Gel and Electric Field-Based Desorption of DNA from PMMA-Coated Silicon Surfaces to Optimize Sequencing Accuracy

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Abstract

Following the success of the Human Genome Project in 2003, DNA sequencing has been applied successfully to carrier screening, detection of inherited disorders, and DNA library preparation. Unfortunately, current sequencing methods are limited to DNA fragments a few kilobases long and result in many inaccuracies due to random fragmentation and the repetitive nature of DNA. One method being explored to combat this inaccuracy is DNA combing, in which a substrate is slowly pulled out of a DNA solution, depositing DNA molecules linearly on its surface. This offers the advantage of controlled cutting of DNA to then be sequenced in an orderly fashion. Polymethyl methacrylate (PMMA)-coated silicon wafers have been successfully used for both DNA combing and cutting; however, they have presented issues in the removal of DNA fragments for subsequent replication and sequencing. The primary goal of this study was to determine an effective method of desorbing DNA from PMMA-coated silicon wafers, specifically using agarose gels and an electric field, an approach that has not been previously investigated. Some samples of DNA-dipped PMMA-coated silicon wafers incubated in an oven overnight, while others sat in a well with buffer and an electric field, with and without a gel, with the hope of removing the DNA from the wafers. A Leica TCS SP2 confocal microscope was used to photograph several areas on each sample before and after DNA was desorbed and ImageJ was used to quantify the percent change in DNA on the samples. With 97.9% DNA removed, the use of an agarose gel and electric field (at .39 V/mm for 15 minutes) provides a significant increase in DNA desorption, a drastic difference from the control which incubated in the oven with no gel and had only 28.4% desorbed DNA, overall improving the applicability of the DNA combing and cutting technique to DNA sequencing

I. Introduction

DNA sequencing first exploded in 1990 with the start of the Human Genome Project which aimed to sequence the entire human genome. This was eventually accomplished through the map-based method and whole genome shotgun sequencing, both of which begin with cutting DNA strands into random fragments and amplifying them using bacterial artificial chromosomes (BACs).[1] This DNA was then "fingerprinted" using restriction enzymes to find common sequences that were then used to assemble the entire chromosome using overlapping fragments.[2] The map-based method generated libraries that spanned the 24 human chromosomes.[3] In both methods, DNA was sequenced using Frederick Sanger's chain termination method. While the Human Genome Project is widely regarded as a success, in reality only about 94% of the sequenced genome has been determined to be completely accurate.[4]

It is essential to minimize error rates in DNA sequencing as its benefits are numerous and significant. The purposes of sequencing range from general uses, like DNA library preparation and the analysis of evolutionary relationships, to potentially lifesaving techniques, including carrier screening and the detection of mutations or inherited disorders and diseases.[5, 6]

Next Generation Sequencing (NGS) refers to modern sequencing techniques and encompasses a variety of platforms that use different sequencing and assembly methods. It has reduced the cost of sequencing the genome significantly from the $300 million that it took to complete the Human Genome Project.[7, 8] This is largely due to eliminating DNA amplification through the transformation of bacterial colonies.[9] While sequencing method varies, all NGS platforms start with the random fragmentation of DNA to pieces ranging from hundreds to thousands of base pairs long, depending on the method implemented.[10] This fragmentation is generally accomplished through mechanical breakage and sonication.[11]

While recent technology has improved sequencing cost and efficiency drastically, there are still major issues with inaccuracies in sequenced DNA, some of which originate from this random cutting of DNA. Assembly algorithms using short reads from random positions have particular difficulty in complex, repetitive regions of DNA.[12] This is demonstrated by the 15% single-subread error rate of the Pacific Biosciences SMRT cell and substitution errors with the Illumina platform.[7] In general, short reads, or cutting the genome into very small fragments, are not currently sufficient for very repetitive areas of DNA.[13]

One method that has been attempted to combat this inaccuracy is DNA combing and cutting. With dynamic molecular combing, a substrate is dipped in a DNA solution and pulled out slowly, depositing the DNA linearly on a surface.[14] The technique works better with hydrophobic surfaces as they emerge from the solution dry, immobilizing the DNA, allowing for controlled cutting of the DNA.[15] This can be done by flowing an enzyme solution through microfluidic channels that are created by soft

lithography (PDMS). Ordered fragmentation helps with the accuracy of genome assembly as now it is known where each fragment came from on the larger DNA molecule.[16]

While previous studies have successfully performed DNA combing and cutting on polymethyl methacrylate (PMMA)-coated silicon wafers, they have not accomplished efficient, complete desorption of DNA fragments from the surfaces for sequencing. Thus, current research should aim to examine various conditions to impose upon the DNA in the hopes of removing it from the silicon to address this gap in knowledge. Due to the negative charge of DNA, one strategic approach focuses on applying an electric field to draw the DNA molecules off of the wafer without damaging them.

Given this, the primary goal of this study was to determine an effective method of desorbing DNA from PMMA-coated surfaces. The successful removal of fragmented DNA would improve the accuracy of sequencing, especially in complex, highly repetitive regions of DNA. A novel approach implemented to address this issue of desorbing the DNA from the polymer coated silicon wafer utilizes agarose gels and an electric field to draw the DNA off the surface.

This setup relies on the same electromotive force that is employed in gel electrophoresis to move the DNA molecules through the gel matrix due to the negative charge of DNA. Some samples of DNA-dipped PMMA-coated silicon wafers incubated in an oven overnight, while others sat in a well with buffer and an electric field, with and without a gel, with the hope of removing the DNA from the wafers. A Leica TCS SP2 confocal microscope was used to photograph several areas on each sample before and after DNA was desorbed and ImageJ was used to quantify the percent change in DNA on the samples. I hypothesized that a gel without an electric field would not impact DNA desorption, but that with the addition of an electric field, DNA desorption would increase significantly.

II. Methods

In order to test the effectiveness of the agarose gel and electric field in removing DNA from polymer-coated silicon surfaces, samples had to be created following an elaborate procedure to ensure the quality and consistency of DNA samples that were desorbed. The procedure, including necessary safety precautions and risks, is as follows.

A. Cleaving Silicon Wafers

Silicon wafers with a Miller Index of [1, 0, 0], denoting the orientation of the crystal plane, were used in order to achieve uniform, perpendicular breaks in the silicon.[17] First, 1 cm x 1 cm squares were scribed on the silicon wafers with a diamond-tipped scribe similar to the FlipScribe™, shown in Figure 1.[18] The flat edge of the silicon was lined up parallel and perpendicular with the bottom blocks on the scribe, and the blocks were screwed in on top of the silicon wafer to keep it stable when scribing. The

position of the diamond cutter was adjusted by twisting the knob at the top of the machine or with metal blocks. These scribed lines made the silicon easier to cleave.

Next, a cleaver was used to break the silicon along the scribed lines. To do this, the lines on the wafer were lined up with the dip on the bottom of the machine and the moving part of the machine was pushed down until the silicon cleaved. Throughout this process, the silicon was on a Texwipe® Cleanroom Cloth to protect the silicon and to prevent damage to the homemade machines.

Figure 1
The instrument used to scribe parallel and perpendicular lines into the silicon wafers, along which the wafers were cleaved.

B. Cleaning Silicon Wafers

Cleaved silicon wafers were first properly cleaned before being coated with polymethyl methacrylate (PMMA) by removing organic residues and silicon dioxide from the surface. The first step in cleaning the wafers was to fill a glass beaker with a 3:1:1 ratio of deionized (DI) water : hydrogen peroxide : ammonium hydroxide. The beaker was covered with aluminum foil with holes poked in it with tweezers to allow for the release of pressure. Then, the solution was heated on a hot plate at approximately 100°C for 3-5 minutes or until boiling. After the solution came to a boil, the solution was heated for an additional 20 minutes. Next, DI water was poured into the glass beaker to cool the solution, and all the liquid was disposed of into the designated waste bottle. The silicon wafers and glass beaker were rinsed with DI water three times each to remove any hydrogen peroxide or ammonium hydroxide. A plastic petri dish was filled with 10 mL of DI water and 1 mL of hydrofluoric acid. The silicon wafers were submerged into the solution and allowed to float for 30 seconds. Lastly, they were triple rinsed with DI water again and allowed to air dry completely.

Several safety measures were taken during this process as the acids and bases used can be extremely harmful if inhaled, ingested, or contacted with skin. Hydrofluoric acid (HF) had to be handled with extreme care by an experienced professional and calgonate (calcium gluconate) was present in any lab in which hydrofluoric acid was used. HF was also contained in plastic as it dissolves glass. Additionally, disposal of chemicals occurred within a fume hood and all hot solutions were cooled before disposal. Chemicals were disposed of into appropriately labeled waste containers and these containers were stored in a safety cabinet that resists fire.

C. Spin-casting Polymethyl Methacrylate

Once silicon wafers were cleaned and dry, they were spun-cast with polymethyl methacrylate (PMMA). First, silicon wafers were spun for 30 seconds on the spin-caster to make sure all of the water had dried off of them. Then, the silicon wafers were spun on the spin-caster once again with pure toluene to dissolve any leftover contaminants not washed off from the silicon cleaning procedure. Toluene was applied to the wafers using a glass pipette and was dispensed onto the wafers until the liquid covered the entire surface. After spinning with pure toluene, the silicon wafer was spun on the spin-caster once with the PMMA solution, which was created by dissolving 15 mg of polymethyl methacrylate in 1 mL of toluene. Finally, the silicon wafers were removed from the spin-caster with teflon tweezers, rather than metal, to avoid scratching the surface.

At all times, toluene and PMMA solution (since the PMMA was dissolved in toluene) were kept away from plastics as toluene dissolves plastic. All containers holding toluene were made of glass.

D. Annealing Samples

After silicon wafers were spun-cast with PMMA solutions, samples had to be annealed overnight in a vacuum oven to produce a solvent-free, moisture-free, and uniform surface. Samples were placed carefully in the oven using plastic tweezers as to not scratch the silicon.

Parts of the oven were hot when inserting and removing samples. Experimenters used proper protective equipment and only used the oven under the supervision of an experienced professional.

E. Making DNA Solutions

Before making the DNA solution, concentrated Lambda DNA and SYBR® Gold Dye were removed from the freezer and given time to defrost. The tube containing DNA was vortexed for 10 seconds as DNA concentration may vary throughout the sample. Appropriate micropipettes were used to place 2 μL DNA, 3 μL SYBR® Gold, and 995 μL of NEBuffer 3.1 into a separate microcentrifuge tube, changing pipette tips each time, to create 1 mL of a 1 μg/mL DNA solution. The solution was vortexed for 10 seconds to ensure an even distribution of DNA throughout the tube.

Several weeks were spent testing various concentrations of both PMMA and DNA solution to create ideal samples. This concentration of DNA was determined to produce the most successful samples, with little to no DNA dimers and a consistent concentration of DNA throughout the sample.

All dyes used are mutagens and teratogens. While making the DNA solution, SYBR® Gold Dye was handled with extreme care and experimenters avoided contact with skin or ingestion of any of the chemicals used.

F. Depositing DNA on PMMA-Coated Wafers

Prior to dipping the PMMA-coated silicon wafers into the DNA solution, the DNA solution was removed from the fridge and incubated in a 60°C oven for 30 minutes to avoid the formation of DNA dimers. Once the solution had been heated, a micropipette was used to move 700 µL of the DNA solution into a teflon well. Using a computer program and a laboratory exclusive dipping machine, shown in Figure 2, the well was placed underneath the sample and the machine dipped the wafer into the DNA solution, pulling out after 2 minutes. After the sample was removed from the machine, it was stored in a petri dish until it was ready for desorption.

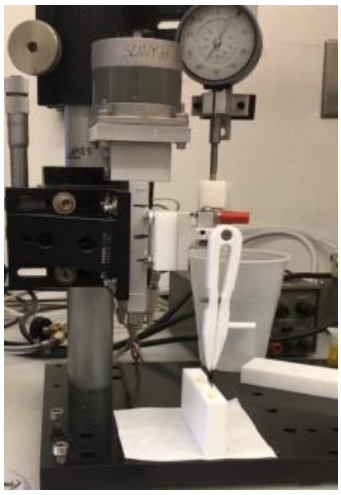

Figure 2
The instrument used to dip each PMMA-coated sample into the DNA solution at a controlled rate.

Many samples were created over the course of this study. Six of the most uniform and quality samples were selected to be desorbed according to the six different treatments.

G. Making 3% Agarose Gel

As half of the samples in this study were desorbed in contact with a gel, three 3% agarose gels had to be made. To create the agarose gel solution, 0.9 g of agarose powder was measured out on a weighing plate using a scale and was poured into a glass beaker. Then, the beaker was filled with 35 mL of NEBuffer 3.1 to create a 30 mL solution as some evaporated when it was heated. The solution was swirled and placed in the microwave for 30 seconds or until the solution was completely clear. When removing the beaker from the microwave, experimenters wore oven mitts as it can be hot. The solution was poured into petri dishes up to about 4 mm deep. After the gel had fully cooled, an X-ACTO® knife was used to cut the solid gels into 1 cm x 1 cm squares so they could cover the entire surface of the silicon wafer samples. Gels were kept submerged in NEBuffer 3.1 and stored in the fridge before use.

The glass beaker and agarose gel solution could be hot right after they were removed from the microwave, so experimenters were careful and used oven mitts during this procedure, in addition to the basic safety equipment.

H. DNA Desorption

Successful DNA-dipped samples were then assigned to several different treatments. Two samples were placed in wells, one covered with a 3% agarose gel made with NEBuffer 3.1 and the other just in NEBuffer 3.1 solution, as a control, and both incubated in a 60°C oven overnight. Four other samples were placed in wells with NEBuffer 3.1 and an electric field parallel to the sample for different lengths of

time (at either .55 V/mm field strength for 10 minutes or .39 V/mm for 15 minutes), with and without a gel. After desorption, all samples were then re-dyed in diluted SYBR® Gold solutions, washed with DNase reaction buffer, and blown dry with nitrogen gas.

While using this setup, it was essential that experimenters did not touch the exposed electrodes when the power supply was on to avoid getting shocked.

I. Confocal Microscopy

All samples were observed using a Leica TCS SP2 confocal microscope before and after desorption. The DNA was seen on a computer through a CCD camera connected to the microscope using a 63x water immersion lens. Photos were taken of eight areas on each sample to get an average of the amount of DNA on each sample before and after desorption.

Certain precautions were taken while using the confocal microscope to prevent any damage. To avoid crashing the lens, experimenters looked closely at the sample at eye level when changing magnification or focus. Additionally, the water lens was cleaned after use to prevent drying spots.

J. Data Analysis

In this study, a Leica TCS SP2 confocal microscope was used to photograph eight areas on each silicon wafer before and after the DNA was desorbed. ImageJ was used to calculate the area covered by DNA on each sample before and after DNA desorption, and the percent change in DNA on the samples was quantified.

III. Results

In this study, both after the PMMA-coated silicon wafers were dipped in the DNA solution and after the attempted DNA desorption, eight photographs from different areas were taken of each sample using a Leica TCS SP2 confocal microscope at 63x magnification. Two examples of these photos for each of the six samples, one before and one after desorption, are shown in Figure 3. Some samples exhibit drying spots from when the buffer evaporated, especially samples (a) and (b) in Figure 3, the ones that incubated in the oven. These spots were minimized by washing the wafers with buffer and blowing them dry after they were re-dyed post-desorption. Several strands of DNA are visible in these samples, indicating that a good portion of the DNA remained after desorption. Comparatively, sample (f) is almost entirely clear of DNA, suggesting that the majority of DNA was successfully removed. Samples (c), (d), and (e) also exhibit nearly empty surfaces, implying that the electric field, regardless of strength or running time, results in more effective DNA desorption.

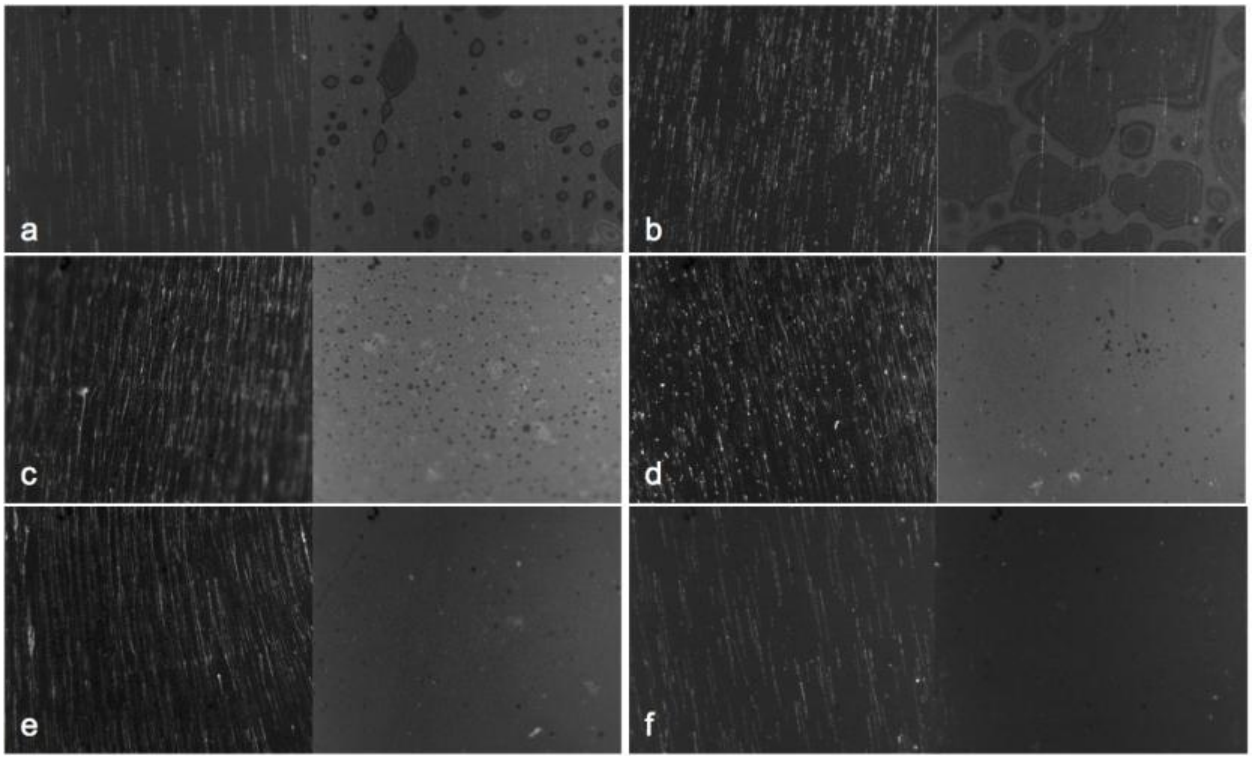

Figure 3

Confocal microscope images (63x) of samples before (left) and after (right) desorption with (a) no gel, (b) only a gel, (c) no gel at .55 V/mm for 10 minutes, (d) a gel at .55 V/mm for 10 minutes, (e) no gel at .39 V/mm for 15 minutes, and (f) a gel at .39 V/mm for 15 minutes.

The computer program ImageJ was used to adjust the threshold of photographs and quantify the amount of DNA present on samples by area before and after desorption, and the percent desorbed was calculated for each sample. This information is displayed in the table shown in Figure 4. The most successful sample, highlighted in red in Figure 4, desorbed with the electric field at .39 V/mm for 15 min. in contact with a gel.

Percent DNA Desorbed		
Condition	Mean	Standard Deviation
60°C no gel for 15 hours - control	28.4%	13.51%
60°C with gel for 15 hours	79.1%	7.57%
.39 V/mm for 15 min. no gel	96.5%	3.19%
.39 V/mm for 15 min. with gel	97.9%	0.92%
.55 V/mm for 10 min. no gel	92.0%	4.76%
.55 V/mm for 10 min. with gel	97.8%	1.84%

Figure 4

Chart displaying the mean and standard deviation percent DNA desorbed on each sample.

IV. Discussion

The sample with the most desorbed DNA was the one with the gel at .39 V/mm for 15 minutes, with 97.9% DNA desorbed. As seen in the graph in Figure 5, this is a drastic difference from the control, which incubated in the oven with no gel and had only 28.4% desorbed DNA. In fact, all of the samples that desorbed with the electric field yielded impressive results at 92.0%, 97.8%, and 96.5% DNA desorbed. Interestingly, between the two that only incubated in the oven, the sample in contact with a gel also had a higher percentage of DNA desorbed at 79.1%.

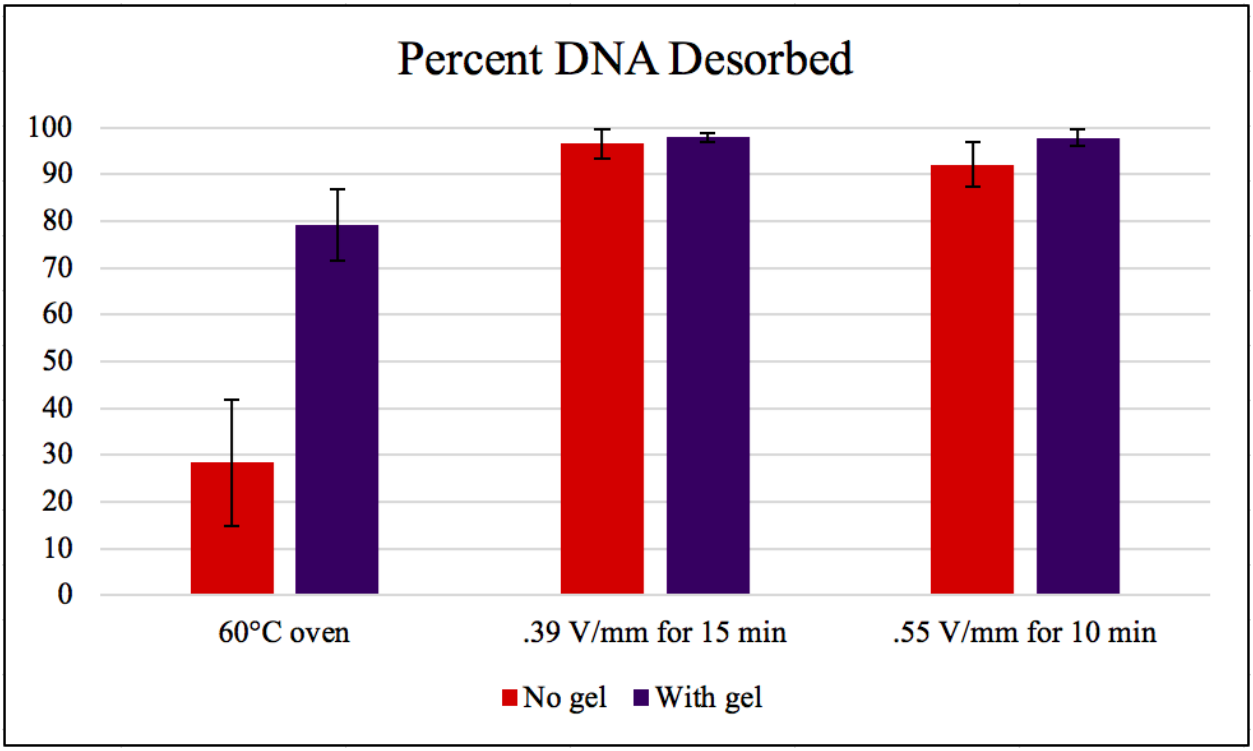

Figure 5

Bar graph showing the percent of DNA desorbed on each sample (left to right: 28.4%, 79.1%, 96.5%, 97.9%, 92.0%, 97.8%) with error bars indicating one standard deviation.

V. Limitations and Ongoing Work

While the collected data strongly demonstrates the efficiency of the electrophoresis technique in removing DNA from the PMMA-coated silicon wafers, other aspects of the technique must be further analyzed to determine its reliability when used in the context of sequencing the desorbed DNA fragments. As the samples exhibited very little DNA present on the surface post-desorption, it can be inferred that this DNA was desorbed into the buffer that surrounded the wafers in the well; however, the condition of the DNA is unknown. Before this method of ordered DNA fragmentation can be applied to sequencing, it must be confirmed that the procedure does not damage the DNA significantly. Damage may occur during desorption as the electric field heats the buffer, causing some to evaporate, making the pH less stable. Although electrophoresis is limited to 15 minutes, there is still the potential for harming the DNA.

Additionally, there is still room for improving the technique to be employed for more than just desorbing the DNA into solution. Currently, with the agarose gel on top of the silicon wafer and the electric field running parallel to the sample, it is unlikely that any DNA ran up into the gel rather than

sideways into the buffer. In my future work, I plan to attempt to create an electric field perpendicular rather than parallel to the sample, so that the DNA may migrate up into the gel where it would be easier to isolate by fragments while knowing where they came from on the DNA molecule. This may also be achieved by placing a gel on the edge instead of on top of the sample where the DNA would run into it. Still, if neither of these options are feasible, fluorescent labels may be used to identify the origin of each fragment even when the DNA is desorbed into solution.

VI. Conclusions

This study provides convincing evidence that electrophoresis, both with and without a gel, is a viable method of removing DNA from PMMA-coated surfaces for sequencing purposes.

- With 97.9% DNA removed, the use of an agarose gel and electric field (at .39 V/mm for 15 minutes) provides a significant increase in DNA desorption.
- The successful removal of DNA from PMMA-coated silicon wafers overall improves the applicability of the DNA combing and cutting technique to DNA sequencing.
- The implementation of DNA combing and controlled cutting to DNA sequencing, made possible by the strategic use of electrophoresis, would eliminate inaccuracies in DNA sequencing caused by the random fragmentation of DNA.
- Higher quality sequencing would result in more reliable DNA research and more successful diagnosis of genetic diseases and disorders.

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