Measuring ligation activity of T4 DNA ligase on nicked DNA with switchSENSE®

Keywords: T4 DNA Ligase I ligation activity I nick sealing I ATP I thermal stability | inactivation

DNA ligases fulfill essential functions in DNA replication, DNA repair, and are standard tools in molecular biology labs. Here, we describe a switchSENSE® assay for the rapid assessment of ligation activity with minimal sample consumption. The ligation activity of T4 DNA ligase is quantified in real-time by observing the ligation of a nick in one strand of a double stranded oligo. Complementary read-outs of dissociation behavior and melting temperature allow the reliable differentiation of ligated and non-ligated DNA oligos.

Using this assay, ligation activity or the influence of ligase inactivation, mutation or inhibition can be determined rapidly, accurately and in real-time for research, development and quality control.

Background

DNA ligases are enzymes that catalyze the formation of a new phosphodiester bond to ligate two dsDNA fragments. They usually require co-factors such as ATP or NAD^+ and Mg^{2+} to join sticky or blunt DNA ends or seal a nick in one DNA strand of a dsDNA template. This class of enzyme is particularly important in DNA replication and DNA repair processes. However, detailed activity studies were challenged by time and material consuming multi-step solution assays and error-prone single-time point analyses thus far.

Here, we describe how the switchSENSE® technology can be utilized to quantify the T4 DNA ligation activity in real-time. The measurement is based on the fast dissociation of short oligomers at room temperature, which can be slowed down by ligation to a longer oligomer. Using this measurement setup, not only ligation activity but also T4 ligase inactivation and inhibition and the influence of co-factors on the ligation process can be investigated in more detail.

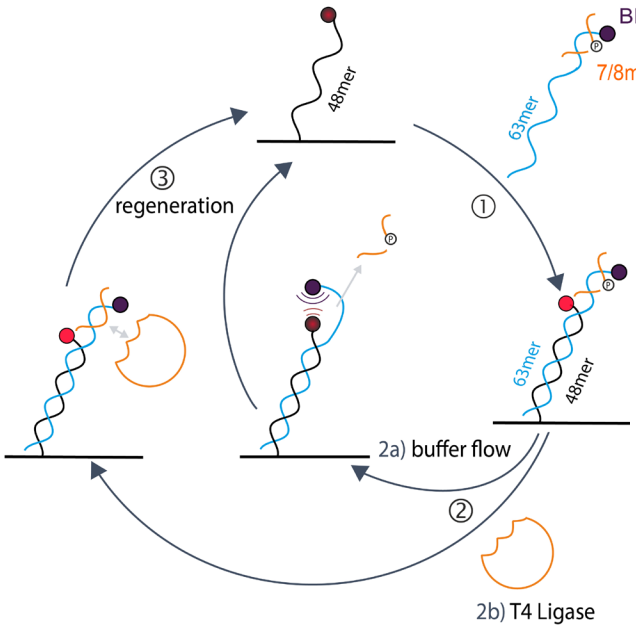


Figure 1 | Schematic ligation activity measurement cycle. ① 63/7/8mer collective hybridization with a black-hole quencher (BHQ) at the 5' end of the 63mer strand. ② TE40 buffer flow for 5 mins with 1 mM ATP and 10 mM $MgCl_2$ and a) without or b) with various T4 ligase concentrations for DNA nick sealing to 15mer. ③ Regeneration of the surface for the next measurement cycle.

Methods

Setup. Measurements are performed in a DRX2 instrument. A 63mer DNA sequence (500 nM), complementary to the 48mer standard nanolevers of an MPC-48-2-R1 biochip plus a 15mer DNA overhang, is pre-mixed with 7mer (5 µM) and 8mer (2 µM) oligos, which are complementary to the overhang. The mixture is hybridized to the immobilized 48mer on the biochip (Figure 1; ① 63/7/8mer hybridization). A phosphate at the 5' end of the 8mer is required for a successful ligation process. A 15mer (2 µM) complementary sequence serves as positive control (63/15mer hybridization). To increase the signal-to-noise ratio, the 63mer strand carries a black-hole quencher (BHQ) for the red dye on the 5' end. The TE40 running buffer (10 mM TRIS-HCl, 40 mM NaCl, 50 µM EDTA, 50 µM EGTA, 0.05 % Tween20) is complemented with 1 mM ATP and 10 mM $MgCl_2$ for all measurements.

Ligation activity measurements are performed at room temperature (25°C) and in static mode (DNA nanolevers are continuously kept in an upright position by a constant -0.1 V negative potential on the electrode). The T4 DNA ligase (NEB, stock concentration: 400,000 U/mL) is injected directly after 63/7/8mer hybridization at various concentrations (0 – 5 U/µL) at a 100 µL/min flow rate (Figure 1; ②). The fluorescence is monitored for 5 minutes. Dissociation of the non-ligated short oligos causes the overhang to collapse which brings the quencher closer to the red dye. This results in a decrease of fluorescence signal, while the signal remains stable with a ligated 15mer. Ligation Activity (1/s) is calculated as the reciprocal of the time constant (1/τ) which is provided by the individual mono-exponential fits of the fluorescence signal curves. For T4 ligase inactivation studies, the buffer solution containing 1 U/µL T4 ligase is heated for 15 mins at 65°C in an Eppendorf ThermoMixer (heat inactivation) or mixed with 25 mM EDTA (EDTA inactivation) prior to **switch**SENSE® measurements, respectively.

Melting experiments are also performed in static mode, applying a temperature ramp of 2°C/min from 20°C – 70°C at a constant 100 µL/min buffer or 2 U/µL T4 ligase solution flow. Oligo sequences and their calculated melting temperatures under 10 mM Mg^{2+}, 50 mM Na^{+} buffer condition (calculated by IDT OligoAnalyzer 3.1) are listed in the table below.

oligo	Sequence 5'-3'	T_M
7mer	CCG GTT T	24.0°C
8mer	phospho-GGA GCC GA	38.3°C
15mer	CCG GTT TGG AGC CGA	63.8°C

Results and discussion

First, the setup is tested for its ability to distinguish between ligated and non-ligated oligos. When washing buffer over non-ligated 7/8mer oligos, a 60% decrease of fluorescence indicates their dissociation, resulting in quenching of the fluorophore by the now closer quencher (Figure 2, green). The signal of the ligated control, a 15mer oligo (blue), remains stable over the entire time, ensued by the very slow off rate of a hybridized 15mer at room temperature. A 2 U/µL T4 DNA ligase solution flow over the 7/8mer oligos results in only 24% signal decrease (red), indicating that the majority of small oligos has been ligated to a full length 15mer which is stable and does not dissociate. Interestingly, T4 ligase binding to the DNA does not affect the fluorescence signal significantly (data not shown). Hence, no referencing for the influence of T4 ligase binding is required for the ligation activity analysis.

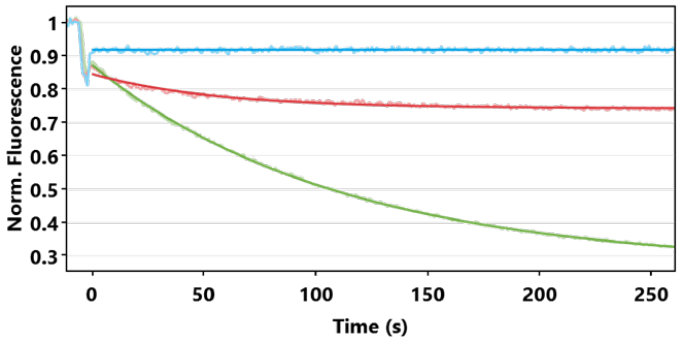


Figure 2 | Fluorescence signal stabilization after T4 ligase binding and ligation. **Red:** 7/8mer oligos stabilized with 2 U/µL T4 ligase and ligated to a 15mer; **blue:** 15mer control; **green:** unstable 7/8mer oligos.

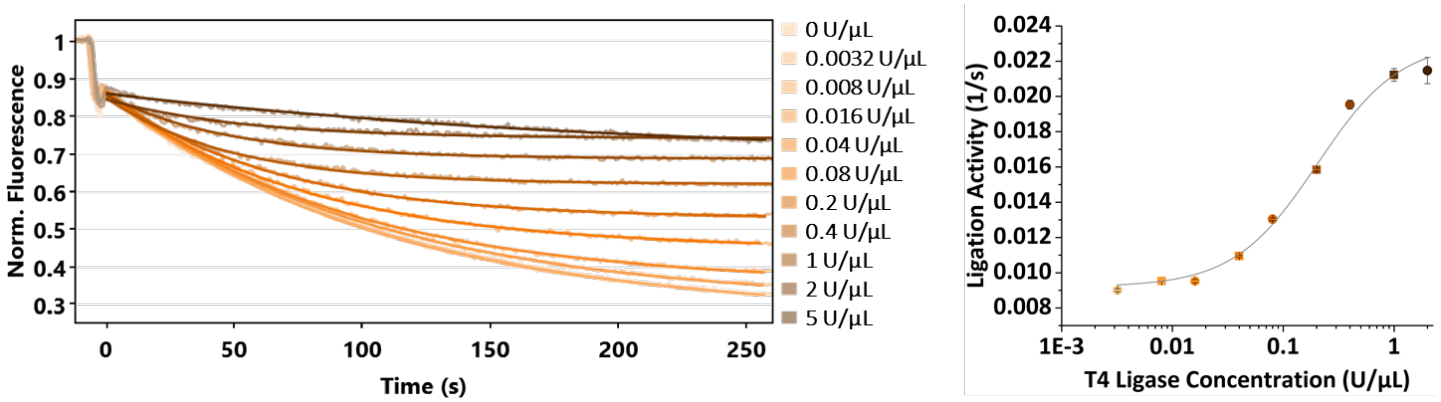
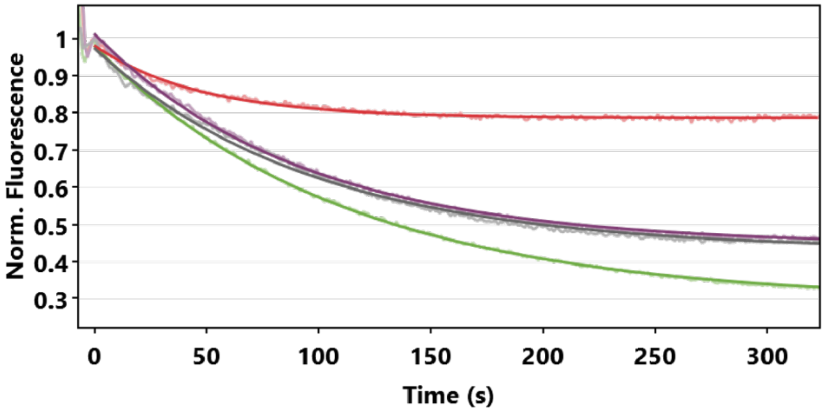


Figure 3 | T4 ligase concentration-dependent fluorescence signal stabilization, indicating increasing ligation efficiency. Ligation activities (1/s) deduced from the mono-exponential fits of the fluorescence signals are plotted against the corresponding T4 ligase concentrations on the right.

Next, the concentration dependence of T4 ligation activity is investigated. The dissociation of 7/8mer in the presence of T4 ligase at different concentrations is monitored by the fluorescence decrease and quantified by the determination of the time constants *τ(off)* of the fluorescence curves, which is interpreted as Ligation Activity. The ligation of the 7mer and 8mer to a 15mer is T4 ligase concentration dependent and can be observed at concentrations as low as 0.04 U/µl and saturates at 1 U/µL T4 ligase (Figure 3).

The setup can be employed to quantify inhibition or inactivation of T4 ligase. Here, 1 U/µL T4 ligase is inactivated by heat or by EDTA. Comparison of the activity to the positive control (non-inactivated 1 U/µL T4 ligase, 100% activity) and to the negative control (0 U/µL T4 ligase, 0% activity) reveals an inactivation of T4 ligation activity to 14.9% (heat inactivation) or 10.7% (EDTA inactivation) of its original activity (Figure 4).

For a multiparameter analysis and to confirm the ligation of the two short oligos to a 15mer, the DRX2 can also be employed for real-time melting experiments. Taking advantage of the increased melting temperature (T_M) of longer oligonucleotides, the determination of T_M after ligation experiments serves as confirmation of successful ligation. Administering a temperature ramp of 2°C/min between 20°C and 70°C, the dsDNA overhang melting curves allow the differentiation between 7mer and 8mer oligos from a 15mer oligo (Figure 5). The melting curves of a 15mer and a 7/8mer after 2 U/µL T4 ligase treatment display the same melting behavior at T_M ~60°C, which



Treatment	Ligation Activity (1/s)	% Ligation Activity
0 U/µL T4 Ligase	0.0090	0%
1 U/µl T4 Ligase	0.0211	100%
1 U/µL T4 Ligase Heat inactivated at 65°C	0.0108	14.9%
1 U/µL T4 Ligase EDTA inactivated	0.0103	10.7%

Figure 4 | T4 ligase inactivation by heat or EDTA. Heat inactivation (65°C for 15 mins) or EDTA inactivation (25mM EDTA) leads to accelerated oligo dissociation. Ligation Activity (1/s) is calculated from the reciprocal of the time constant *τ(off)* resulting from the monophasic fit of the signal response curves. Percent (%) ligation activity is calculated based on the assumption that 0 U/µL T4 ligase shows 0% activity whereas 1 U/µL T4 ligase yields 100% activity.

confirms successful ligation to a 15mer. In contrast, the non-treated 7/8mer shows a T_M of 27.7°C, which is in the expected melting point range of a 7mer and 8mer mixture (see OligoAnalyzer 3.1 T_M calculation table in *Methods*).

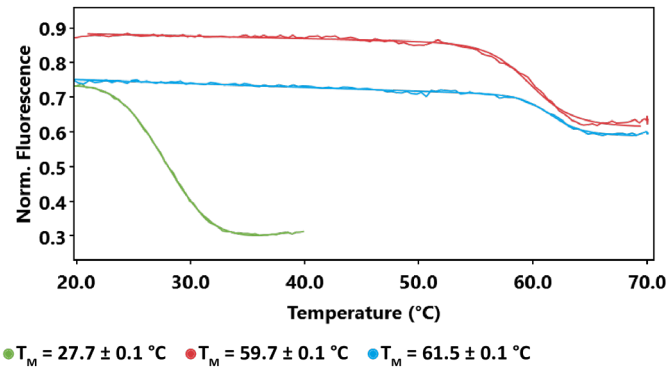


Figure 5 | Thermal stability and melting points (T_M) of short oligos. **Red:** 7/8mer oligos after ligation by 2 U/µL T4 ligase to a stable 15mer; **blue:** 15mer control; **green:** unstable non-ligated 7/8mer oligos.

Conclusions

Here, we present an easy, fast and accurate method to measure DNA ligation activity. The ligation activity can be monitored in real-time while labeling of the enzyme or DNA oligos is not necessary. Additionally, melting experiments can be appended to the same assay for end-point analysis by a complementary read-out. One measurement cycle can be completed in less than 15 minutes.

The described ligation assay provides an innovative and high information content tool to characterize DNA ligases and quantify their ligation activity for research & development and quality control applications. The impact of ligase inactivation, small molecule ligase inhibition or co-factor concentration dependence (ATP, Mg^{2+}) on the ligation activity can be rapidly and reproducibly quantified.

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