

Analysis of Oligonucleotides by Capillary Gel Electrophoresis with the Agilent 7100 Capillary Electrophoresis System

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Abstract

Oligonucleotides cannot be separated according to size by applying conventional capillary zone electrophoresis (CZE) due to their similar mobilities (independent of chain length). However, such analytes can be separated by using a sieving matrix. To do so, the capillary is filled with a sieving matrix (a gel) to achieve a difference in traveling time through the sieving matrix dependent on the size of the analyte molecule. In this study, three different gels, two detector filters and three different neutral-coated capillaries were evaluated by separation of four different standards. In addition, different methods for optimization of separation due to the needs of the analysis are demonstrated.

Introduction

This application note focuses on the analysis of oligonucleotides. By using different parameters such as different gels, filters, temperature and voltage, this analysis can be applied as a starting point and guidance for the analysis of an oligonucleotide sample. The Agilent 7100 capillary electrophoresis system is perfectly suited for capillary gel electrophoresis (CGE) due to programmable washing steps, different wavelength filters and the easy observation of the filling level in the vial. CGE is used for this analysis, as it is perfectly suited to separate analytes due to their difference in size. Even a difference of only one unit can be separated when optimizing the separation parameter.

Experimental

This section sums up the applied consumables, chemicals, methods and their preparations.

Materials

Polyethylene glycol 35000 stabilized (BHA) (8.18892) was purchased from Merck (Darmstadt, Germany). Two different part numbers lots (94646/BCCD4303 and 03557/BCCF4480) of polyethylene glycol 35000 were ordered from Sigma-Aldrich (Darmstadt, Germany). BisTris (0715) was purchased by VWR (Darmstadt, Germany). Boric acid (1.00165), water (1.15333, LC/MS Grade) and Acetonitrile (1.00030) were ordered from Merck (Darmstadt, Germany). All used capillaries (PVA 100 µm (G1600-60419); µSIL WAX 100 µm (197-7202); µSIL DNA 75 µm (199-2602)), filters (260 nm detector filter (G7100-62700) and 280 nm CIEF filter (G7100-68750)) and the DNA Ladder (5190-9029), as well as the RNA resolution standard (5190-9028) were ordered from Agilent Technologies (Waldbronn, Germany).

The Oligo(dT)12-18 Primer (18418-012) was purchased by Thermo Fisher Scientific (Dreieich, Germany). The Test Mix Polyd(A), 40-60mer Oligos (362387) was ordered from Sciex (Darmstadt, Germany).

Method parameters

Table 1 shows the experimental conditions of the acquisition method.

Preconditioning

To prevent contamination and bonding of the gel on the outside of the capillary and on the electrode, the *Wash Inlet Electrode* function followed by a 5-second water dip was applied to the preconditioning, as described in Table 2.

For the same reason, different filling levels of the vials were used. The electrolyte vials were filled with 1 mL electrolyte each and the dip vial with approximately 1.3 mL water. The gel vial contained only 0.5 mL gel. For the sample solution, microvials were used,

and between 10 µL and 40 µL were filled with sample. The user must ensure that the electrode and the capillary are immersed into the sample solution during the injection step. For all vials, care must be taken to ensure that no air bubbles are present.

By applying external pressure the purging time can be shortened to 300 seconds.

Preparation of the electrolyte solutions and gels

The electrolyte solution and gels were prepared as follows:

Electrolyte solution: 200 mM BisTris, 200 mM boric acid

4.18 g of BisTris and 1.24 g of boric acid were weighed together in a 100 mL volumetric flask and filled with water up to the mark. After approximately five minutes in the ultrasonic bath, a clear solution was obtained. Before use, the solution was filtered with a 0.45 µm syringe filter.

Table 1. Experimental conditions.

Parameter	Value
Device	Agilent 7100 capillary electrophoresis system
Firmware	B.07.021
CDS	Agilent OpenLab ChemStation C.01.10
Electrolyte	200 mM BisTris, 200 mM boric acid
Capillary	As described in the individual section/figure – short-end to 33 cm
UV Interface	The “blue” interface (G7100-60310)
Injection	–10 kV, 10 sec. for short-end tests: +10 kV, 5 sec. (or as described in figures and sections)
Detection	260 nm (BW10), Ref off (or as described in figures)
Voltage	–25 kV (for short-end tests: +25 kV) (or as described in figures)
Temperature	30 °C

Table 2. Preconditioning parameters.

Function	Parameter
Wash Inlet Electrode	Inlet: Air Outlet: Air
Flush	600 seconds (Inlet: Gel, Outlet: Water)
Load Inlet Vial	Water
Wait	5 seconds
Wash Inlet Electrode	Inlet: Air Outlet: Air

Polymeric solution (gel)

2.7 g of polyethylene glycol (PEG) was weighed into a 10 mL volumetric flask and filled with the filtered electrolyte solution up to the mark. For obtaining a clear solution, the flask was treated in the ultrasonic bath at least three times for 15 minutes. In between, the flask was shaken by hand about five times. The resulting gel contained 27% polymer, 200 mM BisTris and 200 mM boric acid. The solution was then ready to use.

Polymeric solution (gel) with 20% acetonitrile

2.7 g of polyethylene glycol was weighed and transferred into a 10 mL volumetric flask. 2 mL of acetonitrile was added, and the flask was filled with the filtered electrolyte solution up to the mark. To obtain a clear solution, the flask was treated in the ultrasonic bath at least three times for 15 minutes. In between, it was shaken by hand approximately five times. The resulting gel contained 27% polymer, 20% (v:v) acetonitrile, 160 mM BisTris and 160 mM boric acid. The solution was then ready to use.

In total during the current tests, four gels were prepared:

1. **Gel 1:** PEG, Sigma-Aldrich (part number 94646)
2. **Gel 2:** PEG, Merck (part number 8.18892.1000)
3. **Gel 3:** PEG, Sigma-Aldrich (part number 03557)
4. **Gel 4:** PEG, Merck, part number 8.18892.1000, and 20% acetonitrile

All gels were kept in the refrigerator (2 to 8 °C) after preparation. During the tests, all gels were usable for three weeks. A longer storage time might be possible, but this has not been tested.

Preparation of the standard solutions

- **Standard solution 1:** DNA Ladder Standard (Agilent): 1 mL water was added to the original sample vial and the solution was vortexed for 2 seconds.
- **Standard solution 2:** RNA Resolution Standard (Agilent): 1 mL water was added to the original sample vial and the solution was vortexed for 2 seconds.
- **Standard solution 3:** Oligo(dT)12-18 Primer (Invitrogen): The sample solution was ready to use.
- **Standard solution 4:** Test Mix Polyd(A), 40-60mer Oligos (Sciex): 0.5 mL water was added to the original sample vial and the solution was vortexed for 2 seconds.

Before injection, standard solutions 1, 2, and 4 were diluted 1:2 with water (e.g. 20 µL water + 20 µL standard solution) directly in the microvial and vortexed for approximately 2 seconds. Sample solution 3 was diluted 1:10 with water before use. For this, 3 µL of sample was added to 27 µL of water and vortexed for 2 seconds. Other dilutions are given in the figures.

Results and discussion

In the next sections the following points will be investigated:

- Influence of the DAD filter
- Influence of the kind of gel
- Separation using different kinds of capillaries
- Reproducibility of consecutive injections
- Effect of different capillary lengths
- Influence of temperature and voltage

Tests with 260 nm, 280 nm, and without wavelength filter

The first tests were done without a DAD filter. Gels 1 and 2 were used in a PVA capillary and the separations were carried out over the short end (8 cm) as well as over the long distance (24 cm). It can be seen in Figure 1 that the six peaks of standard 1 are detectable. In the next step, measurements were carried out with filters. For this purpose, a 260 nm filter and a 280 nm filter were installed one after the other in front of the DAD, and all standard solutions were tested using gel 1 and the PVA capillary in each case. Figure 1 compares the results for standard solution 1 for all three detection options. With all three detection methods, six peaks are detectable in principle. The use of a DAD filter is necessary to obtain sharp peaks and good resolution. In principle, both filters are suitable for the application. However, for the separation of the pd(A) oligos present in standard 4, the 260 nm filter is preferred.

Testing of different gels

Four different gels were tested using the PVA capillary and a 280 nm filter. At first, the three different batches of PEG were compared. For standard solution 2, all peaks were resolved. This is especially a challenge for the last two peaks (20mer and 21mer), because there is only 1mer difference in their structure. In conclusion, all three PEGs are suitable for the separation of the oligonucleotides.

For better resolution, the use of 20% acetonitrile (ACN) is recommended. For standard 1, a better resolution was achieved using the ACN gel. However, for standards 2 and 3, there are only marginal differences in resolution using gels with and without ACN. For

standard 4, a significant improvement of resolution was achieved. In summary, the use of ACN in the gel depends on the kind of sample. For some samples with difficult peak resolution, the use of ACN can be advantageous.

Testing different types of capillaries

Three different types of capillaries were tested for separation of oligonucleotides:

- **Capillary 1:** PVA, 100 μm
- **Capillary 2:** $\mu\text{SIL WAX}$, 100 μm
- **Capillary 3:** $\mu\text{SIL DNA}$, 75 μm

For all capillary types, a length of 33 cm in total was prepared and tested using the short-end detection, the 280 nm filter and gel 1. In principle all three capillaries can be applied for the separation of the four oligonucleotides in standard solution 2. The resolution of the peaks is similar for all three capillaries. For capillaries 1 and 2, a better signal-to-noise ratio is achieved. This can be explained by the thicker inner diameter of these capillaries.

In summary, the use of capillaries 1 and 2 is preferred. However, one advantage of capillary 1 is easier handling when installing in the cassette. This is due to the fact that the detection window can be placed more safely and quickly in the alignment interface, because the fixed stopper ensures the correct adjustment in the detector.

Reproducibility of migration times

The reproducibility of the method was tested with six consecutive injections of standard 2 using gel 1, the 280 nm filter and the PVA as well as the $\mu\text{SIL-WAX}$ capillary (illustrated in Figure 2). Good RSDs below 1% were achieved (Table 3). Therefore, both capillaries are usable for reproducible analysis. For both series of measurements, it was found that the reproducibility was improved for injections 7 to 12. The reason for this

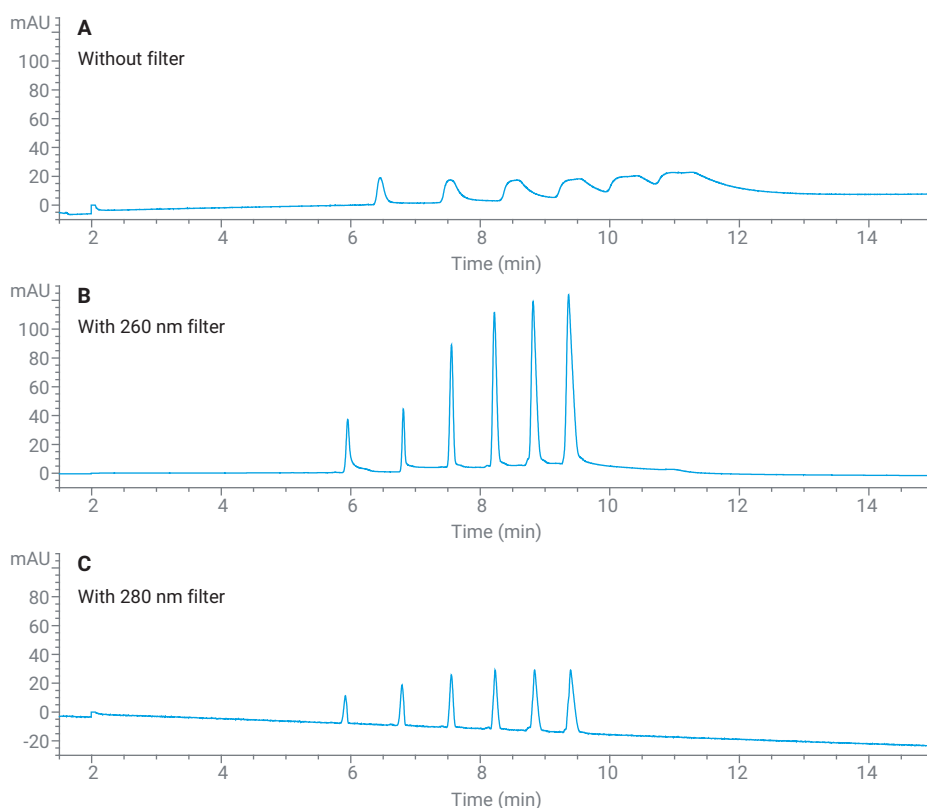


Figure 1. Standard solution 1 (diluted 1:2), short-end, gel 1, applying different DAD filters.

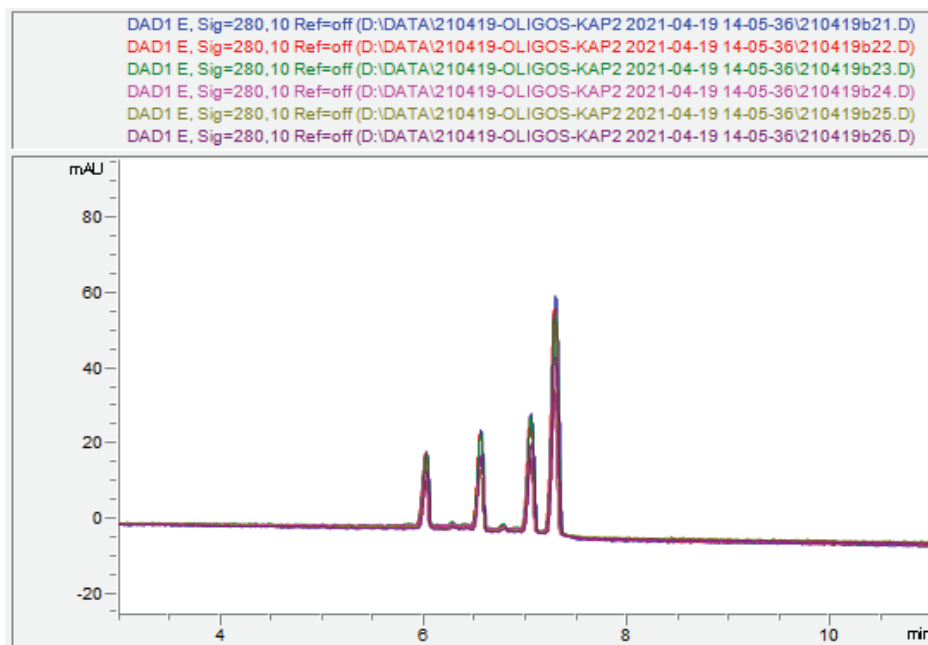


Figure 2. Overlay of consecutive injections using gel 1 and a $\mu\text{SIL-WAX}$ capillary.

could be better capillary conditioning. The relative standard deviation (RSD) of the migration time was calculated for the last peak (21mer).

Effective length

Applying a 33 cm capillary, two different separation lengths can be used. If the sample is injected on the side of the inlet home vial, the separation length up to the detector is 24.5 cm. The sample can also be injected from the other side (the side of the outlet home vial). This side is closer to the point of detection and thus, the resulting separation length is only 8.5 cm (short-end detection).

In this way, two different lengths can be tested comfortably with the same capillary during method optimization. The comparative measurements were performed using gel 1, a PVA capillary and the 260 nm filter. Figures 3 to 5 show the same electropherograms with enlarged sections of peak zones for standard solutions 1 to 3. It can be observed that baseline resolution of the peaks is also achieved over the short-end separation. The use of the longer distance clearly leads to an even better resolution. The disadvantage, however, is the significantly longer analysis time. For standard solution 4, the long side detection is recommended, as can be recognized in Figure 6. In conclusion, both options are applicable for the samples shown without exchanging the capillary. Priorities can be determined by the user.

Table 3. Relative standard deviation.

Test	Capillary	RSD (%) (injection 1-6)	RSD (%) (injection 7-12)	RSD (%) (injection 1-12)
1	PVA	0.90	0.31	0.67
2	μSIL-WAX	0.51	0.11	0.36

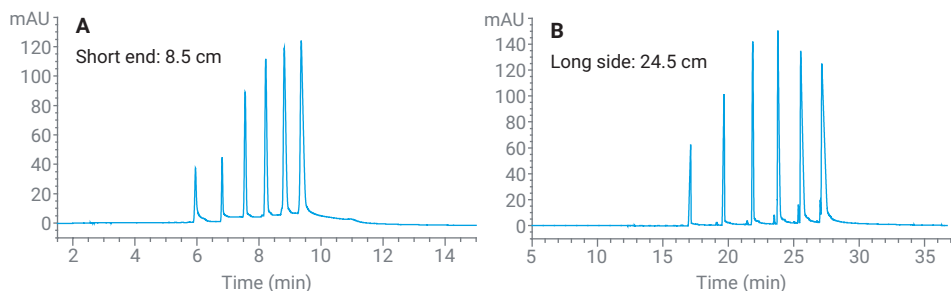


Figure 3. Separation of standard 1 using two different separation lengths.

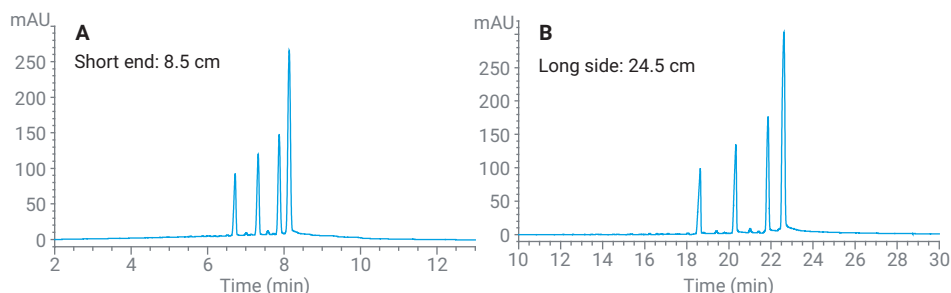


Figure 4. Separation of standard 2 using two different separation lengths.

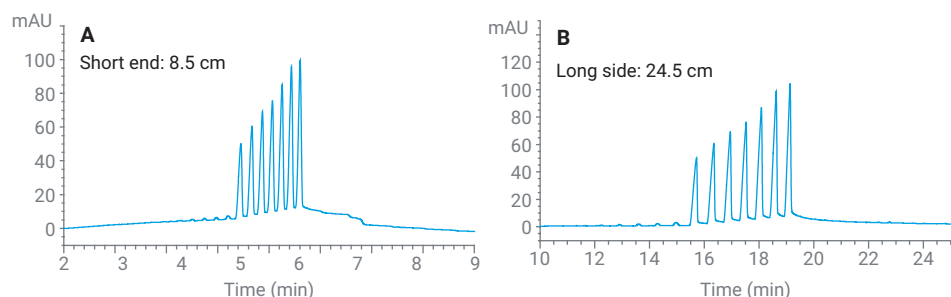


Figure 5. Separation of standard 3 using two different separation lengths.

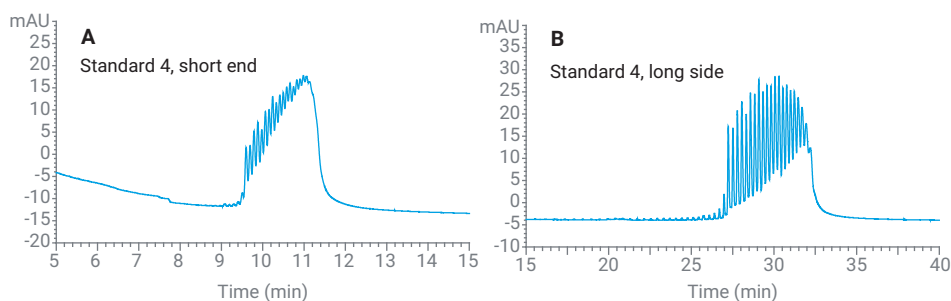


Figure 6. Separation of standard 4 using two different separation lengths.

Influence of temperature

Changing the temperature can be a simple way to optimize a separation. The influence of temperature on the resolution and migration time for standard solution 2 was investigated in the range of 20 to 35 °C. Figure 7 shows that the resolution improves with decreasing temperature, with the resolution of the last two peaks indicated in the figure in each case. On the other hand, due to the higher viscosity at lower temperature, the analysis time also becomes longer. Therefore, the optimal temperature should be selected depending on the type of sample and the intention of the user.

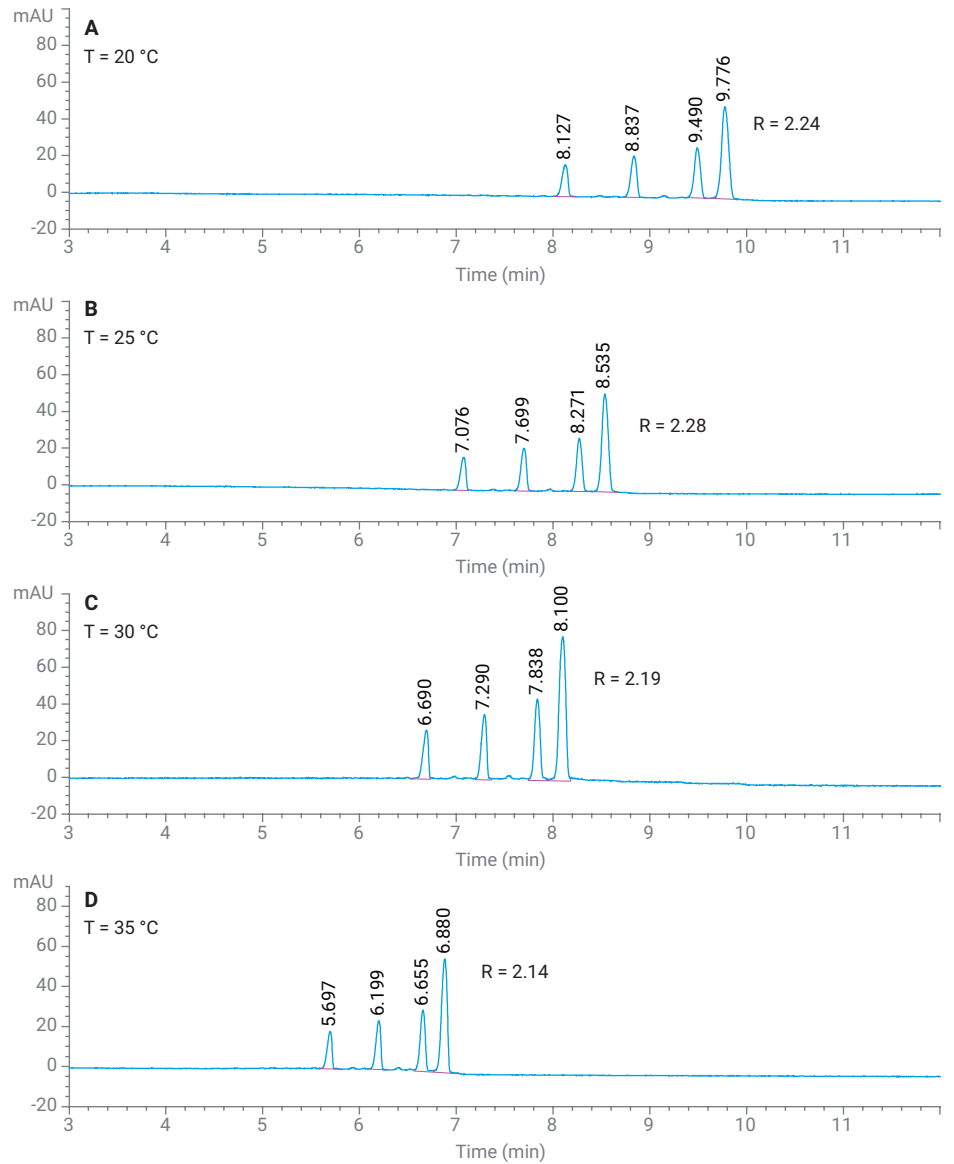


Figure 7. Influence of temperature (using gel 1, a PVA capillary and the 280 nm filter).

Influence of voltage

In Figure 8, the influence of the separation voltage was investigated for standard solution 2. The main interest was on the resolution of the last two peaks, which is written to the right of the last peak, respectively. The enormous influence on the analysis time becomes immediately apparent. Furthermore, it can be seen that the peaks also become sharper with increasing voltage, and thus the loss of resolution caused by the faster separation is moderate. From Figure 8D, it is evident that the combination of a higher voltage with a lower temperature is also interesting. Here, the separation is faster than at 25 kV (see Figure 8B) but the resolution remains practically unchanged. In conclusion, a higher separation voltage could therefore be of interest to users who plan a high sample throughput.

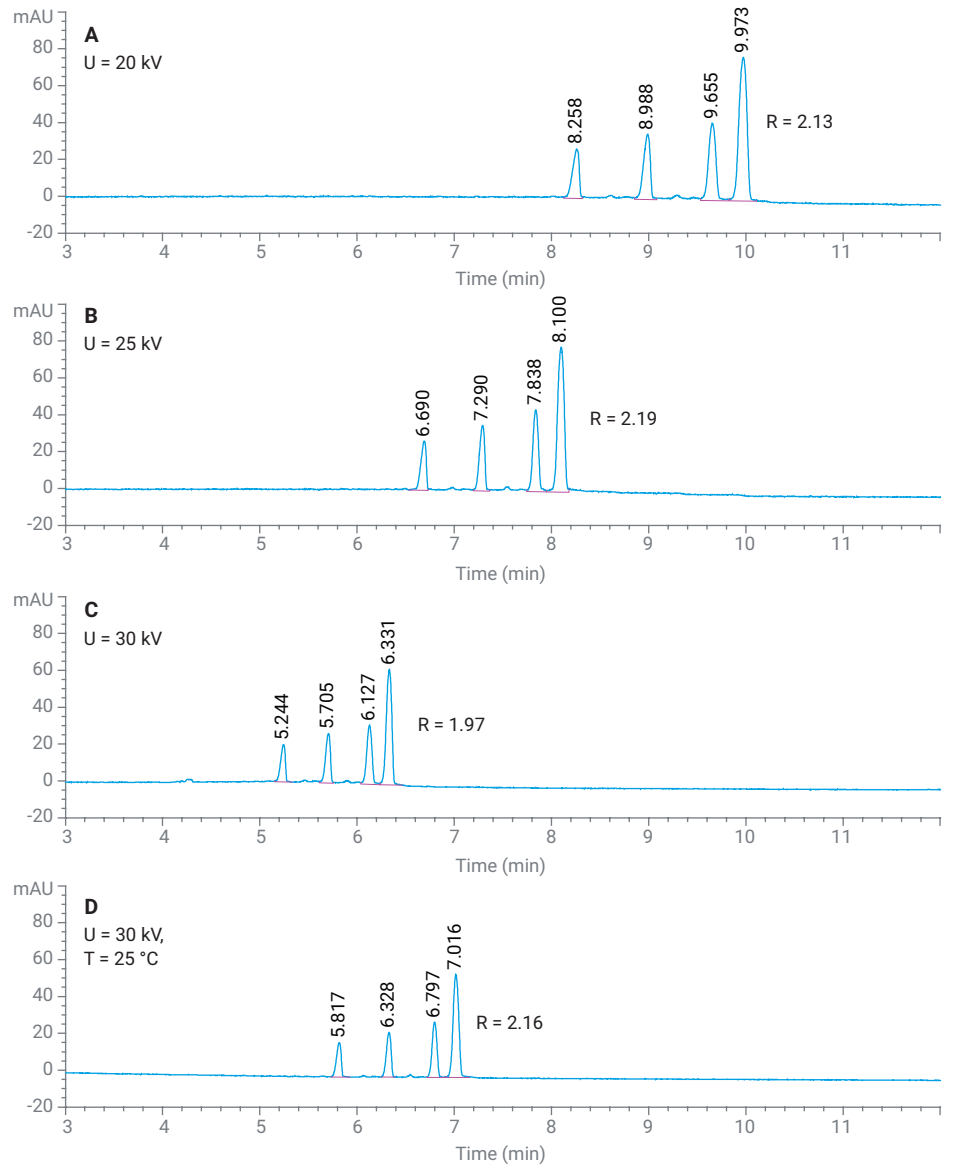


Figure 8. Influence of voltage (using gel 1, a PVA capillary and the 280 nm filter).

Optimization of separation for standard solution 4

Separating the oligos in standard solution 4 is a challenge, as 20 consecutive pd(A) oligos with a relatively long chain (40mer to 60mer) are involved. Therefore, the influence of the capillary length, the addition of acetonitrile and the optimization of the voltage were investigated. For the experiments, the PVA capillary and the 260 nm filter were applied and the electropherograms obtained under different conditions. The best results are presented in Figure 9, where scaling was adjusted in each case. A significant improvement in separation was achieved by using 20% acetonitrile in the gel (see Figure 9B). Finally, an increased voltage of 30 kV with simultaneous reduction in temperature to 25 °C was tested. It is evident from Figure 9C that the best separation was achieved with these changes. If necessary, further optimization could be carried out, e.g. by adjusting the sample concentration and injection or further optimizing the acetonitrile content. Therefore, it has been shown that it is possible to optimize even difficult oligo separations by simply and quickly adjusting the experimental conditions.

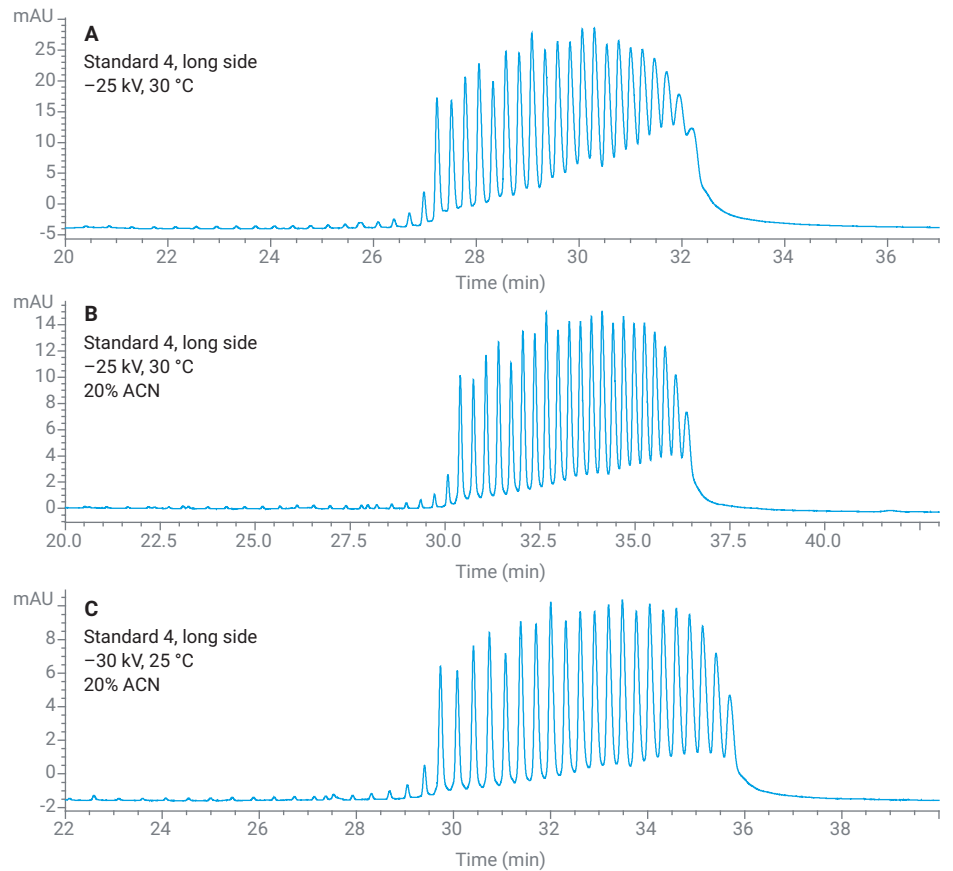


Figure 9. Optimization of experimental conditions for standard solution 4 (diluted 1:2).

Conclusion

The procedure and effects described in *Oligonucleotide Analysis with the Agilent Capillary Electrophoresis System*¹ could be well reproduced using currently available chemicals and materials. The results can be briefly summarized as follows:

Detectability: In principle, the separation could be carried out without a filter. However, because of the poor peak resolution and reproducibility, this is not recommended. By using a 260 nm or 280 nm detector filter, good resolutions were achieved. Both filters are suitable in principle, but a better signal-to-noise ratio was achieved with the 260 nm filter. Especially for pd(A) oligos, the 260 nm filter should be used.

Gels: All three PEGs tested are equally suitable for the application. The addition of acetonitrile can partially improve separation performance. Thus, with the currently purchasable chemicals, the separations can be carried out successfully. The prepared gels can be used for at least three weeks.

Capillaries: The use of the PVA capillary and the μ SIL-WAX capillary is recommended. Good resolution and good detection strength were achieved with both capillaries. The PVA capillary has the advantage of easier handling when installing in the interface. The use of the μ SIL-DNA capillary is not recommended, because the smaller diameter results in significantly lower detection strength.

Reproducibility: The reproducibility of the migration times for the 21mer in standard solution 2 was investigated using the PVA capillary and the μ SIL-WAX capillary. For both capillaries, a good RSD was achieved over 12 injections. The value was always below 1%. Examining the RSD of the peak areas does not make sense because there is depletion from measurement to measurement. This can be justified with the electrokinetic injection, because here only the charged analytes are injected (rather than aliquots of the sample solution).

Optimization: In most cases, the short-end detection was sufficient for successful and rapid separation of the oligos. For standard 4, long side detection was necessary. Furthermore, it was shown that the method can be easily and quickly optimized e.g. by using acetonitrile gel and adjusting voltage and temperature.

The Agilent 7100 capillary electrophoresis system is suitable for a broad range of analyses with oligonucleotides, including pd(A), pd(T) and oligos with mixed bases and different chain lengths.

References

1. Oligonucleotide Analysis with the Agilent Capillary Electrophoresis System. *Agilent Technologies application note*, publication number 5988-4303EN, **2001**.

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