Technical Overview

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Performance Characteristics of the Agilent Oligo Pro II System

Introduction

The Agilent Oligo Pro II system is an automated parallel capillary electrophoresis instrument designed for high-throughput separation and purity analysis of single-stranded DNA (ssDNA) and RNA oligonucleotides (oligos). As oligos are synthesized, small amounts of impurities can be introduced at each step. Different synthesis methods and purification efficiencies can impact the quality of the sample, requiring assessment of the oligo to ensure it is of the purity level needed for downstream applications such as PCR, gene assembly and microarray/hybridization assays.

The Oligo Pro II system provides high resolution and direct detection of oligos using a denaturing gel that allows single-stranded nucleic acid samples to migrate through a capillary and separate by size. UV absorption spectroscopy is used for dye-free detection of the sample. The UV absorption is plotted over time to generate a digital electropherogram of the sample. The system can accommodate a variety of throughputs, with interchangeable arrays allowing for the analysis of 12, 24 or 96 samples simultaneously. Up to three 96-well plates can be loaded and analyzed without user intervention.

Sample preparation and system operation, including both the instrument setup and data analysis, are simple and intuitive. The Oligo Pro II software automates capillary conditioning, sample injection, electrophoresis separation and data processing for fast and easy analysis. For example, sample preparation can be accomplished with a simple nucleic acid dilution to 1-5 µM using deionized (dI) water. Prepared specimens are added to the sample plate, which is subsequently loaded onto the instrument. The injection and separation conditions are selected by the researcher using the controller software.

Experimental

Variables such as the oligo sequence, GC content, modifications and the presence of salt, can affect the injection of the sample. The specifications of the system state that oligos up to 60 nucleotides (nt) in length (60-mer) can be detected with n-1 resolution. When necessary, such as for longer or modified oligos, the system is flexible, allowing for the injection and separation conditions to be easily adjusted. The Oligo Pro II data analysis software automatically displays the purity of the largest peak in the sample and can identify samples meeting user-specified purity standards.

In this technical overview, we demonstrate the capabilities of the Oligo Pro II to analyze ssDNA oligos. The limit of detection of the system was investigated by altering either the sample concentration or the injection conditions. The system can achieve n-1 resolution of poly-dT oligos up to 60-mer with the recommended settings, and up to 100-mer by modifying the separation parameters. Finally, we show that the system can easily detect n-1 impurities as low as 5% of the total sample concentration, and explore the impact that salt has on sample injection.

An Agilent Oligo Pro II system (p/n M5340AA) equipped with an Oligo Pro II 96-Capillary Array Long (p/n A2500-9675-5580) was prepared according to the protocol provided in the user manual[1]. Briefly, the instrument was set up daily by filling one deep-well plate with ssDNA OLIGEL Buffer (p/n DN-465-1000) and a second plate with dI water. OLIGEL ssDNA Gel (p/n DN-415-0250) and Capillary Conditioning Solution (p/n DN-475-1000) were added to the reagent bottles as needed and a full conditioning performed weekly or every 10 runs. Samples were diluted with dI water to a minimum volume of 30 µL for analysis, with an optional mineral oil overlay to minimize evaporation and allow for repeated use.

Agilent Oligo Verification Std 10x Solution (p/n DN-400-0001) (Verification Std) was diluted to a 1x solution and analyzed using the Oligo Pro II to ensure system performance. The Verification Std is composed of six poly-dT oligos of sizes 19, 20, 39, 40, 59 and 60-mers, which can be easily separated from each other, highlighting the n-1 resolution of the system through 60-mer. The 1x Verification Std was also mixed with poly-dT oligos of smaller and larger sizes to investigate the resolution of the system beyond 19- and 60-mer. For the Verification Std, the injection was set at a 3 kV 5 second injection, with a 12 kV 70 minute separation. As the larger oligos were mixed in, the separation time was increased to 90 minutes to ensure detection of the longer fragments.

Single-stranded DNA oligos of random sequence were obtained from Integrated DNA Technologies (IDT). The sequences and related information are listed in Table 1. Each oligo was prepared at 100 µM in IDTE (a 1x TE solution), pH 8.0, and then diluted to lower concentrations in dI water for analysis with the Oligo Pro II. Two-fold serial dilutions of each oligo were prepared and analyzed using a variety of injection parameters. The separation was maintained at 12 kV for 70 minutes.

Results and discussion

Limit of detection

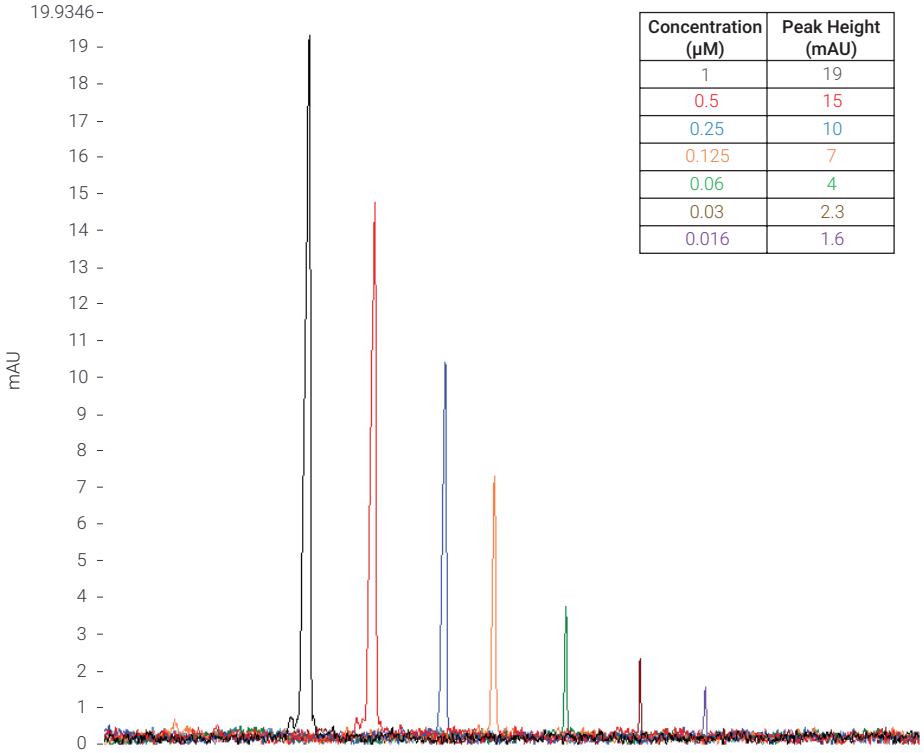
An important benefit of the Oligo Pro II is the ability to adjust the voltage and time of the injection period, allowing for a specific amount of the sample to be injected into each capillary for analysis. The flexibility of the Oligo Pro II system thus allows for detection of samples over a wide concentration range. The recommendation for analysis is generally to begin with a sample at a concentration between 1–5 µM and an electrokinetic injection of 2–5 kV for 3–10 seconds. The sample concentration or the injection parameters should then be adjusted until an optimal peak height of approximately 15–20 mAU is achieved. This peak height will allow for detection of the main peak, as well as any smaller impurity peaks that may be present in the sample.

Table 1. Oligos used for analysis.

Primer	Sequence	Length (nt)	Melting Temperature (°C)	GC Content (%)	Molecular Weight (g/mole)
A	AAAAAGTTTAAACCATTTTGTCCGCGCCGGG	31	63.4	45.2	9519.2
B	AAAAAAGTTTAAACGGAACAGCCCATGTTTTTTCGAGC	38	62.7	36.8	11693.7
C	AAAAAAGTTTAAACTGGTGTTTACGAAGGTTTTACGTTTTCCC	43	61.9	32.6	13245.7
D	5Phos/TTA CTC GGC GTG CTT GGT CT/3Phos	20	59.0	55.0	6265.9

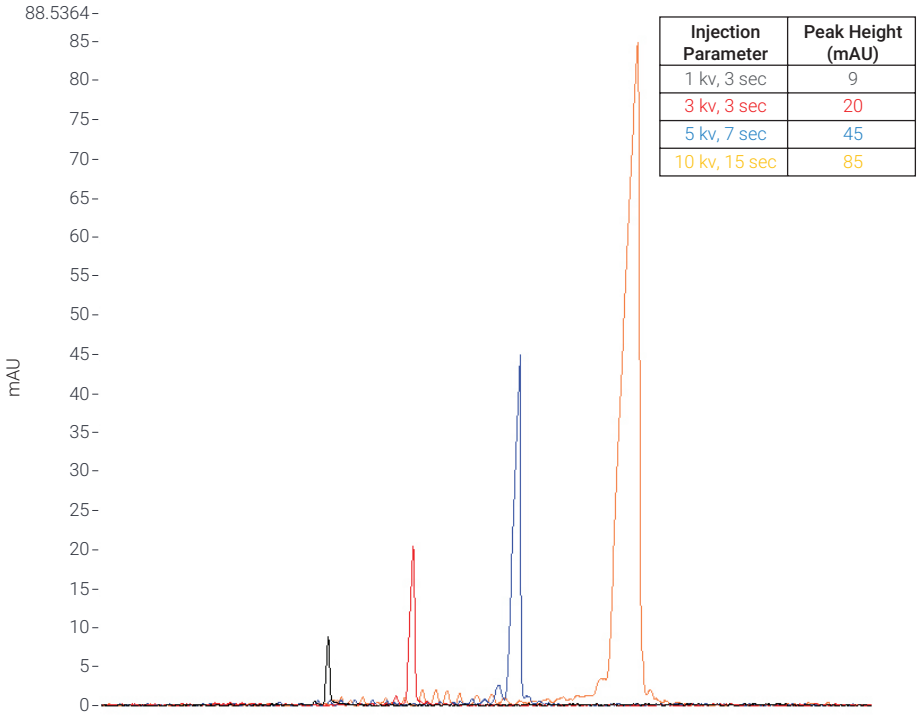
To demonstrate the limit of detection of the Oligo Pro II, serial dilutions of several oligos (Table 1) were analyzed with a consistent injection of 5 kV for 7 seconds. Figure 1 shows an electropherogram overlay of each concentration of Primer D, a 20-mer oligo. The inset table shows the concentration and the corresponding peak height (mAU) of each sample. While the sample can be detected as low as 0.016 µM, an optimal peak height is achieved at 0.5–1 µM to ensure detection of both the main peak and any n-1 impurities.

To further investigate the limit of detection of the Oligo Pro II, the oligos were diluted to a concentration of 2.5 µM and analyzed over a variety of injection conditions. Figure 2 shows an electropherogram overlay of Primer A, a 31-mer oligo, injected for 1–15 seconds with the voltage set between 1 and 10 kV. The lower the injection time and voltage, the less sample is injected into the capillary, and the smaller the peak heights. As the injection time and voltage increases, more sample is injected, and the peak heights become larger. The sequence and composition of a sample can impact how it is injected. The ability to adjust the injection conditions allows the user to determine the best running conditions for each sample type. However, care must be taken to ensure that the sample peak heights are within the specified range, as too low of peak heights may make it so that impurities present in the sample are not visualized. Conversely, too much of the sample injected can cause peak broadening and decrease resolution, or can amplify baseline noise which can be misinterpreted as sample impurities.



Concentration (µM)	Peak Height (mAU)
1	19
0.5	15
0.25	10
0.125	7
0.06	4
0.03	2.3
0.016	1.6

Figure 1. Analysis of a serial dilution of an oligo analyzed on the Agilent Oligo Pro II system, with a 5 kV, 7 second injection. Triplicates of each concentration were analyzed in a separate well of a 96-well plate. For visualization of the serial dilution, several wells were overlaid and spaced apart using the "Project" tool provided with the Oligo Pro II data analysis software. The inset table displays the peak heights of each sample. The optimal peak height for sample analysis with the Oligo Pro II is 15–20 mAU. Thus, the most reliable concentration for analyzing this sample, at this injection, is 0.5–1 µM.



Injection Parameter	Peak Height (mAU)
1 kv, 3 sec	9
3 kv, 3 sec	20
5 kv, 7 sec	45
10 kv, 15 sec	85

Figure 2. An oligo sample diluted to 2.5 µM in dI water was analyzed on the Agilent Oligo Pro II system, with a variety of injection conditions. For visualization of the sample with different injection parameters, several wells from different runs were overlaid and spaced apart using the "Project" tool provided with the Oligo Pro II data analysis software. The inset table displays the peak heights of each sample. The optimal peak height for sample analysis with the Oligo Pro II is 15–20 mAU. Thus, the most reliable injection for analyzing this sample at a 2.5 µM concentration is 3 kV for 3 seconds (red trace). A lower injection (black) impedes visualization of the small impurity peaks present in the sample. A higher injection (blue, yellow), may result in extraneous peaks from baseline noise and peak broadening, decreasing resolution.

n-1 resolution through 60-mer

Single nucleotide resolution can be achieved with the Oligo Pro II for oligos up to 60 nt in length, allowing identification of minuscule nucleic acid impurities (n-1, n-2, etc.). The Verification Std is a mixture of six poly-dT oligos of sizes 19, 20, 39, 40, 59 and 60-mer. The Verification Std was analyzed on the Oligo Pro II to demonstrate the capabilities of the system, with n-1 resolution of oligos through 60-mer (Figure 3).

To further demonstrate the resolution capabilities of the Oligo Pro II, Primer A, a 31-mer oligo with a random sequence, was analyzed. The larger peak observed in the electropherogram is the primary sequence, and the smaller peak likely represents an n-1 impurity introduced during the oligo synthesis process. The Oligo Pro II data analysis software automatically identifies the peaks and provides a purity score to the largest peak in the sample (upper left corner, blue label). The Peak Table (inset) displays information about each peak, including the peak height, corrected peak area, and percent purity. In this example, the main oligo has a percent purity of 93.61% (Figure 4).

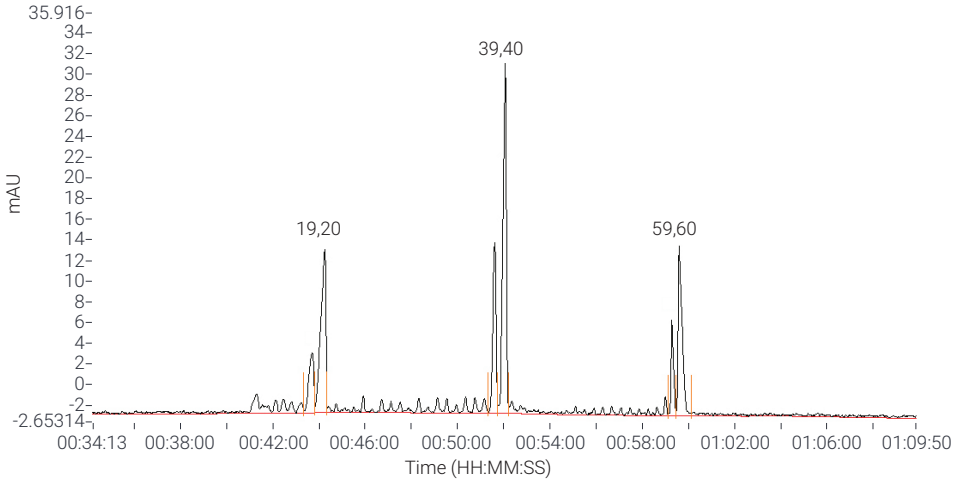


Figure 3. The Agilent Oligo Verification Standard analyzed on the Agilent Oligo Pro II system highlights the resolution capabilities of the system for oligos up to 60 nucleotides in length.

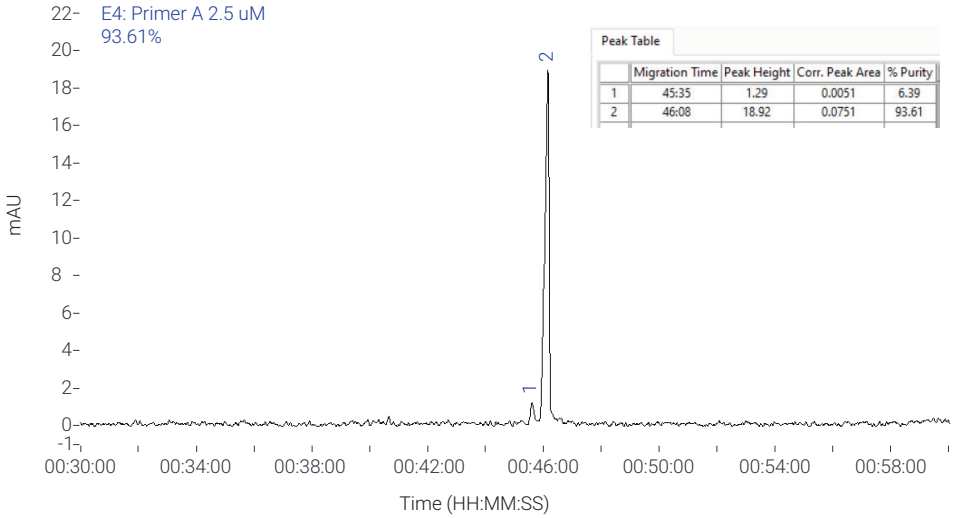


Figure 4. Purity analysis of a random oligo using the Agilent Oligo Pro II system. The highest peak (2) is the expected oligo, while the smaller peak (1) is an n-1 impurity. The inset Peak Table displays the peak height, corrected peak area, and percent purity of each integrated peak. The oligo has a purity of 93.61%, which is also displayed in the upper left corner for quick and easy assessment.

Resolution beyond the Verification Std

The specifications of the Oligo Pro II indicate that the system can achieve n-1 resolution through 60-mer oligos, based on the size comparison relative to the Verification Std. However, the flexibility of the system enables the separation of a variety of samples, by altering the injection and separation parameters. To demonstrate the capabilities of the system to analyze samples with oligo lengths smaller than the Verification Std, 14- and 15-mer poly-dT oligos were analyzed on the Oligo Pro II system individually, blended together (Figure 5A), and mixed with the Verification Std. The Oligo Pro II was easily able to achieve n-1 resolution for oligos as small as 14 nt in length.

To investigate the resolution of the system beyond 60-mer, the Verification Std was mixed with poly-dT oligos of larger sizes. The resolution of the system at 60-mer was first investigated using a mix of 61- through 65-mer poly-dT oligos together with the 59/60 blend of the Verification Std (Figure 5B). As n-1 resolution was easily achieved at each of these sizes, several n + n-1 mixes of larger sizes, from 65- to 100-mers, were analyzed individually and combined with the Verification Std. For best resolution, peak heights of less than 15 mAU were achieved with a sample injection of 3 kV for 5 seconds. Shown in Figure 5C is the Verification Std, with blends of 19/20, 39/40 and 59/60-mers, mixed with n + n-1 blends of 69/70, 79/80, 89/90 and 99/100-mers. The separation was increased to 12 kV for 90 minutes to ensure that each of the oligos in the mix had sufficient time to travel through the capillaries. With the adjusted settings, each n + n-1 mixture was easily resolved, demonstrating that the Oligo Pro II can achieve n-1 resolution of samples through 100-mer.

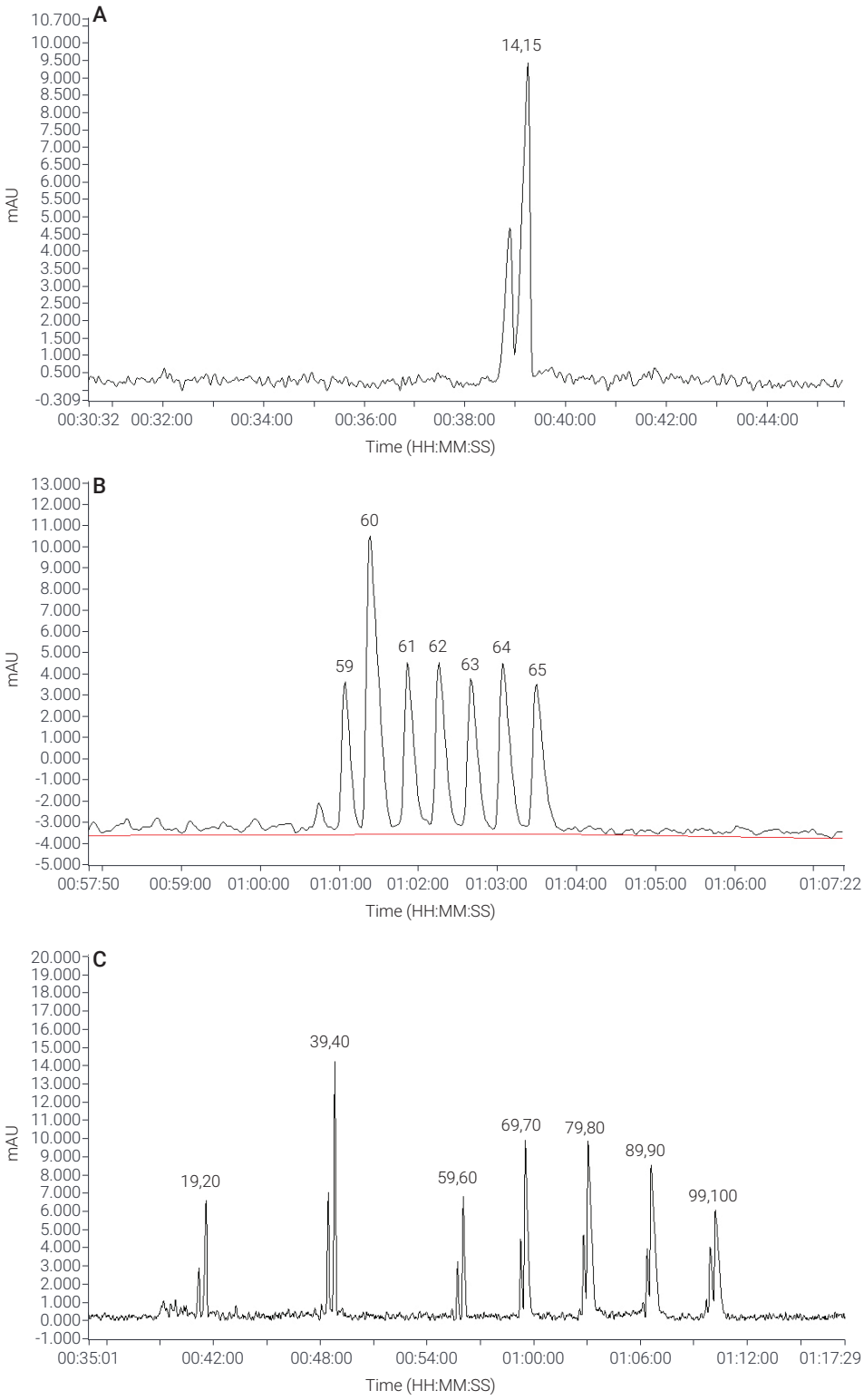


Figure 5. Resolution of poly-dT oligos beyond the 19-60-mers of the Verification Standard on the Agilent Oligo Pro II system. (A) A blend of 14- and 15-mer poly-dT oligos demonstrate the capabilities of the system to resolve smaller fragments. (B) The 59- and 60-mer of the Verification Std mixed with poly-dT oligos of 61- to 65-mer lengths highlight the n-1 resolution of the system. (C) The Verification Std mixed with several poly-dT duplexes indicate that the system can resolve fragments through 100-mers.

Impurity detection

Certain applications require highly purified oligos for best results. The Oligo Pro II can be used to examine the purity of oligos to enable researchers to make decisions about the quality of an oligo for downstream application. To explore the capabilities of the system to identify impurities in an oligo, a 20-mer poly-dT oligo was mixed with known amounts of a 19-mer poly-dT oligo to mimic an n-1 impurity. The injection was set at 5 kV 5 seconds to achieve an optimal peak height of approximately 20 mAU for the 20-mer oligo. The impurity was easily detected when mixed with the 20-mer oligo at 10 and 5% of the concentration of the 20-mer (Figure 6). With the flexible operation of the system, it may be possible to detect even lower amounts of impurities.

Impact of salt on injection

For best results, it is recommended to dilute samples in dI water for analysis with the Oligo Pro II, as the presence of salts can inhibit the injection of the sample. To demonstrate the effect that the presence of salt can have on the injection of a sample, the 10x Verification Std was diluted to a 1x concentration using dI water, 0.1x TE, 1x TE, and 10x TE (Figure 7). The TE concentrations were prepared by performing a 10-fold serial dilution of a 100x TE concentrate (GBiosciences p/n R003) using dI water. The 100x TE concentrate is composed of 1M Tris-HCl and 0.1M EDTA with a pH of approximately 8.0. TE is commonly used for storage and stabilization of nucleic acids. However, the salts in this buffer can interfere with capillary electrophoresis.

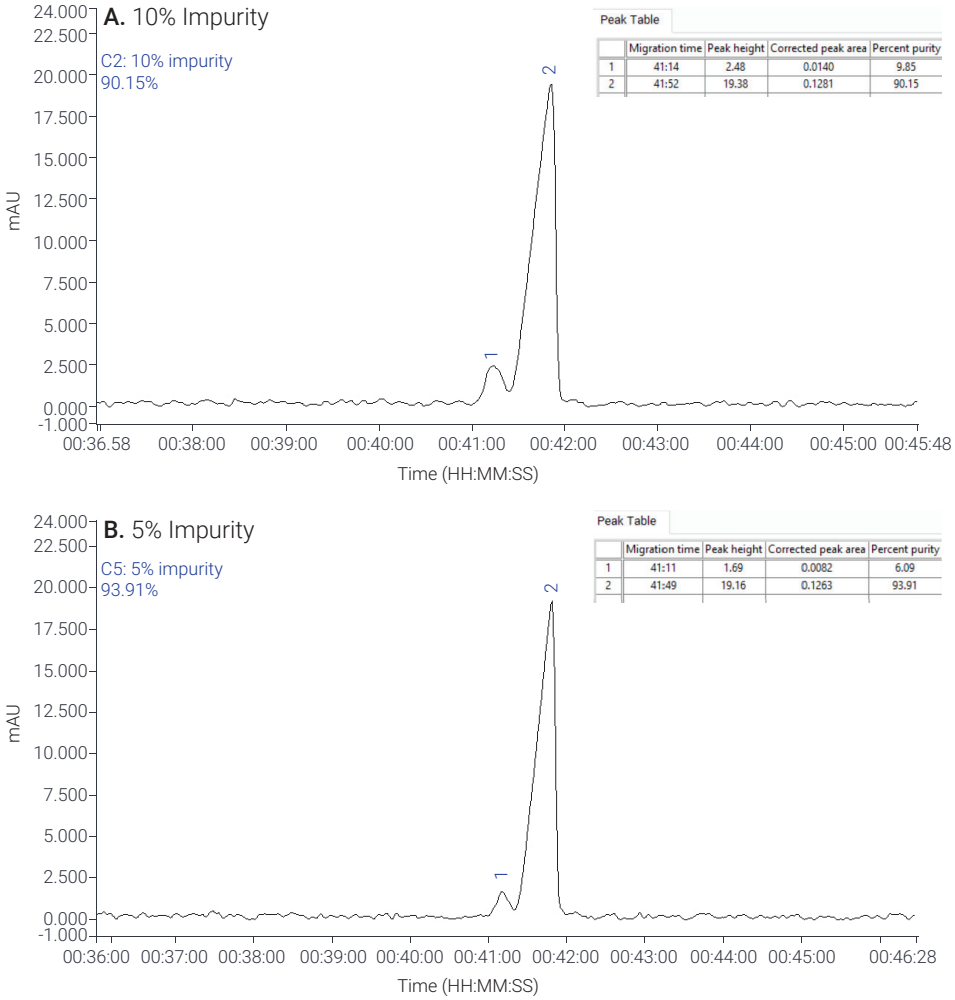
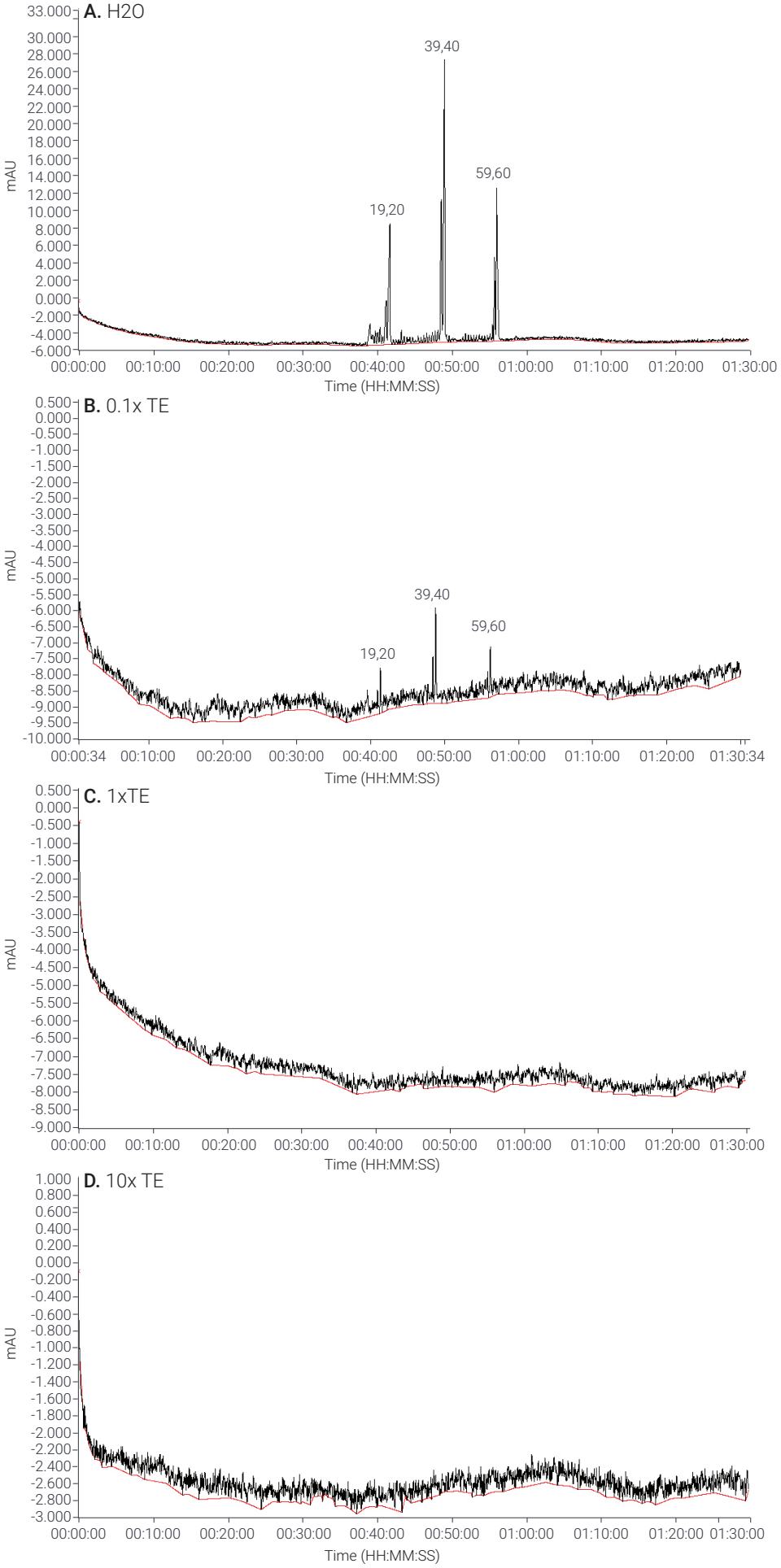


Figure 6. A 20-mer poly-dT oligo at 1 µM concentration was mixed with known concentrations of a 19-mer poly-dT oligo to examine the capabilities of the Agilent Oligo Pro II to identify small amounts of n-1 impurities. The 19-mer impurity peak (1) was easily resolved from the 20-mer oligo peak (2) at both (A) 10% and (B) 5% of the concentration of the 20-mer oligo peak.

As shown in Figure 7, even the addition of 0.1x TE decreased the peak heights of the oligos in the Verification Std. For example, the tallest peak displayed a peak height of approximately 30 mAU when diluted with dI water (Figure 7A), but only 3 mAU when diluted with 0.1x TE (Figure 7B). Dilution with higher amounts of TE completely inhibited injection of the sample, and no peaks were visualized (Figure 7C and 7D). Samples should be desalted and/or prepared in water for analysis on the Oligo Pro II. However, it may be possible to increase the injection voltage and time to allow for an appropriate amount of sample to be injected even if there is salt still present in the sample. The flexibility of the system allows for users to inject the same sample wells multiple times to try different conditions and find the parameters that best fit their sample types.



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Oligo	Water	0.1x TE	1x TE	10x TE
19-mer	5.18	0.76	NA	NA
20-mer	13.99	1.63	NA	NA
39-mer	16.30	1.37	NA	NA
40-mer	32.14	2.94	NA	NA
59-mer	9.81	0.88	NA	NA
60-mer	17.54	1.59	NA	NA

Figure 7. The 10x Verification Std was diluted to 1x with (A) water, (B) 0.1x TE, (C) 1x TE and (D) 10x TE to demonstrate the effect that salt has on sample injection. The presence of even a small amount of salt results in decreased peak heights (B). Larger amounts of salt inhibit injection of the sample altogether (C, D). (E) The average peak height of the oligos of the verification standard when prepared with dI water and varying concentrations of TE, n=4.

Conclusion

The Agilent Oligo Pro II system is ideal for fast and automated assessment of single-stranded nucleic acids. The system specifications state that n-1 resolution for oligos can be achieved up to 60 nt in length, with a verification standard composed of oligos from 19 to 60-mers. In this technical overview, we demonstrate the capabilities of the system to analyze oligos beyond the specifications, from 14-100 nt. Different sample compositions, including the sequence, GC content, modification, and the presence of salt can affect the injection and separation of the sample. The flexibility of the system is highly advantageous allowing the user to alter the sample concentration, electrokinetic injection and separation conditions to identify the best running conditions for different sample types. The Oligo system was able to achieve n-1 resolution of oligos up to 100-mer by simply extending the separation time. While the presence of salt can interfere with capillary electrophoresis, it was further shown that this issue can be alleviated by diluting samples in water or potentially by altering the injection voltage and time to allow for more sample uptake. Additionally, n-1 impurities were easily detected at 5 and 10% of the sample concentration, highlighting the capabilities of the Oligo Pro II system for purity analysis of single-stranded oligos prior to their use in sensitive downstream applications.

References

1. Agilent Oligo Pro II User Manual. Agilent Technologies, document number D0002114 Rev. A, **2020**.

www.agilent.com/genomics/oligo-pro

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