kilobaser

QUALITY REPORT

OF PHOSPHORYLATED OLIGONUCLEOTIDES

Synthesized by Kilobaser one-XT

Abstract

Direct synthesis of phosphorylated DNA strands is the simplest way to prepare short inserts for ligation. An insert is formed by two separately synthesized strands that hybridize to a double-stranded DNA fragment under ligating conditions. In the analysis at hand, strand quality and insert formation were examined. The results of the analysis confirm that Kilobaser one-XT is fully capable of synthesizing phosphorylated strands of the required quality and quantity.

Description

Phosphorylation of oligonucleotides is only required for synthetic oligonucleotides, as they lack a phosphate group at the 5'-end after synthesis. Most applications of synthetic oligonucleotides do not require 5'-phosphorylation. However, it is essential for ligation reactions, since ligase can only join two strands efficiently when the phosphate group is present, as shown in figure 1.[1-4] The most common enzyme used for ligation is T4 DNA ligase, which links DNA ends via 5'-phosphate and 3'-hydroxy groups.

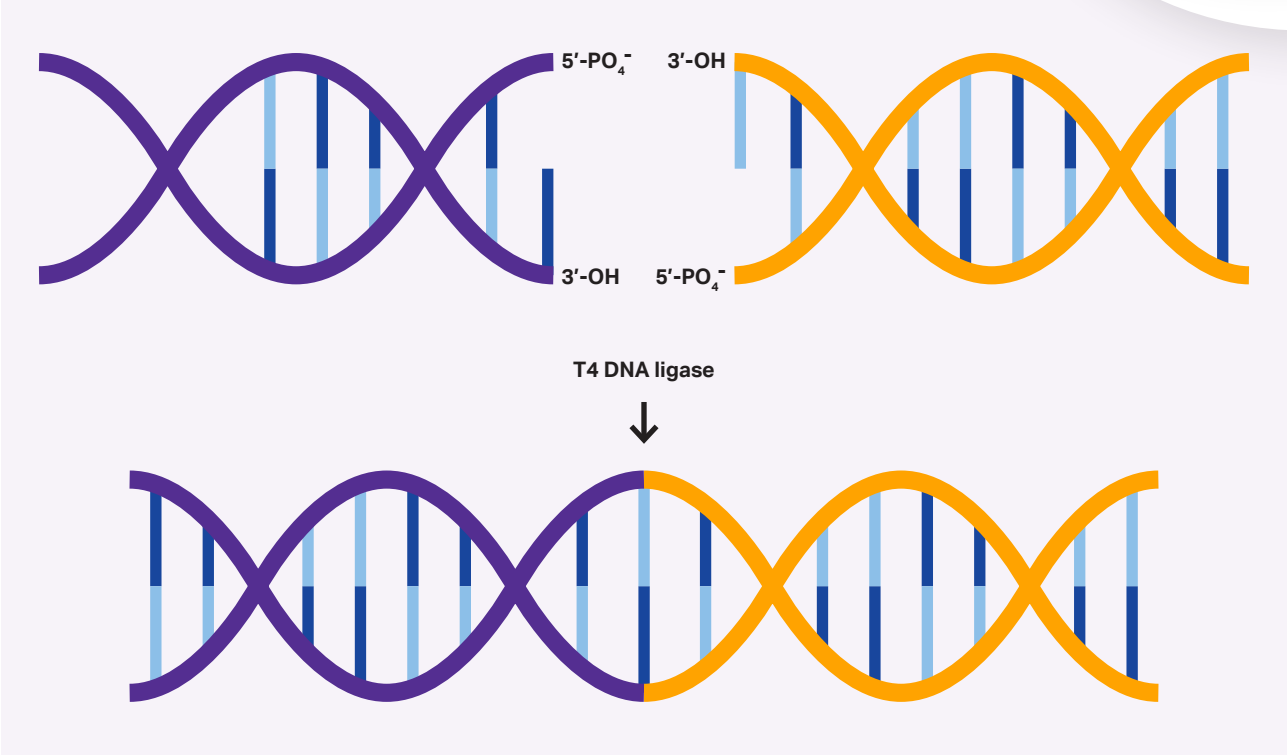

Figure 1: Ligation of DNA by T4 DNA ligase

Thus, ligation efficiency depends on the presence of the 5′-phosphate. There are three ways to phosphorylate the 5′-end of the DNA strand:

1. Phosphorylation by a DNA synthesizer
2. Phosphorylation by kinases
3. Cutting of double strand DNA (dsDNA) by restriction enzymes

Cutting of dsDNA, such as vectors and inserts, by a restriction enzyme results in strand ends with the necessary reactive groups—5′-phosphate and 3′-hydroxy. However, DNA fragments generated by the polymerase chain reaction (PCR) are not all phosphorylated at the 5′-ends, due to the use of synthetic primers. The PCR product needs to be treated separately with a kinase, such as T4 polynucleotide kinase, to introduce the 5′-phosphate. To avoid kinase treatment, primers for PCR may be synthesized directly with the phosphorylation, using a DNA synthesizer. If no PCR is required, the insert itself can be synthesized with the 5′-phosphate.

To synthesize the DNA strand, phoshoramidites containing the respective bases are used as building blocks. The phosphate group for the phosphorylation comes from one phosphoramidite, which consists of the phosphate and protective groups. The protective groups are cleaved off in the final step of the synthesis, resulting in a free phosphate group at the 5′-end of the DNA strand. When this phosphoramidite is applied directly during DNA synthesis, the phosphorylation delivers significantly higher yields than using kinases.

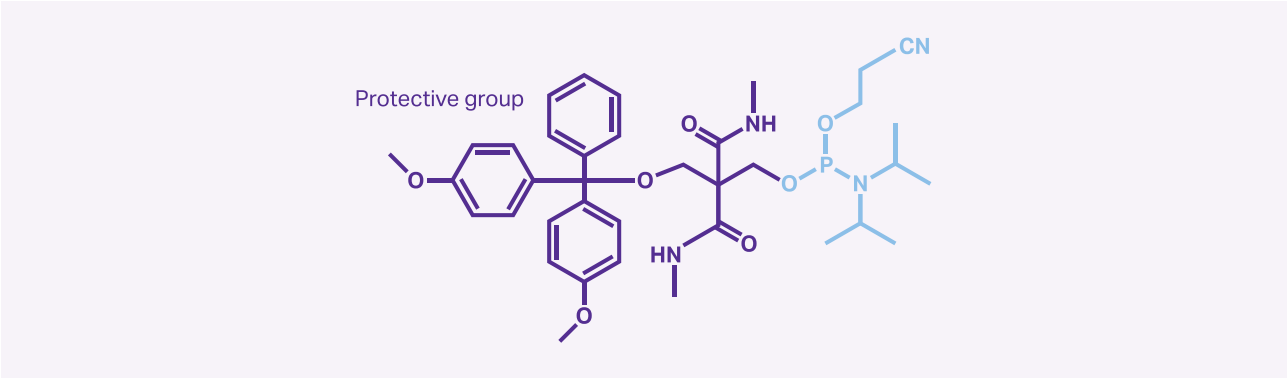

Figure 2: Phosphoramidite for building 5′-phosphorylated oligonucleotides

References:

1. Lehman IR: DNA ligase: structure, mechanism, and function. Science 1974, 186:790-797.
2. Sugino A, Snoper TJ, Cozzarelli NR. Bacteriophage T4 RNA ligase. Reaction intermediates and interaction of substrates. J Biol Chem1977, Mar 10;252(5):1732-8.
3. Pascal JM: DNA and RNA ligases: structural variations and shared mechanisms. Curr Opin Struct Biol 2008, 18, 96-105.
4. Shuman S: DNA ligases: progress and prospects. J Biol Chem 2009, Jun 26;284(26):17365-9.

Application

A plasmid can be edited by insertion of a new DNA fragment into its strand. This is done by first cutting the plasmid strand and subsequently ligating an insert to either end of the cutting site. An overhanging cut is often used to achieve directional insertion. One example of such an insertion is the addition of a reverse primer that needs to be on the reverse strand at the end of the ligation for PCR, as shown in figure 3.

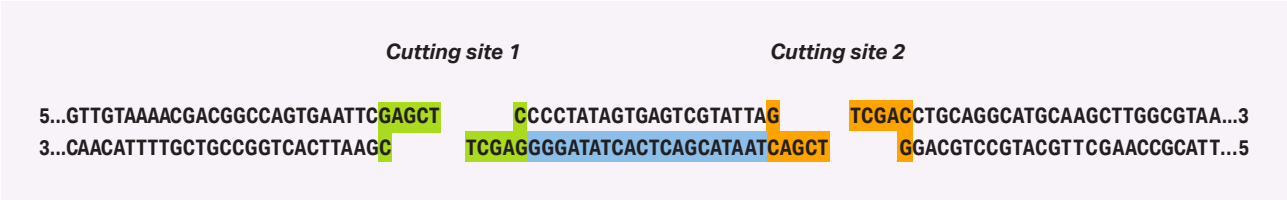

Figure 3: Design of an insert to add a reverse primer into a DNA strand

An appropriate insert is formed by two separately synthesized strands with the phosphorylated 5'-end for ligation. These two strands hybridize to a short strand of dsDNA that features two overhangs for annealing with the cut plasmid. To ligate the insert successfully into the plasmid, the insert has to form a dsDNA strand under ligation conditions.

To prove the successful synthesis of short inserts by Kilobaser one-XT, the two strands were synthesized and analyzed. Afterwards, the formation of a dsDNA strand was tested through melting curve analysis.

Synthesized Oligonucleotides

#	Name	Modification	Sequence
1	Insert with new primer, sense	5'-Phosphorylation	5'-CCCCTATAGTGAGTCGTATTAG-3'
2	Insert with new primer, antisense	5'-Phosphorylation	5'-TCGACTAATACGACTCACTATAGGGGAGCT-3'

Measurements

Device	Method	Outcome
Advion AVANT™ UHPLC System and Advion expression® Compact Mass Spectrometer (CMS)	Reverse HPLC to separate oligonucleotides from each other by their length and modifications Separated oligonucleotides are detected via absorption at 260 nm and analyzed in the mass spectrometer	• Chemical composition of sample • Differentiation of oligonucleotides in length and number of modifications • Identification of modifications • Yield in % of full-length product
Qubit 3.0 Fluorometer	Fluorometric quantification of ssDNA	Yield of synthesis in pmol
Thermocycler - Open qPCR by Chai	Melting curve analysis	Hybridization efficiency

Synthesis 1 Insert with new primer, sense

Sample type Phosphorylated DNA Oligonucleotide
Sequence 5'Phos-CCCCTATAGTGAGTCGTATTAG-3'
Length 22 nt

Chemical composition, mass and purity

After synthesis, the oligonucleotide was analyzed with reverse HPLC, which separates the synthesis products and side products according to their size and chemical polarity. The first results of the analysis can be seen in the chromatogram in figure 4.

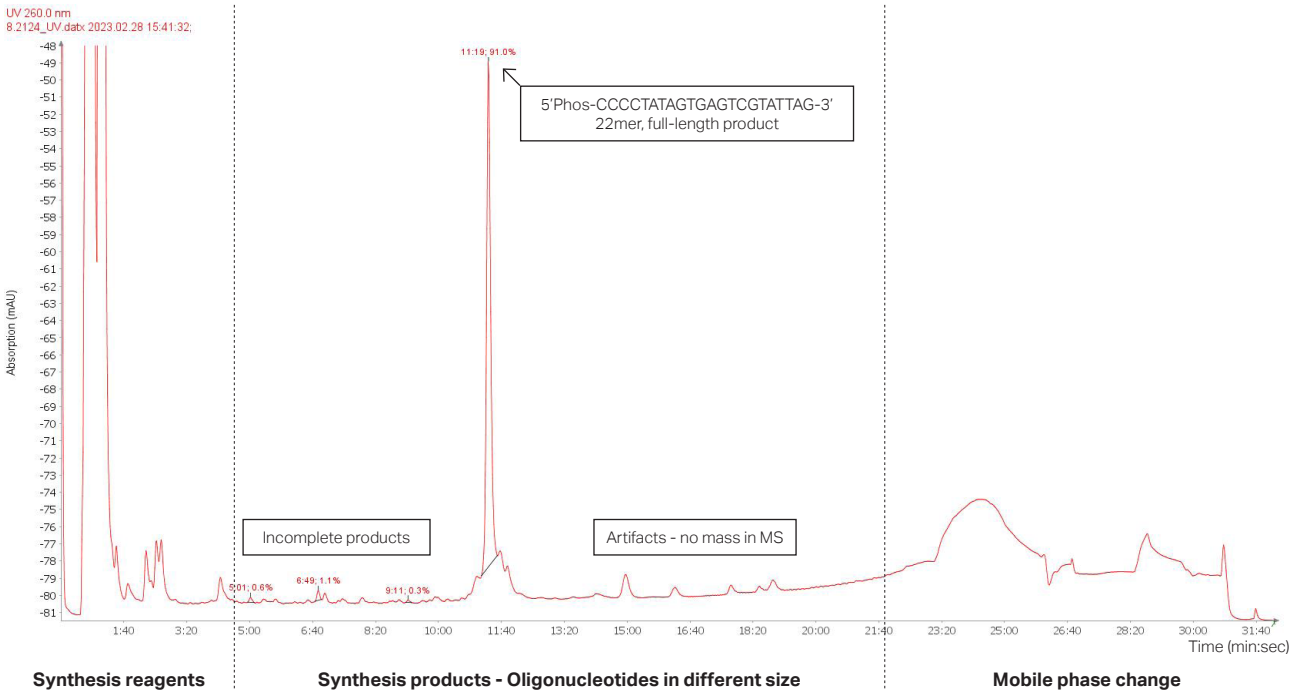

Figure 4: Chromatogram of reverse HPLC collecting absorption at 260 nm overtime

The chromatogram of this synthesis shows the following peaks, from left to right:

- Peaks arriving during the first 5 minutes of the HPLC are mainly caused by synthesis reagents.
- 5 minutes into the HPLC, oligonucleotides of various lengths become visible.
- **The peak at 11:19 is the full-length product and the main product of the synthesis.**
- Shortly before and after this peak, other peaks appear that are not completely separated from the main peak due to the decreasing separation efficiency of the oligonucleotides at this length.
- Peaks arriving after 15 minutes are artefacts of the UV measurements, as these peaks do not show a mass in the mass spectrometer (MS) when measured in parallel by both methods.

After separation via HPLC, the synthesis products were further analyzed through a mass spectrometer to confirm their chemical composition and mass.
The mass spectrum confirms that the main product (see peak at 11:19 in HPLC) is the full-length oligonucleotide, which is also completely deprotected and is thus ready to use for further applications.

Measured Mass = 6803.0 g/mol
Calculated Mass = 6804.4 g/mol

Yield of synthesis
The total yield of functional oligonucleotide – full length, completely deprotected – was determined considering the following values:

- Resuspension volume for the analysis: 20 µL
- Concentration of ssDNA via Qubit Fluorometer: 99.8 ng/µL
- Purification degree based on chromatogram: 91 %
- Molecular weight without phosphate: 6725.4 g/mol

This results in a yield of **270 pmol** of functional oligonucleotide.

Outcome of synthesis
The synthesis performed well, resulting in a final yield of 270 pmol full-length product, equating 91 % of all detectable oligonucleotides. Based on these results, it can be concluded that the synthesis of phosphorylated oligonucleotides of this length was successful.

Synthesis 2 Insert with new primer, antisense

Sample type Phosphorylated DNA Oligonucleotide
Sequence 5'Phos-TCGACTAATACGACTCACTATAGGGGAGCT-3'
Length 30 nt

Chemical composition, mass and purity

After synthesis, the oligonucleotide was analyzed with reverse HPLC, which separates the synthesis products and side products depending on their size and chemical polarity. The first outcome of the analysis is the chromatogram shown in figure 5.

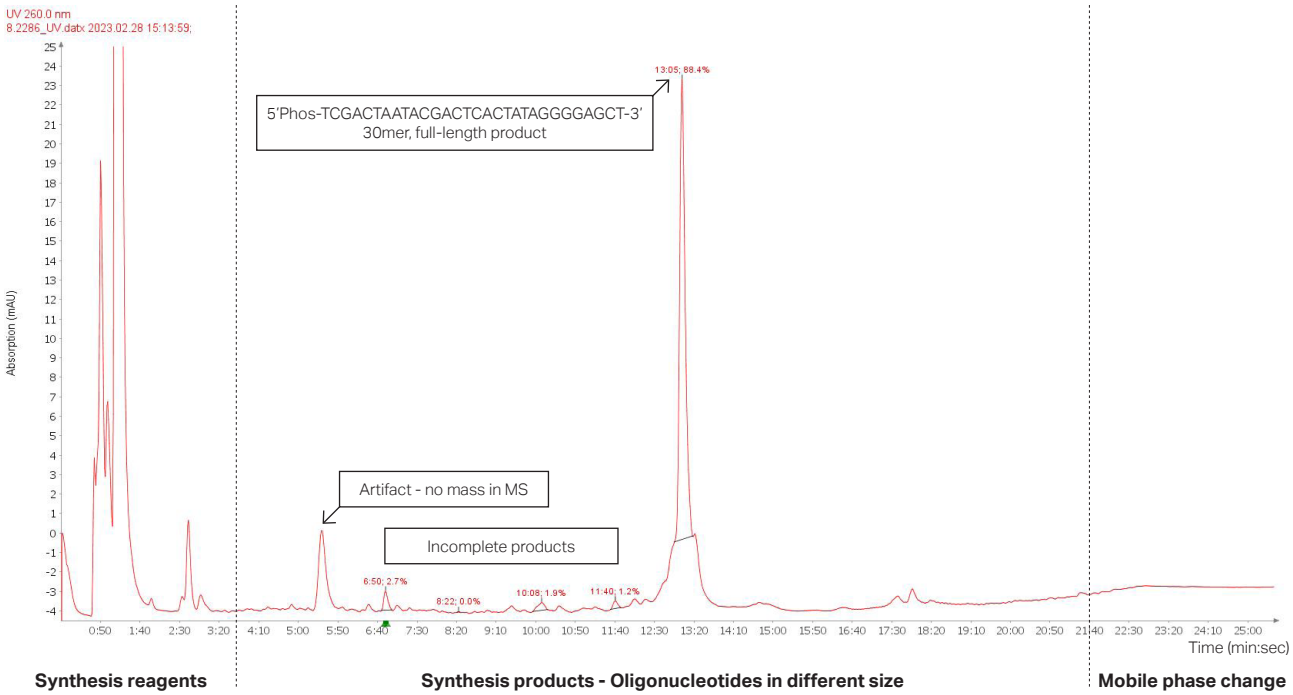

Figure 5: Chromatogram of reverse HPLC collecting absorption at 260 nm overtime

The chromatogram of this synthesis shows following peaks from left to right:

- Peaks arriving during the first 3 minutes of the HPLC are mainly caused by synthesis reagents.
- The peaks arriving after 5 minutes are artefacts of the UV measurements, as these peaks do not show a mass in the mass spectrometer (MS) when measured in parallel by both methods.
- 6 minutes into the HPLC, oligonucleotides of various lengths become visible.
- The peak at 13:05 is the full-length product and the main product of the synthesis.
- Shortly before and after this peak, there are other peaks that are not completely separated from the main peak due to the decreasing separation efficiency of the oligonucleotides at this length.
- Peaks arriving after 14 minutes are artefacts of the UV measurements, as these peaks do not show a mass in the mass spectrometer (MS) when measured in parallel by both methods.

After separation via HPLC, the synthesis products are further analyzed through a mass spectrometer to confirm their chemical composition and mass.
The mass spectrum confirms that the main product (the peak at 13:05 in HPLC) is the full-length oligonucleotide, which is also completely deprotected and is thus ready to use for further applications.

Measured Mass = 9293.8 g/mol
Calculated Mass = 9294.1 g/mol

Yield of synthesis
The total yield of functional oligonucleotide – full length, completely deprotected – was determined considering the following values:

- Resuspension volume for the analysis: 30 µL
- Concentration of ssDNA via Qubit Fluorometer: 42.6 ng/µL
- Purification degree based on chromatogram: 88.4 %
- Molecular weight without phosphate: 9215.1 g/mol

This results in a yield of **122.6 pmol** of functional oligonucleotide.

Outcome of synthesis
The synthesis performed well, resulting in a final yield of 123 pmol full-length product, i.e., 88 % of all detectable oligonucleotides of this synthesis. Based on these results, it can be concluded that the synthesis of phosphorylated oligonucleotides of this length was successful.

Result of hybridization test

Melting curve analysis allows the detection of double strand formation and its stability against heat, represented by the melting point. The melting points of the synthesized oligonucleotides – oligo 1 (insert, sense) and oligo 2 (insert, antisense) - are visible as peaks in figure 6, in which dF/dT is plotted against the temperature.

It can be seen that:

- Each oligonucleotide alone (oligo 1 in purple, oligo 2 in green) forms unspecific double strands at low temperature that disappear quickly with the increase in temperature.
- Mixing the oligonucleotides together forms the insert (see orange graph), a 22 nt long double strand that melts at 60 °C.

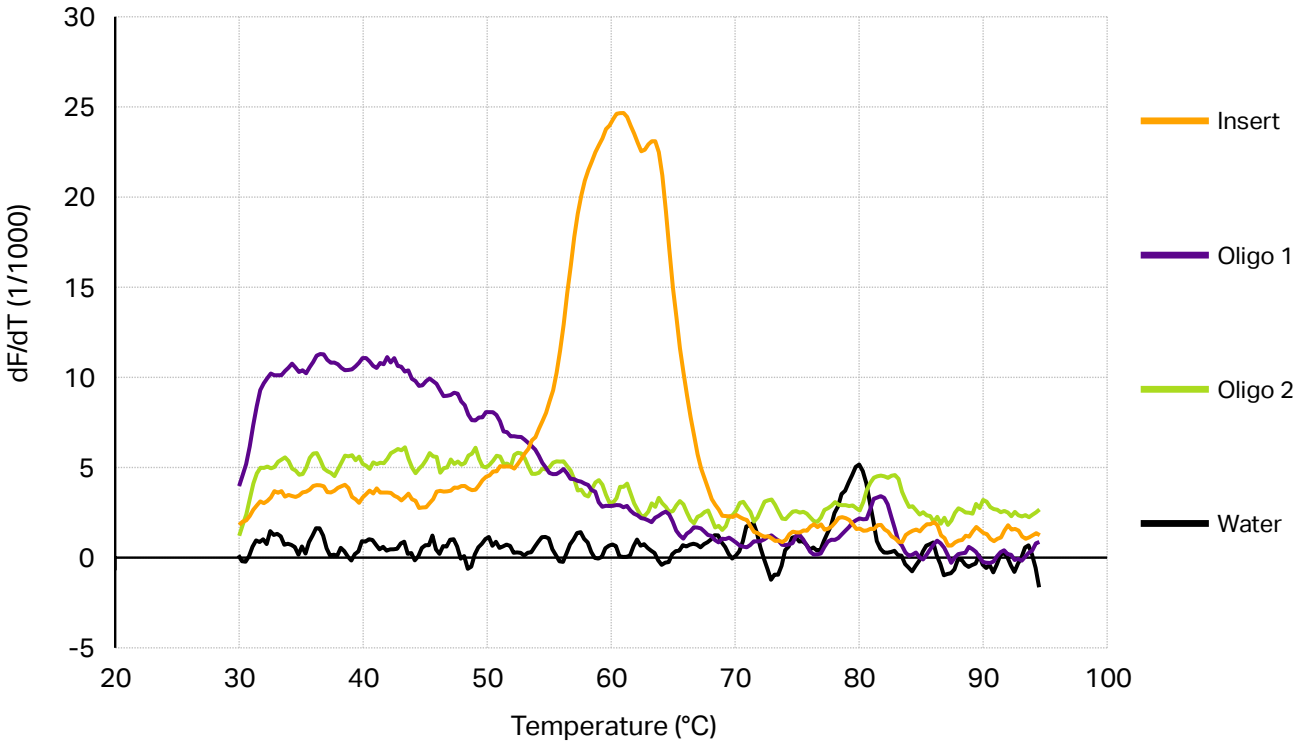

Figure 6: Melting curve analysis of insert sense (oligo 1, purple) and insert antisense (oligo 2, green) alone and mixed.

Outcome of hybridization test

Mixing the parts of the inserts together forms a double strand that is also stable at ligation conditions. This outcome proves that the quality of the oligonucleotides allows the formation of the correct double stand for ligation.