Joining of Difficult to Ligate dsDNA Fragments with Blunt/TA Ligase Master Mix

Introduction

T4 DNA ligase is routinely used to ligate breaks on one strand of a dsDNA molecule (nicks), or to ligate a variety of double-stranded breaks. This includes "cohesive ends", with two or more bases of complementary single-stranded regions (such as those generated by BamHI-HF®), and fully base-paired fragments or "blunt-ends" (such as those generated by EcoRV). Certain ligation substrates are known to be difficult to ligate, with sluggish rates and poor yields even with prolonged incubation periods and high enzyme concentration. Commonly encountered ends that are difficult to ligate include single base overhangs, with 5´-single base overhangs (such as those generated by BstNI) showing almost no ligation (< 5%) by T4 DNA ligase under typical conditions.

The Blunt/TA Ligase Master Mix (NEB #M0367) is a formulation of T4 DNA ligase pre-mixed with its reaction buffer and proprietary ligation enhancers, in a convenient single-tube 2X mixture. In most cloning applications, combining equal volumes of master mix and a solution of vector and insert will provide rapid ligation and high colony yield after transformation equaling, and sometimes exceeding, the Quick Ligation™ Kit. Additionally, the Blunt/TA Ligase Master Mix has been found to give superior ligation yields for particularly difficult to ligate substrates, substantially exceeding that of any other ligase formulations.

(see other side)

Materials

- DNA substrates in nuclease-free water (2 µM in ligatable ends, ~50 ng/µl 20-50 bp dsDNA fragments)
- High concentration T4 DNA Ligase (NEB #M0202)
- T4 DNA Ligase Buffer (10X, Included with High Concentration T4 DNA Ligase)
- Quick T4 DNA Ligase (NEB #M2200)
- Quick Ligation™ Reaction Buffer
- Blunt/TA Ligase Master Mix (NEB #M0367)
- Diluent A (NEB #B8001)
- Nuclease-free water
- Stop solution (50 mM EDTA)

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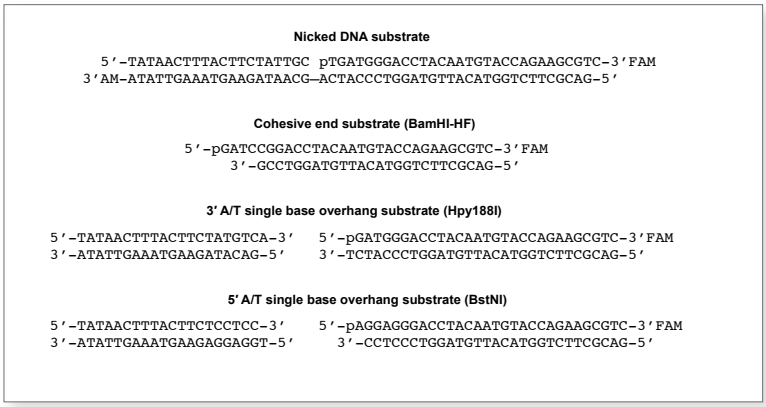
Here, we demonstrate the use of Blunt/TA Ligase Master Mix for the ligation of difficult-to-ligate dsDNA fragments. The substrates tested were prepared from short, synthetic oligonucleotides purchased from IDT, and annealed using standard conditions (Figure 1). Each substrate contains one oligonucleotide 5′-phosphorylated and 3′-FAM labeled to allow ease of quantitation.

- Nicked DNA Substrate: a 50 bp dsDNA with a break on one strand between nucleotides 20 and 21. The 30-base strand was 5′-phosphorylated and 3′-FAM labeled.
- Cohesive-end Substrate: a 30-base strand 5′-phosphorylated and 3′-FAM labeled with a 26-base complement strand, resulting in a substrate with a 4-base, 5′-overhang modeling the DNA ends generated by treatment with BamHI-HF.
- 3′-A/T Single-base Overhang Substrate: a 29-base strand 5′-phosphorylated and 3′-FAM labeled with a 30-base complement strand, and a second fragment with a 21-base oligonucleotide annealed to a 20-base complement strand modeling fragments generated by Hpy188I, a 3′ single-base dA overhang on one fragment and a dT on the other. This substrate also serves as a surrogate for a "TA cloning" substrate, such as insertion of a *Taq* DNA Polymerase amplicon with a 3′ adenosine into a "T-vector", or for adapter ligation during an NGS library prep.
- 5′-A/T Single-base Overhang Substrate: a 30-base strand 5′-phosphorylated and 3′-FAM labeled with a 29-base complement strand, and a second fragment with a 20-base oligonucleotide annealed to a 21-base complement strand modeling fragments generated by BstNI, a 5′ single-base dA overhang on one fragment and a dT on the other.

Figure 1. Substrate sequences tested



Protocol

1. Transfer master mix to ice prior to reaction setup. Mix tube by flicking before use.

2. Combine 5′-phosphorylated dsDNA ligation substrates in water with an equal volume of Blunt/TA Master Mix.

3. Mix thoroughly, by pipetting up and down 7-10 times or by flicking.

4. Incubate at room temperature (25°C) for 15 minutes. Then, place the tube on ice (incubation times can be extended up to 1 hour if yields are still low after 15 minutes).*

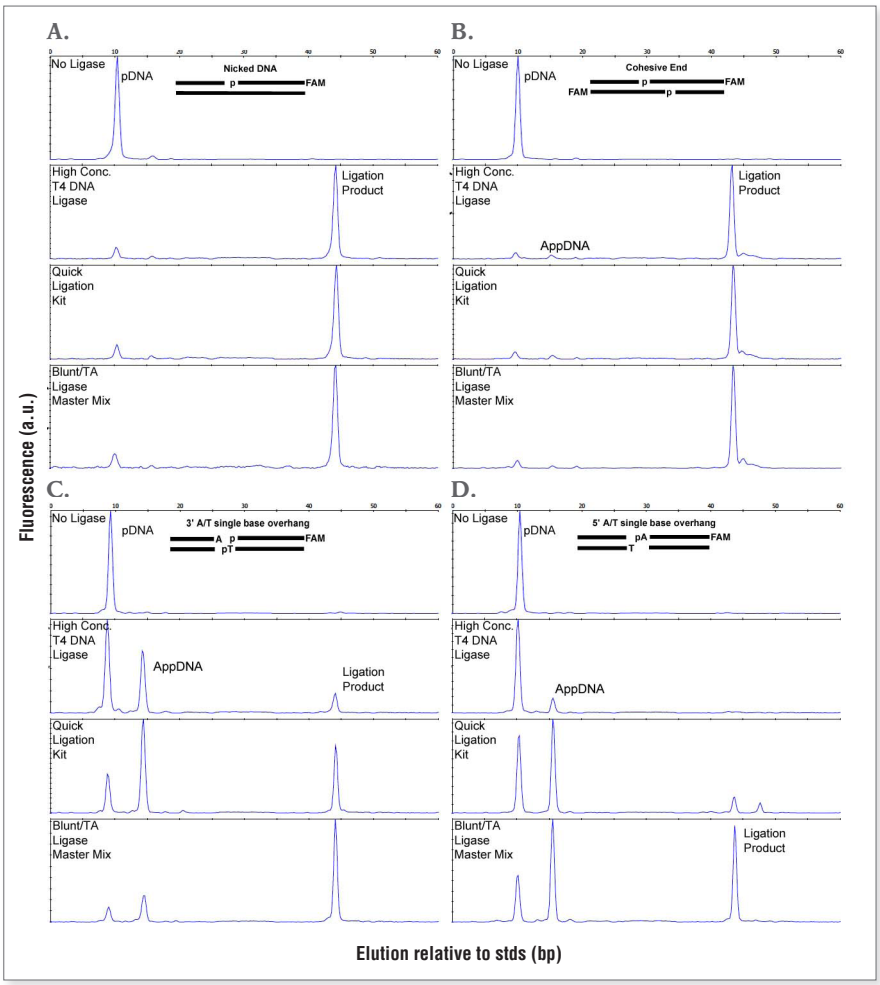
   * Incubation overnight may give improved ligation yields; however, DNA intended for transformation applications should never be incubated longer than 1 hour in the ligation mixture. For overnight incubations and/or prolonged DNA storage, the ligation products should be purified using and appropriate method (e.g., spin column, AMPure® Beads, gel purification) before storage at –20°C.

5. Stop the reaction by adding 10 µl of stop solution (50 mM EDTA). Do not heat inactivate.

Results

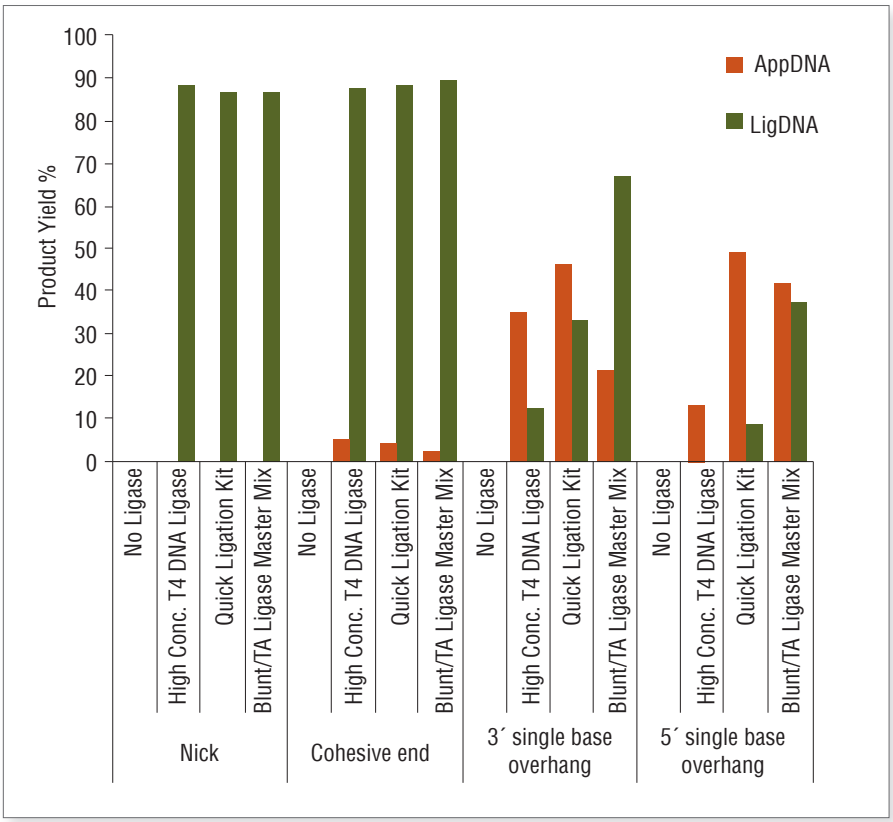
The extent of ligation in each reaction was visualized by detection of the FAM-labeled strands by high throughput capillary electrophoresis, which allows for high accuracy in quantitation and product identification. For both nicked DNA (Fig. 2A) and cohesive ends (Fig. 2B), all three ligase products perform similarly, with high ligation yields in a short reaction time. For the more difficult to ligate 3´-A/T single base overhang (Fig. 2C), ligation produces "AppDNA" in addition to ligated product. AppDNA is a reaction intermediate in ligation where the ligase has transferred an enzyme-conjugated 5´phosphoryladenosine (Ap) group to the 5´-phosphate of the DNA substrate, producing an activated 5´-5´ AppN linkage at the 5´ terminus. In the course of the ligation of an easily ligatable substrate, this intermediate is not often observed. For very difficult ligations, the intermediate step can dissociate prematurely, accumulating in solution as a side product. Improved ligation yields are observed with the Quick Ligation Kit, but the Blunt/TA Master Mix produces even higher yields of ligated DNA. For the exceedingly difficult to ligate 5´A/T-single base overhang (Fig. 2D) substrate, almost no reaction is observed when using high concentration T4 DNA ligase. The Quick Ligation Kit produces predominantly AppDNA, but the Blunt/TA Master Mix again provides superior yields of ligated DNA (Fig. 3).

Figure 2. Blunt/TA Ligase Master Mix provides superior ligation yields for difficult templates



Ligation reactions (100 nM ligatable ends in a 20 µl total reaction volume) were analyzed by capillary electrophoresis (CE) fragment analysis on an Applied Biosystems 3730xl Genetic Analyzer. In all cases, the FAM-labeled DNA oligonucleotides can be observed and quantified by fluorescence. For nick ligations (A) and cohesive ends (B), we see that all three ligase formulations rapidly convert the starting material (pDNA) to ligated product in 15 minutes. For 3´ over- hangs (C), a 15 minute incubation resulted in only partial ligation for high-concentration T4 DNA Ligase and Quick Ligase, with large amounts of unreacted starting material and AppDNA, but the Blunt/TA Master Mix gives superior ligation yields. For the very slow-to- ligate 5´ overhangs (D), incubation for 1 hour resulted in very little product,except when the Blunt/TA Master Mix was used.

Figure 3. Blunt/TA Ligase Master Mix improves yields for ends that typically react slowly



Yields of intermediate (AppDNA) and final ligation product (LigDNA) for all reaction conditions. Nick, cohesive end and 3´ single-base overhang substrates were incubated for 15 minutes; the 5´ single-base overhang was incubated for 1 hour.

Conclusion

When attempting to ligate dsDNA ends that typically react slowly and with poor ligation yield, the Blunt/TA Ligase Master Mix will provide superior ligation yields over other T4 DNA ligase formulations. When ligating more robust substrates (cohesive ends, nicks, blunt ends) the Blunt/TA Master Mix performs similarly to the Quick Ligation Kit in terms of yield and reaction time, and can be substituted for other T4 DNA ligase preparations for convenience.

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