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Evaluation of three commercially available capillary (gel)electrophoresis kits for single stranded DNA oligonucleotide analysis

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Abstract

Three commercially available capillary (gel)electrophoretic kits, the BioCap oligonucleotide capillary kit (A), the single stranded (ss) DNA 100 gel capillary kit (B) and the ssDNA 100-R (replaceable) gel capillary kit (C), were compared with respect to resolution and repeatability of migration time, absolute and relative corrected peak area and resolution. As a sample, a mixture was made of crude synthetic ssDNA oligonucleotides with overlapping sequences with lengths of 16, 17, 18 and 19 nucleotides (n-mer, group 1) and with lengths of 51, 52 and 53 nucleotides (n-mer, group 2). For each kit the injection conditions (kV s), analysis temperature (°C) and analysis voltage (V/m) were optimized with respect to resolution applying a 2^3 factorial design using the analysis conditions as supplied by the respective kit manufacturers as a central point in the experimental design matrix (standard condition). Repeatability results ($n=8$) as obtained with group 1 components showed remarkable differences between the kits, e.g. resolution factors of 2.6, 1.9 and 6.4 were measured in kits A, B and C, respectively. Migration times as measured for the n-mer group 2 component were 11.1, 64.7 and 111.4 min in kits A, B and C, respectively. Kit C was selected to be applied to the analysis of synthetic ssDNA oligonucleotides which is part of the quality control procedure of these components. The method has been validated for this purpose. Such purified oligonucleotides are used as primers in nucleic acid sequence based amplification (NASBA) which is an isothermal nucleic acid amplification technology. At Organon Teknika nucleic acid diagnostics are made using this technology which are marketed under the name NucliSens. © 1998 Elsevier Science B.V. All rights reserved.

Keywords: Electrophoretic kits; Oligonucleotide; DNA

1. Introduction

The specificity of target nucleic acid amplification technologies such as polymerase chain reaction (PCR), nucleic acid sequence based amplification (NASBA), transcription mediated amplification (TMA) and ligase chain reaction (LCR) is determined by the sequences of the ssDNA oligonucleotides which are used as primers, i.e. selection of the target is done by sequence specific hybridisation of the primers [1]. Generally these oligonucleotides are manufactured on a DNA synthesizer applying phosphoramidite chemistry [2]. Each cycle of nucleotide addition includes four steps, i.e. detritylation, coupling, capping and oxidation [3]. The trityl group, that is cleaved-off prior to coupling of the next nucleotide in the synthesis process, is measured to monitor the step-wise coupling efficiency, which is over 0.98. About 2% of support-bound nucleotides fails to undergo addition and consequently 2% of the 5′-hydroxyl groups remain unreacted. These hydroxyl groups are capped by acetylation to prevent

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them from participation in the rest of the synthesis reactions. Chromatographic methods were reported for the removal of the incomplete synthesis products [4]. Capillary gel- electrophoresis is the method of choice for quality control of the synthetic ssDNA primers. Several kits for the analysis of synthetic ssDNA oligonucleotides are commercially available, three of such kits have been evaluated. As a testing sample, a mixture was prepared containing oligonucleotides with lengths approximating the length range of the molecules to be analysed in our final application, i.e. approximately 20 nucleotides for the p2 primer up to approximately 55 nucleotides for the p1 primer [5]. First, the conditions for analysis of ssDNA oligonucleotides were optimized for each kit separately with respect to resolution. The optimization was done using a 2^3 factorial design [6]. Applying these optimized conditions repeatability, which is the appropriate term as data are obtained within 1 day by one operator using one capillary electrophoretic system, was measured with respect to resolution, migration time and for the absolute and relative corrected peak area.

2. Experimental

2.1. Materials and reagents

The BioCap oligonucleotide capillary kit (A) was obtained from Bio-Rad (Hercules, CA, USA), the capillary had a total length of 30 cm (effective length 23 cm)×75 μm I.D. The ssDNA 100 gel capillary kit (B) was obtained from Beckman (Fullerton, CA, USA), the capillary was 58.5 cm (effective length 50 cm)×100 μm I.D. The ssDNA 100-R (replaceable) gel capillary kit (C) was also obtained from Beckman, the capillary was 47.5 cm (effective length 40 cm)×100 μm I.D. Each kit was used with the reagents supplied with the kit. Reagents were prepared according to the instructions in the package inserts. The water used was 18 MΩ quality from Sigma (St. Louis, MO, USA). Oligonucleotides were synthesized at Organon Teknika on an automated model 390Z large-scale DNA/RNA synthesizer (Applied Biosystems, CA, USA).

2.2. Preparation of the oligonucleotide mixture

A mixture was made containing crude synthetic ssDNA oligonucleotides with lengths of 16, 17, 18 and 19 (n-mer group 1) nucleotides and of oligonucleotides with lengths of 51, 52 and 53 (n-mer group 2) nucleotides. The nucleotide sequence of the n-mer group 1 oligonucleotide was 5′-GTC TAG CCA TGG CGT TAG TA-3′. The 16, 17 and 18-mers had the same sequences as the n-mer except the 3, 2 and 1 nucleotides were missing respectively from the 5′-end. The nucleotide sequence of the n-mer group 2 oligonucleotide was 5′-ATT CTA ATA CGA CTC ACT ATA GGG TGC TAT GTC ACT TCC CCT TGG TTC TCT CA-3′. The 51 and 52-mers had the same sequences as the n-mer except that 2 and 1 nucleotides were missing respectively from the 5′-end. The oligonucleotide concentrations in the mixture were 7 (±1) μ*M* in water for each oligonucleotide.

2.3. Electrophoresis

Electrophoresis was performed using a HP^{3d}CE capillary electrophoretic system from Hewlett-Packard (Waldbronn, Germany). Each analysis was done using a fresh sample, i.e. only one injection was done out of the same sample vial. Electrophoresis buffers were changed between two consecutive runs. In the analyses using the BioCap capillary also the gel was changed between two consecutive runs.

2.4. Optimization of analysis conditions

For each kit the experimental conditions were optimized with respect to resolution.

A 2^3 factorial design was used, i.e. the effect on resolution of three parameters, injection time× voltage, analysis voltage and analysis temperature, was investigated at two levels. A schematic overview of the experimental set-up is given in Table 1 and the exact values for each parameter are given in Table 2. One injection was done at each condition, except for the conditions as supplied by the manufacturer (standard condition) which were analyzed in fourfold.

Resolution, R_s, was calculated according to Eq.

Table 1
Schematic representation of the experimental set-up as applied in the optimization study

Run no.	Analyses T (°C)	Analyses$V$ (V/m)	Injection t (V s)
1	+	+	+
2	+	+	−
3	+	−	+
4	+	−	−
5	−	+	+
6	−	+	−
7	−	−	+
8	−	−	−
Standard			
Standard			
Standard			
Standard			

+ = Maximum value; − = minimum value.
Standard = conditions as supplied by the kit manufacturer.

(1), which is similar to the formula for calculation of resolution in chromatography [7].

$$R_s = \frac{t_{m2} - t_{m2}}{\frac{1}{2}(PW_{b1} + PW_{b2})} \quad (1)$$

where t_m = migration time of component (min) and PW_b = peak width at peak base (min).

2.5. Determination of repeatability

Repeatability of the resolution, migration time and for the absolute and relative corrected peak area was measured in 8-fold under the experimental conditions as optimized for each kit.

As the analyses are done on 1 day by one technician and by using one capillary electrophoretic system, repeatability is the appropriate term. Reproducibility has not been investigated, i.e. performing the same analyses on different days by different operators and using different capillary electrophoresis systems.

As a result of differences in electrophoretic mobilities, differences in detection efficiencies occur in CE although components may have the same spectral properties. To correct for this phenomenon the absolute peak area is divided by the migration time, yielding the corrected peak area.

3. Results

3.1. Optimization

In Table 3 the resolution values are given as calculated using the n/n-1-mer peaks of group 1 and group 2 for each capillary kit.

Regression analysis was done on the resolution data obtained with the different capillary kits, assuming a first order linear model, using the n/n-1-mer group 1 components. See Table 4 for the equations describing the relation between the experimental conditions and the resolution. In Table 4 the R^2 value is also given representing the closeness of agreement between the measured data and the applied linear regression model.

Fig. 1 shows the electropherograms as obtained from the testing sample after optimization of each capillary kit. The electropherograms are shown applying different time axes to illustrate differences in resolution as obtained using the different capillary kits.

Table 2
Analysis conditions as applied in the optimization study with (A) Bio-Rad BioCap oligonucleotide, (B) Beckman ssDNA 100, (C) Beckman ssDNA 100-R, i.e. each permutation has been tested

Capillary	Standard condition			Upper value			Lower value		
	A	B	C	A	B	C	A	B	C
Analysis T (°C)	40	30	30	40[a]	35	35	30	25	25
Analysis V (V/m)	400	350	300	500	400	400	300	300	200
Injection (kV s)	0.5	75	35	0.75	100	60	0.25	50	10

[a] Value is equal to standard conditions.

Table 3
Resolution values as calculated using the n/n-1-mer peaks of group 1 and group 2 as obtained in the optimization study with (A) Bio-Rad BioCap oligonucleotide, (B) Beckman ssDNA 100, (C) Beckman ssDNA 100-R

Run no.	Capillary					
	A		B		C	
	Group 1	Group 2	Group 1	Group 2	Group 1	Group 2
1	2.78	1.27	2.07	0.99	2.96	1.16
2	3.42	1.12	2.50	1.09	5.80	1.62
3	1.91	1.22	2.07	0.93	3.00	1.34
4	2.89	1.10	2.90	1.25	5.75	1.73
5	0.95	1.19	1.80	1.14	3.50	1.27
6	2.16	0.93	2.44	0.78	6.82	1.69
7	0.64	1.23	2.00	0.70	6.07	1.76
8	1.63	1.05	2.69	0.88	6.09	1.73
Standard	2.06	0.71	1.91	1.32	6.31	1.61
Standard	1.91	0.73	2.11	1.41	6.53	1.68
Standard	1.90	0.73	2.09	1.66	7.02	1.66
Standard	2.07	0.76	2.63	1.46	7.21	1.12

A = Bio-Rad BioCap capillary.
B = Beckman fixed gel capillary.
C = Beckman replaceable gel capillary.

3.2. Determination of repeatability

3.2.1. Resolution

In Fig. 2 results are shown on repeatability with respect to resolution for n/n-1 mer group 1 and group 2 components. Each bar represents the average resolution value ($n=8$). The error bars represent the 95% confidence limits. With the ssDNA 100-R kit, resolution values are obtained that are approximately twice as high as those obtained with the BioCap oligonucleotide and the ssDNA 100 kits.

3.2.2. Migration time

In Fig. 3 repeatability results are shown with respect to the migration time when using n-mer components of groups 1 and 2, respectively. Each bar represents the average migration time ($n=8$). The error bars represent the 95% confidence limits. Large differences in migration times are measured with the different capillary kits, i.e. with the BioCap oligonucleotide kit separations take only approximately 10% of the time required for separations using the ssDNA 100-R capillary kit. However, separations as obtained with the different capillary kits were been optimized with respect to run time.

Calculation of the migration time as a function of the oligonucleotide length using the data from the oligonucleotide mixture yields equations when assuming a linear relation with $R^2>0.996$ for all three capillary kits. The high R^2 value shows that a linear relation between migration time and oligonucleotide length exists. Although the oligonucleotides of group

Table 4
Coefficients and constants as obtained after linear regression analysis on the resolution data of each capillary kit in the optimization study, with the R^2 value for each equation

Capillary kit	Coefficient			Constant	R^2
	X_1	X_2	X_3		
BioCap oligonucleotide	−0.9600	0.1400	0.0925	−3.005	0.982
ssDNA 100 (fixed)	−0.1295	0.0152	−0.0213	3.194	0.959
ssDNA 100-R (replaceable)	−0.4885	−0.1468	−0.0667	12.116	0.854

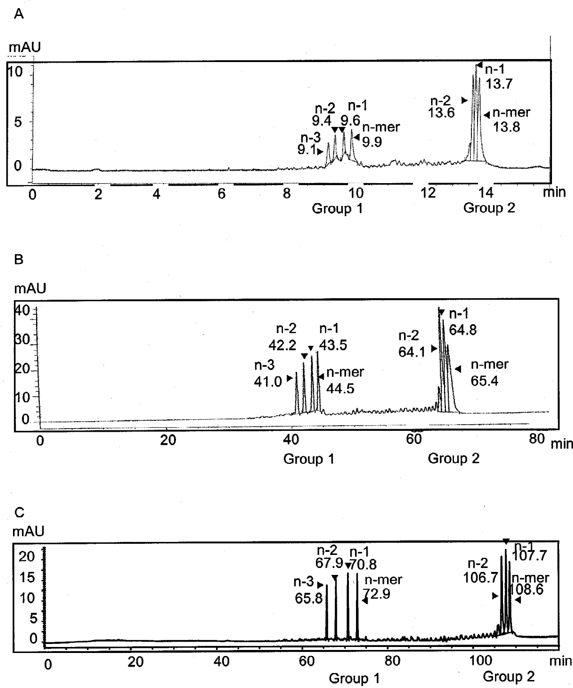

Fig. 1. Electropherograms obtained with the analysis of a synthetic ssDNA oligonucleotide mixture as optimized for each capillary kit. The electropherograms are obtained with (A) the BioCap oligonucleotide kit, (B) the ssDNA 100 kit and (C) the ssDNA 100-R kit. In the electropherograms the lengths of the components are given relative to the full-length component of each group (n-mer). Below the length the respective migration time (min) is given.

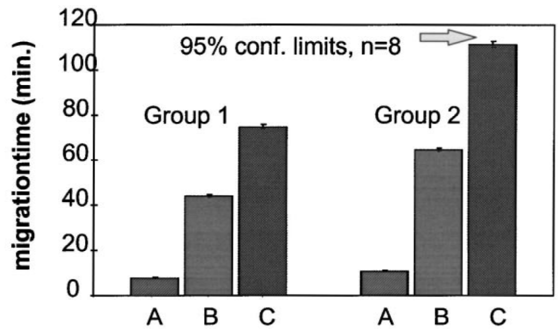

Fig. 3. Average migration times ($n=8$) as measured from oligonucleotide components of the standard mixture with a chain length of 19 nucleotides (group 1) and with a length 53 nucleotides (group 2), obtained with (A) the BioCap oligonucleotide kit, (B) the ssDNA 100 kit and (C) the ssDNA 100-R kit. Error bars represent the 95% confidence limits.

1 have different nucleotide sequences to the oligonucleotides of group 2, their respective mobilities apparently fit very well in the linear relation.

From Fig. 3 it can be seen that the repeatability of the migration time is good as the relative standard deviations, i.e. the standard deviation divided by the mean migration time, are below 1.5% for all three kits.

3.2.3. Absolute corrected peak area

In Fig. 4 our results are shown on repeatability with respect to the absolute corrected peak area for n-mer components of group 1 and 2 respectively.

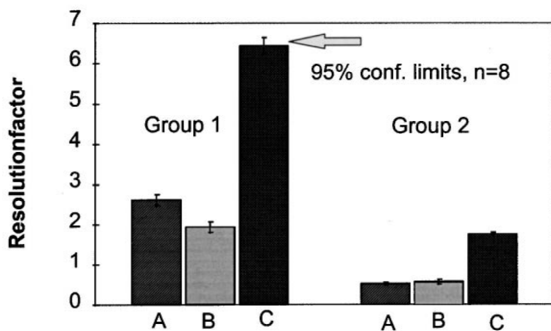

Fig. 2. Average resolution factors ($n=8$) as calculated from oligonucleotide components of the standard mixture with chain-lengths of 18 and 19 nucleotides (group 1) and with lengths of 52 and 53 nucleotides (group 2), obtained with (A) the BioCap oligonucleotide kit, (B) the ssDNA 100 kit and (C) the ssDNA 100-R kit. Error bars represent the 95% confidence limits.

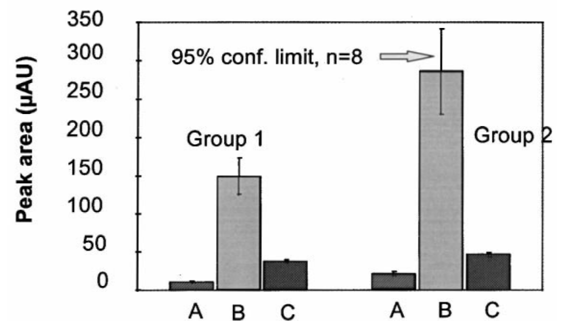

Fig. 4. Average absolute corrected peak area ($n=8$) as calculated from oligonucleotide components of the standard mixture with a chain length of 19 nucleotides (group 1) and with a length of 53 nucleotides (group 2), obtained with (A) the BioCap oligonucleotide kit, (B) the ssDNA 100 kit and (C) the ssDNA 100-R kit. Error bars represent the 95% confidence limits.

Each bar represents the average absolute corrected peak area ($n=8$). The error bars represent the 95% confidence limits. Substantial differences occur in the absolute corrected peak areas obtained with the different capillary kits and these differences do not reflect the differences in injections conditions in terms of V s.

The relative injection efficiency can be calculated be dividing the obtained corrected peak area by the applied injection V s. With the BioCap capillary kit $\sim 10^{-8}$ AU/V s are obtained which is ~5–10 times higher than that obtained with the ssDNA 100 and ssDNA 100-R kits.

Relatively poor repeatability of injection is obtained with the ssDNA 100 capillary kit as is shown by the relatively long error bars.

3.2.4. Relative corrected peak area

Fig. 5 shows the results on repeatability with respect to relative corrected peak area for n-mer group 1 and group 2 components. The relative corrected peak area is the corrected peak area of the peak of interest expressed as percentage of the total corrected peak area. Each bar represents the average relative corrected peak area ($n=8$). The error bars represent the 95% confidence limits. The relative corrected peak area for each component is similar for the different capillary kits, i.e. these values are within 20% of the mean value that is obtained for each components after combination of the data of all three kits. The differences that have been measured in the peak areas presumably are the result of differences in separations.

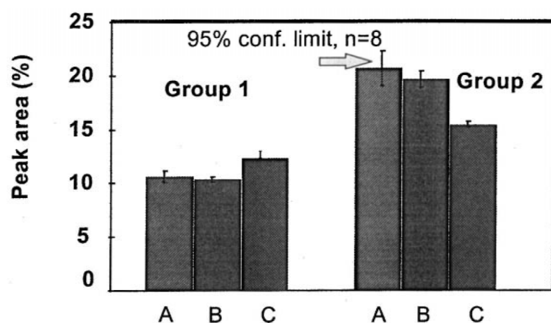

Fig. 5. Average relative peak area ($n=8$) as calculated from oligonucleotide components of the standard mixture with a chain length of 19 nucleotides (group 1) and with a length of 53 nucleotides (group 2), obtained with (A) the BioCap oligonucleotide kit, (B) the ssDNA 100 kit and (C) the ssDNA 100-R kit. Error bars represent the 95% confidence limits.

4. Discussion

The approach that is reported in this article, is, in our opinion, an efficient way of systematically investigating the effects that various experimental parameters have on response, i.e. a minimal number of injections yield a maximum on quantitative information, although some improvements can be done. One improvement would be to perform duplicate analyses under each experimental condition. Each duplicate would give an estimate of the between-run variance at that specific condition. This is preferable to the reported optimization study in which single-fold injections were done except for the standard conditions. The significance of the differences measured applying different experimental conditions have been interpreted relative to the differences measured in the data obtained from four-fold injection under standard condition. This was done assuming that the between-run variance under standard conditions is representative for the between-run variance to be obtained with the other experimental conditions. This assumption has not been verified. To overcome this problem, in future optimization studies we would prefer duplicate analyses at each experimental condition. As the analyses are done using automatic sampling and injection, the extra time required to perform duplicates is not considered to be a serious drawback.

The data obtained in this study also would allow for optimization with respect to migration time or with respect to the combination of migration time and resolution.

In our application we consider maximum resolution as most important, therefore the ssDNA 100-R capillary kit has been selected for the analysis of synthetic ssDNA oligonucleotides. When high throughput is the dominant criterion, the BioCap oligonucleotide analysis kit would be more appropriate.

By combining the linear relation between oligonucleotide length and migration time and the good repeatability of the migration time it should be possible to calculate the length of oligonucleotides

from their migration times. The data reported in this paper confirm the conclusions of [8], in which calibration of a gel-filled capillary is reported for molecular mass determination under specified conditions. It can be expected that the reliability of this method for determination of the lengths of oligonucleotides can be improved by applying an internal standard of known length as this minimizes inaccuracy due to inter-run differences. However, care should be taken with application of this method as separations have been reported of oligonucleotides sharing the same base composition but with different sequences [9,10]. In [10] the existence of secondary structures in oligo-DNAs is strongly suggested even in the presence of 7 *M* urea. Additionally it is suggested in [10] that secondary structures in oligo-DNAs are a cause of decrease in migration time relative to a molecule expected to be linear and having the same base composition even when run in 7 *M* urea.

The results reported here are obtained with three different capillary gel-electrophoresis kits. With the two Beckman kits denaturing conditions are applied as separations are performed in 7 *M* urea. With the Bio-Rad kit separations are performed using a nondenaturing electrophoretic buffer. Although the Bio-Rad kit is run applying nondenaturing conditions, a linear relation is obtained between oligonucleotide length and migration time. These observations suggest that there is probably no secondary structure in the oligonucleotides applied. The suggested absence of internal structure can be explained by the fact that the n-mer group 1 and group 2 oligonucleotides applied in this study represent hybridisation sequences of primers used in NASBA. Oligonucleotides intended for the purpose of hybridisation are designed to have a minimum of internal structure [1].

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References

[1] D.L. Wiedbrauk, D.H. Farkas, Molecular Methods for Virus Detection, Academic, San Diego, CA, USA, 1995, p. 182.
[2] J.W. Efcavitch, Macromolecular Sequencing and Synthesis, Selected Methods and Application, Alan R. Liss, 1988, p. 221.
[3] J.S. Eadie, D.S. Davidson, Nucl. Acids Res. 15 (1987) 8333.
[4] W.J. Warren, G. Vella, in: S. Agrawal (Editor), Methods in Molecular Biology, Vol. 26, Protocols for Oligonucleotide Conjugates, Synthesis and Analytical Techniques, Humana Press, Totowa, NJ, 1994, p. 233.
[5] T. Kievits, B. van Gemen, D. van Strijp, R. Schukkink, M. Dircks, H. Adriaanse, l. Malek, R. Sookanan, P. Lens, J. Virol. Methods 35 (1991) 273.
[6] D.L. Massart, A. Dijkstra, L. Kaufman, Evaluation and Optimization of Laboratory Methods and Analytical Procedures, A Survey of Statistical and Mathematical Techniques, Part II, Experimental Optimization, Elsevier, Amsterdam, 1978, pp. 181–203.
[7] J.C. Janson, L. Ryden (Editors), Protein purification, Principles, High Resolution Methods and Applications, VCH, New York, 1989, p. 35.
[8] A. Paulus, E. Gassmann, J.F. Matthew, Electrophoresis 11 (1990) 702.
[9] A. Andrus, in: S. Agrawal (Editor), Methods in Molecular Biology, Vol. 26, Protocols for Oligonucleotide Conjugates, Synthesis and Analytical Techniques, Humana, Totowa, NJ, 1994, pp. 277–300.
[10] T. Satow, T. Akiyama, A. Machinda, Y. Utagawa, H. Kobayashi, J. Chromatogr. A 652 (1993) 23.