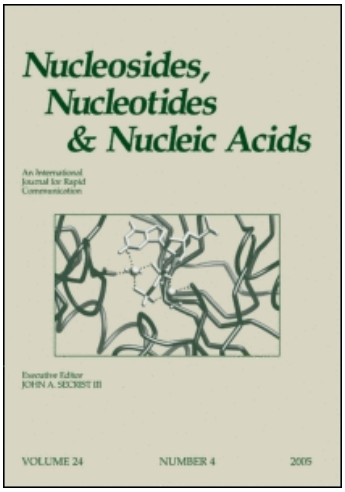

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Oligonucleotides Quality Control Analysis in Free Solution by Capillary Electrophoresis at Acidic pH

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OLIGONUCLEOTIDES QUALITY CONTROL ANALYSIS IN FREE SOLUTION BY CAPILLARY ELECTROPHORESIS AT ACIDIC pH.

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ABSTRACT

A new method for fast, automated and inexpensive oligonucleotides analysis by capillary electrophoresis at low pH is presented. This method does not need any sieving media to resolve a mixture of polynucleotides which are analysed in free solution and separated on the base of composition and not length. This technique has been used to test a large set of standard and modified oligonucleotides thus to be applied in oligos routine quality control.

The use of synthetic oligonucleotides (oligos) is widely diffused in molecular biology and biochemistry [1]. They are extensively utilized as probes for gene isolation, primers in PCR and DNA sequencing [2] and in diagnostics. They are becoming indispensable tools in the study of complex genomes, and in mutations detection; at present their utility is growing due to the recent development of the DNA chip technology which requires a huge numbers of oligos [3]. For all these reasons analysis is necessary in order to determine oligos purity and several approaches have been used including HPLC [4], PAGE, capillary gel electrophoresis (CGE) and recently HPLC coupled to ESI mass spectrometry [5]. HPLC and PAGE analysis results are more costly to obtain than CGE, which is becoming one of the more utilized technique for the separation of oligos [6]. This methodology uses crosslinked gel or liquid high viscosity polymers that are necessary to resolve a mixture of nucleic acids. Around neutrality and at basic pH (which is the traditional condition in the electrophoretic separation of DNA)

a polynucleotide chain carries a net negative charge for each residue. This implies that mass to charge ratio of the whole molecule is nearly constant in a very large mass range.

Thus the electrophoretic separation demands the presence of sieving media.

Here we propose a method for oligo quality control carried out in free solution by using an acidic running buffer. At low pH (pH<5) in fact the four nucleobases (adenine A, cytosine C, guanine G and thymine T) acquire a variable fraction of positive charge due to their different pKa values [7]. The mass to charge ratio and thus the electrophoretic mobility is related to oligos composition and no sieving media are necessary. This method has been already proposed for RNA fragment analysis by bidimensional electrophoresis [8].

We analysed a set of 77 oligonucleotides 20-21 mer in length of variable composition by using 50 mM iminodiacetic acid (IDA) pH 2.3. IDA has been recently proposed as a good running buffer for acidic separations [9] as it presents a $\Delta pK=1$ ($pI=2.23$; $pK_1=1.73$; $pK_2=2.73$). In addition this amphotere exhibits a very good buffering power at pI=2.23.

We applied a very simplified model already proposed in RNA fragment analysis [8] to correlate migration time and composition of an oligo expressed as ratio R between A+C and G+T. Our set of oligos was chosen to have $0.23< R <2.5$.

The analysis were performed in duplicate and triplicate (CV%=0.72). Results are shown in Figure 1 and demonstrate the remarkable correlation between RT and R for this heterogeneous set of macromolecules. To our knowledge this is one of the largest studies for the analytical separation of this class of biopolymers. Although it is not possible to derive the exact composition of an unknown oligonucleotide only from its retention time, we have already demonstrated that closely related oligos (n-1 mer, failed sequences) can be easily separated under these conditions [10].

Tabulated pKa values for free nucleotides are 3.8 for adenine ; 4.5 for cytosine and 2.4 for guanine. Thymine is not protonated even at very low pH. The phosphate on the backbone has a pKa=1.0. Based on these pKa values the best pH region to resolve compositional differences seemed 3.7, which is the pH value proposed recently following theoretical consideration based on the reminded pKa [11].

From our results we observed very good separation at pH 2.3 where no separation seems possible due to the nearly identical charge for A, C and G.

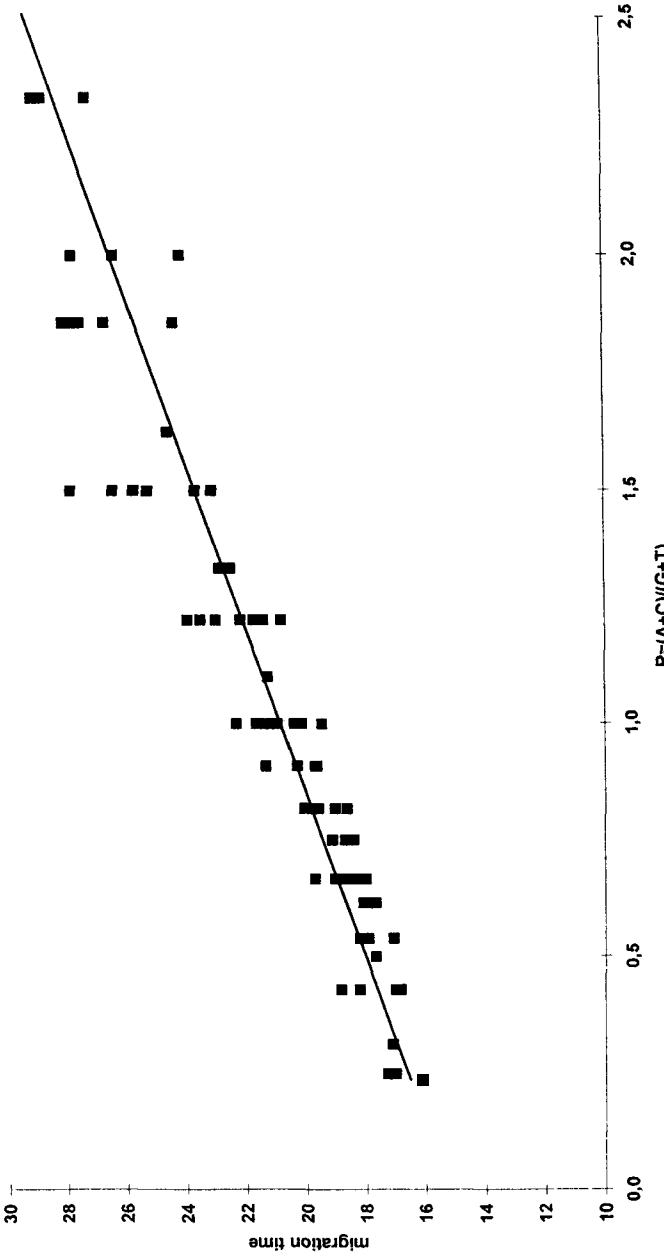

FIG.1 : Relationship between ratio R= (A+C)/(G+T) and migration time of 77 oligonucleotides analyzed by HPCE in 50 mM iminodiacetic acid pH 2.3. The analysis was performed on a BIOFOCUS 3000 system (BIORAD Laboratories Hercules Ca) working in reversed polarity ; it has been used a coated fused-silica capillary tubing (J&W, DB-Wax I.D. 50 µm) with a column length of 50 cm. The sample was injected by pressure into the capillary column (20 psi s) and electrophoresed at a constant voltage (10 kV) at 20°C.

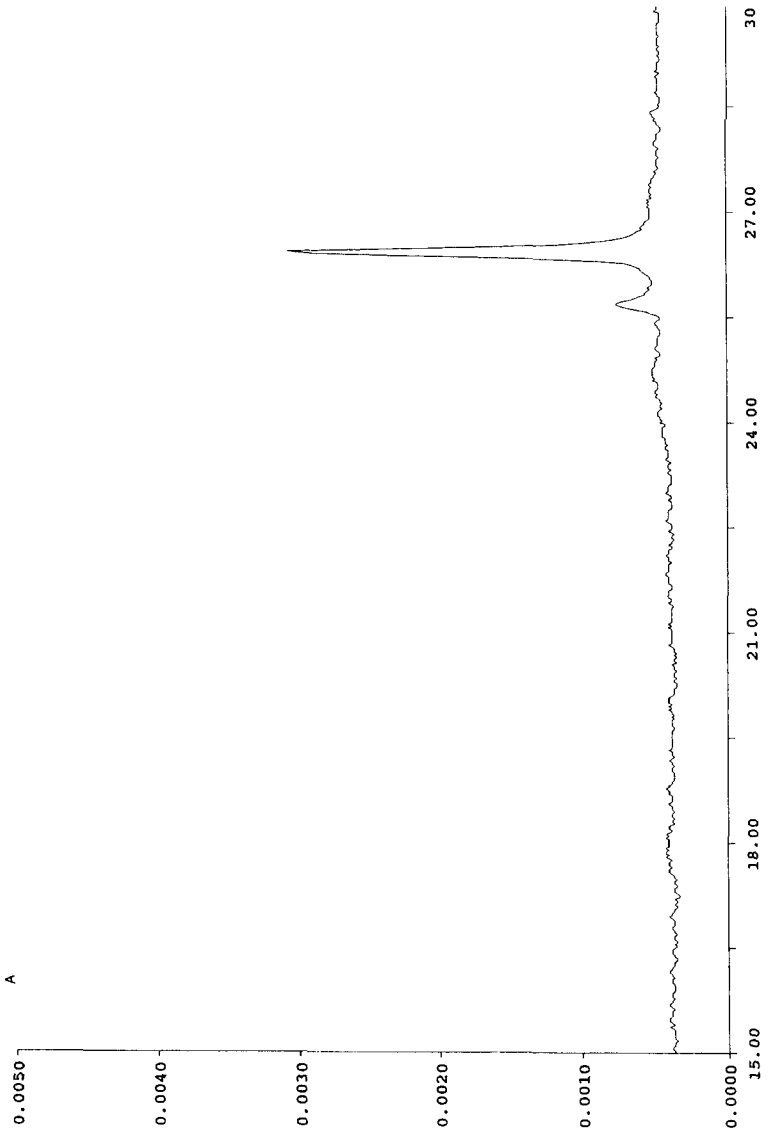

FIG.2 : HPCE separation in 50mM iminodiacetic acid ot two oligonucleotides (A) 16-mer ;(B) 19-mer. The analysis was performed on a BIOFOCUS 3000 system (BIORAD Laboratories Hercules Ca) working in reversed polarity ; it has been used a coated fused-silica capillary tubing (J&W, DB-Wax I.D. 50 μm) with a column length of 50 cm. The sample was injected by pressure into the capillary column (20 psi s) and electrophoresed at a constant voltage (10 kV) at 20°C. Note the presence of an impurity due to oligo synthesis in (A).

These results suggest a different protonation behaviour of nucleobases included in a polynucleotide chain compared with free nucleotides. In this condition it is possible to hypothesize that each nucleobase varies its pKa, within the polynucleotide chain, depending on the proximity of other protonable groups. This might justify a lowering in pKa values thus lowering the optimal pH window for separation.

The analysis of this large set of sample demonstrates the robustness of this method, which can be applied in routine control of both standard and modified oligos. Figure 2 shows an example of analysis control of modified oligos : they belong to a set of aminated synthetic oligos of different length (16-19 mer) and $0.9<R<2.0$.

From these results we can conclude that oligonucleotides analysis by capillary electrophoresis at acidic pH in free solution is a very useful method for routine control test. This technique allows several applications in DNA study especially in the field of mutation detection analysis as it is possible to separate oligonucleotides that differ just for one base.

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