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# Plasmid DNA Bind and Elute Chromatography Using Borosilicate Filters and Filter Holders

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Abstract

## Chapter 1 (Introduction/Motivation)

This research has been completed to determine whether plasmid DNA will bind to a simple borosilicate filter in a reusable filter holder, and whether it can be released in a controlled procedure after a wash has been done to eliminate impurities.

The motivation for this is to help small research groups, both in educational settings as well as in other small research settings be able to study isolated DNA without much of the expense generally associated with purification kits and to remove the limitations on trial numbers imposed by the expense of each individual isolation kit.

Medical research, forensics, and other biological studies often require the examination or multiplication of DNA. In order to examine the DNA, it must be separated from the other cellular components, or lysosomes. Many techniques have been developed by research groups to accomplish the goal of DNA isolation, including through Chromatography. This is any method that uses a stationary phase and a mobile phase [10]. Some types of chromatography include Affinity Chromatography, Size Exclusion Chromatography and Ion Exchange Chromatography.

This study is focused on Ion Exchange Chromatography [3]. This method requires the DNA to be part of a solid phase (in this case, a filter) and a mobile phase (in this case, a series of buffers). The goal of chromatography is to separate a solution based on some characteristic of the particles in the solution. For DNA separation, the separation is based on the electrical conductivity of the ions present [4]. DNA is a polar molecule because of its phosphate backbone. Because it is a polar molecule, it can be bound to a positively charged silicate filter in a chaotropic solution (Image 1) and then released from the filter as the ionic strength is reduced by flowing an elution buffer that changes the pH of the solution (Image 2).

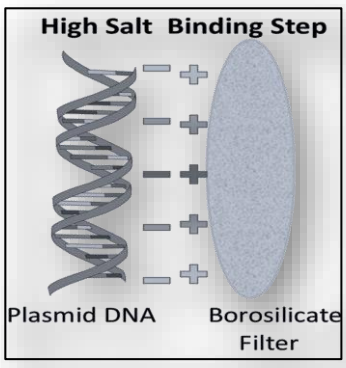


Image 1: DNA molecule is bound to a borosilicate filter when a chaotropic agent is used to lower the pH of the sample and filter.

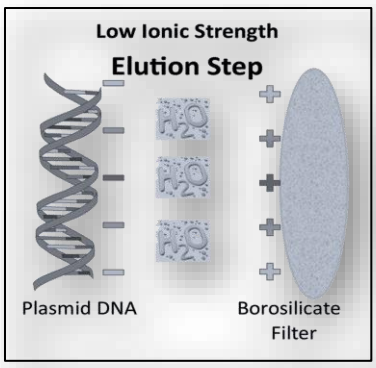


Image 2: After a buffer is allowed to wash away impurities, $H_2O$ is used as an elution buffer which allows the release of the DNA from the filter.

Image 3: AKTA Start Chromatograph used for precise monitoring of the flow rates, column volumes and number of mL of each buffer used [5].

The chromatograph above (Image 3) is the machine used for the tests done in this trial, though the machine is not necessary for every DNA separation. The chromatograph simply allows for a very controlled injection of buffers, DNA samples, and other fluids. It monitors flow rates, total mL of solution, and conductivity of the solution. A special UV sensor also monitors the DNA particles in mobile phase at any time. It produces a chromatogram, giving the researcher information about the binding and release of the DNA. It is also able to fraction off the flow-through into micro-tubes, allowing specific fractions to be analyzed for DNA concentration.

In this study, a chromatograph was used to discover whether plasmid DNA would bind to a simple fiberglass (borosilicate) filter held in a reusable filter, and if the DNA could remain bound to the filter through a gradient wash and then release from the filter at a precise time during the elution stage.

After many preliminary tests, during which the pH of the buffers were balanced and optimized, the flow rate and size of the column volume were established, and the chromatograph preparation and equilibrium were adjusted, a series of 6 consistent tests were completed showing nearly 100% recovery of DNA from injection to isolation. The borosilicate filters have been shown to be a consistent and effective tool for the isolation of plasmid DNA.

This paper will address the basic protocol developed as well as recipes for each buffer used. It will give the results of the research done and some suggestions for continuing research.

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## Chapter 2 (Methods)

Testing was completed using plasmid DNA (double stranded circular DNA from bacteria or single-celled organisms) which was prepared by another research group before-hand using an Omega Biotek midi-prep kit. PcDNA3.1 plasmid was transformed into NEB5-alpha cells, after which a bacterial colony was isolated and used to inoculate a liquid culture.
The protocol used followed the one provided in the Biotek manual [9]. Once the plasmid DNA was prepared, the following method was followed for preparing the chromatograph, binding the DNA to the filter, washing the filter, and eluting the DNA from the filter:

# Protocol for Plasmid DNA isolation using Borosilicate Filters, Swinnex Filter Holder and AKTA Start Chromatograph

## Materials Needed

| |
|---|
| 1. AKTA Start Chromatograph or similar liquid chromatography apparatus with Unicorn software. |
| 2. Whatman Grade GF/B Glass Microfiber Filters, Binder Free 21 mm circles (100 pcs) http://www.gelifesciences.com/webapp/wcs/stores/servlet/catalog/en/GELifeSciences-us/products/AlternativeProductStructure_16176/28418346 |
| 3. Swinnex Filter Holder 13mm https://www.emdmillipore.com/US/en/product/Swinnex-Filter-Holder,-13%C2%A0mm,MM_NF-SX0001300 |
| 4. HBC Buffer (Binding Buffer)<br>**Recipe**<br>5M Guanidine HCL 30% isopropanol<br>For 200mL (Each Run will take<br>140mL $H_20$<br>95.2g Guanidine HCL<br>Then add:<br>60mL isopropanol<br>(7.14M dilutes down to 5M with addition of the isopropanol)<br>**Or Purchase**<br>http://omegabiotek.com/store/product/hbc-buffer/ |
| 5. PE Buffer (Wash Buffer)<br>**Recipe**<br>10mM TrisHCl w/ 80% EtOH<br>For 424.2 mL:<br><br>4.2 mL of 1M Tris Base Stock (6.055 g Tris Base + 2.5 mL HCl + 40 mL $H_2O$ +10 mL $H_2O$)<br>Then add 420 mL 80% EtOH<br>**Or Purchase**<br>https://www.qiagen.com/us/shop/lab-basics/buffers-and-reagents/buffer-pe/#orderinginformation |
| 6. Deionized water |
| 7. 1mL syringes |
| 8. Micro centrifuge tubes |
| 9. 60 µL Plasmid DNA crude or pre-purified sample. |
| 10. Chemwipes |
| 11. Plumbers Tape for wrapping Swinnex threads |
| 12. .05M $NaN_3$ (Sodium Azide) Cleaning Solution |
| 13. 13mm or ½ inch hole punch (sterilized with EtOH) these can be bought at any craft store or online. |

## Step one - Preparing the Filter Holder

Using a 13 mm or ½ inch hole puncher, punch a 13mm circle out of one of the 21mm filter circles (Be sure to handle everything with clean gloves). Open one Swinnex filter holder. Into the side without the membrane support, place 2 small white gaskets, then the filter, then one more small white gasket. Wrap one layer of plumbers' tape around the threads on the other side of the filter holder, and then gently screw the two sides together. They must be screwed tightly together.

## Step Two - Preparing the injection lines:

Remove the thin line from the $NaN_3$ solution while wiping with a chem-wipe. Once the line is wiped, put it into the $H_2O$ bottle (elution buffer).

Remove Buffer A line and sinker filter from $NaN_3$ and wipe and place in the bottle on top of the machine labelled HBC or Gu HCL with 30% IPA (Buffer A - Binding Buffer)

Remove Buffer B line and sinker filter from $NaN_3$ and wipe and place in the bottle on top of the machine labelled PE Buffer or EtOH + Tris HCl (Buffer B - Wash Buffer)

On the front of the machine, there are some connectors for the columns to be installed. Unscrew the black connector exposing the threaded male connector on the line to the right. Onto this connector, place the luer lock threaded female connector which has a non-threaded open end on the opposite side (this will be sitting on top of the machine). On the left side, screw in a male threaded connector with open non-threaded connection on the opposite end. Now you should have two open non-threaded ends available. Place the straight white connector into the line on the right. Insert the prepared Swinnex filter holder in-line with the wider side to the right. Apply pressure to all connections, making sure of tight fittings. If there is any leaking, use plumbers tape to tape the threads of the filter.

| Phase | Variable | Value | Range |
|---|---|---|---|
| Method Settings | ColumnVolume {CV} | 0.500 | [0.100 - 999999.0] |
| | Column | Any | |
| Method Settings,... | FlowRate {ml/min} | 1.0 | [0.5 - 5.0] |
| Prime and Equilibration | Equilibration Volume {CV} | 10.00 | [0.00 - 999999.0] |
| Sample Application | Loop Sample Volume {ml} | 1.00 | [0.00 - 999999.0] |
| Elution and Fractionation | Elution Start at B Concentration {%B} | 0.0 | [0.0 - 100.0] |
| Elution and Fractionation | Elution Fixed Fractionation Volume {ml} | 1.0 | [0.5 - 15.0] |
| Elution and Fractionation | Target B concentration for elution segment_(1) {%B} | 100.0 | [0.0 - 100.0] |
| | volume of elution gradient segment_(1) {ml} | 20.0 | [0.0 - 10000.0] |
| Elution and Fractionation | Target B concentration for elution segment_(2) {%B} | 100.0 | [0.0 - 100.0] |
| | volume of elution gradient segment_(2) {ml} | 5.0 | [0.0 - 10000.0] |
| Sample Application | Sample Volume_1 {ml} | 15.00 | [0.00 - 999999.0] |

Image 4: System Method for AKTA START Chromatograph using Unicorn One software.

Use the above settings for the Unicorn software.

## Step two - Priming the lines:

**Initial Use for a day:**

Prepare a syringe with one ml of HBC Buffer A. Set it near the chromatograph on a chem-wipe.
Open Unicorn 1 software. (If the computer is already on when you begin, you will need to restart the machine. It will not connect to the chromatograph otherwise). When the first selection menu opens, select boxes labelled “system control”, “evaluation”, and “method editor”.

Toggle to the window displaying the system control. Click on the “Connect” button on the top bar. The control center will open.

Click on the “Manual Run” button. A box will pop up asking at what flow rate you want to run the manual run. 1ml/min is good. Accept this amount and Buffer A will begin to flow.

Using the computer control panel. Click on the sample jar (the water), so that Buffer A stops and the water begins to flow through the line and out through the waste valve. Allow this to run for 2-3 minutes, until there are no longer any bubbles traveling through the line.

Now, click on Buffer B. Allow this buffer to flow for 2-3 minutes as well, until there are no longer any bubbles in that line.

Finally, click back onto buffer A. It will begin to flow out through the first waste line. Click on the valve (little circle) opposite the waste line to open the lines carrying the fluid through the filter, the UV sensor and the conductivity sensor and out through the second waste valve. Allow this fluid to flow for 2-3 minutes. This is bringing the pH of the entire machine down to about 4.5. Now, on the machine, turn the injection bar to inject, instead of to load. Allow the fluid in the line to run for exactly one minute. Now, turn the bar back to load, and inject the HBC buffer in the syringe into the port.

Last, switch the valve on the computer so that instead of flowing through the waste valve, the buffer will flow through the fractionation line long enough to expel all bubbles and equilibrate the line to the lower pH.

**Secondary Use in a day:**

Prepare a syringe with one ml of HBC Buffer A. Set it near the chromatograph on a chem-wipe.

On the computer control, click on the “Manual Run” button. A box will pop up asking at what flow rate you want to run the manual run. 1ml/min is good. Accept this amount and Buffer A will begin to flow. Click on the valve which allows buffer A to travel through the filter and the sensors. Allow it to flow for 3 minutes. Now, switch the load/inject bar to inject and allow the fluid to flow for one minute. Switch the bar back to load and push the HBC buffer from the syringe into the port.

Switch the waste valve to fractionate, and allow the fluid to flow for .5-1 ml. Now push stop. The machine is ready.

## Step 3: Method Run

Put fractionation collection tubes into the fractionation machine.

Before beginning a method run, be sure to prepare the DNA sample. It will be made up of 5% pDNA and 95% HBC buffer. Prepare it in a small 1.7 ml tube, and then suck it up into a syringe, using a needle tip. Turn the syringe pointing upwards, and eject the air bubble. Push the fluid so gently up to the tip of the syringe until a small bulge of fluid sits atop the end of the syringe. Now, put the syringe into the port on the machine and, with the bar turned toward LOAD, inject the fluid from the syringe into the port. Leave the syringe in place until the end of the test.

Now, select the method run you need and allow the machine to run the test. At one point, you will be asked to turn the LOAD/INJECT bar to INJECT. Do so. Then you will be asked to turn it back to LOAD. Do so again. Now the program will continue to run to the end of the test.

## Step 4: Cleaning the Chromatograph

Using a pipette, suck 1.2 ml of Sodium Azide out of the jar to the left of the machine. Put it into a small tube. Now suck it up with the syringe.
After all of the testing is finished and the tubes have been labelled and stored, the machine must be thoroughly cleaned with $NaN_3$. Take all three lines (buffer A, buffer B, and water sample) and wipe them with chem-wipes and place them back into the Sodium Azide. Run this carefully through all of the lines. Inject it with the syringe into the coil. Push $NaN_3$ into every waste line and then the fractionation line. This sterilizes and de-salts the machine. Wipe the front injection port off, wipe the fractionation line off. Make sure there is no standing or dripping liquid anywhere. Open the pump chamber and allow the line to hang loosely in front of the pump chamber.

Turn off the machine. Exit from the software.

## Discussion of Above Process

The DNA has been prepared in a solution of HBC buffer (5M Guanidine HCL 30% isopropanol). It cannot be prepared in water or when the sample injection occurs, the DNA flows right through the filter without binding.

The line preparation in step two has a two-fold purpose. First, the lines must be evacuated of any air bubbles or inconsistencies. Second, the lines and the valves and ports must be equilibrated to a much lower pH. The beginning pH of the machine should be around 4.6.

Once the lines are prepared, the method run begins, and the sample has been injected into the machine, the second buffer will begin to increase on a linear gradient, slowly increasing the pH of the system including the filter. Any of the less-electronegative particles still stuck on the filter will begin to come off. The UV sensor may pick up some of these particles, but rest assured that the DNA is still firmly bound to the fitler.

After the wash step, water will elute the DNA off of the filter and into fractionation tubes. It can now be analyzed for concentrations.

The concentrations can be analyzed using two different methods described here:

## Gel Electrophoresis Procedures – Analysis Method 1

1. Make a solution of 1.2 ml 50X TAE pH8 with 58.5 ml $H_20$

2. Add .4179 g of agarose crystals [59.7 ml(sol) x 7 mg/ml(Agarose) = 417.9 mg Agarose]

3. Heat for polymerization 45 sec in microwave

4. Add 7 µl red gel dye

5. Pour the gel into the tray. Insert Comb. Allow to set up for 30 minutes.

6. Select DNA samples and Ladder (1kb) sample

DNA prep: 20µl sample DNA + 4 µl loading dye in micro tubes

5. Centrifuge for 2 seconds (balance position of tubes)

6. Once the gel is set, turn the tray around sideways (make sure comb is near the negative side of the tray so the DNA can move from negative to positive) and fill the side chambers and a thin layer on top of the gel with TAE 1X:

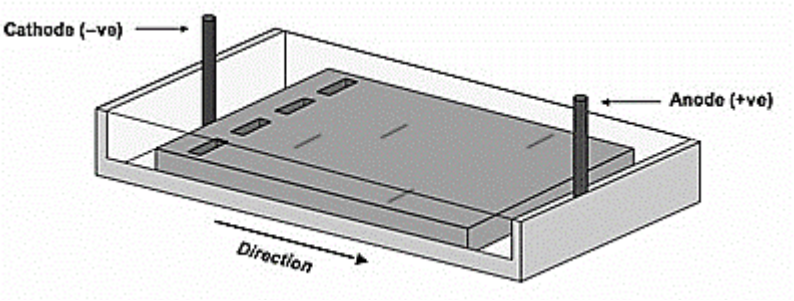


Image 5: Gel Electrophoresis Device [8].

7. Carefully remove the comb and carefully pipette the ladder into the first well made by the comb. Inject each sample into the remaining wells, annotating where each sample has been loaded in a notebook.

8. Connect the lid and plug in the leads to the power supply. 110V for 45 minutes or 150V for 30 min.

9. Remove the tray and set on paper towels. Blot the excess liquid from the gel.

10. Take the gel to the gel analysis camera. Set the gel onto the camera deck and cover with camera cover. Be sure not to turn on the UV light until the entire surface is covered.

11. Turn on computer and open the camera software.

12. Turn on UV light. Click acquire on the computer. Set exposure time to 8 seconds or so. Click acquire again. Save the image for later analysis.

13. Using ImageJ software, found for free at this website: *https://imagej.nih.gov/ij/download.html*, create a selection of the known ladder bands. Three bands will be enough. Using the known DNA masses (given in the ladder information), and the lumens detected by ImageJ, plot a line in Excel or another graph making program. Use excel to display the line's equation. Using that equation and the lumens detected in the sample lanes of the gel, calculate the mass of DNA in each of the sample lanes and bands. Using the found masses, calculate the concentration based on the number of µl injected into the gel. Now, using that concentration, calculate the mass of DNA in the entire fractionation tube based on the number of µl in the tube.

## Discussion of Above Process (Gel electrophoresis)

Using a gel electrophoresis procedure is very standard in the scientific community. The data acquired through the gel testing is accurate and well received. There are a few problems with using a gel to find and identify DNA from a test. First of all, the camera and human eyes both have a very difficult time sensing the light created by less than about 15 ng/ µl concentrations of DNA on the gel. For large samples, the gel is great, but if a dilute sample is being run, then it can be difficult to perceive masses of DNA from gel analysis.

## Spectrophotometry – Analysis Method 2


Image 6: Becker Coulter Spectrophotometer [1].

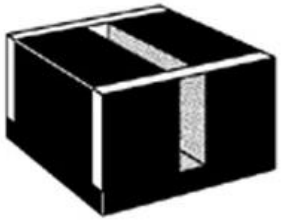

Image 7: Micro cuvette [6]

1. Clean the micro cuvette with 80% EtOH spray. Carefully and thoroughly dry the cuvette with a chem-wipe or an $N_2$ compressed gas hose [11].

2. Using the micro cuvette (image 7) place 100 microliters of the buffer used in the sample needing to be tested. Set the cuvette into the spectrophotometer and close the lid.

3. On the screen, touch the selection labelled DSDNA.

4. Press "Blank"

5. Now remove the cuvette and dump the buffer into the appropriate disposal location (storage container or sink) and re-clean it with EtOH. Dry the cuvette again.

6. Place 100 microliters of the sample into the cuvette. Set it into the spectrophotometer again and close the lid. Press "READ".

7. If the reading comes out to be negative, start over and re-blank the spectrophotometer.

8. The spectrophotometer will give a UV reading at 260 and 280 nm. The number given at 260 nm will be the DNA.

9. Select "View Concentration". The machine will then use the following method to calculate the concentration of the DNA:

"DNA concentration is estimated by measuring the absorbance at 260nm, adjusting the $A_{260}$ measurement for turbidity (measured by absorbance at 320nm), multiplying by the dilution factor, and using the relationship that an $A_{260}$ of 1.0 = 50µg/ml pure dsDNA.

$$\text{Concentration (µg/ml)} = (A_{260}\text{ reading} - A_{320}\text{ reading}) \times \text{dilution factor} \times 50\text{µg/ml}$$

Total yield is obtained by multiplying the DNA concentration by the final total purified sample volume.
DNA yield (µg) = DNA concentration × total sample volume (ml)" [2]

10. Repeat the following steps to test each fractionation collected from the chromatograph that showed particulate in the UV detection on the chromatogram. Make sure to blank in the correct buffer for each fractionation.

## Discussion of Above Process (Spectrophotometry)

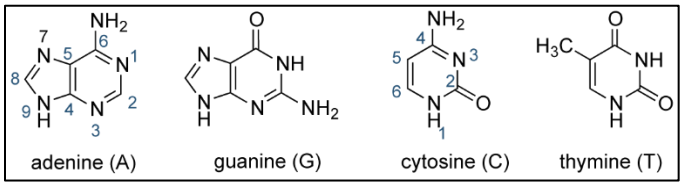


Image 8: Heterocyclic Rings of Nucleotides [7]

Spectrophotometry is a simple way to calculate a quantified mass contained in the DNA Isolation. The spectrophotometer sends light beams of specific wavelengths through a test sample solution. It has a sensor that then calculates the amount of transmitted light and the amount of absorbed light. Heterocyclic rings (Image 8) of DNA absorb light with $\lambda$ = 260 nm. Using the amount of absorbed light, the machine can calculate the concentration of DNA in any sample. By testing the concentration of the DNA prep before it is injected into the chromatograph, and then testing concentration of the elution fractionations from the end of the chromatography run, conservation of mass can be used to determine the percentage of DNA recovered from the initial sample.

Drawbacks of this method are that blanking the spectrophotometer can be very difficult. If it is not blanked correctly, then all of the numbers generated by the machine will be inaccurate. Sometimes those inaccuracies can be VERY inaccurate. If the blanking is done correctly, then the information gleaned from this apparatus is extremely valuable.

# Chapter 3 – Results

Using the above described methods for ion-exchange chromatography the following tests were performed:

**Tests 5.1.3 and 5.1.4**

Procedure:

1. The chromatograph was prepared as was described above.

2. Into 1140 µl of HBC buffer with pH of 4.6, 60 µl of prepared plasmid DNA (this DNA prep was dilute, 4 parts HBC and 1 part DNA in H2O) was added. The dilution was due to limited supply of plasmid. This was then drawn into a 1ml syringe.

3. The tip of the syringe was placed into the loading port on the chromatograph and loaded gently into the sample loop.

4. Using method 8, described above, the sample was injected into the filter in HBC buffer, then a wash was completed using 20 mL of HBC Buffer in linear gradient with PE Buffer, PE buffer going from 0% to 100% and HBC Buffer going from 100% to 0%.

5. Next 15 ml of the elution buffer (milli-Q Water) was injected through the exterior sample line.

The chromatograms produced by these tests:

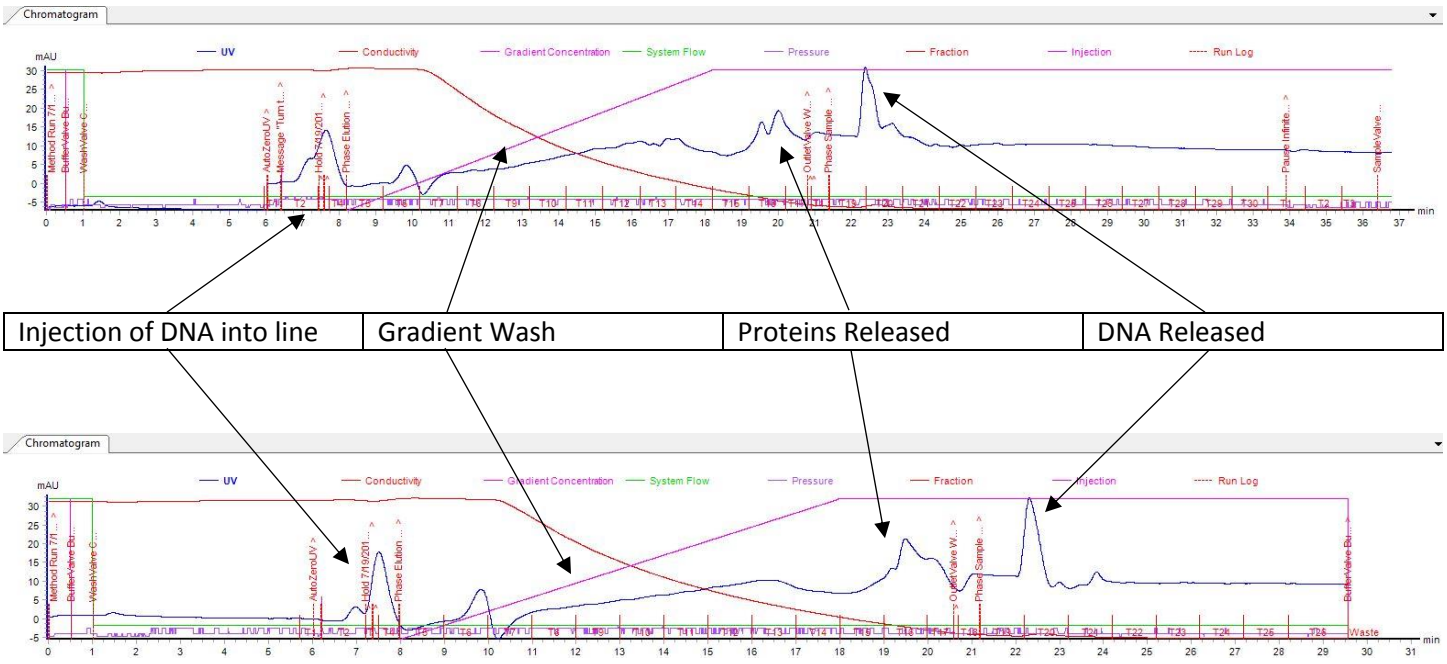


Image 12: Chromatograms produced by the AKTA Chromatograph and Unicorn One Software for tests 5.1.3 and 5.1.4.

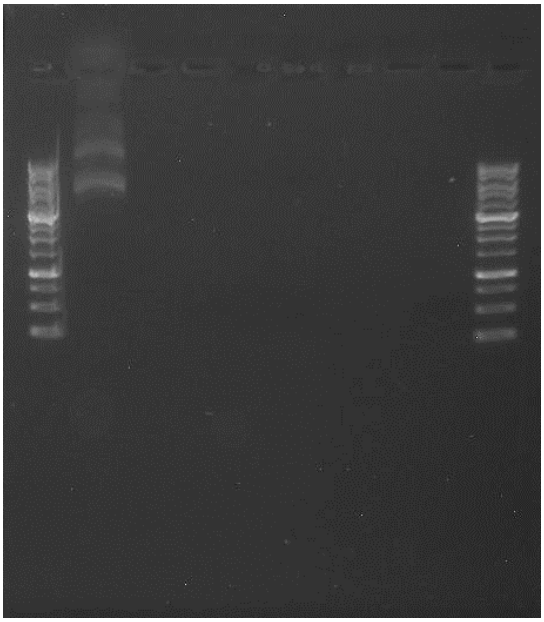

Image 13: Agarose gel from DNA fractions of test 5.1.3 and 5.1.4. The ladders are seen on both sides, and the DNA prep is shown on the second column from the left. Very little light was detected on the rest of the gel due to the very low concentration of DNA injected and the even lower concentrations of DNA recovered. This demonstrated the limitations of the Agarose Gel when working with small quantities of DNA.

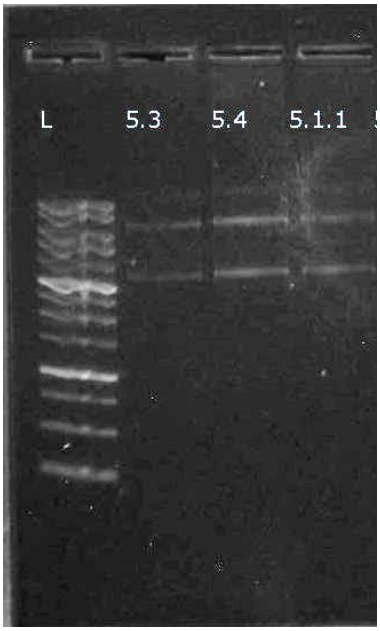


Image 14: Agarose gel from DNA fractions of test 5.3 and 5.4 (Not to be confused with 5.1.3 and 5.1.4-they were different tests). The ladder is seen on the left and the eluted DNA is shown on the second, third and fourth column from the left. This test was completed using a much higher concentration of Plasmid, so the recovered DNA still had high enough concentrations to show up on the Gel.

Spectrophotometry Readings:

| Object | Injected Volume uL | Spectrophotometer Readings for Concentration ng/uL | DNA Recovered ng | Total Injected DNA ng | % Recovered DNA |
|---|---|---|---|---|---|
| DNA Prep | 1000 | 5.88 | | 5800 | |
| | | | | | |
| 5.1.3 (19) | 1000 | 1.945 | 1945 | | 33.53% |
| | | | | | |
| 5.1.3 (20) | 1000 | 2.299 | 2299 | | 39.64% |
| | | | | | |
| 5.1.3 (21) | 1000 | 0.893 | 893 | | 15.40% |
| | | | | | |
| 5.1.3 (22) | 1000 | 1.065 | 1065 | | 18.36% |
| | | | | Total: | 106.93% |

| Object | Injected Vol-ume uL | Spectrophotometer Readings for Concentration ng/uL | DNA Recovered ng | Total Injected DNA ng | % Recovered DNA |
|---|---|---|---|---|---|
| DNA Prep | | 5.88 | | 5800 | |
| | | | | | |
| 5.1.4 (19) | 1000 | 1.278 | 1278 | | 22.03% |
| | | | | | |
| 5.1.4 (20) | 1000 | 3.96 | 3960 | | 68.28% |
| | | | | | |
| 5.1.4 (21) | 1000 | 1.129 | 1129 | | 19.47% |
| | | | | Total | 109.78% |

Image 15: Spectrophotometry readings for tests 5.1.3 and 5.1.4. Obviously, the readings are not 100% correct, because the collected DNA totals more than the injected DNA in both cases. This could be because of faulty equipment, imperfect blanking, though perfection was the goal, or possibly DNA contamination within the chromatography system of which we were not aware (again, the goal is a clean chromatograph and accurate readings).

The results acquired by these two tests as well as multiple other tests, show that, with surety, the borosilicate filters are able to capture DNA molecules, retain them during a wash phase, and release them when eluted with water. Evaluation and perfect quantification of the results are a struggle, but using Real Time PCR, the results can be better determined.

Continuing Research:

This work was initially begun to attempt to isolate DNA without having to buy kits that cost up to $100 per test. The filters were inexpensive (about $60.00 for 100 filters). What we did not anticipate was the cost of making the buffers. Gu HCl is a fairly expensive chemical. Using a chromatograph to complete this analysis required a fairly large amount of these buffers. In the future, it would be great to establish a protocol which would allow for the elimination of the chromatograph, so that this procedure could easily be completed at a high school or a small research facility with limited budgets. Using centrifugation or a vacuum, this same procedure could be completed using mini-centrifuge tubes and just a few very inexpensive supplies.

Another aspect related to this research that would be interesting to study is whether or not this same separation would work with very low quality or crude plasmid samples. For example, high school students often grow bacterial cultures for study, and they also capture DNA from fruits and vegetables. It would be interesting to see if they could then isolate that DNA from impurities, enough to be able to actually study that DNA in downstream processes.

If this protocol can be verified through other studies, it could be used as an inexpensive comparison procedure in the development of new filtration methods in the future.

Overall Conclusion:

This project was completed by a research group including physics students, a high school physics teacher and chemistry students and their professors. For the physics students, this was an enormous undertaking as they did not have the biological background knowledge. The method developed here can allow for biological studies by cross curricular organizations. The protocols were developed for people with even only a little understanding to be able to successfully isolate DNA. When these coordinated efforts happen, the knowledge, abilities and understanding of the different groups can merge and become greater. This type of work will drive the research of the future, will allow for the development of new techniques and may even lead to the development of new fundamental technologies or medical cures.

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