APPLICATION NOTE

dynamic BIOSENSORS

Reverse Transcriptase binding and activity assays with switchSENSE®

Keywords: Reverse transcriptase I template variation | elongation

Reverse transcriptase (RT) is an enzyme found in retroviruses that catalyzes the conversion of single-stranded RNA into double-stranded cDNA. This function of the protein is widely used in the field of molecular biology.
switchSENSE® can be used to investigate the binding and elongation properties of different RTs. Here we analyzed the association of multiple M-MLV RT wild-type concentrations to an RNA template. Subsequently, and as a function of degree of RNA template saturation, the elongation of the DNA primer was measured in real-time.

Background

Reverse transcriptase (RT) also known as RNA-dependent polymerase was discovered in many retroviruses, such as the human immunodeficiency virus (HIV). RT is an enzyme that catalyzes the conversion of single-stranded RNA into double-stranded cDNA. This cDNA can then integrate into the host genome, a mechanism used to enable viral proliferation within the host cell. The same reactions set into motion by the virus within the host cell are widely used in the field of molecular biology to generate cDNA libraries for cloning, to run the RT-polymerase chain reaction or a microarray analysis, to name a few examples. A RT commonly used for such applications is the Moloney Murine leukemia virus (M-MLV) RT. (Baranauskas, 2012)

Various mutant classes of RTs have been generated for a multitude of experimental applications, with the purpose of knocking-out specific side-functions to the relevant main activity of the RT, e.g. RNase H activity. As a guide for the development of the mutant RTs, the effect of corresponding mutations on the RNA template binding and DNA primer elongation rates need to be characterized.

The switchSENSE® technology is a biophysical characterization method very well-suited for such RT binding and activity measurements: different RNA transcript templates are easily hybridized and exchanged on the biosurface, which enables the real-time measurement of the various kinetic parameters. Here we measure the association of different concentrations of M-MLV RT wt to surface-immobilized RNA templates, and the subsequent elongation of a DNA primer.

Methods and Results

Assay Setup and Workflow

All measurements were performed at 37 °C using a switchSENSE® Enzyme Chip (ENZ-54-1-Y1 Chip) with a surface-functionalized DNA strand 54 base pairs in length. The assay setup is shown in Figure 1. For the RT activity measurements, a DNA primer was hybridized onto either an RNA-template (T1) or DNA-RNA-hybrid template (T2). In both cases, the template carried a red dye at the distal end for real-time observation of RT binding and DNA primer elongation. The sequence and length of the overhang were optimized according to required measurement conditions, in addition to buffer composition and assay temperature. All experiments were performed in the following buffer: TRIS 10 mM, 40 mM KCl, 6 mM MgCl2, 0.05 %(w/v) Tween 20, pH 8.0.

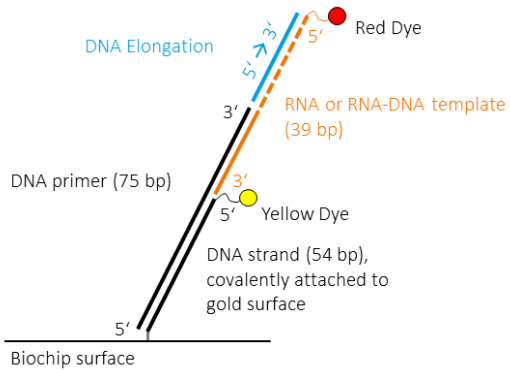

Figure 1 | For all experiments, a standard **switch**SENSE® Enzyme Chip (ENZ-54-1-Y1 Chip) was used. A DNA primer (75 bp) and a template (39 bp), consisting of either RNA only or DNA and RNA, were hybridized onto the 54 bp DNA strand. The solid orange line was either RNA (T1) or DNA (T2) while the dotted orange line is RNA for both used templates. The readout of the red dye, attached to the distal end of the template, was used throughout the measurements.

Chip functionalization

The sequential hybridization of primer and template, each with a concentration of 0.5 µM in TE40, onto the surface-immobilized DNA strand can be observed in real time: first the DNA Primer is hybridized, visible as an increase in fluorescence, since the surface-immobilized DNA strand's increase in rigidity upon duplex formation effectively pushes the yellow dye further away from the surface, reducing the quenching effect due to the dye's proximity to the gold surface (Figure 2). The result of the surface hybridization with the primer is a single-stranded overhang to which the template is hybridized in the subsequent step. The template hybridization can be seen as a decrease in the fluorescence signal, as the hybridizing nucleotides in close proximity to the yellow dye (dye 1) quenches its signal. The LED detection channel, corresponding to the red dye (dye 2), is turned off during template hybridization, as the presence of dye 2 would create a very high background upon excitation. The hybridization rates for both templates – T1 and T2 – are very comparable.

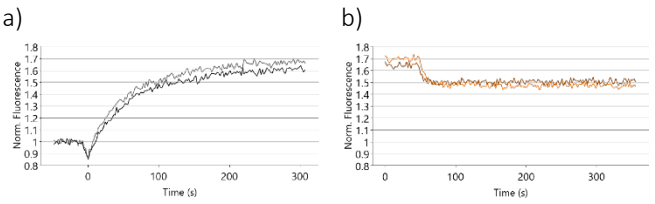

Figure 2 | The fluorescence signal change during the hybridization of a) primer and b) template to an ENZ-54-1-Y1 Chip. Primer hybridization leads to an increase of fluorescence resulting from the increased stiffness of dsDNA compared to ssDNA. The hybridization of template T1 (light orange) and T2 (dark orange) can be observed as a decrease in fluorescence signal intensity induced by the quenching effect of the nucleotides hybridizing in close proximity to the dye. Hybridization curves are comparable for both templates T1 and T2.

Binding of different RT concentrations

Binding experiments, with three different RT concentrations (c1 = 0.96 nM, c2 = 9.64 nM and c3 = 96.40 nM), were performed using T1 as the template and dye 2 as fluorescence readout (Figure 3). An RT concentration of 96.40 nM corresponds to an active concentration of 5.9 U/µL. Association of RT to the overhang is observed as a decrease in fluorescence intensity, with each analyte concentration in a concentration-dependent manner. The RT was associated at a flow rate of 200 µL/min. For the highest and lowest RT concentrations, both measured in duplicates, reproducible results were obtained. For the lowest RT concentration (0.96 nM) only a negligible fraction of surface DNA strands were RT-bound after association. The RT association rate constant k_{ON} to T1 was (7.23 ± 0.11) E+5 $M^{-1} \cdot s^{-1}$. The data was analyzed using a global mono-exponential fit model.

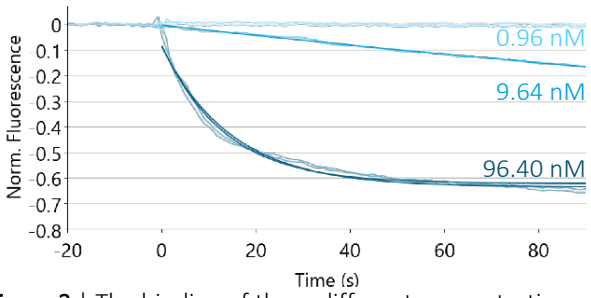

Figure 3 | The binding of three different concentrations of M-MLV RT to template T1 was measured, yielding an association rate constant k_{ON} = 7.23 ± 0.11 E+5 $M^{-1}{\cdot}s^{-1}$ using a global monoexponential fit model.

Primer elongation

For the RT elongation rate measurement, a solution of 200 µM concentration of each of dATP, dTTP, dCTP and dGTP was injected over the RT-bound surfaces – at 0.96 nM, 9.64 nM, and 96.40 nM of RT – using a flow rate of 200 µL/min (Figure 4). For the 0.96 nM RT-bound surface, with the low fraction of bound RT, no elongation was observed, and the fluorescence level stayed constant. For 9.64 nM and 96.40 nM RT-bound surfaces, the incorporation of dNTPs and consequently primer elongation was observed as an increase of fluorescence intensity. The elongated double stranded DNA/RNA is more rigid than the original single stranded overhang, leading to an increase of fluorescence signal caused by the increased distance to the gold sensor surface. For the highest associated RT analyte concentration (96.40 nM), where the plateau in the association curve indicates that all surface DNA/RNA strands are saturated with RT, the increase in fluorescence upon dNTP solution injection is thus an effect of only primer elongation, elongating 100 % of surface DNA strands within seconds (k_{CAT} = 3.52 ± 0.16 bp/s). For the intermediate associated RT analyte concentration (9.64 nM), not all surface DNA/RNA strands are saturated with RT, based on the downward linearly trending association curve. Thus, the fluorescence signal readout combines, in addition to primer elongation, the process of strand hopping of RT from elongated to non-occupied DNA/RNA strands, which is observed as a slower measured elongation rate (k_{CAT} = 1.56 ± 0.09 bp/s). Furthermore, only a fraction of the surface DNA strands are elongated, as the surface-bound RT concentration is gradually reduced, which is observed as a lower fluorescence signal saturation level.

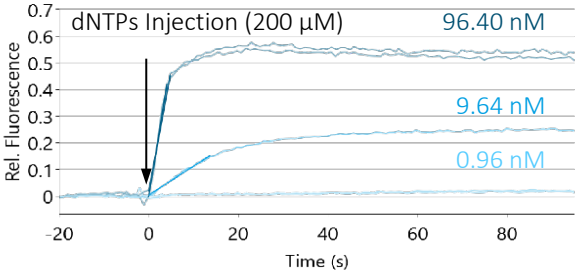

Figure 4 | Measurement of the elongation rate of M-MLV in the presence of 200 µM dNTPs for different saturation levels of RNA with RT: For 0.96 nM RT-bound surface no elongation is observed whilst 96.4 nM is showing full elongation with a rate of k_{CAT}=3.52 ± 0.16 bp/s. For 9.64 nM of RT the observed elongation rate is reduced since strand hopping to non-occupied surface DNA strands occurs (k_{CAT} = 1.56 ± 0.09 bp/s).

Conclusions

switchSENSE® enables the fast and easy exchange of desired DNA and RNA/DNA hybrid templates for the real-time binding and elongation experiments of RT. After surface-immobilization of the template sequences, RT was injected at three different concentrations, and the association rate constant successfully determined: k_{ON} = 7.23 ± 0.11 E+5 $M^{-1}{\cdot}s^{-1}$. The elongation speed was also observed as a function of degree of RT saturation of surface DNA/RNA.

Author contributions

This work was done in collaboration with Dr. M. Schräml (Head of Department Enzyme & Protein Technologies, Roche Diagnostics) developing the assay design and Ute Jucknischke who performed the assay development and conducted the experiments in the lab of Roche Diagnostics. Assay setup, writing and data compilation were done by Hanna Beike from Dynamic Biosensors.

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