Generating Oligonucleotide Probes using Immobilized T4 DNA Ligase minimizes sample loss and saves time

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INTRODUCTION

Fluorescently labeled oligonucleotides (oligos) are often utilized as probes and/or substrates in molecular biology. They provide a safer option compared to radiolabels and are free from the stringent regulatory requirements associated with radiolabel use. Additionally, based on the user's needs and the equipment at their disposal, these fluorescent probes can be tailored to allow for multiplexing either on a gel or through more advanced, high-throughput techniques such as capillary electrophoresis.

Oligonucleotide probes comprised of a fluorescent label and site-specific modification(s) are generated using solid-phase chemical synthesis. Customized oligonucleotide synthesis is available commercially, generally with prompt delivery of the target oligonucleotide. However, challenges such as inefficient synthesis schemes can arise when specific fluorophore/modification combinations require distinct reaction conditions during solid phase synthesis and subsequent cleavage and deprotection from the solid support. These issues result in increased cost and can also lead to much longer lead times when ordering oligonucleotides from commercial vendors.

Alternatively, a ligation strategy (Figure 1) can be employed to generate a dual-modified oligonucleotide from the distinct chemically synthesized fragments, each containing either the fluorophore or the modification. Ligating a fluorophore-containing oligo to a modification-containing oligo allows the user to quickly screen various scaffolds/modifications (e.g., combining a commonly used fluorescently labeled oligo with various modification-bearing oligos). Purification of shorter oligonucleotides is more effective (i.e., one has a greater ability to eliminate N-1 synthetic impurities), resulting in higher purity substrates while minimizing costs and lead times.

The drawback of a ligation-based strategy is the need to remove the enzyme and purify the ligated oligo. Typical yields after a PAGE purification with "crush and soak" isolation purification method can vary widely from <30% to 90% (1). Apart from time lost from these procedures – they can often take more than a day – precious sample can be unnecessarily lost.

To address this, and to facilitate a quick, simple, high-yielding, fluorophore attachment to a modified oligo, we demonstrate here the easy and effective use of Immobilized T4 DNA Ligase (IM T4 DNA Ligase, NEB #M0569). The target oligo contained an expensive and difficult-to-synthesize cyclopyrimidine dimer (CPD) (2) modification to which we attached a 5′ FAM fluorophore to visualize respective enzymatic reactions (Figure 2, page 2). Employing the appropriate stoichiometric ratios of the respective fragments allows a purification-free ligation reaction that is immediately ready for screening via capillary electrophoresis. It is important to note the requirement of a 5′ phosphate in the oligo component that is 3′ in the ligase reaction. The phosphate can be introduced chemically or added using T4 Polynucleotide Kinase (NEB #M0201).

MATERIALS

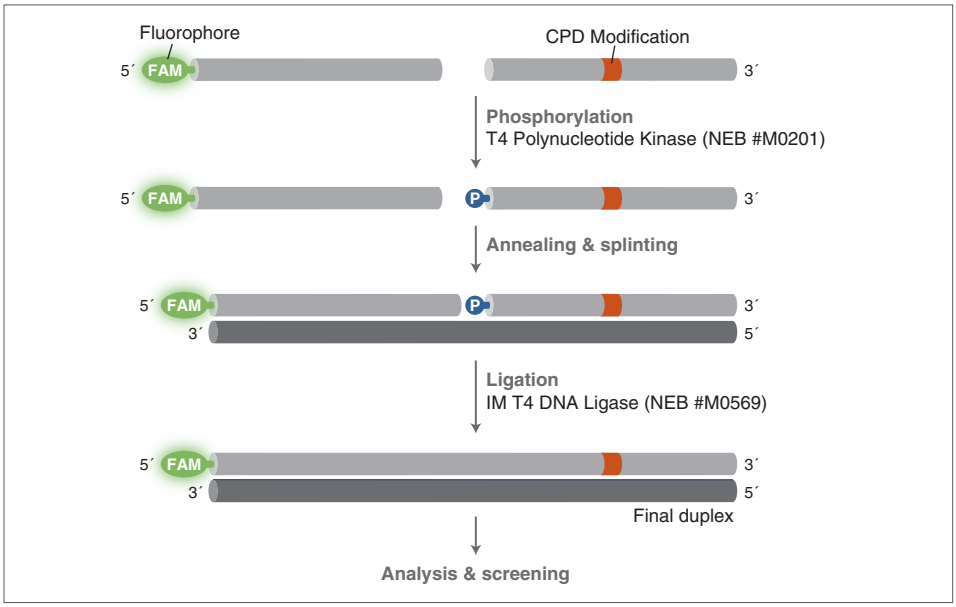
- Immobilized T4 DNA Ligase (NEB #M0569)
- T4 PNK (NEB #M0201)
- Endonuclease IV (NEB #M0304)
- T4 PDG (T4 Endonuclease V) (NEB #M0308)

METHODS

Annealing

10 µl of the CPD-containing oligo with a 5′ phosphate (final concentration 4.7 µM), 31.2 µl of water, 0.4 µl of FAM-DNA (100 µM), 0.9 µl of complementary oligonucleotide (100 µM), and 5 µl of 10X ligase buffer was added to a PCR tube. Annealing was performed by heating the mixture to 80°C for 2 minutes, followed by ramping down the temperature at 0.1°C/sec. to 25°C.

FIGURE 1: **Immobilized T4 DNA Ligase workflow**



Assembly of target oligo is achieved by first phosphorylating the 5′ end of the unlabelled, damage containing oligo. The phosphorylated oligo is then annealed with the fluorophore-containing oligo and splint. Subsequently, Immobilized T4 DNA Ligase is added to seal the nick and create intact duplex ready for screening. The Immobilized T4 DNA Ligase is removed from the newly assembled duplex by placing on a magnetic rack and removing the supernatant.

Ligation

2.5 µl of Immobilized T4 DNA Ligase (NEB #M0569) was added to the annealed components and mixed well by pipetting 10 times. The reaction was left at room temperature overnight. The following day, the reaction tube was placed on a magnetic rack to pellet the IM T4 DNA Ligase and the supernatant was transferred to a fresh tube. No sample loss was observed.

Oligo incubation with T4 PDG and end cleanup with Endonuclease IV

A 20 nM reaction was conducted in 1X T4 PDG reaction buffer for 30 minutes at 37°C with either PDG or no PDG added. 1 µl of Endonuclease IV (Endo IV) (NEB #M0304) was added, and the mixture was incubated for 15 minutes. These samples were then diluted to 5 nM with nuclease-free water and analyzed by capillary electrophoresis (CE).

RESULTS

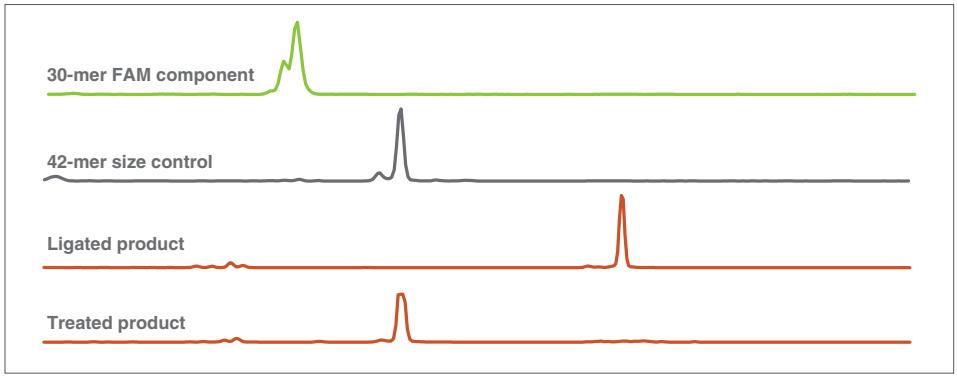
Optimal annealing ratios enable single signal

Optimization of the ratios used for annealing allows for total consumption of the oligo that contains a fluorophore. As shown in Figure 2, a slight excess of unlabeled oligo prevents any unligated fluorophore-containing oligo from complicating the readout. The correct ratios, therefore, result in only a single, full-length peak in the CE trace.

Successful incorporation of CPD-modification

While the consumption of fluorophore-containing oligo confirmed a ligation event, it did not show that the CPD modification was successfully incorporated. However, incubation with T4 PDG and, subsequently, Endo IV to homogenize the product ends confirmed the successful incorporation of the CPD site in the ligated oligo. The presence of a single product indicated no shorter oligos were present with the CPD site, and the complete conversion of the product peak confirmed all fully ligated material did contain the CPD site.

FIGURE 2: **Capillary electrophoresis traces from various points of workflow**



The unligated 30-mer FAM component runs fastest on CE (top). A 42-mer size control is included to match the anticipated reaction product following incubation of target oligo with T4 PDG and Endo IV (second from top). The ligation reaction can be successfully visualized by the shift to a much larger size, that elutes slower on CE, of the ligated product (second from bottom). Finally, enzymatic treatment of the ligated product reveals successful incorporation of the modification that can now be easily visualized on CE (bottom).

TABLE 1: **Oligonucleotide sequences utilized for this workflow**

COMPONENT	SEQUENCE
Damage Containing Oligo	5´ TCAACCAGCTCG**TT**GGACACGCAAGGGCCT 3´
Fluorophore Containing Oligo	5´ FAM-TGGAGATTTTGATCACGGTAACCCATCAGA 3´
Splint/Complement	5´ AGGCCCTTGCGTGTCCAACGAGCTGGTTGATCTGATGGGTTACCGTGATCAAAATCTCCA 3´
Final Duplex	5´ FAM-TGGAGATTTTGATCACGGTAACCCATCAGATCAACCAGCTCG**TT**GGACACGCAAGGGCCT 3´ 3´ ACCTCTAAAACTAGTGCCATTGGGTAGTCTAGTTGGTCGAGCAACCTGTGCGTTCCCGGA 5´

Bold orange indicates dimer

CONCLUSION

Immobilized T4 DNA Ligase enables an efficient approach to generating oligonucleotide probes/substrates containing multiple modifications. We have demonstrated here how this product can attach a fluorescently-labeled oligo to an oligo containing a specific modification in an easy, straightforward manner. Easy removal of IM T4 DNA Ligase using a magnetic rack limits the loss of precious sample, which occurs during typical post-ligation clean-up (a requirement when using soluble enzymes). This process facilitates enzyme screening assays – completing ligation and screening – within a day. We envision this process can be used in various workflows requiring ligation to help minimize purification steps and sample loss.

References

1. Green, M. R. and Sambrook, J. (2019). *Cold Spring Harbor Protocols* (2): pdb.prot100479.
2. https://www.glenresearch.com/reports/gr16-21