Supporting Information

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A molecular beacon for quantitative monitoring of the DNA-polymerase reaction in real-time

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Oligonucleotides. Hairpin-template was purchased in double HPLC quality (Iba GmbH, Göttingen, Germany). The nucleotide sequence was 5'-GGC CCG U^{TAMRA} AG GAG GAA AGG ACA TCT TCT AGC AU^{FAM} A CGG GCG GTC AAG TTC ATG GCC AGT CAA GTC GTC AGA AAT TTC GCA CCA C-3' where underlines identify the stem sequences. TAMRA and FAM are linked as 5,6 regioisomeric mixtures to C5 of 2'-deoxyuridines. The employed primer (5'-d(GTGGTGCGAAATTTCTGAC)-3') was synthesized using standard automated DNA synthesizer technology and PAGE purified before use.

Enzymes, reagents and vectors. Klenow fragment (exo⁻, KF⁻) was purchased from New England Biolabs (Beverly, MA, USA) HIV-1 reverse transcriptase was purchased from Roche Molecular Biochemicals (Mannheim, Germany). AZT-5'-O-triphosphate (AZT TP) was purchased by Iba GmbH. Nevirapine was obtained through the AIDS Research and Reference Reagent Program, NIAID, NIH. Vector pQKlenowExo⁻ was kindly provided by Dr. S. Brakmann, Leipzig.^[1] Vector pQE30Xa was purchased from Quiagen (Hilden, Germany).

General procedure of DNA polymerase reactions. 50 µl reactions were carried out at 37 °C and contained 200 nM hairpin-template, 300 nM primer, 10 mM MgCl₂, DNA polymerase and varying amounts of dATP, dCTP, dGTP, and dTTP in reaction buffer. Hairpin-template and primer were preannealed in the respective reaction buffer excluding MgCl₂ by heating to 95°C for 4 min and subsequent cooling to room temperature over one hour. dNTP and enzymes were added at 0°C and mixture was transferred into black 96 well plates (Corning incorporated, Corning, NY, USA). Multiwell plates were preincubated for 2 min at 37°C in a Fluoroscan FL multiplate reader (Labsystems GmbH, Frankfurt, Germany) and started through MgCl₂ addition. FAM fluorescence was excited at 485 nm and emission was measured at 518 nm in different time intervals. Raw data were exported to Microsoft Excel and analyzed.

Primer extensions and kinetic measurements using KF⁻. Reactions were conducted in Tris-HCl (50 mM, pH 7.3) and DTT (1 mM) using 1.4u KF⁻ (unit as defined by the supplier) as described above. Initial reaction velocities (v_i [F_{518}/min^{-1}]) were obtained from measured fluorescence data by determination of the slope of the first linear portion of individual reaction graphs and plotted versus dNTP concentration (see Figure 1).

Figure 1. Michaelis-Menten plot of kinetic measurements using KF⁻. (*): measured v_i -values. Line graph corresponds to nonlinear curve fit.

A K_M -value was obtained by nonlinear curve fitting using the Michaelis-Menten equation (see above). Radioactive control experiments were conducted under the same conditions as fluorometric experiments with addition of appropriate amounts of α -³²P dCTP. Reactions were stopped after 2 min by fourfold dilution with gel loading buffer (80% formamide, EDTA (20 mM)) and subsequently heated to 95 °C for 5 min. DNA products were separated from radioactive dNTPs by denaturing 6% polyacrylamide gel electrophoresis containing 8M urea, transferred to filter paper, dried and quantified by phosphorimager technology.

HIV-1 RT inhibitor profiling. Reactions were conducted in Tris-HCl (50 mM, pH 8.3) and KCl (50 mM) using 2.1u HIV-1 RT (unit as defined by the supplier) as described above. Inhibitors were added to mixture before preincubation. IC₅₀ values for inhibition of enzyme activity were obtained by measuring initial velocities at different inhibitor concentrations as described above. For analysis, obtained v_i were plotted against the logarithm of the corresponding inhibitor concentrations and analyzed by non linear curve fitting.

Expression of recombinant KF. pQKlenowExo⁻ (transferring ampicillin resistance) harbors an 1.8-kb fragment coding for N-terminal 6-His-tagged KF⁻ under control of a T5-promotor and a double lac-operator sequence allowing effective repression before IPTG-induction.^[1] *E. coli* M15 was used as expression strain of recombinant KF⁻. To increase transcription repression during growth, M15 cells harboring plasmid pREP-4 (transferring kanamycin resistance) coding for lac repressor were used. For expression, 50 µl of overnight cultures grown in LB medium^[2] were added to 930 µl super broth^[3] in 2.2 ml 96-deepwell plates (Eppendorf, Hamburg, Germany). Both media included ampicillin (100 µg/ml) and kanamycin (25 µg/ml). Cultures were sealed with Airpore tape sheets (Quiagen), grown at 37 °C to a optical density (OD₆₀₀) of 0.7 - 0.8 and induced by addition of 20 µl IPTG (50 mM, resulting in 1 mM final concentration). After 4 hours, cells were harvested by centrifugation. Expression kinetic was monitored by SDS-PAGE (see Figure 2).

Figure 2. Kinetic of KF⁻ expression. 1) Length marker. 2) commercially purchased KF⁻. 3) lysate of noninduced control. 4-6) lysates of expression cultures 1, 3, and 5 hours after IPTG-induction.

For lysis, pellets of 1 ml cultures were subjected to one freeze-thawing step, resuspended in 500 µl lysis-buffer (50 mM NaH₂PO₄, 300 mM NaCl, pH 8.0 containing 1.5 mg/ml lysozyme) and shaken for 45 min at 4 °C. Lysates were cleared by centrifugation and directly used for primer extensions. Control lysates of M15 cultures harboring the vector pQE30Xa without KF⁻-coding insert were prepared identically.

Primer extensions using lysates of transformed *E. coli* cells. Fluorescent reactions were conducted under same conditions as used for purified KF⁻. MgCl₂ was included in annealing mixture and reactions were started with dNTP solution (200 µM end-concentration). Instead of enzyme, 1 µl cleared lysate of 1 ml cultures was employed. For radiometric analysis of primer extensions, primer was 5'-labeled with [γ -³²P]ATP using T4 polynucleotid kinase (Stratagene) according to the procedure recommended by the supplier. Reactions were conducted identically and contained 200 nM primer and 400 nM hairpin-template. Reactions were stopped after 10 min by fourfold dilution with gel loading buffer (80% formamide, EDTA (20 mM)) and subsequently heated to 95 °C for 5 min. Product mixtures were analyzed by 14% denaturing polyacrylamide gel electrophoresis containing 8 M urea, transferred to filter paper, dried under vacuum and visualized by autoradiography.

References

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