Twist-Engineered T4 DNA Ligase

OVERVIEW

T4 DNA ligase's ability to ligate DNA with blunt or sticky ends has led to its widespread application in various molecular biology techniques. For instance, it plays an important role in gene synthesis and the construction of recombinant DNA molecules. Owing to its reliability and efficiency, T4 DNA ligase is extensively used for adapter ligation in next-generation sequencing (NGS) sample preparation workflows[1].

T4 DNA ligase is the most widely used option due to its availability and generally acceptable performance. Although, some of the limitations of ligase are evident, especially in assays that demand efficient ligation for complex library preparation. Adapter ligation, a common step in library construction, is essential for introducing sequencing adapters. Without efficient adapter ligation, molecules may not be properly amplified during downstream PCR steps, resulting in their loss from the pool and diminishing overall sensitivity.

Ligation inefficiencies are mitigated by increasing reaction parameters such as adapter concentration, ligase concentration, reaction duration, and substrate concentration. Additional strategies include the incorporation of viscous crowding agents like polyethylene glycol (PEG). Although marginally effective, these strategies can negatively affect the overall assay by increasing the cost or decreasing the assay's usability and throughput.

To fundamentally address the limitations of wild-type T4 ligase, Twist has engineered a T4 DNA ligase variant with improved performance.

RESULTS

IMPROVED LIGATION CONVERSION

The Twist-Engineered T4 DNA Ligase was engineered to more efficiently convert A-tailed dsDNA substrate into an amplifiable double-ligated product. To assess the conversion efficacy, we used 15 ng of end-repaired, dA-tailed cfDNA substrates in a 30-minute ligation reaction with varying ligase mass. As shown in Figure 1, Twist-Engineered T4 DNA Ligase outperforms wild-type at all enzyme masses tested from 250 to 1000 ng. This demonstrates that it can more efficiently ligate TA sticky ends onto and produce amplifiable molecules when compared to wild-type ligase and that the engineered ligase is less sensitive to mass input at this condition.

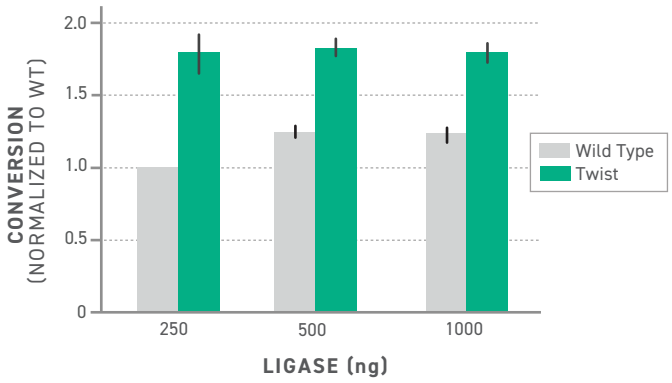


Figure 1. Ligation efficiency with various ligase mass inputs. Conversions are normalized to conversion achieved at 250 ng wild-type T4 DNA ligase. Reactions were performed in quadruplicates (N=4). Conversions were assayed with NGS via over-sequencing (duplicate rate of >30%) and unique reads were quantified.

FASTER LIGATION WITH LESS LIGASE

Reviewing Figure 1, it is evident that saturation was quickly achieved within a 30-minute ligation reaction period employing 15 ng of substrate and 250 ng of ligase. The Twist-Engineered T4 DNA Ligase can create double-stranded products with higher yields and more quickly than wild-type T4 ligase. To quantify the activity of these ligases, we used 25 ng of input A-tailed substrate and performed additional titrations with varying reaction times and ligase input quantities. As demonstrated in Figure 2, Twist-Engineered T4 DNA Ligase converts more amplifiable molecules quicker than wild-type ligase. In many cases, the Twist-Engineered T4 DNA Ligase converts more molecules in 5 minutes than wild-type ligase converts in 30 minutes.

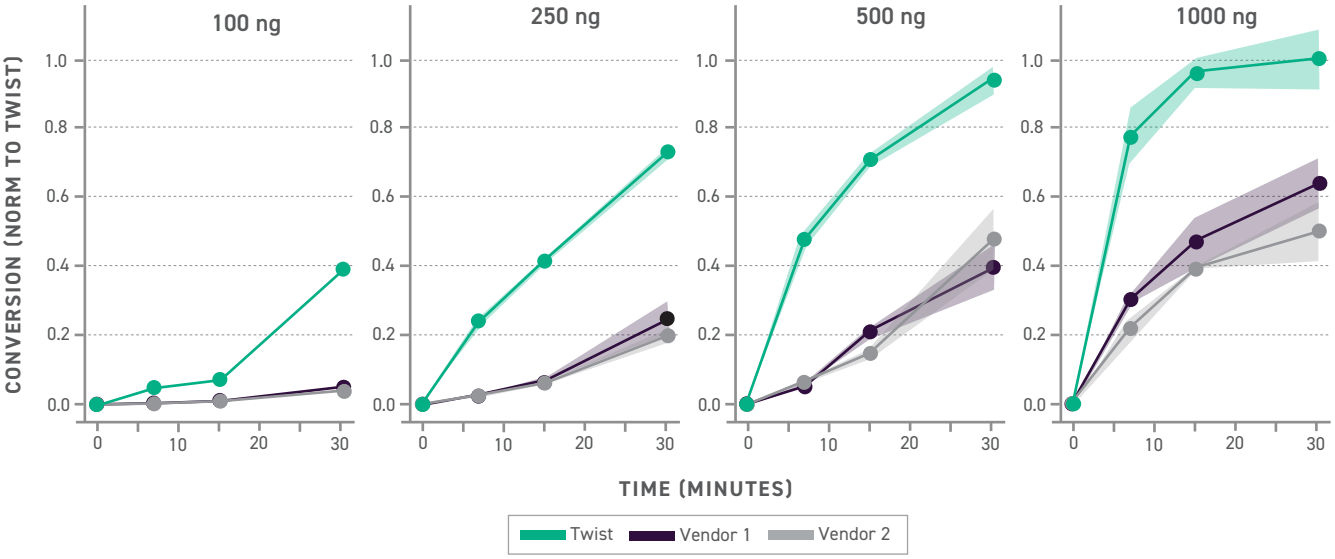


Figure 2. Ligation efficiency with various ligase mass inputs, vendors, and time points. Reactions were carried out with 100, 250, 500, and 1000 ng of ligase from 3 vendors (wild-type T4 DNA ligase from 2 different vendors and Twist-Engineered T4 DNA Ligase). Conversions are normalized to conversion achieved at 30 minutes with 1000 ng Twist-Engineered T4 DNA Ligase. Ligation reactions were done in replicates. After 7, 15, and 30 minutes of incubation, reactions were heat-inactivated at 65°C for 10 minutes and assayed using qPCR with primers specific to the adapters attached by ligation.

IMPROVED SEQUENCE REPRESENTATION

With a higher conversion of the substrate, we observe that sequence bias at the adapter-insert junction is reduced. Past research has shown that T4 DNA ligases exhibit sequence bias for terminal positions on DNA substrates[2]. To evaluate sequence uniformity at the insert junction, we looked into the first 4 cycles of subsampled 1M sequencing reads from respective human NGS libraries serving as a proxy for general ligation motif preference on the DNA substrate. We see a more uniform and well-represented 4-nt population in libraries ligated with Twist-Engineered T4 DNA Ligase (**Figure 3**).

To evaluate bias on the adapter side of the junction, we used a set of 32 defined UMIs and looked into how well-represented each of the sequences in the full set is. Consistent with our finding above, Twist-Engineered T4 DNA Ligase provides a more uniform representation of the 32 unique UMIs when compared with wild-type ligase where certain sequences can be as low as 40% of the mean (**Figure 4**).

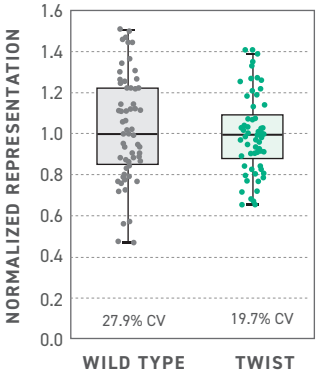


Figure 3. DNA Base Representation in the first 4-nt motif of NGS sequencing reads. NGS sequencing was done on cfDNA substrate after Twist UMI ligation following the protocol listed in the Materials and Methods section. All possible 4 sequencing bases (NNNN) from the start of each read1 or read2 were counted. Representation based on read counts per motif was calculated and normalized to the mean. Each dot represents a particular 4-nt motif in the pool.

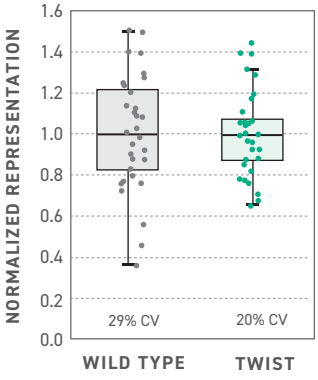


Figure 4. Distribution of 32 sequences in Twist UMI Adapter System after ligation and NGS sequencing. NGS sequencing was performed on cfDNA substrate after Twist UMI ligation following the protocol listed in the Materials and Methods section. Representation based on read counts for each UMI detected was calculated and normalized to the mean. Each dot represents 1 UMI in the pool.

CROWDING AGENT OPTIONAL

Efforts to improve speed and efficiency have utilized viscous crowding agents like polyethylene glycol (PEG) to increase the local concentration of substrates and drive the reaction forward. However, such crowding agents may increase variability or are difficult for automated dispensing systems to use. Furthermore, using crowding agents in cloning reactions necessitates a purification step prior to transformation, which increases hands-on time and processing. We investigated the efficacy of respective ligases on cfDNA substrate in the presence of 0%, 2.5%, and 5% PEG 8000. We referenced the electropherogram traces to identify 3 consecutive peaks: substrate, substrate + 1 adapter, and substrate + 2 adapters bilaterally. As demonstrated in Figure 5, the Twist-Engineered T4 DNA Ligase can convert most of the substrate to the desired double-ligated peak independent of crowding agent input.

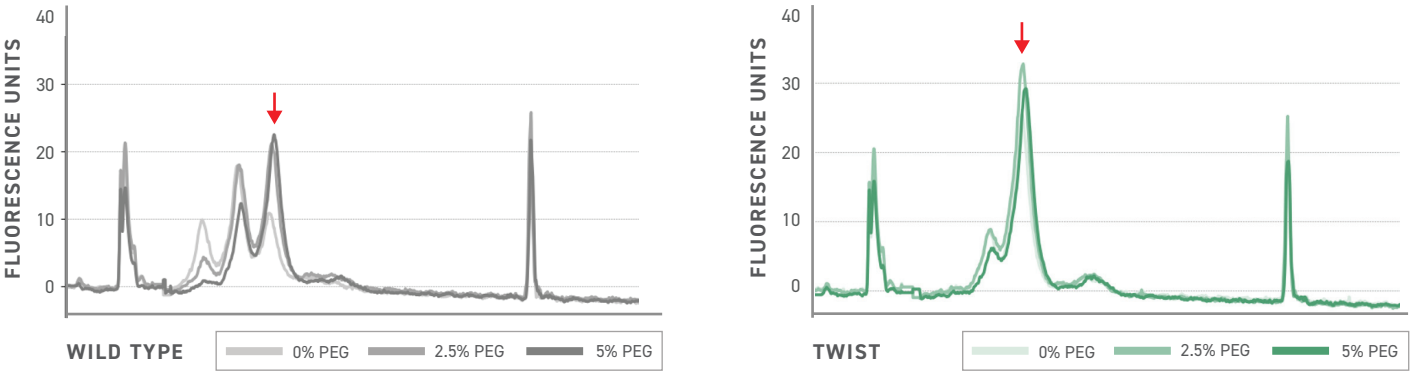
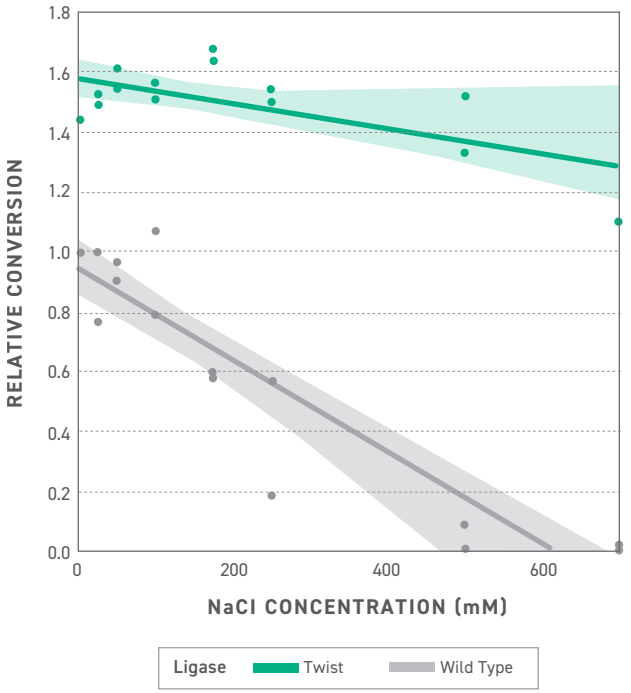


Figure 5. Ligation efficiency with and without polyethylene glycol. Ligation reactions were carried out with the optimal mass of ligases (1000 ng for wild-type and 500 ng for Twist) and 15 ng of input substrate. 1 uL of post-reaction material is analyzed via capillary gel electrophoresis with the 2100 Bioanalyzer High Sensitivity Kit. Raw fluorescence units are plotted against time and the double-ligated peak is denoted by the red arrow.

HIGHER SALT TOLERANCE

The Twist-Engineered T4 DNA Ligase was engineered to be more tolerant to impurities and potential carryovers like salt from earlier processing steps. This enables reaction workflows to proceed without a prior purification process. We demonstrate this insensitivity by showing its high conversion in ligation reactions with increasing NaCl concentrations (**Figure 6**).

Figure 6. Ligation efficiency at various NaCl concentrations. Ligation reactions were carried out with an optimal mass of ligases (1000 ng for wild-type and 500 ng for Twist). Conversions are normalized to conversion achieved at 15 minutes with wild-type T4 DNA ligase at 0 nM NaCl. Ligation reactions were done in replicates. After 15 minutes of incubation, reactions were heat-inactivated at 65°C for 10 minutes and assayed on qPCR with primers specific to the adapters attached by ligation.



BETTER PH TOLERANCE

In addition to the influence of salt concentration, pH is a critical factor that governs enzyme activity for a ligation reaction. Tolerance to pH variations enables one-pot reactions and facilitates drop-ins to existing workflows. To investigate our enzyme's pH tolerance, we made buffers spanning a range of pH values from 7 to 9 for use in the ligation reaction and assessed the resultant conversion. The Twist-Engineered T4 DNA Ligase consistently exhibits higher performance across all pH values tested from pH 7 to 9 (**Figure 7**).

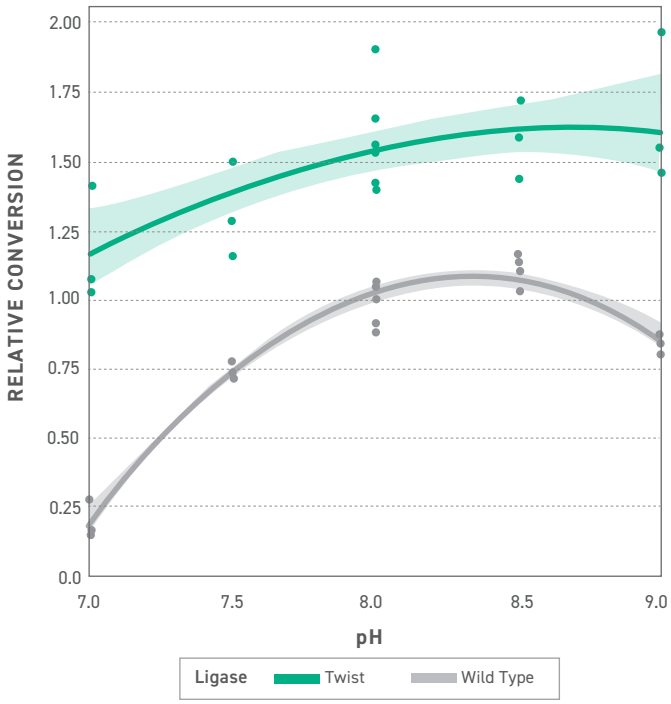


Figure 7. Ligation efficiency with a pH range of 7 to 9. Ligation reactions were carried out with an optimal mass of ligases (1000 ng for wild-type and 500 ng for Twist). Conversions are normalized to conversion achieved at 15 minutes with wild-type T4 DNA ligase at pH 8. Ligation reactions were done in replicates. After 15 minutes of incubation, reactions were heat-inactivated at 65°C for 10 minutes and assayed on qPCR with primers specific to the adapters attached by ligation.

IMPROVED STORAGE STABILITY

Longer enzyme shelf life can reduce waste and the frequency of enzyme replacement, which may lead to improvements in supply chain efficiency. To investigate stability, we incubated Twist-Engineered T4 DNA Ligase alongside wild-type T4 DNA ligase at room temperature (25°C) for 30 days. Residual activity was measured post-incubation with the protocol highlighted in the Materials and Methods sections. From qPCR results, Twist-Engineered T4 DNA Ligase retains 83% of its original activity after a 30-day incubation period (**Figure 8**). Based on accelerated aging estimations, the ligase would in theory have a shelf life of over 3 years.

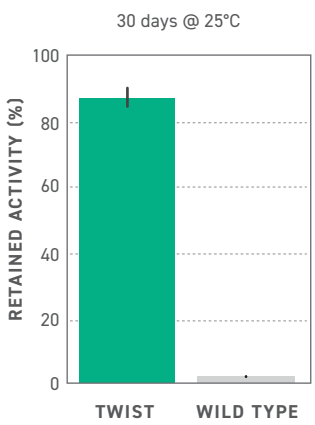


Figure 8. Residual activity observed after 25°C incubation for 30 days. Wild-type and Twist-Engineered T4 DNA Ligase were incubated at 0.5 mg/ml final concentration in a static incubator held at 25°C. Ligation reactions were done in replicates after 30 days following the protocol detailed in the Materials and Methods section and residual activity was calculated from individual ligases that were not aged.

CONCLUSION

The modifications introduced to the T4 DNA ligase improved performance across various parameters and conditions. From increased conversion efficiency, fast reaction times, improved pH and salt tolerance, to longer shelf life, the Twist-Engineered T4 DNA Ligase outperforms its wild-type counterpart. This ligase can be seamlessly integrated into existing workflows and will streamline processing time while also offering substantial benefits in reliability and versatility.

MATERIALS AND METHODS

For each enzyme property test, cfDNA Pan-Cancer Reference Standard v1 and Twist UMI Adapter System TruSeq Compatible were used as substrates and adapters for ligation conversion, respectively. cfDNA Reference Standard was end-repaired, dA-tailed, and bead-purified with enzymes and buffers from Twist cfDNA Library Prep Kit before being pooled together as substrates. Ligation reactions were performed under the following conditions unless otherwise stated: 20 µl of final reaction with 1x Twist T4 DNA Ligase Reaction Buffer with 15 ng of input cfDNA substrate incubated at 25°C for 15 minutes and heat-inactivated at 65°C for 10 minutes. Post-ligation reactions were analyzed by capillary electrophoresis with 2100 Bioanalyzer (Agilent) or diluted for SYBR-green qPCR with QuantStudio™ 6 Real-Time PCR (Thermo Fisher Scientific).

The Twist-Engineered T4 DNA Ligase was purified by over-expressing a recombinant gene with a hexahistidine tag from an *E. coli* strain. Wild-type T4 DNA ligase used in the comparison was purchased from different vendors.

REFERENCES

1. Head, S. R. et al. Library construction for next-generation sequencing: Overviews and challenges. BioTechniques. 56, 61-77 (2014).

2. Bilotti, K. et al. Mismatch discrimination and sequence bias during end-joining by DNA ligases. Nucleic Acids Res. 50, 4647–4658 (2022).