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DNA Separation Methodology Based on Charge Neutralization in a Polycationic Gel Matrix

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A novel method for separation of DNA fragments is here reported, based on migrating the polyanionic DNA fragments in a polycationic polyacrylamide gel, made by incorporating positively charged monomers (the Immobilines used for creating immobilized pH gradients) into the neutral polyacrylamide backbone. Separations can be operated under two working conditions: either against a gradient of positive charges, to allow the various DNA fragments to reach a steady-state position along the migration path and condense (focus) in an environment inducing charge neutralization, or in a plateau gel (i.e., in a gel containing a constant level of positive charges from anode to cathode). In this last case, separation is still obtained due to differential charge modulation of the various DNA fragments. In the 100−1000-bp length, it is shown that separation can be obtained even for fragments differing in length by <0.5%, as shown in the splitting of a 656- and 659-bp doublet, that could not be resolved by conventional polyacrylamide gels. In the 10−100-bp range, it is shown that the present method can resolve single nucleotide polymorphisms, i.e. fragments of identical number of nucleotides but differing by one base substitution. In this last case, separations are obtained only in gradient gels containing a much steeper gradient of charges (0−20 mM Immobiline p*K* 10.3 and p*K* 12, as opposed to gradients of only 2−4 mM positive charges for larger size fragments). This novel methodology represents a marked improvement over existing techniques and appears to hold promises for applications in diverse fields, such as molecular biology, forensic medicine, and genetic screening.

We have recently reported a novel SDS-PAGE method for mass separation of proteins. Unlike conventional SDS-PAGE,[1,2] in which fractionation by mass of SDS-laden polypeptide chains is obtained in constant concentration or porosity gradient gels, this unorthodox method, called "SDS-PAGE focusing", exploits a "steady-state" process by which the SDS−protein micelles are driven to stationary zones along the migration path against a gradient of positive charges affixed to the neutral, minimally sieving, polyacrylamide matrix.[3] As the total negative surface charge of such complexes matches the surrounding charge density of the matrix, the SDS−protein complex stops migrating and remains stationary, as typical of steady-state separation techniques. As a result of this mechanism, the proteins are separated in an unorthodox way, with the smaller proteins/peptides staying closer to the application point and larger proteins migrating further down toward the anodic gel end. Steady-state conditions were demonstrated, since pattern stability could be guaranteed for at least 24 h of "focusing". Our methodology appears to be a particular case of the general theory of adsorption of charged polymers as Z-ions.[4]

A preparative version of this novel methodology has also been reported, by which it was further demonstrated that such separations could be engendered not only in a chemical gel, i.e., in a solid matrix, but also in an entangled solution of cationic polymers, polymerized in the absence of cross-linking agents.[5] For impeding migration of these charged polymers in the electric field, viscosity gradients and low voltages had to be adopted.

Although our technique has been termed a "focusing phenomenon", it might be argued if it is a genuine "focusing" or not, especially in regard to isoelectric focusing[6] (or isopycnic centrifugation[7]), where a true focusing process takes place, since the sample can be applied anywhere in the separation column, even uniformly distributed through it. The final steady state will be completely independent from the mode of sample application. Here, on the contrary, it should be emphasized that the sample can only be applied at one point along the separation path, i.e., at the point of minimum (positive) charge of the surrounding matrix (or liquid polymer): upon migration toward the other extreme of the column, i.e., toward the point of highest charge, each species in the sample will be captured and condensed at a point of "null net charge" of the complex. Thus, uniform sample application or loading at the opposite extreme of the column will not be possible.

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(1) Shapiro, A. L.; Vinuela, E.; Maizel, J. V. *Biochem. Biophys. Res. Commun.* **1967**, *28*, 815–829.

(2) Weber, K.; Osborn, M. *J. Biol. Chem.* **1969**, *244*, 4406–4412.

(3) Zilberstein, G.; Korol, L.; Antonioli, P.; Righetti, P. G.; Bukshpan, S. *Anal. Chem.* **2007**, *79*, 821–827.

(4) Dobrynin, A. V.; Rubinstein, M. *Prog. Polym. Sci.* **2005**, *30*, 1049–1118.

(5) Zilberstein, G.; Korol, L.; Righetti, P. G.; Bukshpan, S. *Anal. Chem.* **2007**, *79*, 8624–8630.

(6) Righetti, P. G. *Isoelectric Focusing: Theory, Methodology and Applications*; Elsevier: Amsterdam, 1983; pp 1−386..

(7) Meselson, M.; Stahl, F. W.; Vinogradov, J. *Proc. Natl. Acad. Sci. U.S.A.* **1957**, *43*, 581–585.

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From this point of view, perhaps, the term "focusing" might not be appropriate and in reality the technique should more correctly be called "arrested migration of an injected plug".

The technique has worked so remarkably well for proteins and peptides only because they are present as mixed micelles with the SDS surfactant, which coats the polypeptide chain, effectively masking its zwitterionic character and rendering it polyanionic in nature, to a point of very-nearly constant charge to mass ratio (1.4 g of SDS bound/g of protein[8]). Clearly this separation mechanism should work quite well for DNA fragments, since they are intrinsically polyanionic at all pH values above pH 5 (below, in the pH 2−3 range, they would also exhibit a zwitterionic character, due to progressive protonation of three (A, C, G) out of four bases (T having no basic groups capable of being protonated[9]).

The present report deals with the separation of DNA fragments in polycationic gel matrixes. Some unique results will be presented, namely, (a) the possibility of achieving baseline resolution above 600 base pairs (bp), where all other gel-based electrophoretic techniques fail, and (b) the possibility of separating oligo-DNAs having the same length but differing in one base along the chain [the so-called single nucleotide polymorphism (SNP)], where no separation occurs by any known electrokinetic transport mechanism.

EXPERIMENTAL SECTION

Chemicals and Materials. Basic Immobilines with various pK_a values (8.5, 9.3, 10.3, >12), were purchased from Amersham Pharmacia Biotech (Uppsala, Sweden). Fluka (Buchs, Switzerland) provided acetic acid, boric acid, Stains All, urea, Tricine, and Tris. Acrylamide/bisacrylamide solution (Catalog No. 161−0156) was from Bio-Rad (Hercules, CA). The DNA ladders Φ174/HinfI and PBR322/AluI were from Fermentas GMBH (St. Leon-Rot, Germany). The following 35-mer oligonucleotides (SNPs) were synthesized and purified via HPLC by PRIMM (Milano, Italy): oligo 35−0 TATTCTGACCCCTGACGTTAGTCAAATCCATTTAT; oligo 35−1 TATTCTGACCGCTGACGTTAGTCAAATCCATTTAT; oligo 35−2 TATTCTGACCGCTGACGTTCGTCAAATCCATTTAT; oligo 35−3 TATTCTGACCGCTGACGTTCGTCAAATCCATCTAT.

Casting of Polycationic Gels. Gels are cast with a two-vessel gradient mixer, as routinely performed for pouring immobilized pH gradient gels.[10] Minigels (8 × 7 cm, 0.75 mm thick) are cast in the BioRad system either singly or in a six-unit casting chamber. All gels are supported by a Mylar film (Gel Bond) for easy handling when opening the cassette. The typical gel formulations consist of 4% T polyacrylamide (3,3% cross-linker) in the presence of a gradient of positively charged ions, i.e., basic Immobilines. We have tried different basic species, but the preferred gel formulation adopts the pK 10.3 and pK >12 Immobilines, since these species will be fully protonated at the standard running pH (0.12 M Tris/acetate pH 6.4). Different slopes of basic Immobiline gradients have been tested, in the intervals 2−4 and 0−20 mM. Immediately after casting, the gels are placed in an oven at 50 °C and polymerization is continued at this temperature for 1 h.[11] For running, we have tried both vertical and horizontal systems. The preferred configuration has been a horizontal setup in the Multiphor II chamber (Amersham-GE). The buffers in the electrolyte reservoirs were 0.1 M Tris/tricine pH 8.3 as a cathodic buffer and 0.1 M Tris/acetate pH 6.4 as an anodic buffer. Gels have been run for various periods of time, from 45 min up to 24 h, in order to test for steady-state "focusing" conditions. In some cases, the system has been run not in a presence of a gradient of basic Immobiline but by adopting a plateau (constant concentration) level. Typical running conditions: 300 V with forced cooling at 10 °C. DNA was revealed by silver staining[12] for ds DNA or with Stains All for oligonucleotides.

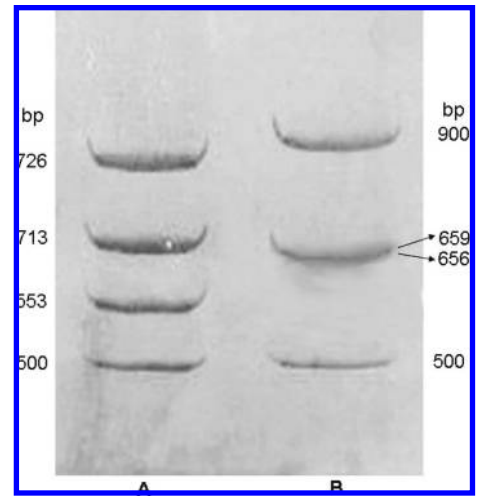

Figure 1. Standard DNA electrophoresis in 6% polyacrylamide gel of a mixture of DNA fragments A, Φ174/HinfI, and B, PBR322/AluI. Note the coalescing into a single zone of the 656−659-bp fragments. Electrophoretic conditions: 7cm long gel, 0.1 M Tris/acetate pH 6.4 in presence of 6 M urea, 400 V, 1.5 h run.

Capillary Zone Electrophoresis (CZE). For CZE of SNPs, runs were made in different background electrolytes such as 100 mM Tris/boric acid pH 8.5, 50 mM Tris/acetate pH 4.5, or 30 mM Tris/formate pH 3.4. In some runs, 1 mM EDTA and 6 M urea were added and other uncharged sieving liquid polymers were tested,[13] such as hydroxypropylcellulose (HPC), average M_r 139 kDa, up to 1% concentration. A Hewlett-Packard CZE instrument (Agilent Technologies, Waldbronn, Germany) was utilized, under the following settings: fused-silica capillary: Lt = 33 cm (245 cm effective length), i.d. = 50 μm, detection at 260 nm.

RESULTS

Figure 1 shows the separation of a mixture of DNA fragments covering the 500−900-bp range in 6% polyacrylamide. It can be appreciated that, whereas for all fragments differing in lengths by >20 bp (upper size ladder) baseline resolution is obtained, for the close pair 656−659, differing by only 3 bp, no separation can be obtained and the two peaks coalesce into one zone. Except

(8) Pitt-Rivers, R.; Impiombato, F. S. A. *Biochem. J.* **1968**, *109*, 825–833.
(9) De Wachter, R.; Fiers, W. In *Gel Electrophoresis of Nucleic Acids: A Practical Approach*; Rickwood, D., Hames, B. D., Eds.; IRL Press: Oxford, 1982; pp 77−116..
(10) Righetti, P. G. *Immobilized pH Gradients: Theory and Methodology*; Elsevier: Amsterdam, 1990; pp 127−139..
(11) Righetti, P. G.; Ek, K.; Bjellqvist, B. *J. Chromatogr.* **1984**, *291*, 31–42.
(12) Merril, C. R.; Washart, K. M. In *Gel Electrophoresis of Proteins: A Practical Approach*; Hames, B. D., Ed.; Oxford University Press: Oxford, 1998; pp 53−84..
(13) Gelfi, C.; Orsi, A.; Leoncini, F.; Righetti, P. G. *J. Chromatogr.* **1995**, *689*, 97–105.

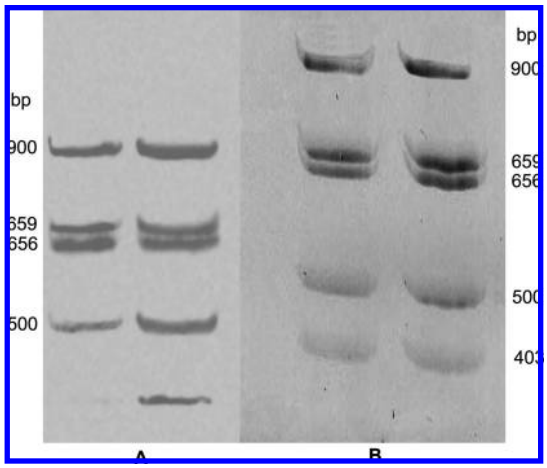

Figure 2. A: separation of the PBR322/AluI fragments in a 6% T polyacrylamide gel containing a 2−4 mM gradient of p*K* 10.3 Immobiline. Conditions: 7 cm long gel, 0.1 M Tris/acetate pH 6.4 in presence of 6 M urea, 400 V, 2 h. B: same as (A), but in presence of a constant concentration (2 mM) of p*K* 10.3 Immobiline (differential migration under nonfocusing conditions). Note, in both cases, the baseline separation between 656- and 659-bp fragments.

for DNA-sequencing in polyacrylamide gels[14] (where readings can still be made up to this length, also by exploiting the four different fluorescence signals of the four, properly labeled, bases), when running DNA restriction fragments in a rather large size distribution, in polyacrylamide gels or in CZE in sieving polymers,[15] it is well-known that such a resolution cannot be obtained. On the contrary, when the same sample is run in our cationic polyacrylamide matrix, in the presence of a gradient from 2 to 4 mM Immobiline, p*K* 10.3, excellent baseline resolution is obtained for the doublet 656−659 bp (Figure 2A). It is quite interesting that the same band splitting is obtained also in a plateau gel, i.e., in a gel containing a constant concentration (2 mM) of Immobiline p*K* 10.3 from cathode to anode (Figure 2B), in agreement with previous findings in the case of SDS-PAGE gels.[5] No other conventional electrophoretic method, whether performed in Nusieve agarose, or in polyacrylamide matrixes, or in CZE in presence of any type of sieving liquid polymers could achieve this kind of separation.

Another interesting application is the detection of SNP, which is one of the most frequently occurring genetic variations in the human genome, with the total number of SNPs reported in public databases currently exceeding 9 million.[16] SNPs in general represent short nucleotide segments, having the same length but differing by a single base. Due to the fact that DNA fragments exhibit essentially the same charge to mass ratio,[17] the separation of fragments of different length can only be obtained in sieving matrixes. In turn, oligonucleotides of identical length cannot possibly be separated by the same sieving mechanism. We have run, in a 0−20 mM p*K* 10.3 and p*K* 12 Immobiline gradient gel, the four oligomers of identical length (35-mers) reported in the Experimental Section, called 35-0, 35-1, 35-2, and 35-3 and

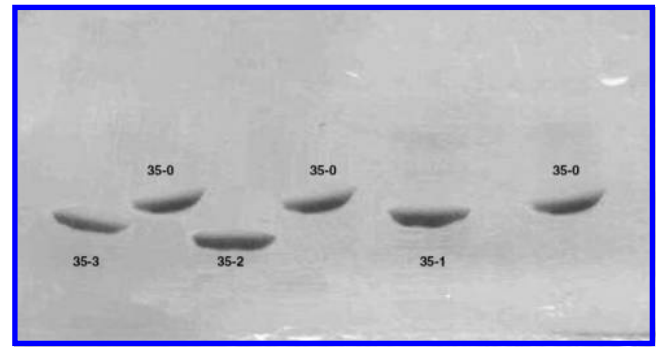

Figure 3. Focusing of SNPs. Four oligomers, called 35-0, 35-1, 35-2. and 35-3, of identical length (35 bases) and differing by 1, 2. or 3 bases, respectively, have been focused in a 6% T polyacrylamide gel containing a 0−20 mM gradient of a mixture of Immobilines with p*K* 10.3 and p*K* 12. Conditions: 7 cm long gel, 0.1 M Tris/acetate pH 6.4 in presence of 6 M urea, 300 V, 18 h.

representing 1, 2, or 3 base substitutions as compared to the 35-0 mer. As shown in Figure 3, good resolution is obtained among the four oligonucleotides. We have run the set of four 35-mers in CZE, in a number of control experiments, i.e., in plain 100 mM Tris−borate buffer, in the same buffer added with 1 mM EDTA, or in the presence of 6 M urea or admixed with sieving liquid polymers selected among cellulose derivatives, such as hydroxypropyl celluloses. Figure 4A gives a typical run, representing four individual runs overlaid: it is seen that their mobilities coincide in a single peak. In Figure 4B, it is seen that no separation of the mixture of four 35-mers occurs even in the presence of 1% HPC. The same single peak was obtained also when running the mixture of four 35-mers in either Tris−formate buffer, pH 3.4 (panel C) or in Tris−acetate buffer, pH 4.5 (panel D).

DISCUSSION

Three important points should be underlined when considering the novel methodology here reported. The first one regards an anomalous distribution of focusing points of different fragments of DNA: the larger DNA fragments stop their migration in the point with small concentration of immobilized charges and smaller fragments migrate further down toward the anodic end where concentration of immobilized charges is large.

The second one regards the increment in resolution obtained in the 100−1000-bp interval, a region of great interest in regard to genome sequencing and to screening of genetic defects. It is well-known that, once the length differences between two neighboring DNA fragments are below the critical level of 1%, separations are extremely difficult if not impossible.[18,19] (except for DNA sequencing gels, which however exploit special techniques reading the four different bases labeled with four different flourophors). In our case, we can fully separate two fragments differing in size by only 3 bp over a total length of ~660 bp, i.e., by only 0.45%. As shown in Figure 1, and as amply demonstrated in current literature, such separations are not quite possible with conventional electrophoretic techniques. And this occurs in polyacrylamide gels, which, over this fragment length region, represent

(14) Garoff, H.; Ansorge, W. *Anal. Biochem.* **1981**, *115*, 450–458.
(15) Dovichi, N. J.; Swerdlow, H. P.; Wu, S.; Harke, H. R. *J. Chromatogr.* **1990**, *516*, 61–67.
(16) Altshuler, D.; Brooks, L.; Chakravarti, A.; Collins, F.; Daly, M.; Donnelly, P. *Nature* **2005**, *437*, 1299–1320.
(17) Stellwagen, N.; Gelfi, C.; Righetti, P. G. *Biopolymers* **1997**, *42*, 687–703.
(18) Lu, H.; Arriaga, E.; Chen, D. Y.; Dovichi, N. J. *J. Chromatogr., A* **1994**, *680*, 497–501.
(19) Dovichi, N. J. In *Analysis of Nucleic Acids by Capillary Electrophoresis*; Heller, C., Ed.; Vieweg: Braunschweig, 1997; pp 236−253..

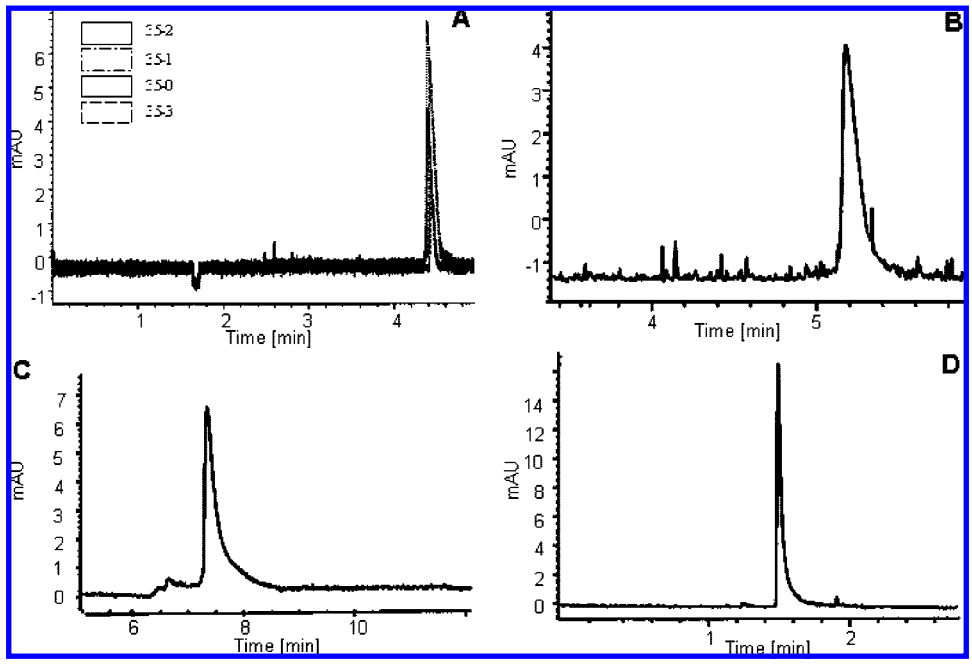

Figure 4. A: Separation of four oligomers, called 35−0, 35−1, 35−2, and 35−3, of identical length (35 bases) and differing by 1, 2, or 3 bases, respectively, via CZE in 100 mM Tris/borate + 1 mM EDTA, pH 8.5, 15 kV × 3 s, 25 °C, $U = 15$ kV. Detection at 260 nm. The four 35-mers have been injected individually and the four different runs overlapped. B: 100 mM Tris/borate pH 8.5 + 1% HPC, 10 kV × 6 s, 35 °C, $U = 15$ kV. C: 30 mM Tris/formate pH 3.4 + 6 M urea, 10 mbar × 3 s, 25 °C, $U = -10$ kV. D: 50 mM Tris/acetate pH 4.5, 20 mbar × 3 s, 25 °C, $U = -25$ kV. For panels B−D, the four oligomers have been injected as a mixture.

the best performing matrixes. When one refers to CZE in sieving liquid polymers, the situation worsens if one adopts the UV-transparent polymers of election for these separations, namely cellulose derivatives. As shown by Barron and Heller,[20] when using HEC of 139 000 M_w in the semidilute concentration regime (i.e., above the overlap concentration, c^*) no separation between a pair of adjacent fragments (271−281 bp, thus differing by 10 bp in length) in the ΦX174-*Hae*III restriction fragments could be obtained in 3% HEC, well above the c^* of 1.8 for this polymer. Barely apex resolution could be engendered for the pair 271/281 when using HEC of M_w 1 315 000 at 0.15% concentration (well above its c^* of 0.037%). It should also be noted that this last polymer, at this concentration, is extremely viscous and quite difficult to be injected and extruded from a capillary lumen. In the upper size region, moreover, the situation rapidly worsens: e.g., baseline separation is barely obtained between two fragments, 2027 and 2322 bp (pertaining to a nonstoichiometric mixture of λ-*Hind*III and ΦX174-*Hae*III), differing in size by ∼300 bp.

The third point concerns the efficient separation of SNPs in either cationic gels or liquid polymers, which opens up the possibility of performing SNP genotyping by electrophoretic methods. SNPs are defined as single nucleotide variations at a specific location of the genome that are found in more than 1% of the population.[21] The average overall frequency of SNPs in the human genome is ∼1/1000 bp. In general, SNPs occur much less frequently in coding regions of the genome than in noncoding regions.[22] SNPs in noncoding regions, although not altering encoded proteins, serve as important genetic or physical markers for comparative or evolutionary genomic studies. However, when present in coding regions, SNPs can cause alterations in protein structure and hence function, leading to development of diseases or changes in responses to a drug or environmental toxin. Hence, SNPs have been used as molecular markers in many disease genetics and pharmacogenomic studies.[23,24] Today, SNP genotyping strategies typically involve two steps: allele discrimination and allele detection. Allele discrimination can be done by primer extension, hybridization, ligation, or enzymatic cleavage. As for the detection, most SNP detection methods today exploit mass spectrometry, fluorescence (when performing direct sequencing via, e.g., capillary array electrophoresis) or via FRET (fluorescence resonance energy transfer) via molecular beacons or via chemiluminescence.[25] The method here reported could represent a valid addition to the array of SNP detection technologies presently in use, as it is characterized by use of simple instrumentation and simple detection methods, such a silver staining.

CONCLUSIONS

With the present report, we are adding two new voices to the polyphonic choir of DNA electrophoretic methods: (a) in the upper register, 100−1000 bp, the modulation of mobility brought about by the complexation of the polyanionic DNA with the polycationic gel matrix, under charge neutralization conditions, leading to condensation (focusing) and arrest of migration (in gradient gels), or under differential charge reduction (in constant charge density gels), both conditions leading to baseline separation in DNA fragments differing in lengths by less than 0.5%; (b) in the lower

(20) Barron, A. E.; Heller, C. In *Analysis of Nucleic Acids by Capillary Electrophoresis*; Heller, C., Ed.; Vieweg: Braunschweig, 1997; pp 93−124..
(21) Brookes, A. J. *Gene* **1999**, *234*, 177–186.
(22) Li, W. H.; Sadler, L. A. *Genetics* **1991**, *129*, 513–523.
(23) McCarthy, J. J.; Hilfiker, R. *Nat. Biotechnol.* **2000**, *18*, 505–508.
(24) Guezey, C.; Spigset, O. *Curr. Top. Med. Chem.* **2004**, *4*, 1411–1421.
(25) Kim, S.; Misra, A. *Annu. Rev. Biomed. Eng.* **2007**, *9*, 289–320.

register, 10−100 bp, for SNPs analysis, by differential modulation of mobility between pairs of oligomers of identical size, but differing by a single-base substitution, leading to a final focusing in steeper gradient gels. Both represent a marked improvement over existing techniques and appear to hold promises for applications in diverse fields, such as molecular biology, forensic medicine, and genetic screening.

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