

A Molecular Beacon for Quantitative Monitoring of the DNA Polymerase Reaction in Real-Time**

Daniel Summerer and Andreas Marx*

The design of new assay formats that allow fast parallel screening of enzyme function plays a pivotal role in the discovery of new products and reagents ranging from industrial processes to diagnostics.^[1] Furthermore, drug discovery and protein engineering are increasingly performed by combinatorial approaches, in which progress is tightly linked to the development of suitable screening systems capable of measuring desired enzyme properties in a high-throughput parallel fashion.^[1]

DNA polymerases are involved in all DNA syntheses occurring in nature. This decisive role in biological key processes has made these enzymes to attractive drug targets.^[2] Furthermore, DNA polymerases are the workhorses in

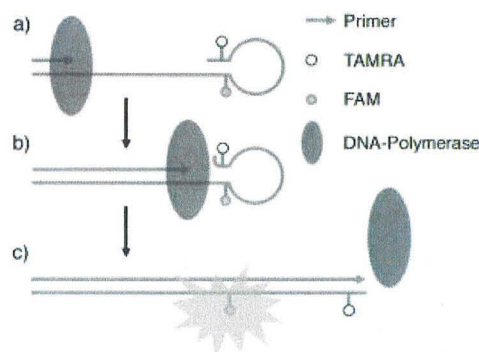
[*] Dr. A. Marx, Dipl.-Chem. D. Summerer
Kekulé-Institut für Organische Chemie und Biochemie
Universität Bonn
Gerhard-Domagk-Strasse 1, 53121 Bonn (Germany)
Fax: (+49)228-73-5388
E-mail: a.marx@uni-bonn.de

[**] Financial support of this project by the DFG is gratefully acknowledged. We thank Dr. S. Brakmann for providing the plasmid pQKlenowExo⁻ and Professor Dr. M. Famulok for his continuing support.

numerous important molecular-biological core technologies such as the ubiquitous polymerase chain reaction (PCR), cDNA cloning, genome sequencing, and detection of nucleotide variations within genes.^[3,4] Nevertheless, emerging drug resistances and the need to tailor DNA polymerases for new technologies, for example, faithful genome-wide nucleotide variation detection, fuel the search for further developments.^[4,5] Several approaches to screen DNA polymerases have been reported, all of which have considerable drawbacks,^[6] these include the use of laborious gel-based methods and/or rely on the employment of radioactive isotopes for product detection.^[6a,b] Several fluorescent-based assays are described, which are nevertheless restricted to specific DNA polymerases (such as thermostable enzymes used in PCR)^[7] or endpoint measurements requiring several cost-intensive auxiliary reagents and proteins for signal generation.^[6c-f]

Herein we report a new assay format that translates the proceeding DNA synthesis into a fluorescent signal in real-time. The method uses commercially available reagents and allows quantitative monitoring of enzymatic DNA synthesis in a multiwell plate-reader format even in crude lysates of DNA-polymerase overexpressing cells. Thus, this assay surpasses known methods to screen these important enzymes.

We designed a DNA reporter molecule in such a way that the template strand forms a stable hairpin structure (Scheme 1 a). The stem is equipped with a dye (carboxyfluorescein, FAM) the fluorescence of which is quenched through



Scheme 1. Assay design for real-time observation of the DNA polymerase reaction. a) The template probe labeled with fluorophor (carboxyfluorescein, FAM) and acceptor (*N,N'*-tetramethyl-rhodamine, TAMRA) has a hairpin extension in closed conformation before start of reaction. b) While extension proceeds, the DNA polymerase opens the stem and prevents reannealing by DNA duplex formation. c) The increase in the distance between the two labels is reported by restoration of FAM emission.

resonance energy transfer (RET) by a quencher (*N,N'*-tetramethylrhodamine, TAMRA) brought in close proximity by the hairpin formation.^[8,9] Both, fluorophor and quencher are attached to the C5-position of 2'-deoxyuridines, which can be bypassed by DNA polymerases.^[10] An extended 3'-end of our template hairpin construct allows binding of a primer strand to its complementary site (Scheme 1b). A DNA polymerase triggers opening of the hairpin stem as primer extension proceeds, which results in spatial separation of fluorescent and quencher dyes accompanied by restoration of FAM-emission (Scheme 1).

First, we tested the Klenow fragment of *E. coli* DNA polymerase I (*exo*⁻ mutant, KF⁻), an enzyme extensively used as a model for DNA polymerase mechanisms and function.^[11] We carried out primer-extension reactions in 96-well-plate format using a fluorescence kinetic reader with automated liquid dispenser. In principle, standard reactions were initiated by addition of MgCl₂ to a mixture of DNA polymerase and hairpin-template primer complex in the appropriate reaction buffer comprising all four deoxynucleoside triphosphates (dNTP). The reaction was monitored by measurement of FAM-fluorescence intensity at different time intervals. Figure 1a illustrates nicely the applicability of the concept and exhibits that fluorescence increases with prolonging incubation time. Noteworthy, is that in the absence of dNTPs, magnesium ions, or enzyme, no fluorescence increase was observed, strongly indicating that the time-dependent signal generation is a result of DNA polymerase function.

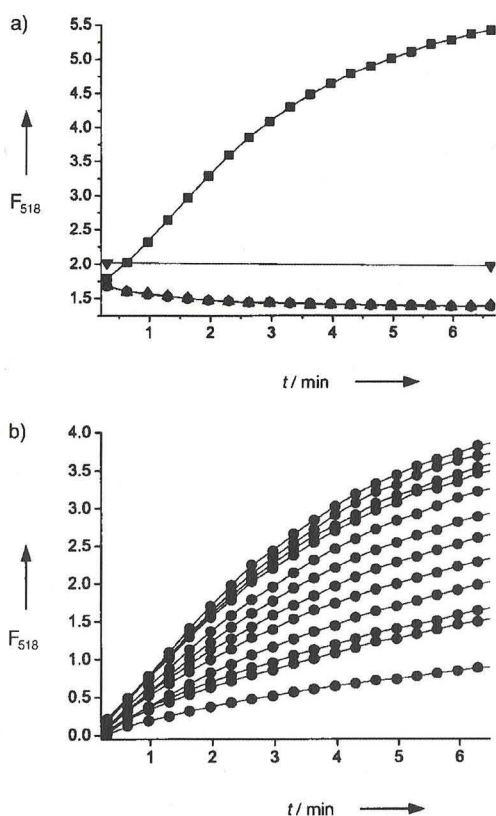


Figure 1. Fluorescent real-time monitoring of DNA synthesis promoted by KF⁻ using the hairpin-template probe. a) Dependence of essential KF⁻ reaction parameters. (■): Reaction conducted at 37°C employing 200 nM hairpin-template primer complex, KF⁻, 62.5 μM of each dNTP and 10 mM MgCl₂ in standard reaction buffer.^[13] FAM fluorescence intensities were measured at 518 nm. Blind measurements were made under the same reaction conditions but without dNTP (●), without KF⁻ (▲), or without MgCl₂ (▼). Presented graphs correspond to raw data without correction for photobleaching or other machine-dependent effects. b) Time courses of reactions employing different dNTP concentrations. Reactions contained KF⁻ and dNTP concentrations ranging from 1.25 μM (lowest curve) to 37.5 μM (highest curve). Data are results of multiple experiments. For clarity, the error bars are not shown. A measurement of an equivalent reaction mix without KF⁻ was conducted in parallel and subtracted from the data from the experiments including KF⁻.^[13]

The kinetics of enzymatic DNA synthesis exhibit Michaelis-Menten dependence of substrate concentration thus, apparent Michaelis-constant (K_M) values can be derived from experiments conducted with a given amount of enzyme and varied substrate concentrations.^[12] We therefore investigated the KF⁻-catalyzed reaction using various dNTP concentrations. The fluorescence-time curves thus obtained show an initial linear signal increase and that with increasing substrate concentrations the reaction rate reaches a maximum (Figure 1b). Remarkably, we obtained a K_M value ($3.6 \pm 0.2 \mu\text{M}$) that compares well with that measured independently using a conventional radiometric assay ($3.9 \pm 1.1 \mu\text{M}$).^[13]

We explored whether the assay is suitable for detecting the interactions of DNA polymerases with inhibitors, for this we employed HIV-1 reverse transcriptase (HIV-1-RT) as a model system. In current HIV drug therapy two classes of RT-inhibitors are in use, differing in their mode of action.^[5] Non-nucleoside RT inhibitors (NNRTIs) are believed to allosterically inhibit RT function, while nucleoside RT inhibitors (NRTIs) are first transformed to 5'-O-triphosphates by cellular processes and then incorporated into the nascent DNA strand by the RT to cause chain termination. To validate whether our assay format rapidly identifies inhibitors of HIV-1-RT acting by both mechanisms, we studied nevirapine and 3'-azido-2',3'-dideoxythymidine triphosphate (AZTTP) which are well characterized NNRTIs and NRTIs, respectively.

Our assay permits real-time monitoring of HIV-1-RT inhibition independent from the mechanism of action (Figure 2). Inhibition profiles from which IC₅₀-values could be obtained were recorded in a single run comprising parallel conducted measurements. Furthermore, the resulting IC₅₀-values of nevirapine ($0.13 \pm 0.03 \mu\text{M}$) and AZTTP ($2.10 \pm 0.22 \mu\text{M}$) correspond well with reported data indicating the suitability of the assay for faithful inhibitor characterization.^[14]

The presented setup should be ideally suited for parallel screening of libraries of DNA polymerase variants in high throughput. In first studies along this line we tested the

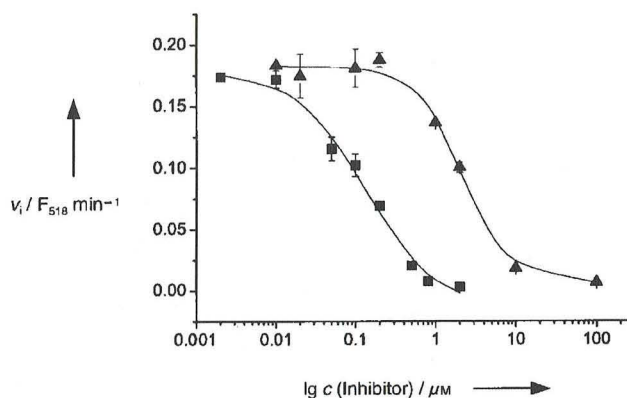


Figure 2. HIV-1-RT inhibitor profiles. Reactions were conducted using HIV-1-RT, 200 nM hairpin-template primer complex, and 50 μM of each dNTP in the presence of inhibitors at different concentrations. (▲) AZTTP, (■) nevirapine. An equivalent blind reaction mix in the absence of RT was conducted in parallel and subtracted from the data from the experiments including RT.^[13] Initial apparent reaction rates (v_i) were obtained from data by determination of the slopes of the initial linear portion of individual reaction graphs. Data are results of multiple experiments.^[13]

feasibility of the molecular probe to sense enzymatic activity in crude lysates of DNA polymerase overexpressing cells. Such a capability would greatly simplify and speed up the parallel screening of mutant libraries without the need for tedious and cost-intensive purification steps. Nevertheless, this is a challenging task, since the presence of a vast number of DNA-binding and -modifying proteins in bacterial-cell lysates sets high demands on the robustness of assay formats relying on a DNA reporter molecule. To demonstrate this possible application, we employed the molecular probe in fluorescent-primer extension reactions with the lysates of KF⁻-overexpressing *E. coli* cells.^[13] Strikingly, significant fluorescence increase was observed exclusively when lysates of expression-induced cells harboring KF⁻-coding vectors were employed (Figure 3 a).

Lysates obtained from induced cells harboring vectors without KF⁻-coding sequences failed to trigger fluorescence increase in the investigated timeframe. To confirm that signal generation indeed results from DNA synthesis catalyzed by expressed KF⁻, we repeated these experiments employing ³²P-labeled primers with subsequent analysis by denaturing polyacrylamide gel electrophoresis (Figure 3 b). The results show that primer extension is only observed when lysates from induced cells carrying KF⁻-coding vectors are employed, strongly indicating that the observed signal generation indeed arises from the desired DNA polymerase function.

In summary, we report here on a new assay format based on a single-molecular probe, which allows quantitative real-time

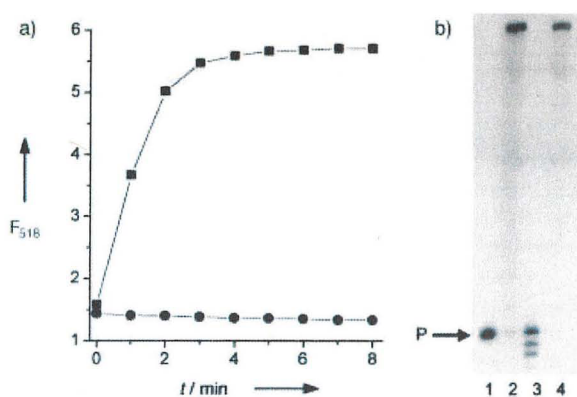


Figure 3. Primer extension reactions promoted by crude lysates of KF⁻-overexpressing *E. coli* cultures using the hairpin-template probe. a) Fluorescence measurement: reactions were conducted at 37°C employing 200 nM hairpin-template primer complex, 200 μM of each dNTP, 10 mM MgCl₂, and lysate of expression-induced *E. coli* cultures in standard reaction buffer.^[13] (■) Reactions employing lysate of *E. coli* cells harboring the KF⁻-coding vector pQKlenowExo⁻ 4 h after induction of expression. (●) Reactions employing lysate of *E. coli* cells harboring the noncoding vector pQE30Xa 4 h after induction of expression. Data are results of multiple experiments. Presented graphs correspond to raw data without correction for photobleaching or other machine-dependent effects. b) Polyacrylamide gel electrophoretic analysis of primer-extension reactions using ³²P-labeled primer template probe. The reaction time was 10 min in all cases. Lane 1: ³²P-labeled primer template probe and dNTPs in KF⁻-reaction buffer (P = primer). Lane 2: Reaction employing commercially purchased KF⁻. Lane 3: Reaction employing lysate of *E. coli* cells harboring noncoding pQE30Xa 4 h after induction of expression. Lane 4: Reactions employing lysate of *E. coli* cells containing KF⁻-coding vector pQKlenowExo⁻ 4 h after induction of expression.^[13]

monitoring of DNA synthesis catalyzed by DNA polymerases. The setup enables the rapid identification and quantitative characterization of small molecules interfering with DNA-polymerase function. Furthermore, we experimentally demonstrated that the presented reporter system is able to monitor DNA polymerase function in real-time directly from lysates of DNA polymerase overexpressing cells. To our knowledge these demands are not met by any of the known methods. Taking these superior properties together, we strongly believe that the presented assay format will find wide applications.

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