for 45 sec) and analyzed by melting curve measurement from 30-94°C.  
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### 10 11 12 **3 Results and discussion**

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15 Previously, we discovered a thermostable DNA polymerase (KlenTaq M1) with RT  
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17 PCR activity [13] by directed enzyme evolution. An overview of the mutations in the  
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19 KlenTaq domain (dark blue) is shown in Figure 1A on a ribbon representation of the  
20  
21 crystal structure [19]. Here, by using the scaffold of KlenTaq M1 we constructed a full  
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23 length Taq DNA polymerase (Taq M1) with the respective amino acid mutations of  
24  
25 the KlenTaq M1. Taq M1 was over-expressed in *E. coli* cells and purified by Ni-NTA  
26  
27 affinity chromatography followed by a gel filtration (see Figure 1b).  
28  
29

30  
31 First, we tested if the mutations of KlenTaq M1 domain influence the activity of the  
32  
33 added N-terminal attached nuclease domain. Therefore we used a stable DNA  
34  
35 hairpin structure to which a radioactive labelled cleavage substrate anneals at the  
36  
37 complementary site (Figure 2A). This structure harbours a displaced 5' end and a  
38  
39 frayed 3' primer terminus and has been shown to be the preferred substrate for  
40  
41 cleavage by the nucleases of Taq DNA polymerase and *E. coli* DNA polymerase I  
42  
43 [15]. Figures 2B, C show the time-dependent cleavage of the 22 nt substrate  
44  
45 resulting in the cleaved shorter product. Taq M1 exhibits similar nuclease activity  
46  
47 than the wild-type Taq DNA polymerase (Taq wt). Thus, it appears that the mutations  
48  
49 in the polymerase domain have little if any effect on the nuclease activity.  
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53 Next, we investigated the PCR activity of Taq M1 compared to Taq wt (Figure 3). For  
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55 this, we amplified a 100 nt long DNA template which was diluted tenfold stepwise  
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57 from 1 nM to 10 fM concentration of template. The resulting real-time PCR  
58  
59 amplification curves using SYBRgreen I were measured and are shown in Figure 3.  
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3 By comparing the threshold-crossing points (Ct) between Taq wt and Taq M1 we  
4 found very similar Ct values and thus the same PCR sensitivity as for Taq wt  
5  
6  
7  
8 (Figure 3C).  
9

10 To investigate the ability of Taq M1 for reverse-transcription (RT) in comparison with  
11 Taq wt we first conducted primer extension reactions. A 20 nucleotide 5'-<sup>32</sup>P-  
12 phosphate labelled DNA primer strand was annealed to its complementary site on a  
13 30 nt RNA template strand. As control we used the respective DNA template. We  
14  
15 further conducted the reactions at different temperatures ranging from 60-72°C to  
16  
17 find an optimal RT temperature. After 10 minutes incubation we analyzed the reaction  
18  
19 products by denaturing PAGE. The control reaction in the presence of the respective  
20  
21 DNA template yields with both employed enzymes the expected 31 nt long full-length  
22  
23 product (Figure 4, C = control reaction). Both enzymes add an additional nucleotide  
24  
25 in non-templated manner as it has been observed for 3'-5'-exonuclease deficient  
26  
27 DNA polymerases before [20-22]. On the contrary, in reactions employing the RNA  
28  
29 template the wt enzyme extends the primer by seven nucleotides and only at  
30  
31 temperatures below 65°C. Surprisingly at higher reaction temperatures no extension  
32  
33 products were visible at all. Whereas using the same reaction conditions, mutant M1  
34  
35 reverse transcribes the RNA template significantly more efficient and produces the  
36  
37 full-length product. Interestingly the reverse transcription efficiency is significantly  
38  
39 reduced at temperatures higher than 70°C. Furthermore, by comparing the RT  
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41 activities between the Taq M1 with the previously evolved KlenTaq M1 we observed  
42  
43 a higher RT activity of the Taq variant which may be due to an increased processivity  
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45 which is known for Taq DNA polymerases compared to their nuclease lacking  
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47 variants (data not shown) [23].  
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60 Next, we performed real-time RT-PCR experiments employing the 3569 nt long RNA  
genome from bacteriophage MS2. The RNA template was diluted stepwise from 10

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3 nM to 10 fM. In chosen set-up a 100 nt RNA target-sequence had to be first reverse  
4 transcribed within 30 min and subsequently amplified according to a standard one-  
5 step RT-PCR protocol (Figure 5). Interestingly, we found that only the mutated Taq  
6 M1 is able to efficiently process the RNA target. Taq wt in contrast showed only low  
7 PCR activities and amplified the reverse transcribed RNA target resulting in Ct value  
8 differences of more than ~10 cycles depending on the RNA template concentration.  
9  
10 These results corroborate previous findings of a low intrinsic RT activity of Taq DNA  
11 polymerase [11, 12]. Inspired by the finding of the temperature dependence of RT  
12 reaction (see Figure 4) we conducted real-time RT PCR experiments at different  
13 temperatures (ranging from 60-72°C) during the RT step (see Figure 6) and found a  
14 clear temperature dependence of the RT which is in good agreement to the  
15 previously conducted RNA primer extensions (see Figure 4). The RT optimum  
16 reaction temperature with the lowest Ct value is between 63-68°C. The efficiency  
17 drastically drops when the RT temperature is below 63°C or higher than 70°C.  
18  
19

20 After having obtained these promising results, we next validated these findings in the  
21 detection of pathogenic RNA obtained from natural sources in a case study. To test  
22 the performance of the newly generated TaqM1 enzyme in an established virus real-  
23 time RT-PCR TaqMan assay the enzyme was adapted for use in an assay for the  
24 detection of Dobrava virus [17]. The real time RT-PCR assay for Dobrava virus has  
25 an analytical sensitivity of  $10^2$  molecules when using the Roche Kit containing an  
26 aptamer blocked Tth DNA polymerase. We found that the TaqM1 enzyme did not  
27 perform well in the real-time RT-PCR assay using a Tris-HCl  $(\text{NH}_4)_2\text{SO}_4$ -based buffer  
28 (pH 9.2) employed in the previous real-time PCR and the extension assays. Better  
29 results were obtained with an analytical sensitivity of  $10^3$  molecules in a less basic 50  
30 mM bicine buffer (pH 8.2). Comparison of the efficiencies of the Dobrava assays ( $E$   
31  $=10^{(-1/\text{slope})-1}$ ) of 0.56 and 0.61 for Tth based kit and TaqM1 in bicine buffer, however  
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3 clearly indicate that the novel TaqM1 enzyme shows a real-time RT-PCR  
4 performance comparative to the aptamer blocked Tth DNA polymerase.  
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7  
8 Finally, we set out to investigate the ability of Taq M1 to reverse transcribe RNA  
9 targets that form stable secondary structures [18]. Therefore, we chose a well  
10 described RNA sequence, which is forming a stable G-quadruplex motif and  
11 conducted real time RT-PCR experiments in comparison with a commercial available  
12 kit (Titan One Tube RT-PCR System, Roche) that contains an enzyme mix  
13 comprising AMV reverse transcriptase (RT) and a thermostable DNA polymerase  
14 blend (see Figure 8). Both set-ups show a strong amplification signal at standard RT  
15 temperatures (55 °C) using an RNA template unable to form a G-quadruplex motif, as  
16 a positive control (see Figure 8a, left side). When applying increased RT  
17 temperatures (70 °C) nearly the same PCR curve is obtained for Taq M1 whereas the  
18  $C_t$  value of the commercial kit increased from 7 to more than 10 indicating an  
19 inactivation of the thermosensitive AMV RT. Using the G-quadruplex forming RNA  
20 target the commercial system was neither able to amplify at standard RT  
21 temperatures (here 55 °C) nor at 70 °C. On the contrary, Taq M1 showed amplification  
22 at both temperatures. These experiments clearly demonstrate the benefits from being  
23 able to perform reverse transcription at higher temperatures by Taq M1. Thus, this  
24 novel enzyme has a high potential for the detection of secondary structure prone  
25 RNA molecules found in RNA viruses or tm-RNA in bacteria [24, 25].  
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#### 51 52 53 **4 Concluding remarks**

54 We successfully combined a nuclease domain to a previously described N-terminal  
55 shortened mutated Taq DNA polymerase [12] that has significantly increased reverse  
56 transcriptase activity without significantly compromising polymerase and nuclease  
57 function of the resulting chimera Taq M1. It is shown that Taq M1 has similar PCR  
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3 activity as the Taq wt enzyme. Furthermore, the mutations in the polymerase domain  
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5 have little effect on the activity of the attached nuclease domain. We demonstrate  
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7 that Taq M1 can be used for reverse transcription of RNA targets at high  
8  
9 temperatures (~60-70°C). The nuclease domain of Taq M1 renders this enzyme  
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11 highly suitable for any probe based detection methods. We demonstrated this in the  
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13 detection of RNA pathogens from natural sources. Noteworthy, without laborious  
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15 optimisation of parameters comparable detection sensitivities than commercially  
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17 available one-step RT-PCR systems, which are usually based on enzyme blends,  
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19 were found. We think that the system might be further enhanced by optimizing  
20  
21 reaction buffer composition, reaction conditions like pH and reagent concentrations.  
22  
23 Further advancements of RNA detection by one-step RT-PCR might be feasible in  
24  
25 particular of complex RNA targets with highly stable secondary structure motifs in  
26  
27 which reverse transcription at high temperatures is of urgent need. The scaffold of  
28  
29 Taq M1 could serve as the basis for further progress along these lines employing  
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31 directed enzyme evolution [26-30].  
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43 *4A008 of the BMVg is gratefully acknowledged.*  
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## 5 References

- [1] Schmaljohn, C., Hjelle, B., Hantaviruses: a global disease problem. *Emerg. Infect. Dis.* 1997, 3, 95-104.
- [2] Palese, P., Influenza: old and new threats. *Nat. Medicine* 2004, 10, 82–87.
- [3] Morens, D. M., Folkers, G. K., Fauci, A. S., The challenge of emerging and re-emerging infectious diseases. *Nature* 2004, 430, 242-249.
- [4] De Paula, S. O., Fonseca, B. A., Dengue: a review of the laboratory tests a clinician must know to achieve a correct diagnosis. *Braz. J. Infect. Dis.* 2004, 8, 390-398.
- [5] Mullis, K. B., Faloona, F. A., Specific synthesis of DNA in vitro via a polymerase-catalyzed chain reaction. *Methods Enzymol.* 1987, 155, 335-350.
- [6] Holland, P. M., Abramson, R. D., Watson, R., Gelfand, D. H., Detection of specific polymerase chain reaction product by utilizing the 5'-3' exonuclease activity of *Thermus aquaticus* DNA polymerase. *Proc. Natl. Acad. Sci. USA* 1991, 88, 7276-7280.
- [7] Strerath, M., Marx, A., Genotyping – From genomic DNA to genotype in a single tube. *Angew. Chem. Int. Ed. Engl.* 2005, 44, 7842-7849.
- [8] Lynch, J. R., Brown, J. M., The polymerase chain reaction: current and future clinical applications. *J. Med. Genet.* 1990, 27, 2-7.
- [9] Bustin, S. A., Mueller, R., Real-time reverse transcription PCR (qRT-PCR) and its potential use in clinical diagnosis. *Clin. Sci.* 2005, 109, 365–379.
- [10] Nazarenko, I. A., Bhatnagar, S. K., Hohman, R. J., A closed tube format for amplification and detection of DNA based on energy transfer. *Nucleic Acids Res.* 1997, 25, 2516-2521.
- [11] Myers, T. W., Gelfand, D. H., Reverse transcription and DNA amplification by a *Thermus thermophilus* DNA polymerase. *Biochemistry* 1991, 30, 182-192.

- 1  
2  
3 [12] Jones, M. D., Foulkes, N. S., Reverse transcription of mRNA by *Thermus*  
4 *aquaticus* DNA polymerase. *Nucleic Acids Res.* 1989, *17*, 8387-8388.  
5  
6  
7  
8 [13] Sauter, K. B. M., Marx, A., Evolving thermostable reverse transcriptase activity in  
9 a DNA polymerase scaffold. *Angew. Chem. Int. Ed.* 2006, *45*, 7633-7635.  
10  
11 [14] Barnes, W. M., The fidelity of Taq polymerase catalyzing PCR is improved by an  
12 N-terminal deletion. *Gene* 1992, *112*, 29–35.  
13  
14 [15] Lyamichev, V., Brow, M. A. D., Varvel, V. E., Dahlberg, J. E., Comparison of the  
15 5' nuclease activities of Taq DNA polymerase and its isolated nuclease domain.  
16 *Proc. Natl. Acad. Sci. USA* 1999, *96*, 6143-6148.  
17  
18 [16] Summerer, D., Rudinger, N. Z., Detmer, I., Marx, A., Enhanced DNA Polymerase  
19 Mismatch Extension Fidelity by Directed Combinatorial Enzyme Design *Angew.*  
20 *Chem. Int. Ed.* 2005, *44*, 4712-4715.  
21  
22 [17] Weidmann, M., Schmidt, P., Vackova, M., Krivanec, K., *et al.*, Identification of  
23 genetic evidence for dobrava virus spillover in rodents by nested reverse transcription  
24 (RT)-PCR and TaqMan RT-PCR. *J. Clin. Microbiol.* 2005, *43*, 808-812.  
25  
26 [18] Halder, K., Wieland, M., Hartig, J. S., Predictable suppression of gene  
27 expression by 5'-UTR-based RNA quadruplexes. *Nucleic Acids Res.* 2009, epub.  
28 PMID: 19740765  
29  
30 [19] Li, Y., Korolev, S., Waksman, G., Crystal structures of open and closed forms of  
31 binary and ternary complexes of the large fragment of *Thermus aquaticus* DNA  
32 polymerase I: structural basis for nucleotide incorporation. *EMBO J.* 1998, *17*,  
33 7514-7525  
34  
35 [20] Clark, J. M., Novel non-templated nucleotide addition reactions catalyzed by  
36 procaryotic and eucaryotic DNA polymerases. *Nucleic Acids Res.* 1988, *16*, 9677–  
37 9686.  
38  
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3 [21] Holzberger, B., Marx, A., Enzymatic synthesis of perfluoroalkylated DNA. *Bioorg.*  
4  
5 *Med. Chem.* 2009, 17, 3653-3658.  
6  
7  
8 [22] Kranaster, R., Marx, A., Taking Fingerprints of DNA Polymerases: Multiplex  
9  
10 Enzyme Profiling on DNA Arrays. *Angew. Chem. Int. Ed. Engl.* 2009, 48, 4625-4628.  
11  
12 [23] Wang, Y., Prosen, D. E., Mei, L., Sullivan, J. C., Finney, M., Vander Horn, P. B.,  
13  
14 A novel strategy to engineer DNA polymerases for enhanced processivity and  
15  
16 improved performance in vitro. *Nucleic Acids Res.* 2004, 32, 1197-1207.  
17  
18 [24] Kuo, K. W., Leung, M. F., Leung, W. C., Intrinsic secondary structure of human  
19  
20 TNFR-I mRNA influences the determination of gene expression by RT-PCR. *Mol.*  
21  
22 *Cell. Biochem.* 1997, 177, 1-6.  
23  
24  
25 [25] O'Grady, J., Ruttledge, M., Sedano-Balbas, S., Smith, T. J., *et al.*, Rapid  
26  
27 detection of *Listeria monocytogenes* in food using culture enrichment combined with  
28  
29 real-time PCR. *Food Microbiol.* 2009, 26, 4-7.  
30  
31  
32 [26] Ghadessy, F. J., Ong, J. L., Holliger, P. Directed evolution of polymerase  
33  
34 function by compartmentalized self-replication. *Proc. Natl. Acad. Sci. USA* 2001, 98,  
35  
36 4552-4557.  
37  
38  
39 [27] Xia, G., Chen, L., Sera, T., Fa, M. *et al.*, Directed evolution of novel polymerase  
40  
41 activities: mutation of a DNA polymerase into an efficient RNA polymerase. *Proc.*  
42  
43 *Natl. Acad. Sci. USA* 2002, 99, 6597-6602.  
44  
45  
46 [28] Vichier-Guerre, S., Ferris, S., Auberge, N., Mahiddine, K., *et al.*, A population of  
47  
48 thermostable reverse transcriptases evolved from *Thermus aquaticus* DNA  
49  
50 polymerase I by phage display. *Angew. Chem. Int. Ed. Engl.* 2006, 45, 6133-6137.  
51  
52  
53 [29] Patel, P. H., Loeb, L. A., Multiple amino acid substitutions allow DNA  
54  
55 polymerases to synthesize RNA. *J. Biol. Chem.* 2000, 275, 40266-40272.  
56  
57  
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2  
3 [30] Summerer, D., Rudinger, N. Z., Detmer, I., Marx, A., Enhanced fidelity in  
4 mismatch extension by DNA polymerase through directed combinatorial enzyme  
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7  
8 design. *Angew. Chem. Int. Ed. Engl.* 2005, 44, 4712-4715.  
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7 Figure 1. (A) Taq M1 mutations mapped on a ribbon representation of Taq DNA  
8 polymerase (PDB code 1TAQ, [19]). KlenTaq domain (deep blue) and nuclease  
9 domain (light blue) of Taq M1 are depicted. (B) SDS-PAGE gel of purified Taq DNA  
10 polymerases.  
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19 Figure 2. Nuclease activity (A) Hairpin structure of template and 22 nt substrate  
20 (bold). The arrow indicates the expected cleavage position based on reported studies  
21 on *E. coli* DNA polymerase I and Taq DNA polymerase [15]. (B) Reaction products  
22 separated by denaturing PAGE. (C) Product formation (quantified ratio of product to  
23 the sum of product and substrate) after certain time periods (0, 5, 15, 30, 60 min).  
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33 Figure 3. PCR activity test of Taq wt compared to Taq M1.

34 (A) and (B) Real-time PCR curves of a template dilutions series using Taq wt (A) and  
35 Taq M1 (B) including a negative control without template (dashed line). Generally, all  
36 reactions were performed in triplicates. (C) Ct values vs. detected DNA template  
37 molecules.  
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48 Figure 4. Reverse transcription primer extension of Taq M1 compared to Taq wt  
49 under equal reaction conditions. M = Marker, reaction mix without enzyme. C =  
50 control reaction with the corresponding DNA template. Incubation (10 min, 10 nM  
51 enzyme concentration) was carried out at different temperatures ranging from 60-  
52 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,  
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60 72.0 °C).

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3 Figure 5. Real-time RT PCR activity test of Taq wt compared to Taq M1.

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

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 ranging from 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 a  
reverse transcription step at 55°C or 70°C for 15 min, respectively.

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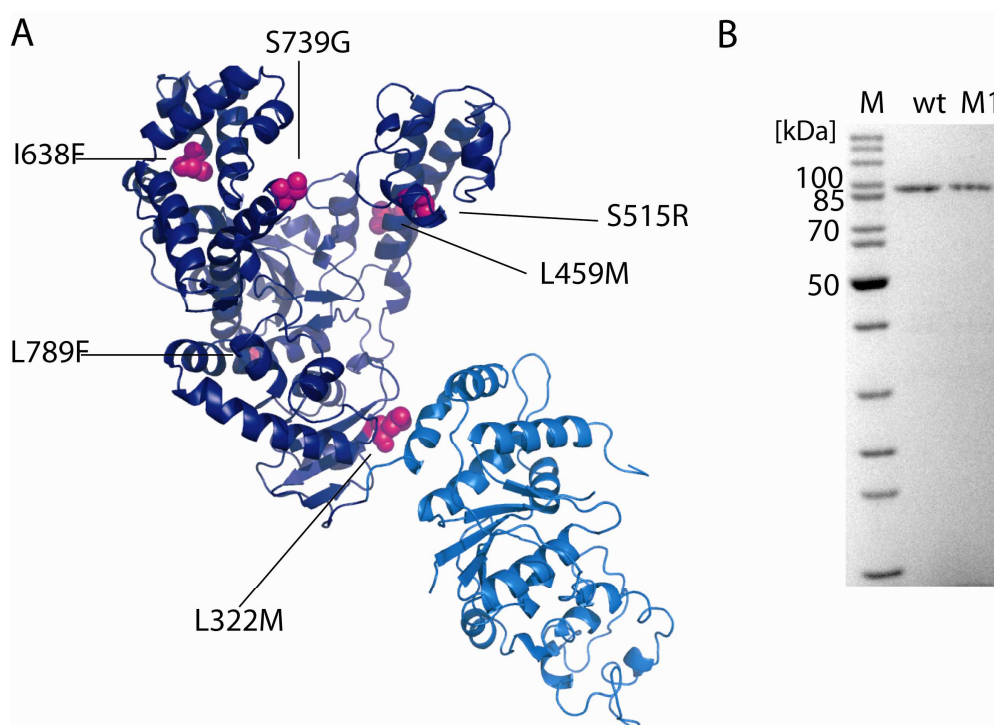


Figure 1. (A) Taq M1 mutations mapped on a ribbon representation of Taq DNA polymerase (PDB code 1TAQ, [19]). KlenTaq domain (deep blue) and nuclease domain (light blue) of Taq M1 are depicted. (B) SDS-PAGE gel of purified Taq DNA polymerases.



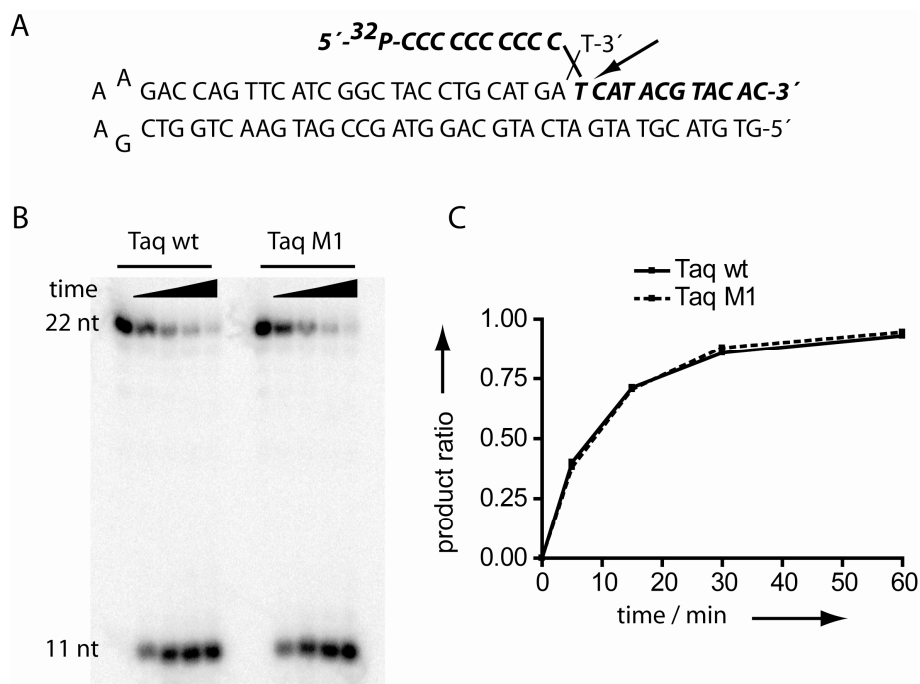


Figure 2. Nuclease activity (A) Hairpin structure of template and 22 nt substrate (bold). The arrow indicates the expected cleavage position based on reported studies on *E. coli* DNA polymerase I and Taq DNA polymerase [15]. (B) Reaction products separated by denaturing PAGE. (C) Product formation (quantified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

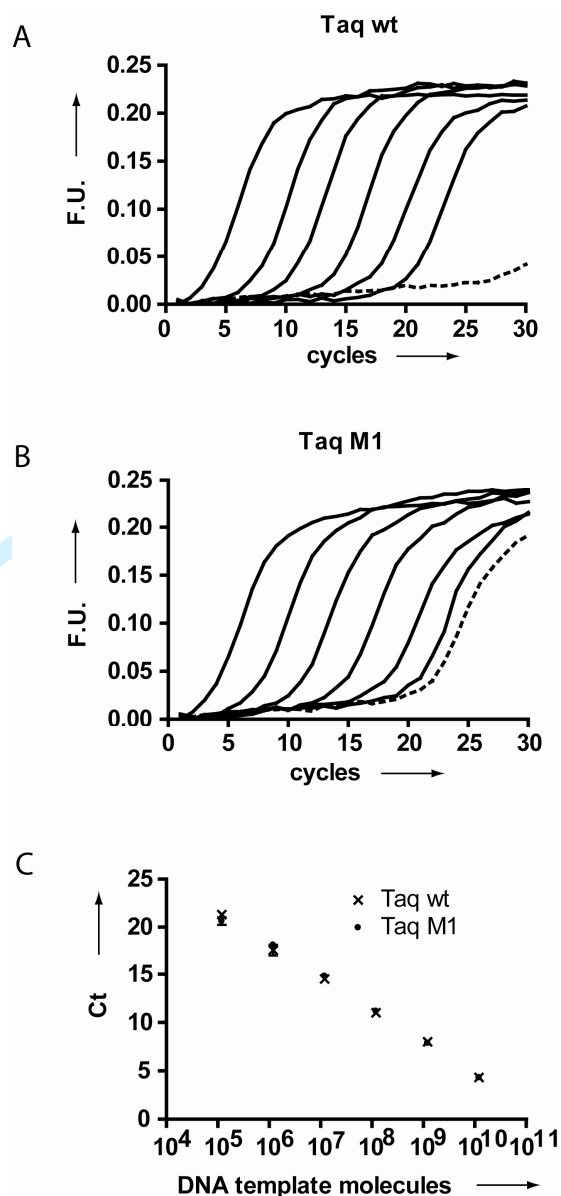


Figure 3. PCR activity test of Taq wt compared to Taq M1.

(A) and (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.

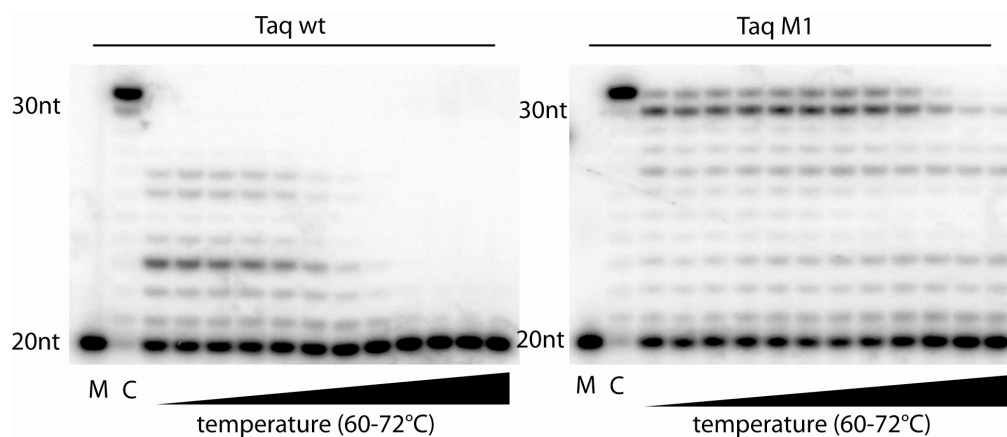


Figure 4. Reverse transcription primer extension of Taq M1 compared to Taq wt under equal reaction conditions. M = Marker, reaction mix without enzyme. C = control reaction with the corresponding DNA template. Incubation (10 min, 10 nM enzyme concentration) was carried out at different temperatures ranging from 60-72 °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).

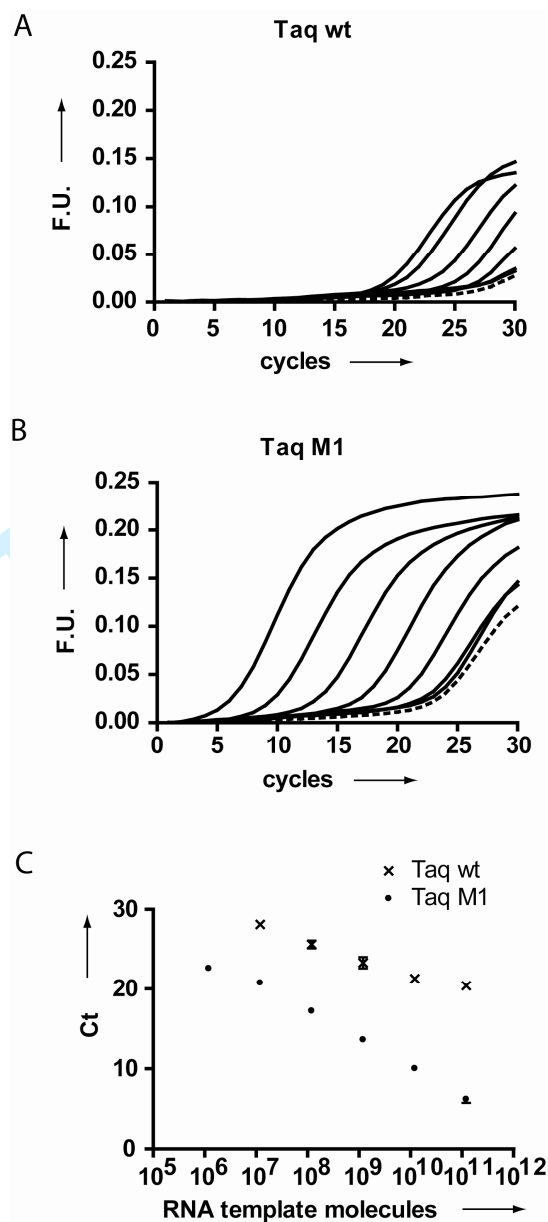


Figure 5. Real-time RT PCR activity test of Taq wt compared to Taq M1.

(A) and (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.

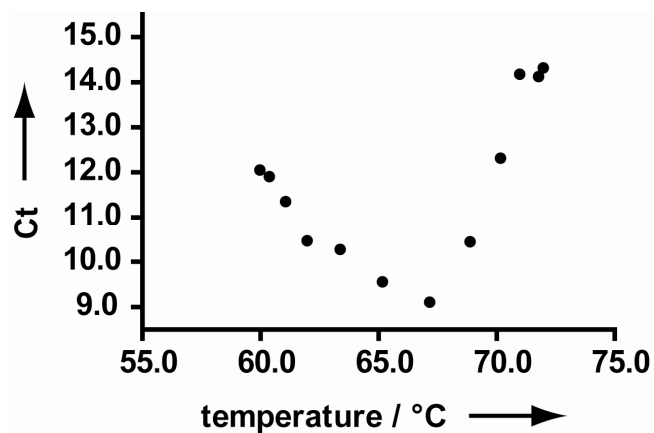


Figure 6. Temperature dependence of Taq M1 reverse transcriptase activity.

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 ranging from 60-72°C.

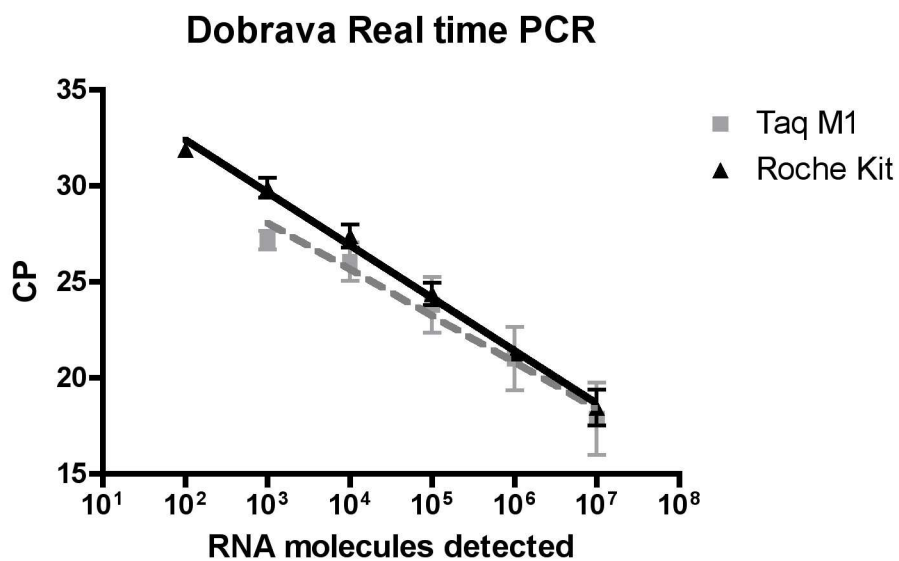


Figure 7. Dobrava virus detection by one step real-time RT PCR. Crossing points (CP) are plotted against RNA molecules detected. Each regression line was calculated from a triplicate data set.

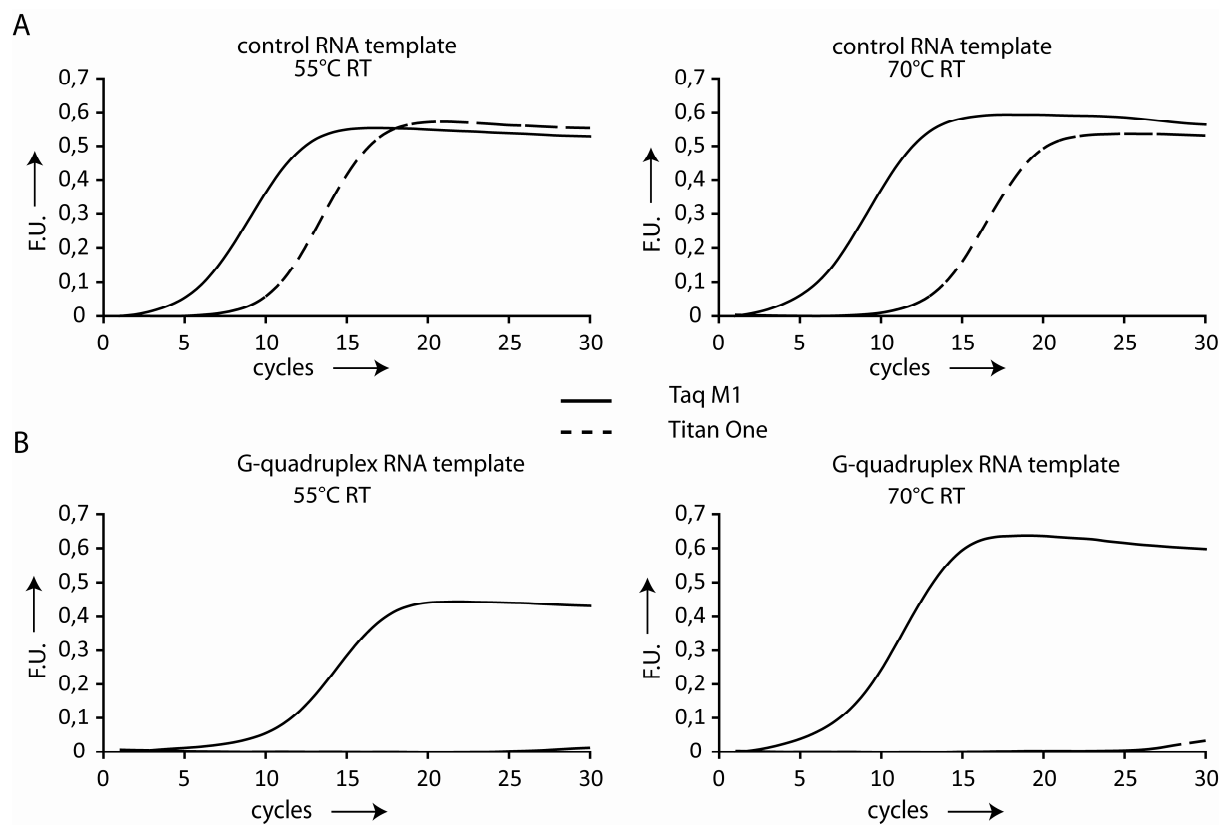


Figure 8. Real-time RT PCR using a RNA template that forms secondary structure (G-quadruplex motif) (B) compared to a quadruplex-free control template (A). Amplification curves are deriving using Taq M1 (solid line) or a commercial kit with a reverse transcription step at 55°C or 70°C for 15 min, respectively.