Covaris®

Ligation Yield is Enhanced by AFA-energetics™

Importance of Ligation in Biotechnology and Molecular Biology

Ligation is used in many processes covering different applications. A ligation reaction is used to covalently join DNA or RNA fragment ends with the help of ligase. Examples of applications that rely on ligation reactions as essential part of the process:

- *Ligation in NGS*: linker/adaptor to fragment ligation; circularization of transposase/cleavase products/fragments
- *CRISPR-Cas9*: circularization of DNA fragments for target specificity screening (*CircleSeq*)
- *Biotechnology*: cloning of gene constructs into vectors; 'splint' reaction for RNA synthesis; Gibson reaction for genome synthesis.

Ligation of Double-Stranded DNA Fragments

Joining two DNA fragments in a ligation reaction involves a quaternary complex, that contains the DNA ligase, two DNA fragments (5' phosphorylated ends), and ATP. for two pieces of DNA to be joined in a ligation reaction.

- *In vivo*, ligases join nicks in double-stranded DNA. The DNA ends are immobilized, waiting for the enzyme to ligate them together (i.e., intramolecular ligation).
- *In vitro*, ligases are used to join two separate DNA fragments which may or may not have self-complementary overhanging ends (i.e, intermolecular ligation).

Ligation of Blunt DNA Fragments: Balancing Speed and Efficiency

- Fragments with sticky ends are easier to ligate as compared to blunt-ended fragments as such fragments are fixed in place.
- Blunt-ended fragments or fragments with single nucleotide overhangs are dependent on collision events in which two fragment ends and enzyme meet.
- Since ATP is presented in vast excess the limiting factor is the number of DNA fragment end interactions in presence of DNA ligase.

Since ligation reactions of blunt DNA fragments are inefficient, mainly two methods are presently used to increase the ligation yields:

Ligation Method	Advantage	Disadvantage	Mitigation
Ligation at low temperature (8 to 14C)	Lowering molecular movement	Enzyme activity is reduced	
Ligation at ambient temperature in presence of viscous buffers (PEG)	Decreasing molecular movement and enzyme is almost fully active	Decreased diffusion of enzyme and DNA due to viscosity; cage effect	AFA: micro-mixing

Ligation reactions of fragments with blunt-ends or T/A overhangs are typically performed at room temperature, where the ligase is active, but are limited in product yield because molecular movement prevents the formation of a quaternary complex (ligase : fragment 1 : fragment 2: ATP). To overcome this, researchers either lower the temperature and incubate for longer or add a crowding reagent (i.e. PEG) to the ligation buffers to reduce molecular movement. Though crowding reagents increase ligation efficiency at shorter time points, they also can reduce the diffusion of reactants in the buffer.

Hypothesis: AFA-induced micromixing will counter the reduced diffusion in buffers with crowding agents.

In standard ligation reactions, double-linkerized products are unfavored as compared to single-linkerized fragments. This bias is 'overcome' by high linker to fragment ratios, which can result in downstream problems such as linker dimer formation, non-quantitative linker-removal, resulting in index-hopping due to linker carry-over in Illumina sequencers (***Figure 1***). Hence, low linker-fragment ratios (e.g., 10:1) are desirable without compromising linker-ligation yields.

Experimental setup Model System to Study Ligation

Joining two DNA fragments in a ligation reaction involves a quaternary complex, that contains the DNA ligase, two DNA fragments (5' phosphorylated ends), and ATP. for two pieces of DNA to be joined in a ligation reaction. The model system to study AFA-enhanced ligation was as follows: A 400 bp PCR fragment (5' phosphorylated primers) was amplified as the acceptor substrate. A 3'-A overhang was added (Taq polymerase incubation in presence of dATP @ 72C, 30 min), following purification via a spin column. Linkers/adapters were prepared by hybridizing a 60 and a 61 nt synthetic oligonucleotides, resulting in a double-stranded adapter. This adapter contains a 5' phosphate and a 3' T overhangs only on one end, whereas the other end is blocked from ligation by a 3' phosphate. Reactions were performed in the Covaris 96 plate oneTUBE. Reactions that were done in presence of AFA, were done on the Covaris E220 Focused-ultrasonicator. Standard ligation reactions using commercial kits were performed at recommended conditions. All reaction volumes were 20 µl.

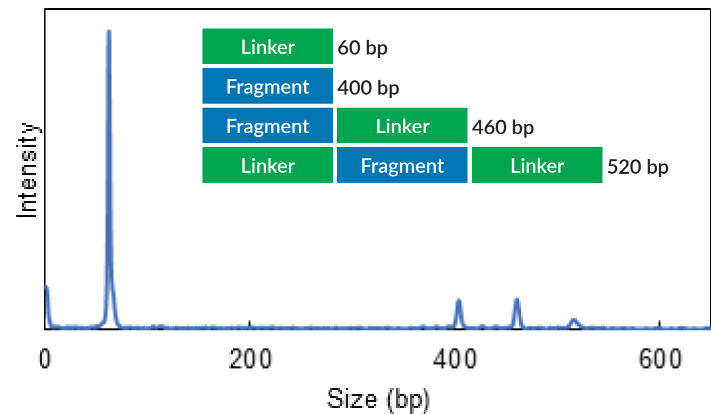

Figure 1. Representative graph of the raw data produced when using gel electrophoresis to measure ligation. When 60 bp linkers are ligated to a 400 bp fragment, a product peak of 460 and 520 bp appears, accounting for fragments with 1 and 2-linkers attached. The magnitude of these peaks can be correlated to an amount, and are used to estimate product yield.

AFA-Energetics Enhances Ligation Compared to Standard Kits

- When AFA is applied to quick ligation kits, which have PEG in the buffer, the yield of 2-linker fragments in increased over time.
- Double-ligated linker fragment yields are significantly increased in presence of AFA treatment.
- AFA-treatment of ligation reactions can be used to increase ligation efficiencies and to decrease reaction times.
- AFA-assisted ligation plus an optimized ligation buffer converts >50% of the input fragments into double-linkerized fragments as compared to <15% when using commercial ligation kits.

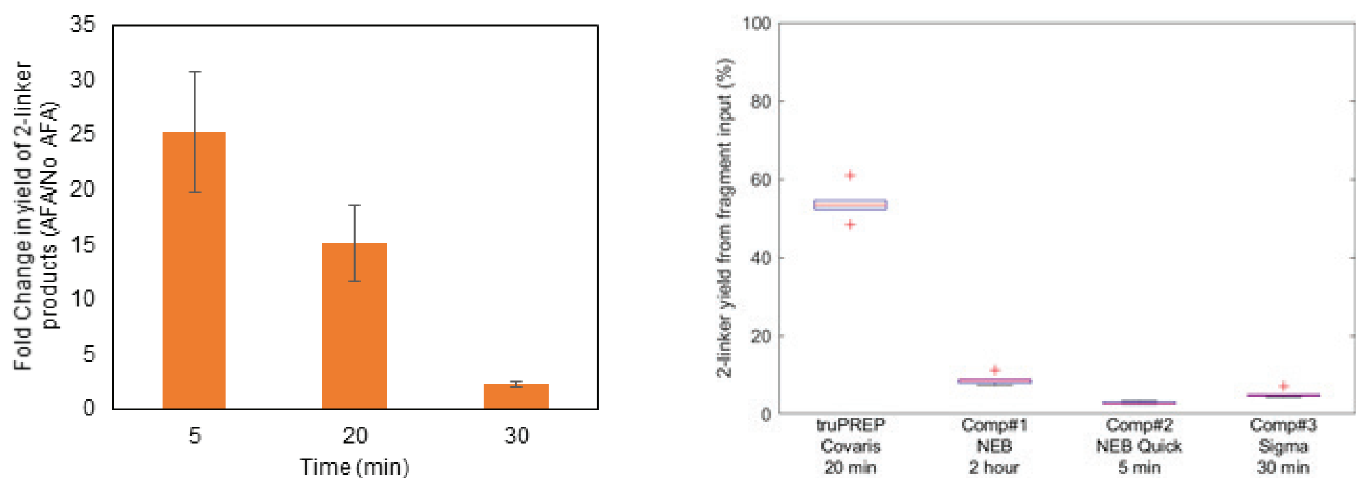

Figure 2. (***left***) Fold change in two-linker fragment yield when the quick ligation kit from NEB is used with and without AFA. (***right***) A comparison of the final percent yield of DNA fragments with 2-linkers using AFA-mediated ligation done in the truPREP™ Ligation kit from Covaris and 3 competitor kits run according to the manufacturer's instructions.

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