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High-Frequency Alternating-Crossed-Field Gel Electrophoresis With Neutral or Slightly Charged Interpenetrating Networks to Improve DNA Separation.

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Abbreviations: APS, ammonium persulfate; **BIS,** N,N'-methylene bisacrylamide; **bp,** base pair; **EDTA,** ethylene diaminetetraacetic acid; **HFAC-GE,** High-Frequency Alternating-Crossed-Field Gel Electrophoresis; **IPN,** interpenetrating network; **kbp,** kilobase pairs; **Mbp,** megabase pairs; **PFGE,** pulsed field gel electrophoresis; **TBE,** Tris-Boric Acid-EDTA; **TEMED,** N,N,N',N'-tetramethyl-ethylenediamine; **Tris,** tris(hydroxymethyl) aminomethane

Keywords: DNA electrophoresis, cyclic migration, hernias, polyacrylamide, agarose

Toward improving DNA separations, this work reports the effects of high-frequency square-wave AC fields superimposed perpendicular to the DC separation field on DNA migration in both polyacrylamide-based interpenetrating networks (IPNs) and in agarose networks.

Compared to standard polyacrylamide networks, IPNs allow the separation of larger DNA (9000 bp vs. 5000 bp at 5 V/cm). In novel polyacrylamide-based IPNs, an AC field of 5 Hz increased the maximum separable DNA size. This effect was increased to larger DNA sizes with increasing electric-field strength up to and apparently beyond the power supply-limited maximum electric-field strength of 48 V/cm. The AC field also increased mobility. These two results combine to yield a reduction in separation time of up to a factor of 20 in novel polyacrylamide-based IPNs. When negatively charged acrylic-acid groups were incorporated into the IPNs, the use of the AC field changed the DNA-network interaction which altered the size dependence of DNA mobility.

In agarose networks, an AC field of 50 Hz increased the separable size range; however, there was no increase in DNA mobility. There was no change in size dependence of mobility in an AC field when charges were introduced into the network. Based on results in the literature, possible mechanisms were examined for the effects of the AC field on DNA separation.

1 Introduction

Gel electrophoresis is currently the most widely used method for the analysis of biological macromolecules, especially for the separation of DNA [1]. DNA electrophoresis is characterized by excellent resolving power and small sample requirements. It is the basis for many important techniques in molecular biology including DNA restriction fragment mapping, DNA sequencing, Southern blotting, DNA fingerprinting and DNase footprinting [2, 3]. It is the focus of considerable research, especially as the central tool in the Human Genome Project [4], an international effort to generate a map of, and then sequence, the entire human genome.

Restriction fragments to about twenty-five thousand base pairs (25 kbp) of DNA in length can be separated relatively quickly using a constant electric field. All larger DNA have essentially the same mobility and cannot be separated by a constant electric field. The limit of constant-field separation varies with the network and the electric-field strength. In a 1 % agarose network the limit of separation with a very low electric-field strength of 1 V/cm is about 25 kbp; at 6 V/cm this limit is reduced to 15 kbp [5]. In a 4 %T polyacrylamide network, with a field strength of 4 V/cm, the size limit is around 5 kbp (Boyd, unpublished results). At present it is only possible to separate DNA above these size limits by using pulsed electric fields that reorient the DNA by switching the electric field direction, restoring a size dependence to its mobility [6]. Unfortunately, this technique is very slow since the DNA must be reoriented each time the field is switched to effect a separation. It is thus desirable to find alternate methods to separate DNA more quickly than in the pulsed field regime.

By changing the interaction between the DNA and the network it is possible to decrease the separation time. Ma and Yeung applied ultrasound to DNA migrating through agarose networks and found a modest increase in mobility of about 20 % with a corresponding decrease in separation time [7]. They were limited by the power and homogeneity of the ultrasonic field. It would be desirable to develop a means to

similarly alter DNA-network interaction without the use of ultrasound by employing high-frequency alternating-electric fields. By high frequencies, we mean frequencies above those used for pulsed field gel electrophoresis (PFGE) which typically range from 0.004 to 0.1 Hz [8], depending on DNA size.

There have been a few reports in the literature concerning high-frequency pulses or sine-wave fields to improve DNA separation. The technique of superimposing a sine-wave field in parallel with the DC field both in an agarose slab gel [9-11] and in a linear polyacrylamide filled capillary [12] resulted in a moderate increase in DNA mobility for DNA up to 23 kbp. As a way of detrapping very large DNA (50 kbp - 5.7 Mbp) molecules during pulsed field gel electrophoresis, short secondary pulses were superimposed on the primary pulsed field [13, 14]. These secondary pulses decreased dispersion and thus decreased the separation time. Pulsed field separations have also been applied to DNA sequencing with limited success [15-18].

Alteration of the DNA-network interaction requires an understanding of how DNA migrate through a polymer network and how DNA separation occurs. Under a constant electric field, DNA has at least three migration regimes in agarose gels as defined by Magnúsdóttir et al. [19]. They measured the boundaries of these regimes by examining the conformation and orientation of DNA as it migrates through a polymer network. Conformation and orientation are intrinsically linked to the way DNA migrate through a polymer network and to its ability to separate. Each regime is determined by the ratio of the radius of gyration (R_G) to the average mesh size (ξ) and by the electric-field strength. DNA for which R_G/ξ is small ($R_G/\xi < 1.0 - 1.7$ depending on field strength) undergo migration as isotropic coils without orientation. This is generally termed the Ogston sieving regime. DNA with intermediate values of R_G/ξ (to 1.8 - 9.5 depending on field strength) migrate as anisotropic deformed coils, or by the classical reptation mechanism in which the DNA molecule migrates end-on in

a snake-like manner through the polymer network. At low field strengths, very long DNA also migrate by classical reptation. This migration regime at intermediate R_G/ξ, and for very long DNA at low field strengths is called the non-geometration regime. At higher electric-field strengths, long DNA follow a cyclic pattern, oscillating between compact and extended conformations as they migrate through a polymer network [20-22]. This regime is called geometration [23]. The DNA is elongated into a U-shape by the electric field when it is hooked by an obstacle in the polymer network; it contracts when it slides off the obstacle [24-28]. The onset of geometration is marked by the existence of loops or hernias in the DNA chain which contribute to the cyclic motion. In polyacrylamide-based networks the correlation of R_G/ξ derived for agarose loses its meaning as the mesh size is smaller than the persistence length of the DNA, although the migration regimes may still be correct.

For example, Magnúsdóttir's correlation predicts that at 30 V/cm, assuming a generous mesh size of 30 nm, all DNA larger than 660 base pairs (a contour length of 225 nm or 4.5 persistence lengths) will migrate by geometration, while in 1% agarose the onset of geometration occurs at 4.7 kbp. However, one would expect the geometration-migration regime border in polyacrylamide-based networks to shift to much larger DNA sizes than in agarose networks as the ability of the DNA to become hooked by an obstacle is greatly reduced in a tight network. While we have evidence from Brownian-Dynamics simulations that DNA above a certain size in tight gels will undergo a migration pattern similar to geometration (results to be published), it is not clear where the boundaries of the migration regimes are in polyacrylamide-based networks.

In agarose networks, the mobility dependence on DNA size is strongest in the isotropic and anisotropic coil regimes. It is less strong in the classical reptation regime and becomes essentially independent of size somewhere in the geometration regime [29]. Loss of the size dependence of the mobility occurs as DNA size and the electric-

field strength increase and the DNA spend more time in the elongated state leading to a more size-independent velocity. The situation is similar in acrylamide networks. The size limit of DNA separation is thought to occur for reasons similar to that in agarose [30].

Therefore, the focus of this work is to improve the separation time of DNA by extending the range of DNA sizes that can be separated without complete reorientation and by increasing the electric-field strength that can be used to separate DNA near the limit of constant-field separation. Both of these goals are accomplished with High-Frequency Alternating-Crossed-Field Gel Electrophoresis (HFAC-GE). We use an alternating square-wave electric field, placed perpendicular to the constant separation field, at frequencies between 1 and 50 Hz to change the interaction between the DNA and the network without completely reorienting the DNA. We apply it to both our novel polyacrylamide-based interpenetrating networks (IPNs) and to agarose networks. The polyacrylamide-based IPNs either contained negatively charged acrylic acid groups in one of the two polymerization steps, or were neutral. Polyacrylamide networks were initially used because it was then possible to control network composition, especially when charged groups were added to the network. The charged groups were used to cause some of the network fibers to move in the AC field further altering the DNA-gel interaction.

2 Materials and Methods

2.1 Materials

Acrylamide, N,N'-methylene bisacrylamide (BIS), N,N,N',N'-tetramethyl-ethylenediamine (TEMED), tris(hydroxymethyl) aminomethane (Tris), bromophenol blue, xylene cyanol FF, glycerol and boric acid were purchased from Aldrich. Anionic co-monomer acrylic acid was purchased from Polysciences Inc. Ammonium persulfate (APS) and ethylene diaminetetraacetic acid (EDTA) were purchased from Fisher. Dimethyldichlorosilane was purchased from Sigma. LE (low electro-osmosis, $-m_r <$

0.122) agarose was purchased from Boehringer Mannheim. Gel Bond and Gel Bond PAG, plastic gel-support materials, HEEO (high electro-osmosis, $-m_r \geq 0.30$) agarose, and Gold (very low electro-osmosis, $-m_r \leq 0.05$) agarose were purchased from FMC Corporation. The value of $-m_r$ is a measure of the amount of electro-osmosis that occurs under defined conditions [31]. 500 bp Molecular Ruler (500-8000 bp) and 1 kbp Molecular Ruler (1000-15000 bp) were purchased from Bio Rad. DNA Molecular Weight Marker III (120 - 21200 bp) was purchased from Boehringer Mannheim. All reagents were used as received. Distilled water was filtered (0.2 μm) and deionized (17.9 MΩ-cm resistivity) with a Barnstead Nanopure II unit.

2.2 Polyacrylamide-Based IPNs

An IPN consists of two molecularly distinct networks having different properties or compositions, that are intertwined on a molecular level. We use IPNs to generate a network with specific concentrations of charged groups on one of the distinct networks that make up the IPN. All of the IPNs were synthesized in two free-radical solution-polymerization steps. The monomers (acrylamide, acrylic acid), crosslinker (BIS) and initiator (APS) for the first network were dissolved in 0.65x Tris-Boric Acid-EDTA (TBE) aqueous buffer at pH 8.0 [32], and degassed by stirring at 0 °C under house vacuum for 1 hour. After degassing, 0.05 v/v% TEMED was added to the solution to accelerate the rate of free-radical production from APS degradation, allowing the free-radical polymerization reaction to proceed. The monomer solution was then quickly filtered through a hydrophilic 0.22 μm filter during injection into the gel molds. The molds consisted of two glass plates separated by a Teflon gasket 0.5 mm thick and a sheet of Gel Bond PAG to which the gel bound. The reaction was allowed to go to completion, at least one hour. before the gaskets were opened. The gels were weighed and set out to dry for 24 hours. The weights of the gel before and after drying were used to determine the composition of the monomer

solution for the second network. The composition is reported in terms of the following variables.

$$\%T_m = \frac{\text{total moles of monomer and crosslinker}}{\text{volume of water (ml)}} * 100 \quad (1)$$

$$\%X = \frac{\text{moles of crosslinker}}{\text{moles of total monomer and crosslinker}} * 100 \quad (2)$$

$$\%C = \frac{\text{moles of charged monomer}}{\text{moles of total monomer and crosslinker}} * 100 \quad (3)$$

The variable $\%T_m$ can be converted into the commonly used variable %T by multiplying $\%T_m$ by the average molecular weight of the monomers in solution:

$$\%T = \frac{\text{total mass of monomer and crosslinker}}{\text{volume of water (ml)}} * 100 \quad (4)$$

The monomer solution for the second network was prepared in the same manner as that for the first except that instead of buffer, only water was used, and the reaction was initiated with heat instead of TEMED. The second monomer solution was injected on top of the dried gels in the reassembled molds. The first network was allowed to reswell in the second monomer solution for at least 4 hours until the solution was completely absorbed. The free-radical reaction was then initiated by heat using a thermostatic temperature bath set at 55 °C. The molds were maintained at 55 °C for at least 18 hours to allow the reaction to achieve its full extent of conversion. The completed networks were stored in their molds at 4 °C for less than 7 days before use.

The top plate of the mold and the gasket were removed. The bottom plate, the supporting gel bond and the gel were placed in the electrophoresis apparatus. The gasket for electrophoresis, Teflon spacers to exclude the well-gel monomer solution from the electrode wells and the top plate were put in place and sealed with a very small

amount of vacuum grease. The top plate was held in place by thumb screw clamps. The monomer solution for the well gel was prepared analogously to the monomer solution for the first polymerization of the interpenetrating network. It was degassed by stirring at 0 °C under house vacuum for 1 hour. After degassing, 0.05 v/v% TEMED was added to the solution. The monomer solution was then quickly filtered through a hydrophilic 0.22 μm filter into the space between the end of the IPN and the Teflon spacers. The plastic comb used to form the sample wells was put in place and the well gel was allowed to polymerize for at least one hour. Cooling water was recirculated to equilibrate the gel to 20 °C.

2.3 Agarose Networks

Agarose networks were prepared directly in the electrophoresis cell. The technique for preparing a 1.0% agarose network is outlined below as an example. 0.100 g FMC Gold agarose was dissolved in 10 ml 0.65x TBE buffer (pH 8.0) [32] by heating to 90 °C. The sample was cooled slightly and poured into the heated apparatus (~ 55 °C). The plastic comb used to form the sample wells was put in place and the gel was allowed to set for one hour before electrophoresis. Cooling water was recirculated to equilibrate the gel to 20 °C. The preparation of agarose IPNs required mixing equal weights of the dry FMC Gold agarose and FMC HEEO agarose together before dissolution in the buffer.

2.4 Apparatus

The apparatus was designed and built specifically for HFAC-GE and is shown in Figure 1. The electric field was generated using a square electrode array of 12 electrodes powered by two power supplies, one to power the positive half of the electrode array, the other to power the negative half, with the field distributed across the electrode array by a stiff resistive divider. A resistive divider was used instead of the more flexible digital-to-analog converter-amplifier combination for each electrode as described by Birren et al. [8, 33] due to the high cost of the latter at the field strengths

required. The switching circuit used to change the direction of the electric field was driven by a square wave from a function generator. The frequency of field switching was controlled by the frequency of the square wave. Each switch in the switching circuit was designed to switch up to 5 kHz using fast MOSFETs. The power supplies were operated in constant-voltage mode. The current was monitored and recorded on a chart recorder.

The electrophoresis-gel box (Fig. 1), based on a horizontal sequencing apparatus described by Brumley & Smith [34], had a water-cooled Plexiglas base with a rubber gasket to created a seal between the base and the glass bottom plate from the gel mold. The gel, attached to a sheet of Gel Bond, sat on the glass plate surrounded by a Teflon gasket. The four-piece Plexiglas top plate sat on top of the gasket and the gel, and was held in place by thumb-screw clamps. The electrode array, consisting of 12 solid platinum electrodes, was placed into the buffer surrounding the gel through holes in the top plate. The entire gel box was placed inside an interlock box to prevent accidental shock during operation.

2.5 DNA Electrophoresis

DNA samples were prepared by pipetting 0.8 μl 6x DNA loading buffer [32], consisting of 0.25% bromophenol blue, 0.25% xylene cyanol FF and 30% glycerol in water, DNA to make 158 μg/ml in final volume of 3.6 μl, and 0.65x TBE buffer (pH 8.0) [32] as required to reach a total volume of 3.6 μl.

When the well gel or agarose network had completely polymerized, the Teflon spacers and the sample well comb were removed. TBE buffer was added to the space around the gel, the electrode array was put in place, and the power supplies were turned on for approximately 30 minutes until the current stabilized to remove any charged remnants of the polymerization reaction. The buffer was replaced and 1.8 μl of a DNA sample was pipetted into each of the 6 central sample wells. The electrode array was put in place and the electric field was turned on.

The DNA was visualized by staining with ethidium bromide after electrophoresis. Mobilities were measured from a photograph of the gel.

3 Results and Discussion

3.1 Polyacrylamide-Based IPNs

Polyacrylamide-based IPNs were used to separate DNA between 500 bp and 15000 bp with and without an AC field. Experiments were performed using two networks. The charged polyacrylamide-based IPNs were 0.100 $\%T_m$ (7.19 %T) and 1.00 %X in the first polymerization and 0.050 $\%T_m$ (3.75 %T), 1.00 %X and 2.00 %C in the second polymerization. The neutral polyacrylamide-based IPNs were 0.100 $\%T_m$ (7.19 %T) and 1.00 %X in the first polymerization and 0.050 $\%T_m$ (3.60 %T) and 1.00 %X in the second polymerization. Experiments with each type of IPN were performed with and without the use of a perpendicular square-wave AC field. In an effort to reduce the separation time, these separations were typically done at relatively high field strengths (up to 48 V/cm).

3.1.1 Separable DNA Size Range in Polyacrylamide-Based IPNs

A comparison of these separations at four frequencies is shown in Figure 2. The maximum separable size without the AC field in these polyacrylamide-based IPNs is larger than that possible in regular polyacrylamide networks (9000 bp vs. 5000 bp at 5 V/cm). This is most likely due to changes in the structure of the IPN network during the drying step, resulting in a larger mesh size. Drying and rehydration of polyacrylamide networks has been shown to allow the separation of larger DNA [35]. The presence of the AC field increases the size range of DNA that can be separated even further with 5 Hz yielding the largest effect. The use of the AC field also increases the mobilities in both the charged and the uncharged networks. This effect could be similar to that noticed by Kotaka et al. [10]. They generated a periodically-applied reversed-field component by superimposing a sine-wave AC field in parallel with a DC field. A mobility peak was observed around a frequency of about 10 Hz in

0.5% agarose over a wide range of molecular weights. At that gel concentration the fastest orienting elements of DNA molecules respond to changes in field direction in about 0.1 seconds, independent of the molecular weight [36]. These fast-orienting components may be loops or hernias that escaped from the tube and are aligned in the electric field [37]. The formation of these hernias is associated with the onset of the geometration migration regime. The hernias allow the DNA to become hooked on the polymer network. Therefore, the reversal of the electric field direction may enhance the mobility by unhooking the hernias formed as a part of geometration and thereby reducing the effective friction between the DNA and the network resulting in an increase in mobility [22]. In our experiments the field is not reversed, rather it is shifted by 90° which should have a similar effect on the hernias, although it should depend on their orientation with respect to the tube and with respect to the electric field. The unhooking of hernias is equivalent to reducing their effectiveness of formation. Therefore it should also result in an increase in the size range of separation as the onset of geometration and thus the maximum separable size, should be shifted to larger DNA sizes.

Figure 3 shows the effect of the AC field on the maximum separable DNA size at various field strengths. The presence of the AC field increased the maximum separable size at all field strengths in both the charged IPNs and the neutral IPNs. The AC field had a small effect on the maximum separable size at 5 V/cm, however at high field strengths (48 V/cm) the presence of the AC field significantly increased the size range of DNA that could be separated (6000 bp without the AC field vs. >12000 bp with the AC field at 48 V/cm). The largest DNA separable with the polyacrylamide-based IPNs (>10 kbp) was separable only at 48 V/cm with the use of an AC field. The maximum separable size actually *increased* with increasing field strength. Brownian Dynamics simulations of DNA in agarose networks under a constant electric field revealed a reduction in hernia formation coupled with an increase in DNA alignment in

the direction of the electric field at high electric-field strengths [24]. It is the alignment of DNA molecules in the direction of the electric field that leads to the loss of size dependence of the mobility. However, the reduction in hernia formation results in an increase in DNA mobility and a shift to larger DNA sizes at which the onset of geometration occurs, and thus a shift to larger DNA sizes of the maximum separable DNA size. In agarose networks as the electric-field strength increases with or without an AC field the process of DNA alignment is dominant over the process of hernia formation. In polyacrylamide-based IPNs without the AC field the process of DNA alignment is also dominant. We hypothesize that the mesh size of the polyacrylamide-based IPNs is small enough with respect to the persistence length of the DNA that when the AC field is applied, the effects of orientation with increasing electric-field strength are significantly reduced due to a tortuous path chosen by the DNA. However, the mesh size is large enough to allow a form of geometration and hernia formation. Thus when the AC field is applied, hernia formation is reduced via the retraction mechanism described above, and as the electric-field strength increases, this effect increases as shown by Deutsch. However, DNA alignment is reduced by the AC field which causes the DNA to follow a more tortuous path through the IPN but not to such an extent that the mobility is reduced.

The net effect in polyacrylamide-based IPNs is to increase the maximum separable DNA size with increasing electric-field strength. This effect is not size dependent. There should be a maximum effect at some field strength larger than 48 V/cm for these networks when the hernias are mostly unhooked and retracted. This hernia retraction plays a central role in PFGE [38]. Using Monte Carlo simulations, Duke showed that the rapid retraction of hernias upon field reversal helps the chain to collapse, allowing a U-shape to form. However, the increase in separable size range is probably not due to a PFGE mechanism of partial or total reorientation of the DNA in the AC field, as one would then expect a *decrease* in mobility rather than the increase

seen here for all DNA sizes. Also, in polyacrylamide-based IPNs in which the pore size is much smaller than in agarose networks, it is not clear whether DNA can undergo the same type of reorientation that is at the heart of PFGE. Although DNA reorientation may contribute to the improvement in separation, it does not explain effects such as the increase in maximum separable size with increasing field strength.

3.1.2 Separation Time in Polyacrylamide-Based IPNs

The increase in the maximum separable DNA size at high field strengths when using the AC field, coupled with the increased DNA mobility when using the AC field, allows a reduction in the separation time for DNA at the larger end of the separable size range without DNA reorientation. Without the AC field, this DNA can only be separated at lower field strengths. The separation times were calculated in each experiment according to:

$$t_{sep} = \frac{(BW_2 + BW_1)}{4(\mu_2 - \mu_1)E} \tag{5}$$

where BW is the band width, μ is the mobility and E is the electric-field strength. The reduction in separation time in these networks by the use of the AC field is illustrated by examining 9000 bp DNA. In our experiments with polyacrylamide-based IPNs, 9000 bp DNA was only separable without an AC field using a field strength of 5 V/cm, but with an AC field, it was separable using a field strength of greater than 48 V/cm. This resulted in a 95% decrease (30 min. vs. 600 min.) in the separation time at a resolution of 1000 bp. As can be seen in Figures 4a & 4b, for all sized DNA under all conditions, the separation time decreased as the field strength increased. In charged and neutral IPNs for all DNA sizes at all field strengths for which a comparison could be made, the AC field decreased or had no effect on the separation time (Fig. 4c & 4d for 26 V/cm). The error bars are large for the charged IPNs due to dispersion caused by electro-osmosis.

3.1.3 Charged Groups in Polyacrylamide-Based IPNs

The incorporation of negatively charged acrylic acid groups in the network, intended to cause the network to oscillate in the AC field and change the interaction between the DNA and the network, resulted in significant electro-osmotic flow during electrophoresis. The electro-osmotic flow dramatically reduced the DNA size range of separation at low field strengths (Fig. 3: 9 - 10 kbp vs. 3 - 3.5 kbp) and resulted in an increase in the separation time at all field strengths (results not shown). However at high field strengths, attainable through the effects of the AC field, the separation time in the charged network was only slightly longer than in the neutral network. At high electric-field strengths the forces due to electro-osmotic flow are much smaller with respect to the electrophoretic forces at these field strengths, and thus the separation is affected to a much lesser extent.

Even though the addition of charged groups to the network did not improve the separation time or the maximum separable DNA size, when combined with the AC field, the charges did alter the size dependence of the separation. This can be seen by plotting the ratio of the difference in mobility with and without the AC field to the mobility without the AC field (the differential mobility ratio) as function of DNA size, shown in Figure 5. For the neutral network, the AC field does not cause the network to move, and no effect on mobility is observed. In the case of the charged network however, one can see a large change in mobility when the AC field is applied, which increases with increasing molecular weight. This indicates a change in the molecular weight dependence of the interaction between the charged network and the DNA in the AC field that is neither present in the charged network without the AC field, nor in the neutral network either with or without the AC field. Although the precise nature of this interaction is unknown at this time, there are two possibilities to describe this behavior: first, the charged network fibers oscillate in the AC field and interact with the DNA changing the way it migrates through the network, and second the oscillating DNA

interacts with the charges on the network in such a way as to change the way the DNA migrates through the network. This is not the same mechanism linked to the increase in the maximum separable DNA size with increasing field strength as that phenomenon occurs in both neutral and charged networks.

As shown in Figure 5, frequencies of 1, 5 and 50 Hz all had a similar effect on the differential mobility ratio. A frequency of 5 Hz appeared to be optimal, although the errors in the differential mobility ratio in the charged networks were large, making it is difficult to separate results obtained at one frequency from another. The effect of field strength on the interaction is shown in Figure 6. There is no change in the relative mobility at 5 V/cm and at 48 V/cm when charges are incorporated into the network. We hypothesize that there is no effect at 5 V/cm because the field strength is too weak to cause the change in DNA-network interaction required to alter the size dependence of the mobility, or that it is too weak to move the charged network fibers. The altered interaction may require some degree of slackness in the DNA chain and at 48 V/cm the chain tension is too large for the altered interaction to occur.

3.2 Agarose Networks

3.2.1 Separation Time in Agarose Networks

Despite the improvement in mobility and the size range of separation with the use of the AC field and the alteration of the size dependence of the mobility in the charged polyacrylamide-based networks, the mobilities in acrylamide networks are generally very low when compared with agarose networks for comparable DNA sizes. This is due to differences in the network structure. Agarose networks consist of bundles of helical chains bonded together by interchain hydrogen bonds. Agarose also tends be slightly hydrophobic, adding to the tendency for these bundles to form. The chain bundles can be thick, and are thus very stiff. This leads to a large mesh size with respect to polyacrylamide-based networks, facilitating higher DNA mobilities. The separation time of 9000 bp DNA in a polyacrylamide-based IPN with an AC field is

still slower than a constant field separation in an FMC "Gold" agarose network (30 min. in polyacrylamide-based IPN at 48 V/cm vs. 20 min. in FMC's "Gold" agarose at 13 V/cm). We therefore attempted HFAC-GE in agarose networks try to take advantage of the improvement in the separation time using the AC field.

In "uncharged" (0.25% FMC Gold) agarose networks, the AC field does not affect the mobility in the same manner as in polyacrylamide-based IPNs (Fig. 7a). While it increases the size range of separation at all field strengths, the mobility is only significantly increased at 13 V/cm. The effects of the AC field on the process of hernia formation are evidently much smaller in agarse networks than in polyacrylamide-based IPNs. Therefore, DNA alignment becomes dominant over hernia formation in agarose at a much lower field strength, resulting in no change in mobility with the application of the AC field above 13 V/cm.

The effects of the AC field may also be related to partial or total DNA reorientation rather than to a reduction of hernia formation. By extrapolating double-stranded DNA pulsed-field electrophoresis methodologies for agarose networks, one calculates that a pulsed field with alternation times of about 100 msec would be required for the optimum separation of 10 kbp DNA in 1 % agarose [39]. This corresponds to a perpendicular square-wave AC field frequency of 5 Hz.

We saw the largest effects on the size range of separation in agarose networks at 50 Hz (results not shown). As with the polyacrylamide-based IPNs, the separation time is decreased as shown in Figure 7b - d, although it is not significantly at decreased 26 V/cm for DNA sizes beyond those that could be separated in the absence of the AC field. Similar results were obtained in 1% agarose (results not shown) with the predictable effect that the mobilities were lower and the size range of separation was smaller due to the more concentrated network. In agarose networks there is no significant increase in the size range of separation with increasing field strength as was the case with polyacrylamide-based networks. However, the AC field appears to

prevent a decrease in size range of separation that occurs without the AC field as shown in Figure 8, consistent with partial or total DNA reorientation in the AC field.

3.2.2 Agarose "Interpenetrating Networks"

To investigate further the alteration in size dependence of the DNA mobility, we also attempted to generate IPN-like networks using agarose similar to those of polyacrylamide-based IPNs with charges incorporated into the network. The agarose "IPNs" were prepared as described in the Materials and Methods section. As in the polyacrylamide-based IPNs, addition of HEEO agarose ostensibly allowed some of the network fibers to have more charge than others, and thus to move more readily in the electric field compared with the Gold agarose network fibers. As with charged polyacrylamide-based IPNs, the addition of charges to the agarose networks did not improve the separation time or the maximum separable DNA size. However, as can be seen in Figure 9, there is no alteration of the size dependent gel-DNA interaction as found in the polyacrylamide-based networks. This suggests that the alteration of the size dependence for charged polyacrylamide-based IPNs is due to either the flexibility of the network or to the mesh size of the network. The stiffness of the agarose network may change the ability of the charged network fibers to move in the AC field. Agarose networks have been shown to move in an AC field [40, 41], but only at much lower frequencies at the field strengths we used, or at much higher field strengths at the frequencies employed.

4 Concluding remarks

We have used an alternating square-wave AC field perpendicular to the DC separation field to improve DNA separation in both novel polyacrylamide-based IPNs and in agarose networks. We find that an AC field of 5 Hz in the IPNs results in an up to 95 % decrease in the separation time near the limit of constant field separation. We also found that the size range of separation increased with increasing electric-field

strength when the AC field was used in the IPNs. In agarose networks we found that an AC field of 50 Hz resulted in a decrease of the separation time of up to 80 % near the limit of constant-field separation. The decrease was nearly absent below the limit of constant-field separation. In the IPNs we hypothesize that the improvement is due to a reduction in hernia formation. In agarose we propose that the improvement is also due to a reduction in hernia formation, but that it is dominated by effects of DNA alignment at higher field strengths.

When charged groups were incorporated into the polyacrylamide-based IPNs, the use of the AC field changed the DNA-network interaction which altered the DNA size dependence of the mobility. If the mechanism can be elucidated, it may be exploitable to improve DNA separations. To this end we are currently using Brownian Dynamics simulations to explore this altered mobility dependence on DNA size as well as the mechanism for the improvements in separation time. It would also be desirable to perform linear dichroism experiments to determine the borders between migration regimes similar to those of Åkerman and Jonsson [19, 22, 29]. These experiments would verify or refute our hypotheses concerning the mechanism for separation-time improvement in polyacrylamide-based IPNs.

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Figure Legends:

Figure 1. Details of HFAC-GE apparatus. (a) Side view of the gel box. (b) Top view of the gel box. (c) Schematic of the apparatus.

Figure 2. Effect of a square-wave AC field of various frequencies on DNA mobility in neutral IPNs and in charged IPNs. "no AC" indicates a standard DC separation. The electric-field strength is 26 V/cm. (a) Neutral IPNs were 0.100 %T_m and 1.00 %X in the first polymerization and 0.050 %T_m and 1.00 %X in the second polymerization (see text for definitions). (b) Charged IPNs were 0.100 %T_m and 1.00 %X in the first polymerization and 0.050 %T_m, 1.00 %X and 2.00 %C in the second polymerization. Error bars represent the standard deviation of three experiments. Lines are drawn to guide the eye.

Figure 3. Effect of electric-field strength and perpendicular square-wave AC field on maximum separable DNA size in neutral IPNs and in charged IPNs. IPN compositions are given in Figure 2. Error bars represent the standard deviation of three experiments, most lie within the data points. Lines are drawn to guide the eye.

Figure 4. Separation time: (a) as a function of electric-field strength for a neutral IPN without the AC field; (b) as a function of electric-field strength for a neutral IPN with a 5 Hz perpendicular square-wave AC field; (c) as a function of DNA size for a neutral IPN at 26 V/cm; (d) as a function of DNA size for a charged IPN at 26 V/cm. IPN compositions are given in Figure 2. Error bars represent the standard deviation of three experiments, most lie within the data points. Lines are drawn to guide the eye.

Figure 5. Effect of charged groups and perpendicular square-wave AC field frequency on differential mobility ratio in neutral IPNs and in charged IPNs. Differential mobility ratio = $(\mu_f - \mu_0) / \mu_0$. The electric-field strength is 26 V/cm. IPN compositions are given in Figure 2. Error bars represent the standard deviation of three experiments. Lines are drawn to guide the eye.

Figure 6. Effect of charged groups and electric-field strength on relative mobility in neutral IPNs and in charged IPNs. Differential mobility ratio = $(\mu_5 - \mu_0) / \mu_0$. The AC frequency is 5 Hz. IPN compositions are given in Figure 2. Error bars represent the standard deviation of three experiments. Lines are drawn to guide the eye.

Figure 7. Effect of electric-field strength and perpendicular square-wave AC-field on mobility and separation time in FMC's Gold agarose networks. All networks are 0.25% Gold agarose. Error bars represent the standard deviation of three experiments. Lines are drawn to guide the eye.

Figure 8. Effect of electric-field strength and perpendicular square-wave AC field on maximum separable DNA size in FMC's Gold agarose and in Gold/HEEO agarose "IPNs". All networks are 0.25% agarose. Agarose IPN is 50% Gold agarose and 50% HEEO agarose. Error bars represent the standard deviation of three experiments. Lines are drawn to guide the eye.

Figure 9. Effect of charged groups on relative mobility. Differential mobility ratio = $(\mu_f - \mu_0) / \mu_0$. The electric-field strength is 26 V/cm. All networks are 0.25% agarose. Agarose "IPN" is 50% Gold agarose and 50% HEEO agarose. Error bars represent the standard deviation of three experiments. Lines are drawn to guide the eye.

Figure 1

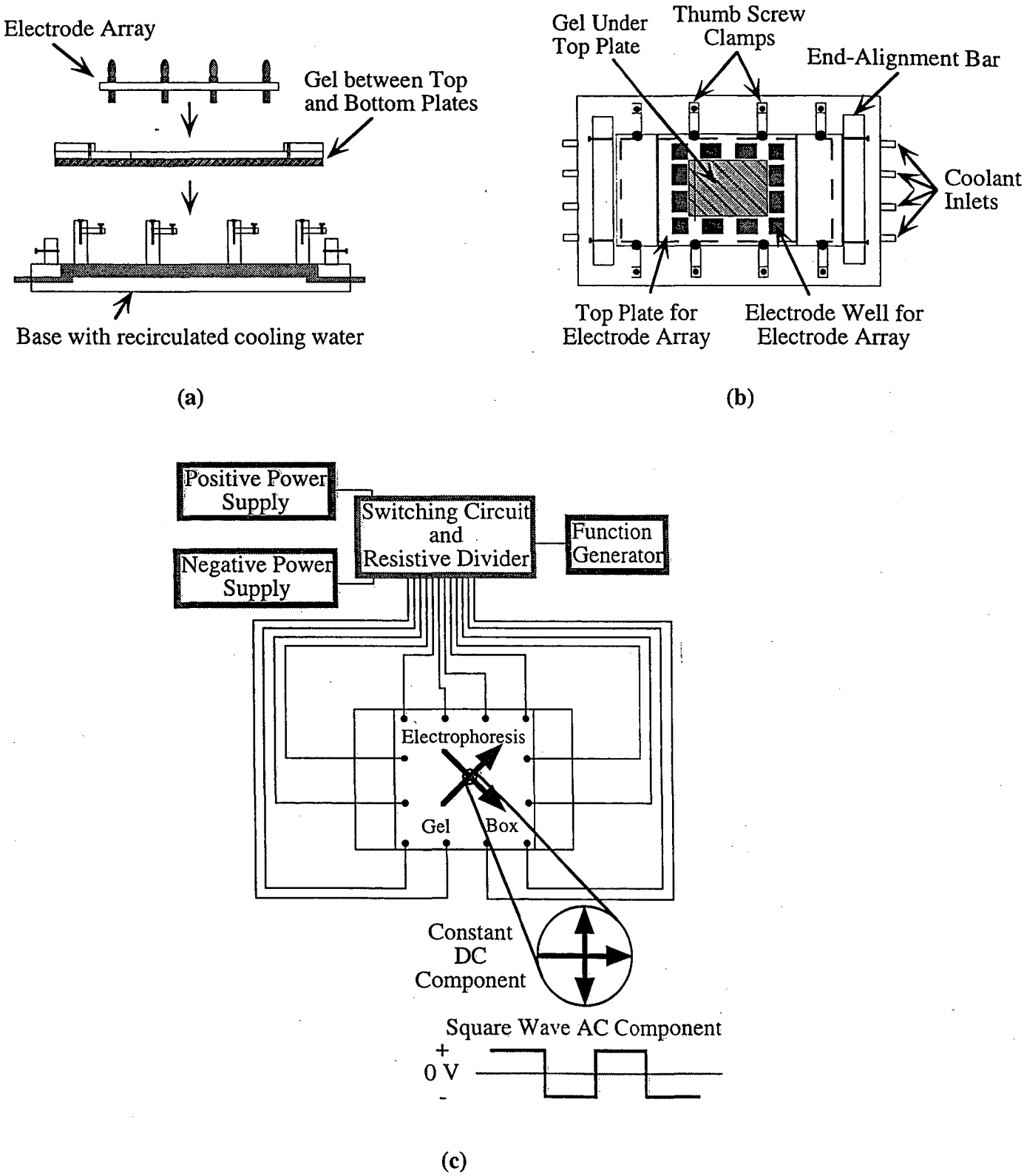
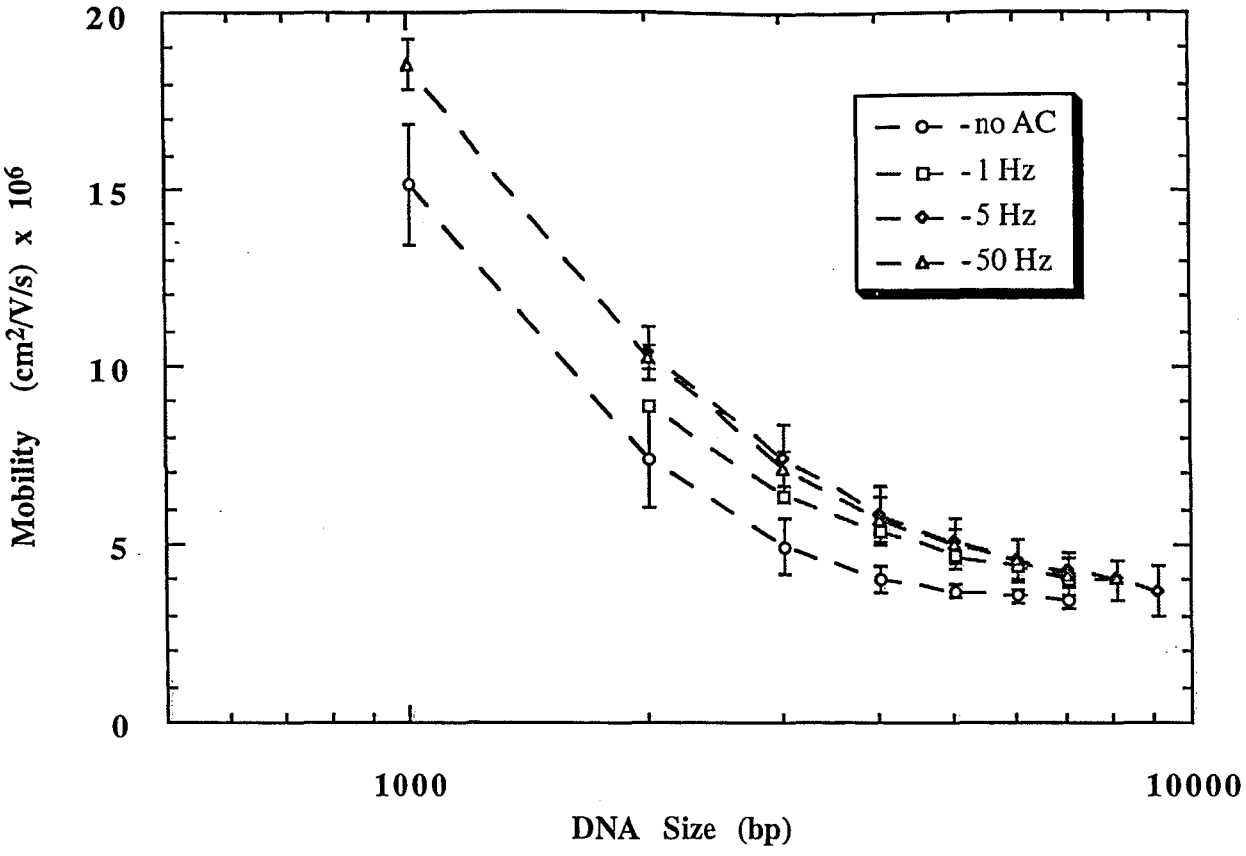

Electrode Array
Gel between Top and Bottom Plates
Base with recirculated cooling water
(a)
Gel Under Top Plate
Thumb Screw Clamps
End-Alignment Bar
Coolant Inlets
Top Plate for Electrode Array
Electrode Well for Electrode Array
(b)
Positive Power Supply
Switching Circuit and Resistive Divider
Function Generator
Negative Power Supply
Electrophoresis
Gel
Box
Constant DC Component
Square Wave AC Component
+
0 V
-
(c)

Figure 2

(a)


20
15
10
5
0
Mobility (cm²/V/s) x 10⁶
1000
10000
DNA Size (bp)
-no AC
-1 Hz
-5 Hz
-50 Hz

(b)

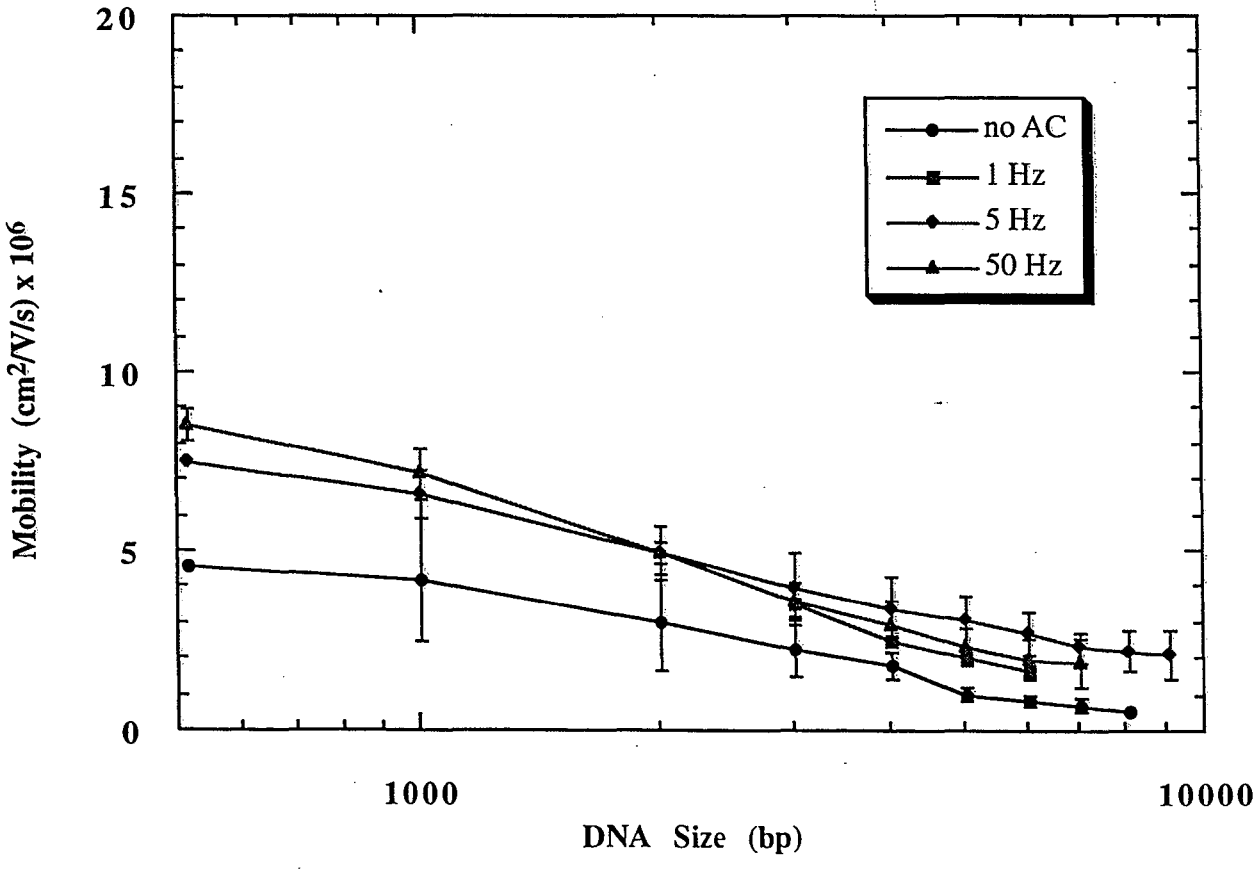

20
15
10
5
0
Mobility (cm²/V/s) x 10⁶
1000
10000
DNA Size (bp)
no AC
1 Hz
5 Hz
50 Hz

Figure 3

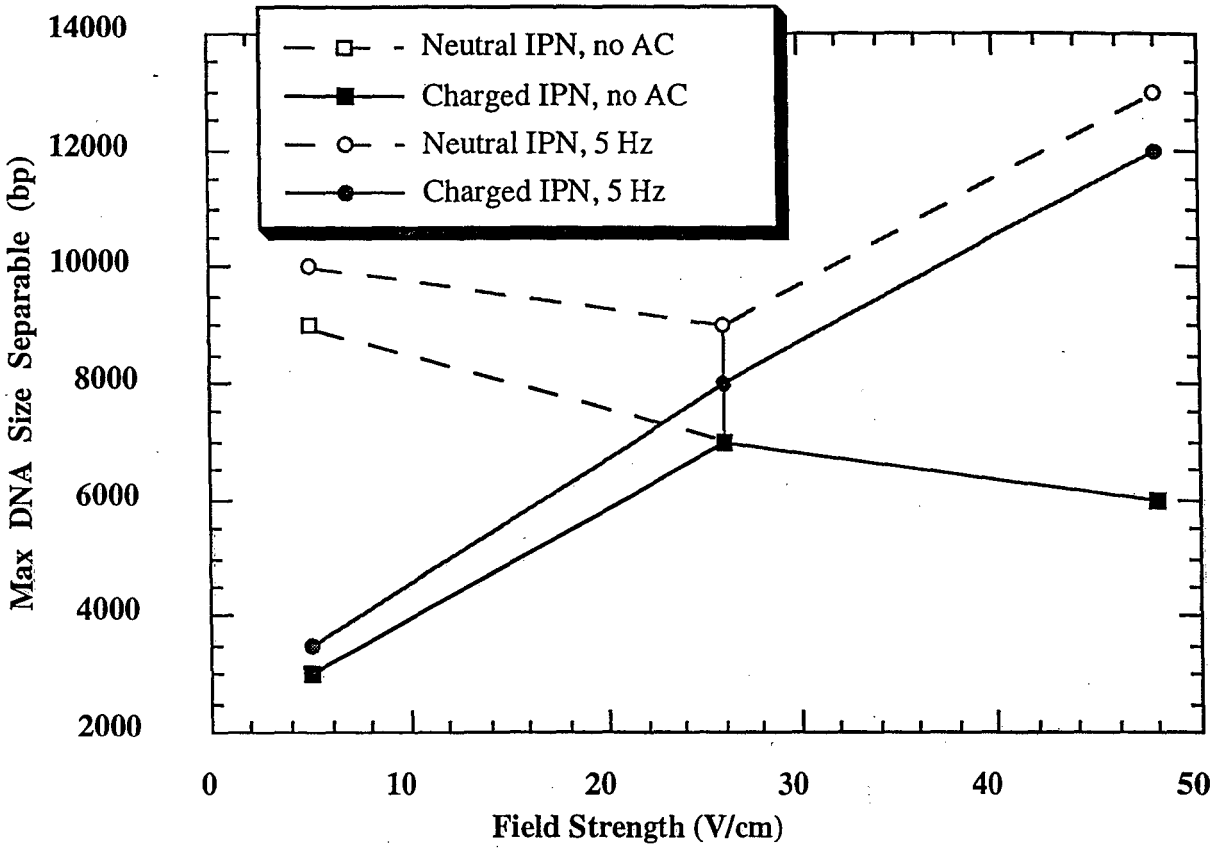
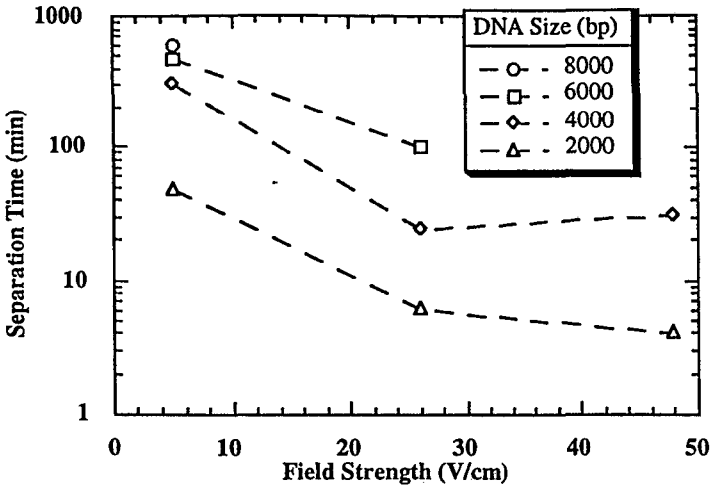
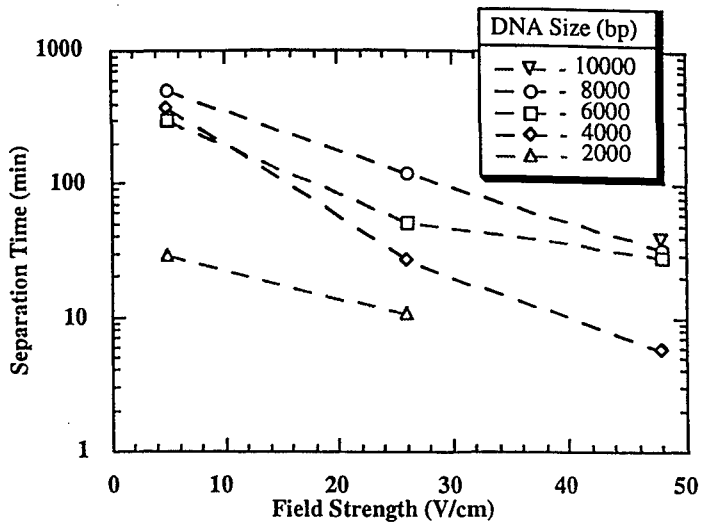

Neutral IPN, no AC
Charged IPN, no AC
Neutral IPN, 5 Hz
Charged IPN, 5 Hz
Max DNA Size Separable (bp)
14000
12000
10000
8000
6000
4000
2000
0
10
20
30
40
50
Field Strength (V/cm)

Figure 4



(a)



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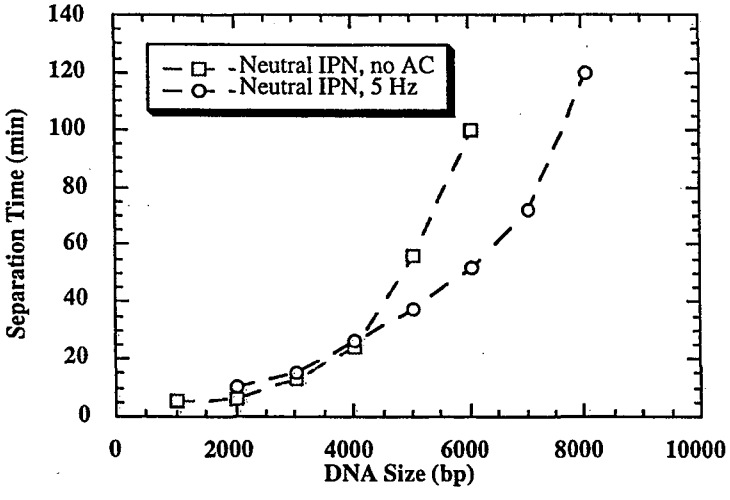

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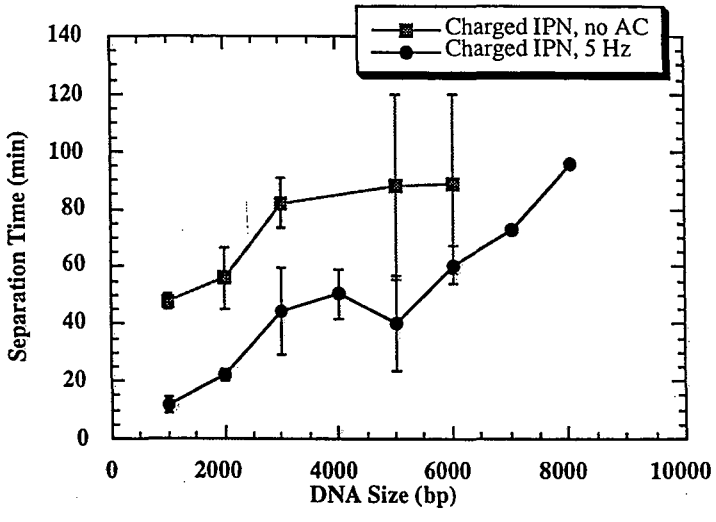


(d)

Figure 5

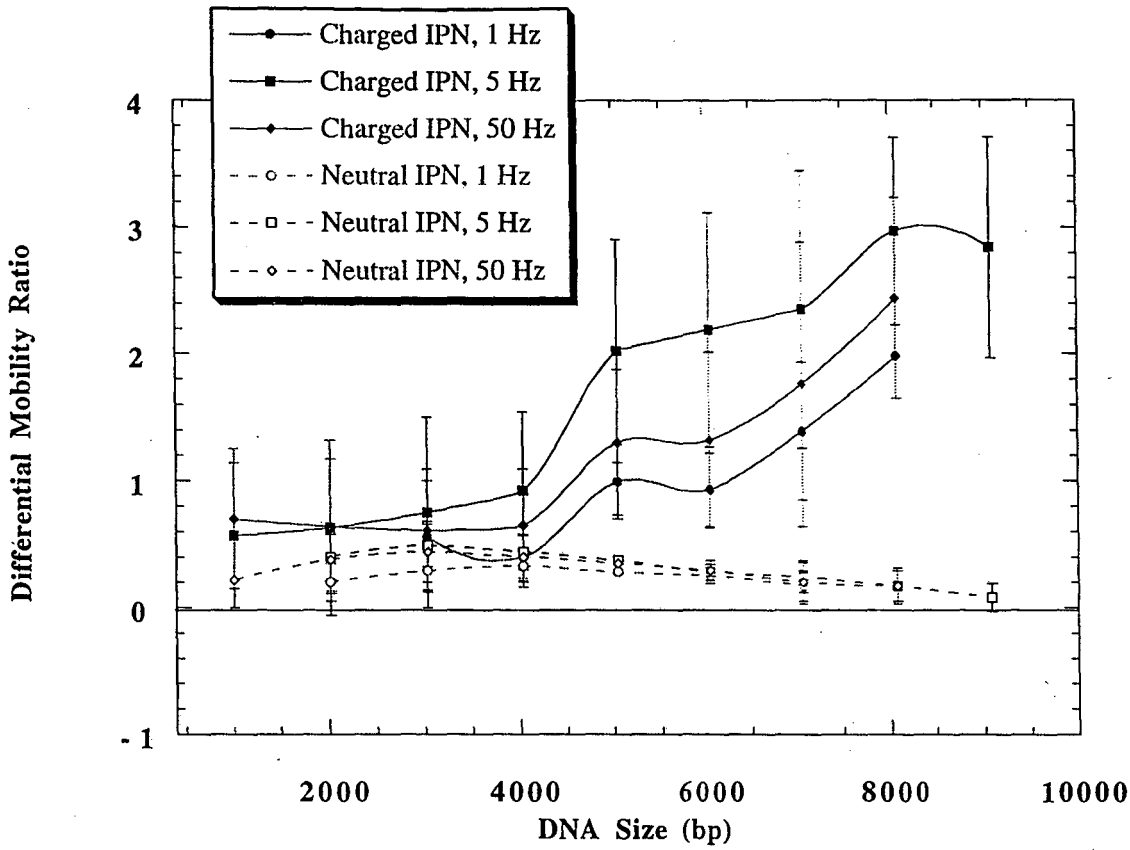

Charged IPN, 1 Hz
Charged IPN, 5 Hz
Charged IPN, 50 Hz
Neutral IPN, 1 Hz
Neutral IPN, 5 Hz
Neutral IPN, 50 Hz
Differential Mobility Ratio
4
3
2
1
0
- 1
2000
4000
6000
8000
10000
DNA Size (bp)

Figure 6

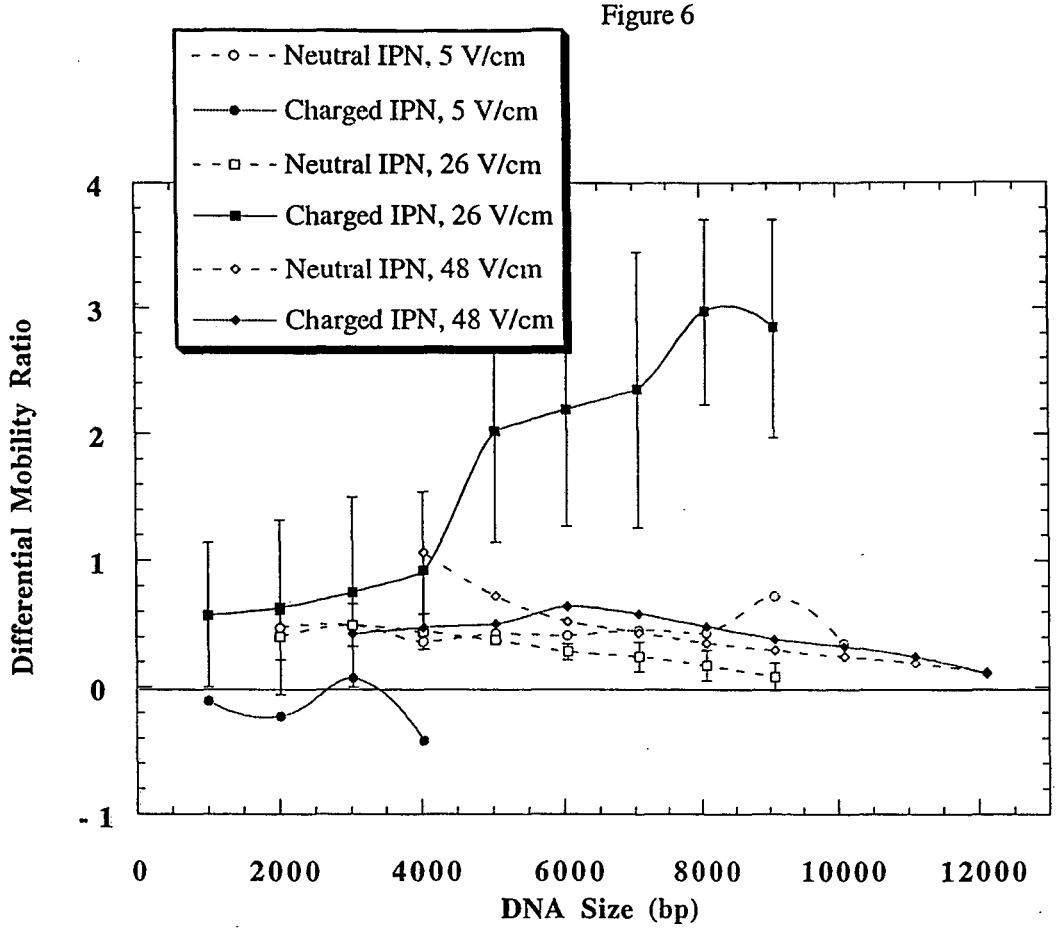
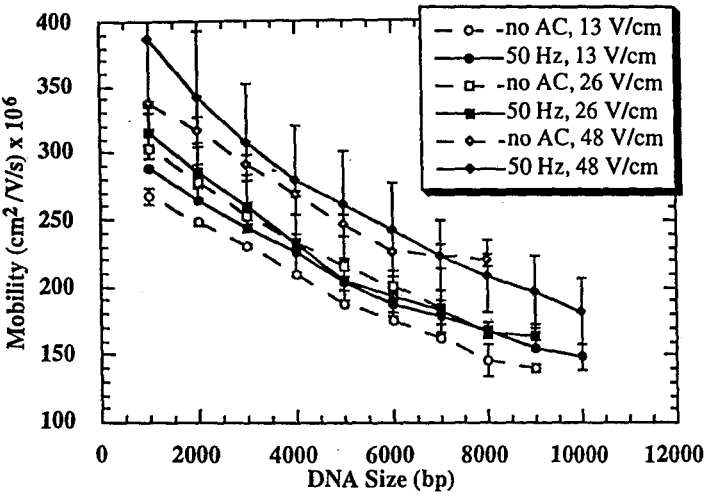
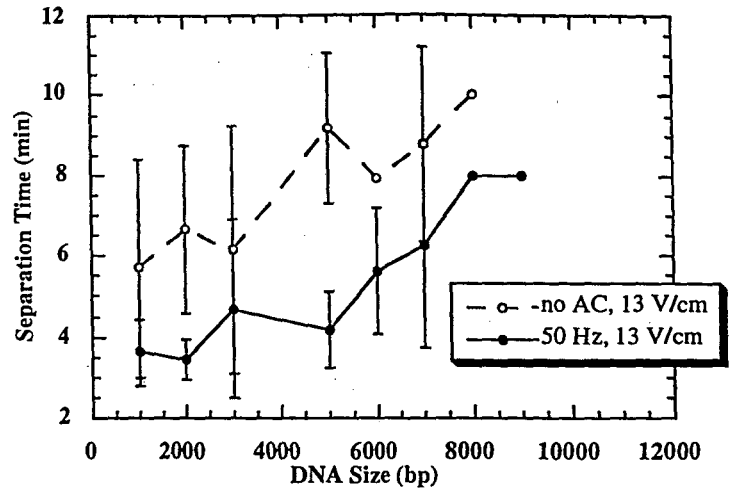

Neutral IPN, 5 V/cm
Charged IPN, 5 V/cm
Neutral IPN, 26 V/cm
Charged IPN, 26 V/cm
Neutral IPN, 48 V/cm
Charged IPN, 48 V/cm
Differential Mobility Ratio
4
3
2
1
0
- 1
0
2000
4000
6000
8000
10000
12000
DNA Size (bp)

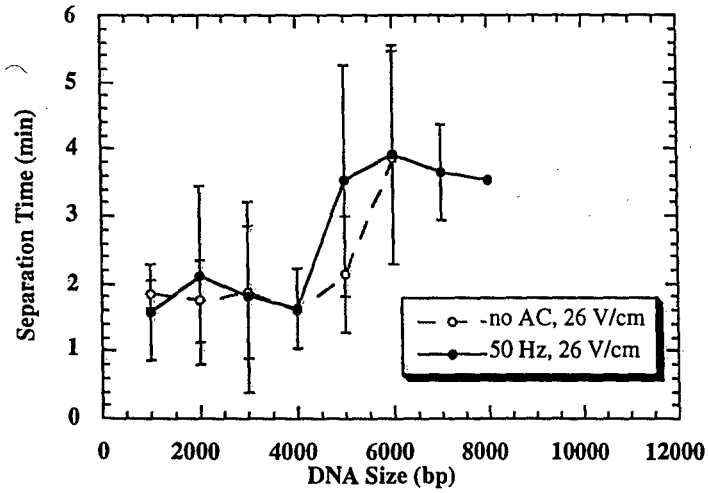
Figure 7



(a)



(b)



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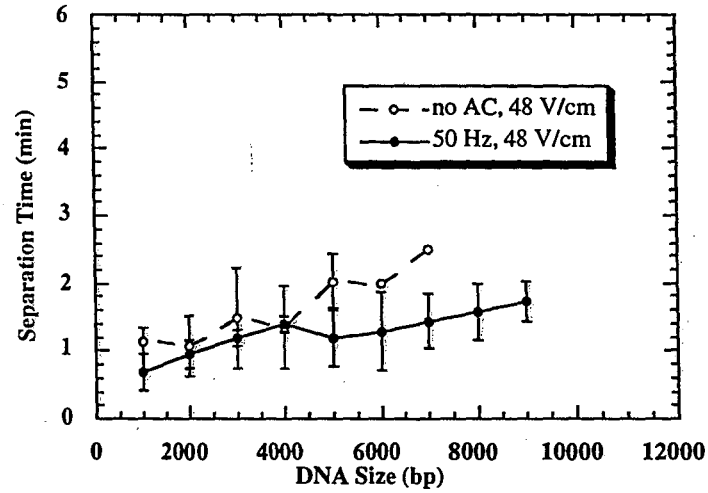


(d)

Figure 8

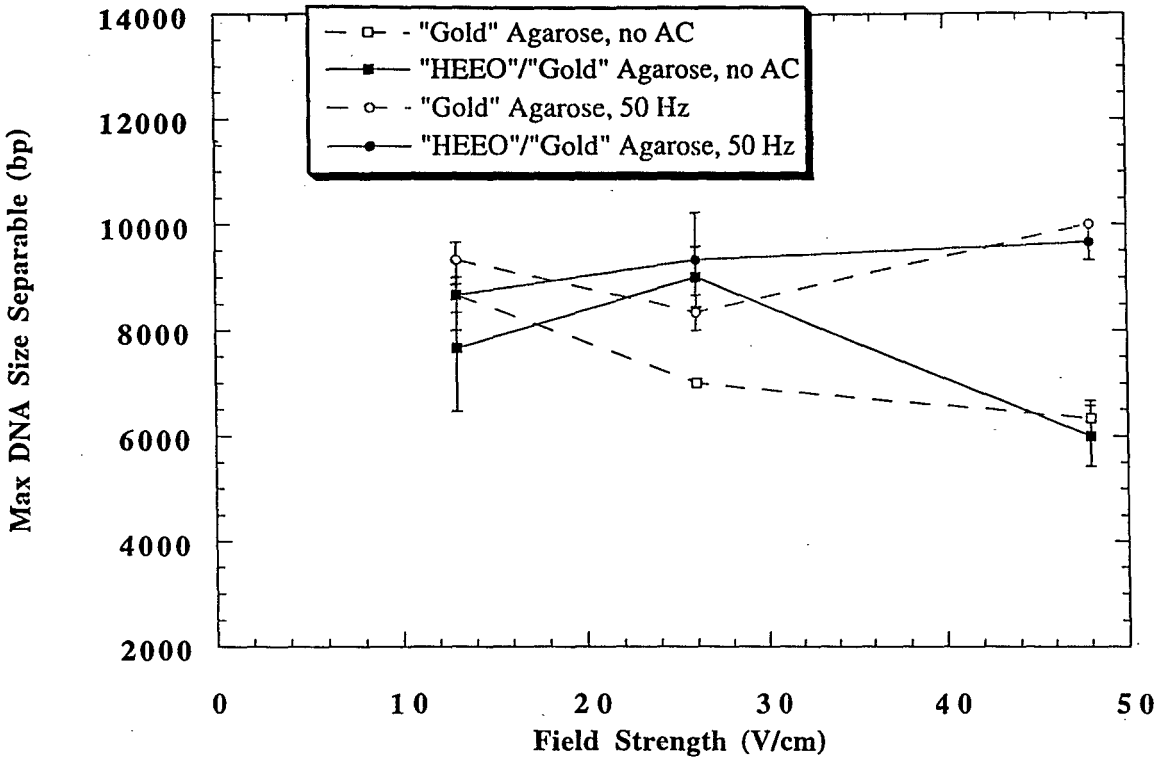
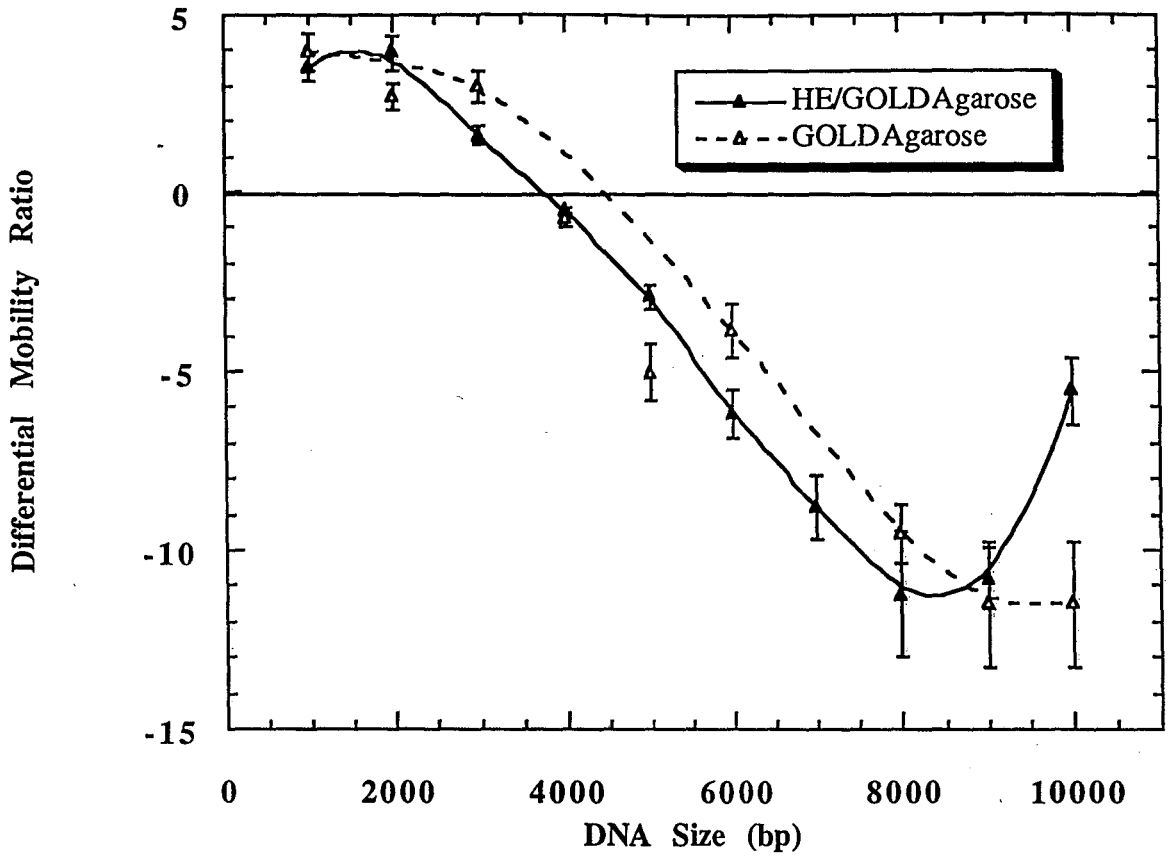

"Gold" Agarose, no AC
"HEEO"/"Gold" Agarose, no AC
"Gold" Agarose, 50 Hz
"HEEO"/"Gold" Agarose, 50 Hz
Max DNA Size Separable (bp)
14000
12000
10000
8000
6000
4000
2000
0
10
20
30
40
50
Field Strength (V/cm)

Figure 9


5
0
-5
-10
-15
Differential Mobility Ratio
0
2000
4000
6000
8000
10000
DNA Size (bp)
HE/GOLDAgarose
GOLDAgarose

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