

Comparisons between Multiplexed, Absorbance-Based Capillary Electrophoresis, Capillary Electrophoresis, and Ion Exchange Chromatography for Analysis of n-1 Oligonucleotide Impurities

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Abstract

Capillary electrophoresis (CE) and ion exchange HPLC (IEC) with UV absorbance detection are commonly employed techniques for the assessment of synthetic oligonucleotide impurities. Advantages of CE include small sample consumption and high resolving power. In addition, it is possible to perform CE in a multiplexed capillary array format, significantly improving sample throughput compared to single channel instruments.

In this study, we will evaluate the separation resolution and reproducibility of multiplexed CE-UV, CE and IEC for different lengths and ratios of n mer and n-1 mer oligonucleotides from 20 mer to 90 mer lengths to compare the different techniques. In addition, preliminary results will be presented for a new multiplexed CE method employing a low pH separation matrix capable of resolving same length oligonucleotides possessing different sequences.

Objectives

- Evaluation of the separation resolution and quantitative accuracy of multiplexed CE-UV, CE-UV, and IEC for various ratios of n mer and n-1 mer oligonucleotides as a function of length
- Correlation of n-1 purity data between multiplexed CE-UV, CE-UV and IEC oligonucleotide analysis methods
- Investigation of a low pH separation matrix possessing alternative selectivity to traditional size-based CE matrices

Materials and Materials

Materials

Different lengths of purified oligonucleotides of known concentrations were obtained from Integrated DNA Technologies (Coralville, IA).

Capillary Electrophoresis

Single capillary CE separations were performed using a Beckman MDQ system (Fullerton, CA). The detection wavelength was set to 254 nm. An eCAP DNA capillary (20 cm effective length, 31 cm total length, 100 µm I.D.) was used with eCAP ssDNA 100R separation gel and buffer. Samples were prepared at 10 µM total concentration in water and injected electrokinetically at -5 kV for 3 sec. The CE separations were performed at 13.7 kV (10 µA current) for 30 – 50 min, depending on oligonucleotide length.

Ion Exchange HPLC

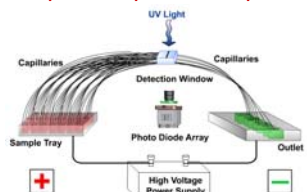
Ion exchange HPLC separations were performed using a Dionex DNAPAC PA-100 column (2 x 250 mm) (Sunnyvale, CA). Conditions: flow rate 0.3 mL/min; column temperature 60 C; run time 33.3 min; post time 9 min; injection volume 10 µL; sample concentration 10 mM in water; sample temperature 5 C; detector wavelength 270 nm; curve shape linear. Mobile Phase A: 25 mM Tris, pH 8.5, 10% ACN; Mobile Phase B: 25 mM Tris, pH 8.5, 1 M NaCl, 10% ACN; Mobile Phase C: 10% ACN. Gradient Pump Settings: 0.0 min - 65% A, 35% B; 33.3 min - 15% A, 85% B; 34 min - 5% A, 95% B; 35 min - 5% A, 95% B; 36 min - 65% A, 35% B; 42.2 min - 65% A, 35% B; 43 min - 0% A, 0% B, 100% C; 110 min - 0% A, 0% B, 100% C (method goes to 100% C on last run only).

Multiplexed Capillary Electrophoresis

Multiplexed CE separations were performed using a cePRO 9600™ 96-capillary electrophoresis instrument utilizing UV absorbance detection (CombiSep, Ames, IA). The detection wavelength was set to 254 nm. The instrument was fitted with 75-µm i.d., 192-µm o.d., fused silica capillaries of 55 cm effective length and 80 cm total length. Data processing and instrument control were performed with CombiSep's cePRO Manager® software package. Oigel™ separation matrix and run buffer were from CombiSep.

A 96-well plate was prepared consisting of various ratios of n and n-1 mer oligonucleotides of various lengths from 19mer to 90mer. The total sample concentration of each mixture was 2 µM. Samples were simultaneously injected electrokinetically at -2 kV for 5 sec. The CE separation was performed using a voltage gradient program (-10 kV for 30 min; 10 min ramp to -13 kV; total separation time 70 min).

Principles of Multiplexed CE-UV Operation



- UV light passing through the detection windows of a 96-capillary array is imaged onto a linear photodiode array detector
- Capillary inlet arrangement enables sample injection from 96-well plates
- Capillary outlets bundled to a common reservoir for pressure filling with gel
- 96 individual CE separations are performed in parallel with simultaneous UV detection

cePRO 9600™ Multiplexed 96-Capillary Electrophoresis System



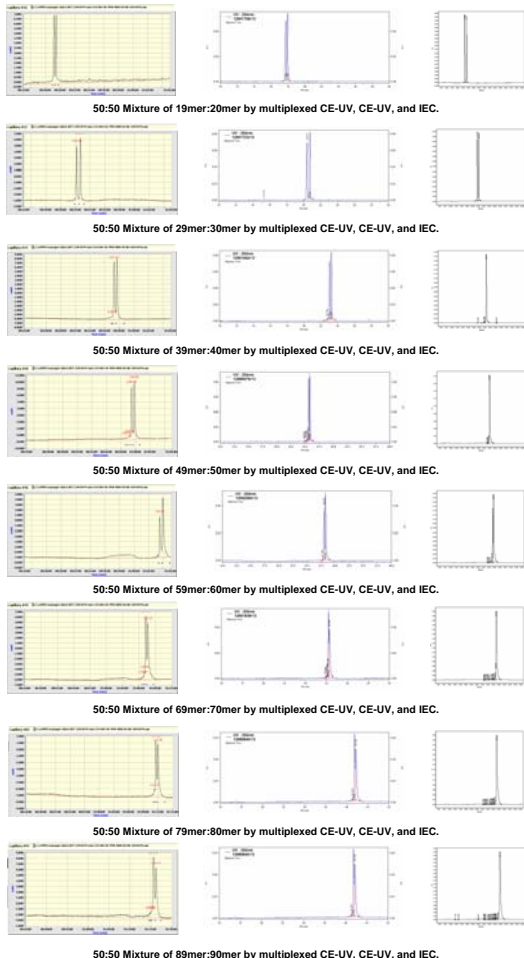
- Fixed wavelength UV detection using a mercury lamp source
- Four position slide-out stage can interface to a robotic arm

Sample Arrangement for Evaluation of n-1 mer Impurities vs. Length

Sample	19mer	20mer	21mer	41mer	42mer	100mer	101mer	307mer	308mer	400mer	500mer	700mer
A	14.20	19.20	19.20	19.20	19.20	19.20	19.20	19.20	19.20	19.20	19.20	19.20
B	19.20	19.20	19.20	19.20	19.20	19.20	19.20	19.20	19.20	19.20	19.20	19.20
C	46.00	46.00	46.00	46.00	46.00	46.00	46.00	46.00	46.00	46.00	46.00	46.00
D	46.70	46.70	46.70	46.70	46.70	46.70	46.70	46.70	46.70	46.70	46.70	46.70
E	79.60	79.60	79.60	79.60	79.60	79.60	79.60	79.60	79.60	79.60	79.60	79.60
F	89.00	89.00	89.00	89.00	89.00	89.00	89.00	89.00	89.00	89.00	89.00	89.00

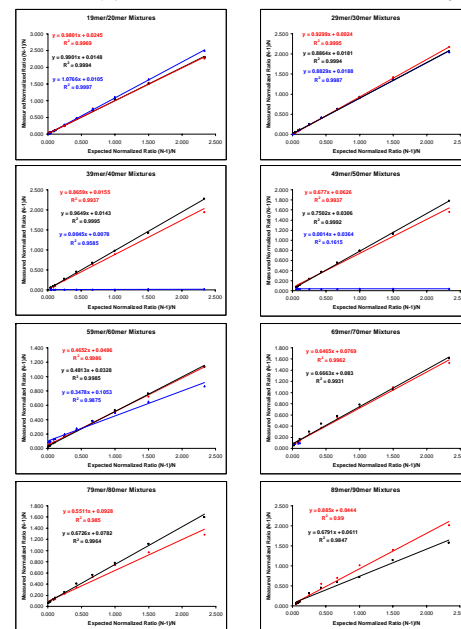
Results

Comparison of N-1 Separation Resolution vs Length

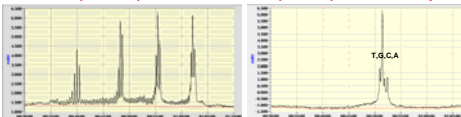


Correlation Between Methods for Measured N-1 / N Mixtures

- The measured n-1 / n ratio (normalized to n) is plotted for each method vs the n-1 / n ratio expected from the prepared sample plate
- For each length, results are plotted for multiplexed CE-UV (red), CE-UV (black), and IEC (blue)
- Similar slopes indicate method correlation; a slope of 1.0 indicates correlation between the measured and expected n-1/n ratio
- The IEC method and column employed could not resolve n-1 from n at 40mer, 50mer and 70mer-90mer lengths



Low pH CE Separation Matrix with Sequence Specific Selectivity



- Mixtures of poly (dT) 20mers-80mers (left) and a 46mer terminated by T.G.C. or A (right) separated with the low pH gel matrix.
- Sequence of 46mers: 5' (T,G,C,A)G CCA GTA GCA TGA CCC TGG CCC TTC TAC AGG ATT AAC CAG T-3'

Summary

- Multiplexed CE-UV provides a rapid, high resolution approach for the characterization of oligonucleotide purity
- Multiplexed CE-UV provides similar information to CE-UV and IEC regarding oligonucleotide purity with a 48-fold improvement in sample throughput
- Similar n-1 resolution was obtained by multiplexed CE-UV and CE-UV for 19mer-90mer oligonucleotide lengths; IEC resolution varied depending upon oligonucleotide length and sequence
- The IEC method employed could not resolve the n-1 species at 40mer and 50mer lengths, or above 60mer length
- A new low pH CE gel matrix could resolve same length oligonucleotides with single base sequence differences