kilobaser

QUALITY REPORT

OF 100MER DNA STRANDS

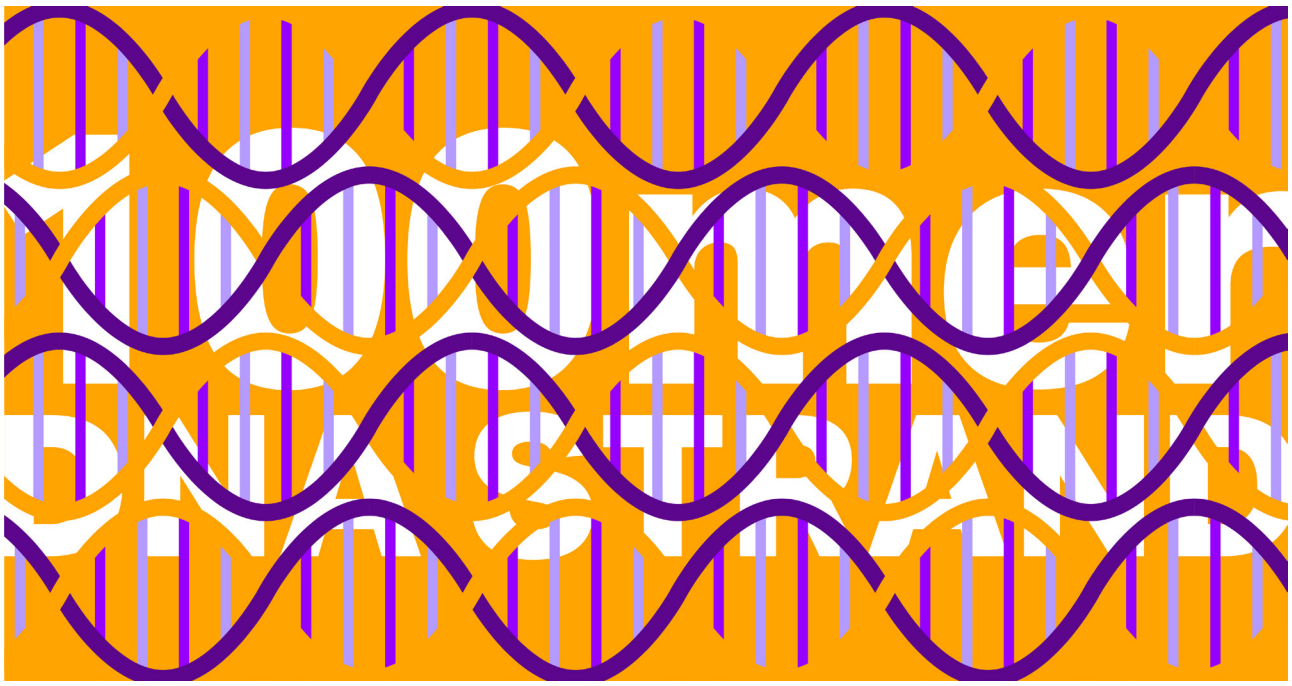
Synthesized by Kilobaser one-XT

Abstract

So far, we used to not recommend synthesis beyond 50mer as we lacked the data to guarantee sufficient quality and quantity of longer DNA strands. Here, we present the synthesis and application of a 100mer oligonucleotide. It was successfully used as a template for a polymerase chain reaction (PCR), confirming that its quantity and quality are well suited for PCR.

Description

100 to 200 nucleotides are the known limit of chemically synthesized DNA strands, making them difficult to order. The synthesis takes time and its yield is significantly lower. In addition, the bases may be damaged by repeated exposure to the reaction conditions. Our workaround for long oligonucleotides used to be the synthesis of smaller oligonucleotides that are then assembled and ligated into one strand. However, this workaround relied on the use of phosphorylated oligonucleotides and was only possible with one of our cartridges. A simpler, more direct approach is presented here.



Synthesis and application of 100mer strands

Each Kilobaser cartridge is capable of synthesizing one 100mer DNA strand. The resulting 100mer-strand, like any other synthesized oligonucleotide, is contaminated with incomplete DNA strands (missing one or more bases) and byproducts of the cleavage and deprotection process. Due to the length, however, the percentage of these contaminants is significantly higher and affects the application of the 100mer strand. Therefore, the following post-synthesis steps are required to improve the quality of 100mer strands.

1. Purification
The OliPure HIC Purification Kit removes these small molecular byproducts, allowing quantification at 260nm absorbance and the use of the 100mer in PCR, as the byproducts may otherwise inhibit the polymerase.

2. PCR
The desalted 100mer strand is then used as a template in the PCR to increase the yield and correct the errors in the sequence, as shown in Figure 1. For PCR, two primers—forward primer and reverse primer—need to be additionally synthesized.

Quantity and quality were determined for three 100mers and two primers. In case of the primers, the quality of the primers was determined by HPLC prior to their use in the PCR. Thus, the result of the PCR was mainly dependent on the quality of the 100mer oligonucleotides. Only the correct sequence at both ends of the 100mer template allows the binding of both primers and the subsequent amplification. At the end of the PCR, the majority of the DNA should be present as double-stranded DNA, as shown in Figure 1.

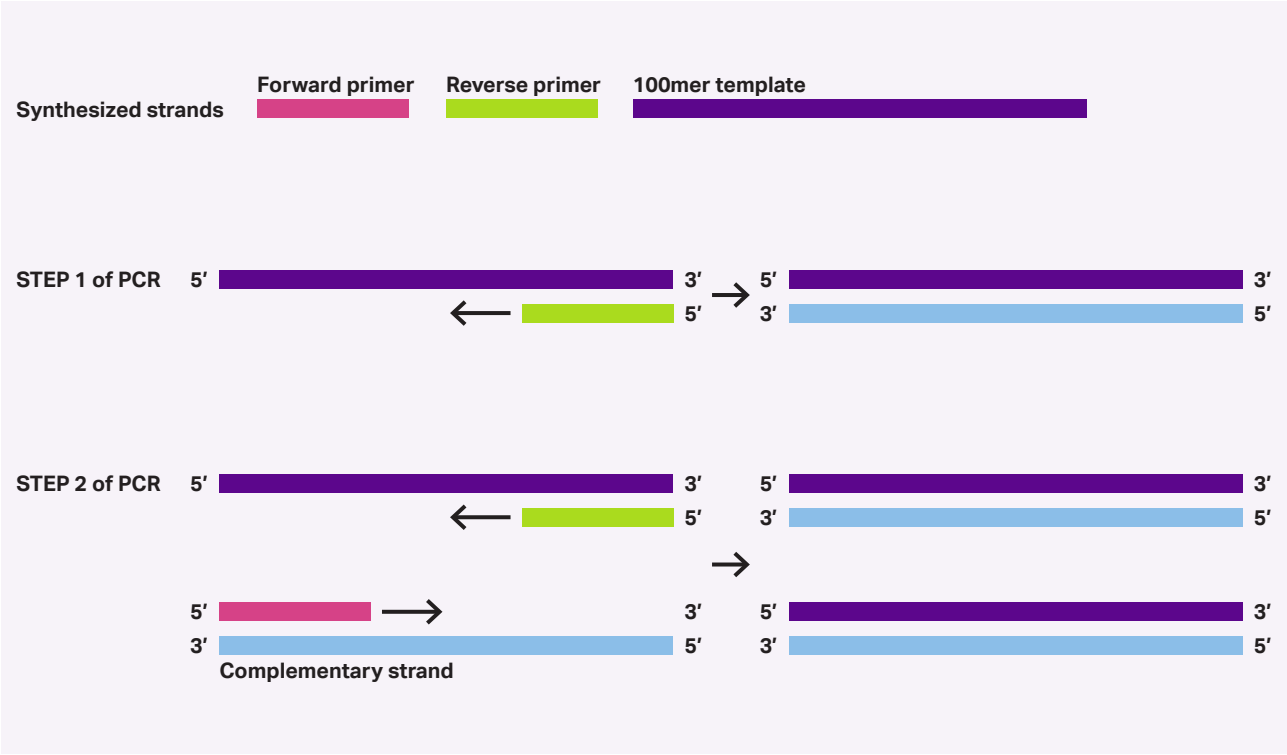


Figure 1: 100mer strand as template in the PCR – after binding of the reverse primer, the polymerase generates a second strand that is complementary to the 100mer strand. The forward primer binds to the second strand, allowing amplification of both strands. Only the DNA strands to which both primers bind sufficiently are amplified by the PCR, increasing the number of full-length products.

Synthesized Oligonucleotides

#	Name	Sequence 5′ → 3′
1	Forward primer	GCTGCAGGTTATCGCACCTGTTCGGTCCAC
2	Reverse primer	GCAAACTCGCGCACAAACTCGTGTTCGACAG
3	100mer template	GCTGCAGGTTATCGCACCTGTTCGGTCCACTGTTTCGGTCTTCGGTCCACT-GTTTTCGAACACGACACGCTGTCGAACACGAGTTTGTGCGCGAGTTTGC

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; limited to oligonucleotides > 40nt. Separated oligonucleotides are detected by absorption at 260nm and analyzed in the mass spectrometer.	• Chemical composition of sample • Differentiation of oligonucleotides in length and number of modifications • Identification of modifications • Detection of defective and truncated products • Yield in % of full-length product
Implen NanoPhotometer® NP80 microvolume photometer	ssDNA quantification by absorption at 260nm; Absorption spectra: 200nm – 900nm	• Yield of synthesis in ng/µL • Purification degree
Invitrogen™ Qubit™ 3 Fluorometer	Fluorometric quantification of ssDNA	• Yield of synthesis in ng/µL
Thermocycler - Open qPCR by Chai	Melting curve analysis SYBR Green qPCR	• Melting points • Hybridization efficiency

Sum up of synthesis outcome

Yield per synthesis
Synthesizing a 20mer oligonucleotide with the Kilobaser one-XT typically yields 300pmol of nominal DNA. This yield is expected to decrease with longer synthesis due to a higher degree of capped strands. In fact, the yield after synthesis is reduced by 50% for 100nt.

Quality of oligonucleotides
Since HPLC analysis only works for oligonucleotides smaller than 40nt, the quality of the 100mer strand was instead determined by hybridization to a complementary oligonucleotide. The formation of a stable dsDNA strand indicates the presence of the correct sequence, which was successfully shown for all synthesized 100mer strands. These results indicate that 100mer strands synthesized by a Kilobaser one-XT are well suited for use as templates for PCR.

Reverse primer

Sample type	Unmodified DNA oligonucleotide
Sequence	5'-GCAAACTCGCGCACAAACTCGTGTTCGACAG-3'
Length	31nt
Analysis by	Reverse HPLC

Chemical composition, mass and purity

The chromatogram (Figure 2) of this synthesis shows the following peaks from left to right:

- Peaks appearing during the first 5 minutes of HPLC are mainly caused by synthesis reagents.
- After 5 minutes of HPLC, oligonucleotides of different lengths are visible.
- **The peak at 19:40 indicates the full-length product and the main product of the synthesis.**

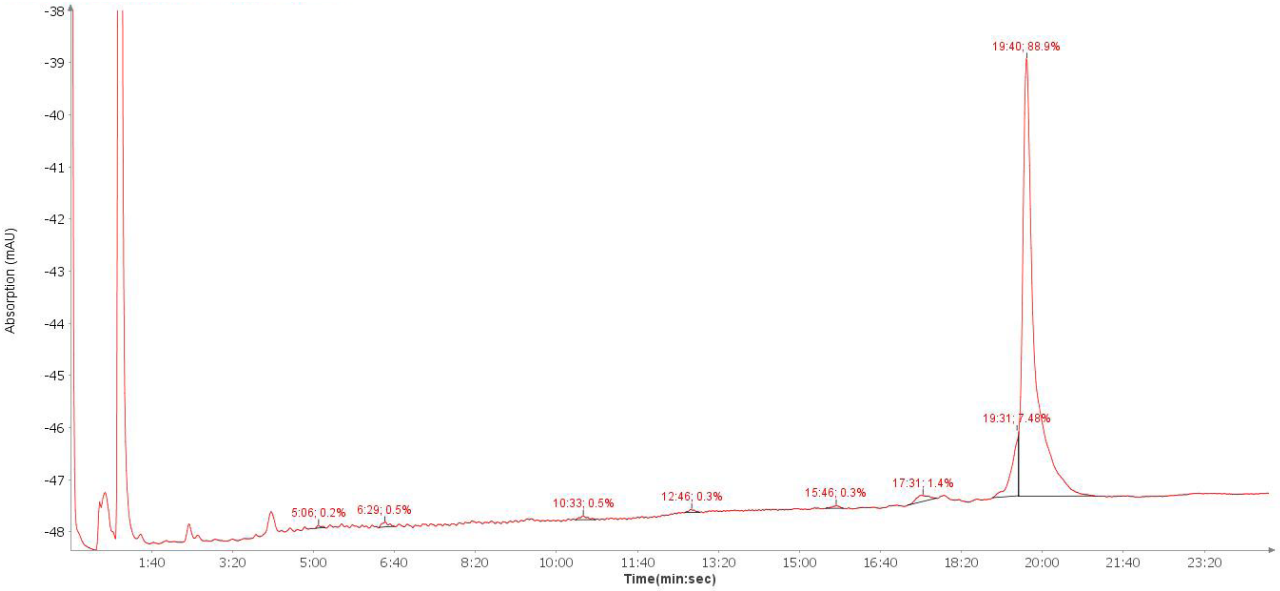


Figure 2: Chromatogram of the reverse HPLC – absorption at 260nm

The mass spectrum confirms that the main product (the peak at 19:40 in HPLC) is the full-length oligonucleotide, which is also completely deprotected and thus ready to use for further applications.

Measured molecular weight:	9476.8 g/mol
Calculated molecular weight:	9474.2 g/mol

Yield of synthesis

- Yield after synthesis: 302 pmol
- Recovery rate of purification: 74.7 %
- Purification degree: 88.9 %
- Final concentration: 69 ng/µL

Forward primer

Sample type	Unmodified DNA oligonucleotide
Sequence	5'-GCAAACTCGCGCACAAACTCGTGTTCGACAG-3'
Length	30nt
Analysis by	Reverse HPLC

Chemical composition, mass and purity

The chromatogram (Figure 3) of this synthesis shows the following peaks, from left to right:

- Peaks appearing during the first 5 minutes of HPLC are mainly caused by synthesis reagents.
- After 5 minutes of HPLC, oligonucleotides of different lengths are visible.
- **The peak at 18:46 indicates the full-length product and the main product of the synthesis.**

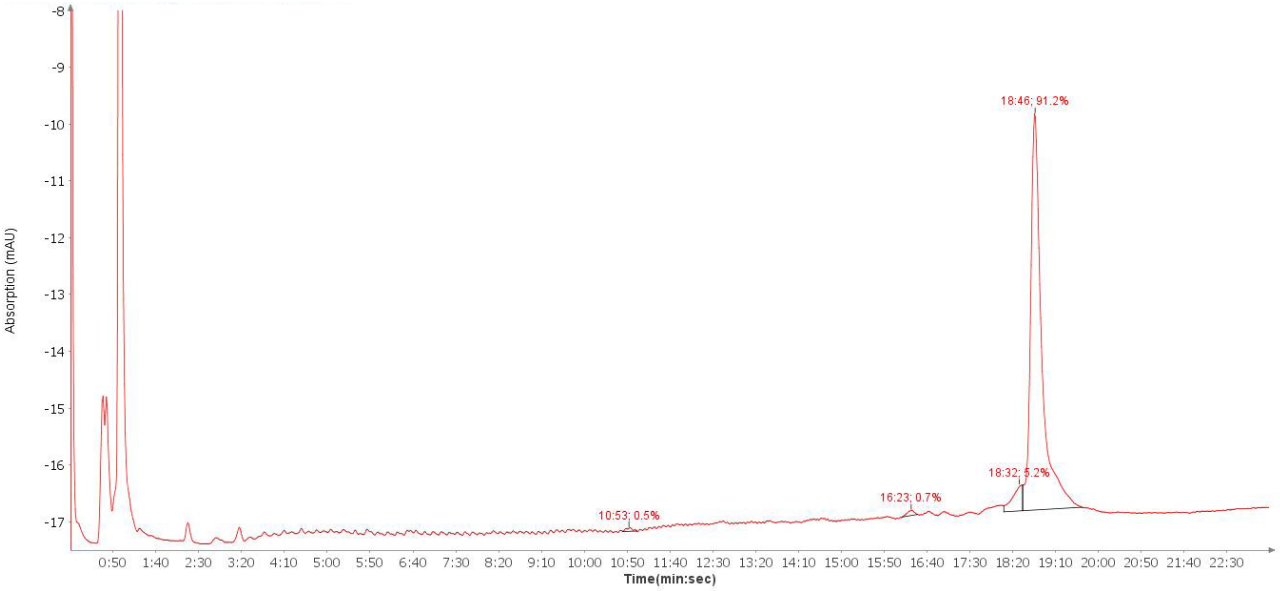


Figure 3: Chromatogram of the reverse HPLC – absorption at 260nm

The mass spectrum confirms that the main product (the peak at 18:46 in HPLC) is the full-length oligonucleotide, which is also completely deprotected and thus ready to use for further applications.

Measured molecular weight:	9150.8 g/mol
Calculated molecular weight:	9150.0 g/mol

Yield of synthesis

• Yield after synthesis:	311 pmol
• Recovery rate of purification:	74.9 %
• Purification degree:	91.2 %
• Final concentration:	71.0 ng/µL

100mer template

Sample type	Unmodified DNA oligonucleotide
Sequence	5'-GCTGCAGGTTATCGCACCTGTTCGGTCCACTGTTTCGGTCTTCGGTCCAC TGTTTTCGAACACGACACGCTGTCGAACACGAGTTTGTGCGCGAGTTTGC-3'
Length	100nt
Analysis by	Melting curve analysis + PCR

Hybridization assay to confirm the quality of synthesized oligonucleotides

Melting Curve Analysis (MCA) allows detection of double strand formation and its stability to heat, represented by the melting point.
The melting points of the mixture of synthesized 100mer template and forward and reverse primers (30nt each) were determined before and after polymerase chain reaction (PCR) and are shown in Figure 4 below.

It can be seen that:

- Before the PCR occurs, each of the single strand 100mers forms unspecific double strand formations that melt at 50°C.
- In case of synthesis 3, an additional double strand is visible that melts at 80°C, indicating the binding of the reverse primer. The additional peak at 90°C might be a remnant of the measurement.
- After PCR, all 100mers appear as double strands that melt around 85°C.

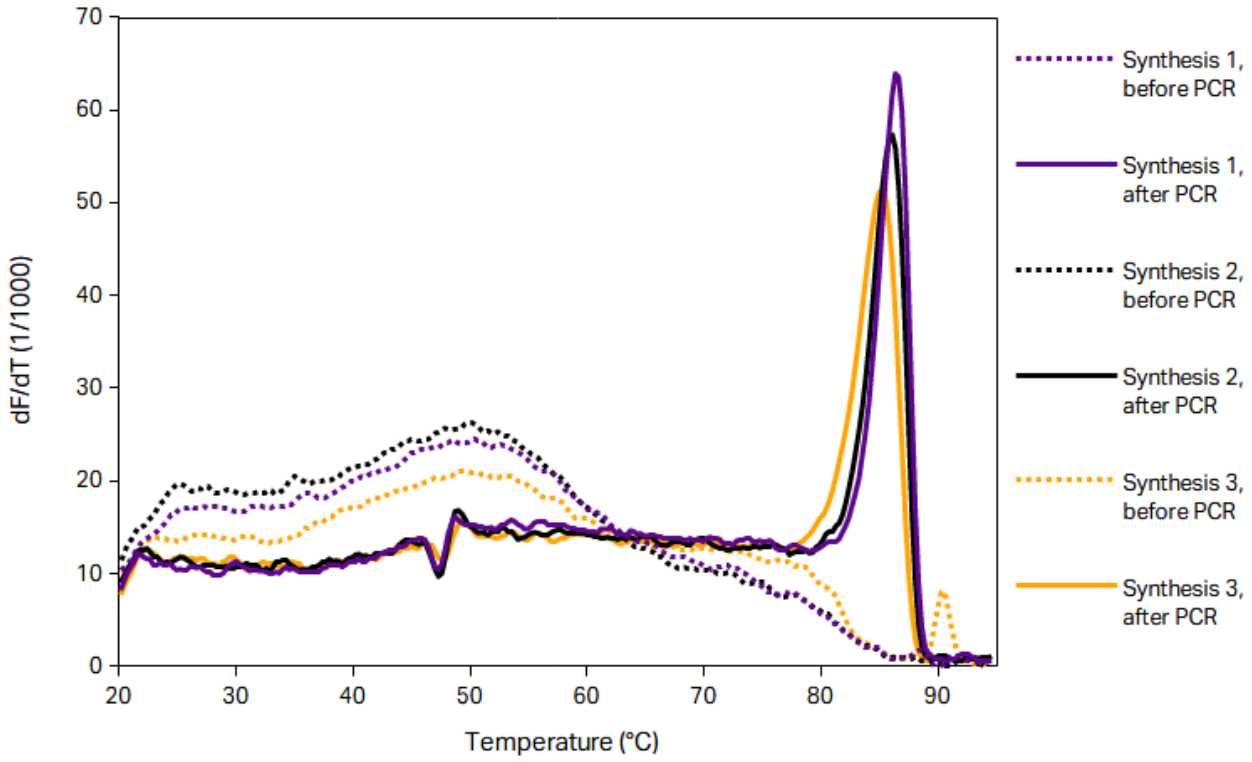


Figure 4: Melting curve analysis of three 100mer strands with PCR primers before (dotted line) and after PCR (continuous line); dF/dT (derivative of fluorescence signal) is plotted versus temperature.

Quantity and quality of synthesis

Synthesis 1:

Yield after synthesis:	151 pmol
Recovery rate of purification:	73.3 %
Quality of synthesis:	Successful hybridization of primers
Final concentration:	121.5 ng/µL
Final yield:	95 pmol

Synthesis 2:

Yield after synthesis:	165 pmol
Recovery rate of purification:	68.5 %
Quality of synthesis:	Successful hybridization of primers
Final concentration:	115.8 ng/µL
Final yield:	83 pmol

Synthesis 3:

Yield after synthesis:	167 pmol
Recovery rate of purification:	70.8 %
Quality of synthesis:	Successful hybridization of primers
Final concentration:	113.4 ng/µL
Final yield:	81 pmol

Outcome of synthesis

As predicted, the synthesis of each of the 100mer oligonucleotides resulted in a yield that was significantly lower than the typical yield of 300pmol for the synthesis of a primer. This reduction in yield is due to a higher degree of capped strands in the synthesis process.

The quality of the 100mer oligonucleotides was determined by hybridization to two 30nt primers for PCR. Since the hybridization resulted in only one stable dsDNA strand, it can be concluded that the majority of the strands are of the correct sequence.

This high quality was true for all three syntheses performed on different instruments, demonstrating reliable reproducibility of the synthesis as well as the high standard of our synthesizers. The results prove that the 100mer strands synthesized by a Kilobaser one-XT are well suited for use as templates for PCR.