Lab BCH2333

Section: 17 (Thursday evening, 1st week)

Lab 5: Isolation and Characterization of Bacterial DNA

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Team #: 12

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Purpose and Techniques:

The purpose of this lab is to extract DNA from *E.coli* cells. We used the melting technique to determine the effect of using a salt and a denaturing agent on the structure of a commercial source of DNA from *E.coli* cells. We also prepared a sample of DNA in the lab and used the same melting technique to compare how the homogeneity and integrity of the DNA changes. The hyperchromic effect is used as a parameter for this comparison. Furthermore, we also determined the purity of our prepared DNA sample at absorbance values of 234nm, 260nm and 280nm. The ratios of these absorbances was calculated and compared to a known ideal range to determine whether the sample was contaminated. Agarose gel electrophoresis is used for plasmid mapping and the enzymes EcoR1 and Hind 111 were used for this analysis in order to cut the plasmid at different restriction sites. The bands were compared against a known molecular weight marker, to determine the weight of each strand.

R1:

Ultraviolet spectroscopy (UV) can be used as a measure for the purity of a DNA preparation. Since there are purine and pyrimidine bases present in both DNA and RNA their maximum UV absorption is at 260nm. The best absorbance at this wavelength would be between 0.4-0.6. If the absorbance is higher than this, we dilute the sample with water. Since absorption varies due to changes in pH, we use the ratio of UV absorption of a DNA preparation at 260nm and 280nm to characterize DNA. The A_{260}/A_{280} ratio should ideally be between 1.8 and 1.9. This ratio is a function of the relative amounts of the different nucleotides in a specific DNA preparation. If the ratio is higher than this range, then it is probably due to RNA contamination. However, for protein contamination, if the ratio of A_{234}/A_{260} is above 0.5, then there is evidence that there is protein contamination of the DNA preparation.

Table 1: UV absorbance of the *Ecoli* DNA preparation at wavelengths of 234, 260 and 280nm. The table below shows the UV absorbance of the Ecoli DNA sample at three different wavelengths, with and without dilution. The dilution factor was 0.5 (100µL of DNA sample and 900µL of water were added, instead of 200µL of the DNA sample and 800µL of water), since the absorbance at 260nm without dilution was approximately twice the ideal range. The ideal range of absorbance at 260nm is 0.4-0.6. The DNA sample has a final volume of 1mL in 15mM citrate buffer and has a pH of 7.0. The spectrophotometer is blanked with the water and citrate buffer, prior to the measurement of absorbances. The calculation for the ratios of absorbance at 260 and 280nm for the diluted sample are shown below the table. Only the absorbance of the undiluted sample at 260nm is considered since that is the maximum absorbance wavelength and hold most importance.

With Dilution		Without Dilution	
Wavelength (nm)	Absorbance	Wavelength (nm)	Absorbance
234	0.769	260	1.24
260	0.663		
280	0.441		

Calculation for the dilution factor:

A_1: Absorbance of undiluted DNA sample (at 260nm) = 1.240

V_1: Unknown volume of DNA sample in diluted preparation=?

A_2: Required (ideal) absorbance of DNA sample (at 260nm) = 0.45

V_2: Volume of DNA sample added (in undiluted sample) = 200µL

$A_1 * V_1 = A_2 * V_2$

$1.240 * V_1 = 0.45 * 200$

$V_1 = (0.45 * 200)/ 1.24$

$V_1 = 72.5\ \mu L$

Dilution factor= V_1/V_2

= 72.5µL/200µL

= 0.3625

The above value is the factor by which our DNA sample should have been diluted in order to lower the absorbance. However, we did not use this exact value and used an approximation instead. The dilution factor that we used in the actual experiment was **0.5** and we added 100µL of the DNA sample and 900µL of water.

Calculation for the ratios:

A_{260}: Absorbance of prepared (diluted) DNA sample at 260nm= 0.663

A_{280}: Absorbance of prepared (diluted) DNA sample at 280nm= 0.441

Ratio= A_{260}/ A_{280}

Ratio= 0.663/0.441

Ratio= 1.503

A_{234}: Absorbance of prepared (diluted) DNA sample at 234nm= 0.769

A_{260}: Absorbance of prepared (diluted) DNA sample at 260nm= 0.663

Ratio= A_{260}/ A_{280}

Ratio= 0.769/0.663

Ratio= 1.16

Comment:

The above calculations show that the ratio A_{260}/A_{280} is much lower than the 1.8-1.9 ideal range and the ratio A_{234}/A_{260} is much higher than the 0.5 cut-off. This tells us that the DNA preparation was not pure and was probably contaminated with protein since the calculated ratio of absorbances is a lot higher than 0.5. The sample is probably not RNA contaminated since the calculated ratio is not above the 1.8-1.9 range but it is instead below.

R2:

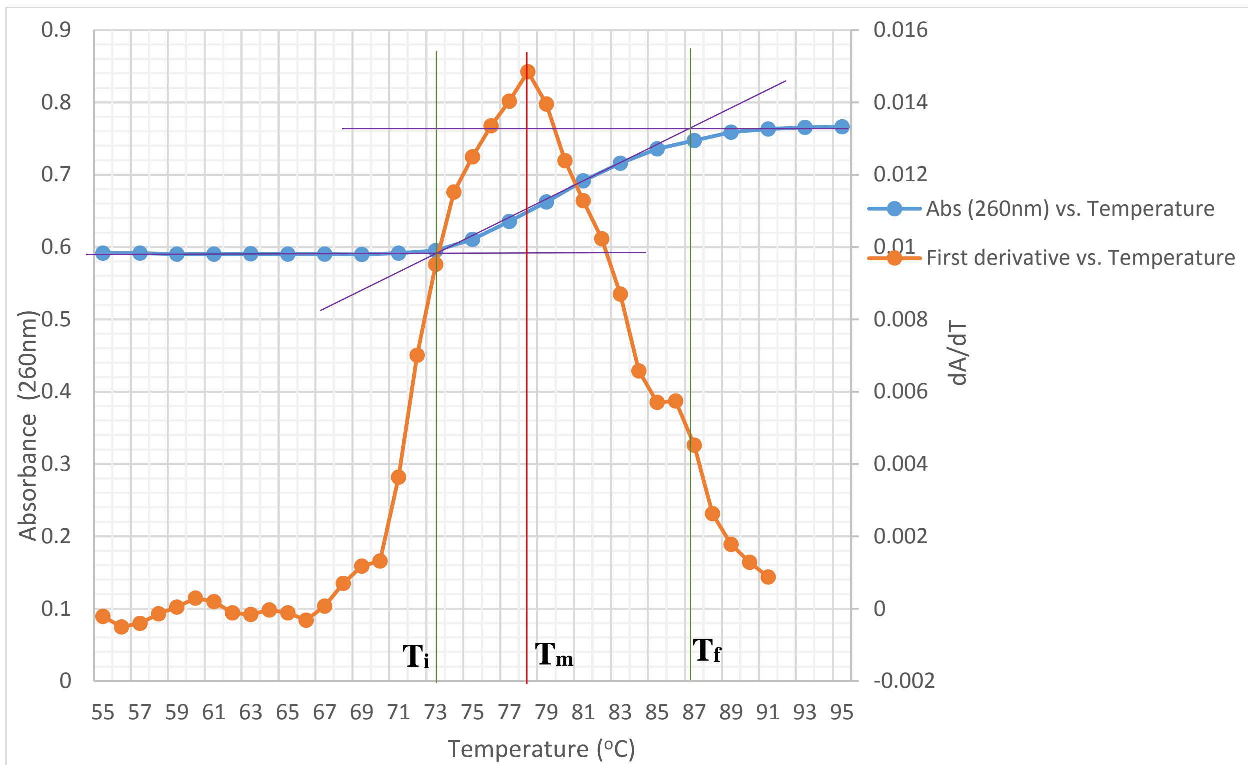


Figure 1: DNA melting curve demonstrating the variation in absorbance (at 260nm) with temperature of a sample of commercial *E.coli* DNA (solution a). The graph above shows how the absorbance of a solution containing 50µL of commercial *E.coli* DNA with a 1:20 dilution factor in 950 μL of 15 mM citrate buffer at pH 7, changes with temperature. The change in absorbance values can be attributed to the process of denaturation.The absorbances are measured using a Cary 100Bio spectrophotomer at 260nm, because that is the maximum wavelength of absorbance for most DNA samples. The melting curve and the first derivative curve are both plotted using group nine's data. The blue curve in the graph above is the melting curve where absorbances are plotted against increasing temperature. The orange curve is the plot for the first derivative (dA/dT) of the measured absorbances, against increasing temperature. The labelled vertical line T_i is the initial temperature which indicates the beginning of the hyperchromic shift, T_f is the final temperature which indicates the end of the hyperchromic shift, and T_m is melting temperatures or inflexion point for the DNA sample. The absorbances A_s and A_d, not shown on the graph above are the absorbance values for the single and double-stranded DNA corresponding to T_f and T_i respectively. T_i, T_f and T_m for this DNA sample have values 73.52, 87.47 and 80.17°C respectively.

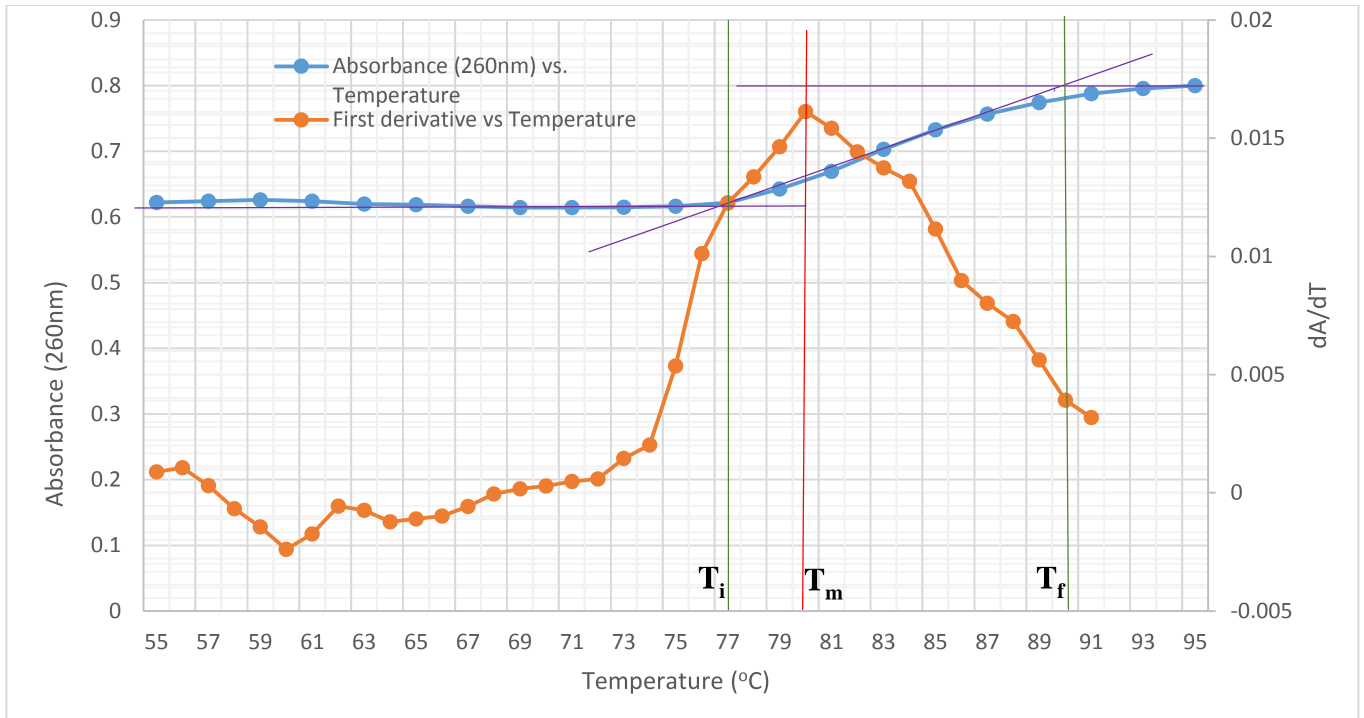


Figure 2: DNA melting curve demonstrating the variation in absorbance (at 260nm) with increasing temperature of a sample of commercial *E.coli* DNA with a low salt concentration (solution b). The graph above shows how the absorbance of a solution containing 50µL of commercial *E.coli* DNA with a 1:20 dilution factor in 950 μL of 15 mM citrate buffer at pH 7 with 0.03 M $NaClO_4$, changes with temperature. The change in absorbance can be attributed to the process of denaturation. The absorbances are measured using a Cary 100Bio spectrophotomer at 260nm, because that is the maximum wavelength of absorbance for most DNA samples. The melting curve and the first derivative curve are both plotted using group ten's data. The blue curve in the graph above is the melting curve where absorbances are plotted against increasing temperature. The orange curve is the plot for the first derivative (dA/dT) of the measured absorbances, against increasing temperature. The labelled vertical line T_i is the initial temperature which indicates the beginning of the hyperchromic shift, T_f is the final temperature which indicates the end of the hyperchromic shift, and T_m is melting temperatures or inflexion point for the DNA sample. The absorbances A_s and A_d, not shown on the graph above are the absorbance values for the single and double-stranded DNA corresponding to T_f and T_i respectively. T_i, T_f and T_m for this DNA sample have values 77.82, 90.73 and 82.50ºC respectively.

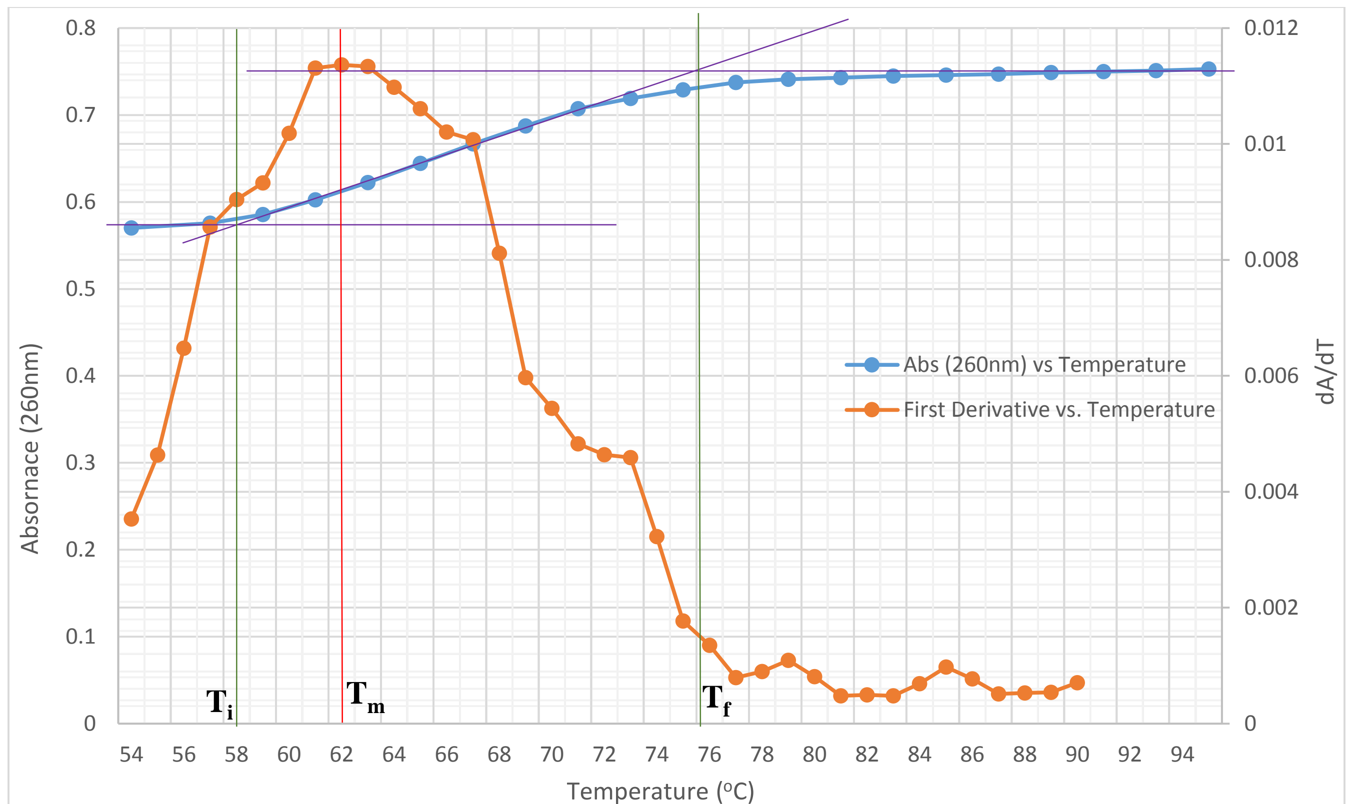


Figure 3: DNA melting curve demonstrating the variation in absorbance (at 260nm) with increasing temperature of a sample of commercial *E.coli* DNA with a high salt concentration (solution c). The graph above shows how the absorbance of a solution containing 50μL of commercial *E.coli* DNA with a 1:20 dilution factor in 950 μL of 15 mM citrate buffer at pH 7 with 4.75 M $NaClO_4$, changes with temperature. The change in absorbance can be attributed to the process of denaturation. The absorbances are measured using a Cary 100Bio spectrophotomer at 260nm, because that is the maximum wavelength of absorbance for most DNA samples. The melting curve and the first derivative curve are both plotted using group seven's data. The blue curve in the graph above is the melting curve where absorbances are plotted against increasing temperature. The orange curve is the plot for the first derivative (dA/dT) of the measured absorbances, against increasing temperature. The labelled vertical line T_i is the initial temperature which indicates the beginning of the hyperchromic shift, T_f is the final temperature which indicates the end of the hyperchromic shift, and T_m is melting temperatures or inflexion point for the DNA sample. The absorbances A_s and A_d, not shown on the graph above are the absorbance values for the single and double-stranded DNA corresponding to T_f and T_i respectively. T_i, T_f and T_m for this DNA sample have values 58.07, 76.05 and 64.92ºC respectively.

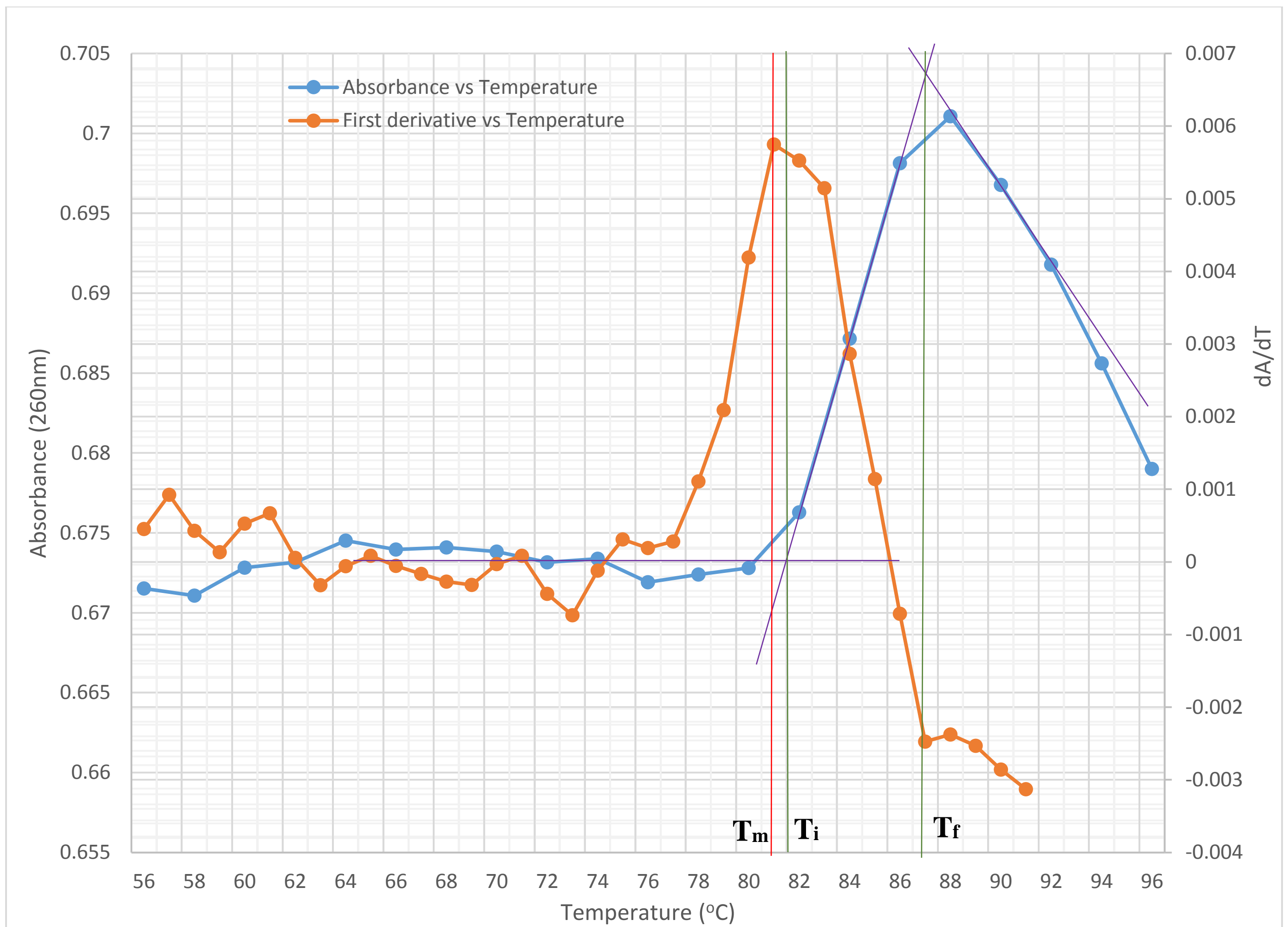


Figure 4: DNA melting curve demonstrating the variation in absorbance (at 260nm) with increasing temperature of DNA extracted experimentally (solution d). The graph above shows how the absorbance of a solution containing 200µL of a preparation of extracted *E.coli* DNA with a 1:5 dilution factor in 800 μL of 15 mM citrate buffer at pH 7, changes with temperature. The change in absorbance can be attributed to the process of denaturation. The absorbances are measured using a Cary 100Bio spectrophotomer at 260nm, because that is the maximum wavelength of absorbance for most DNA samples. The melting curve and the first derivative curve are both plotted using group ten's data. The blue curve in the graph above is the melting curve where absorbances are plotted against increasing temperature. The orange curve is the plot for the first derivative (dA/dT) of the measured absorbances, against increasing temperature. The labelled vertical line T_i is the initial temperature which indicates the beginning of the hyperchromic shift, T_f is the final temperature which indicates the end of the hyperchromic shift, and T_m is melting temperatures or inflexion point for the DNA sample. The absorbances A_s and A_d, not shown on the graph above are the absorbance values for the single and double-stranded DNA corresponding to T_f and T_i respectively. T_i, T_f and T_m for this DNA sample have values 81.18, 87.22and 83.42°C respectively.

Table 2: DNA melting results obtained from the melting curves of absorbance variation at 260nm with increasing temperature. The table below shows the values for the melting temperature (T_m), the hyperchromicity (h%), the change in temperature (ΔT) and the ratio (h/ΔT) obtained for the four sample solution using data from the melting curves. Absorbances are measured at 260nm. Solution a contains 50µL of commercial E.coli DNA with a 1:20 dilution factor in 950 µL of 15 mM citrate buffer at pH 7, solution b contains 50µL of commercial *E.coli* DNA with a 1:20 dilution factor in 950 *µL* of 15 mM citrate buffer at pH 7 with 0.03 M $NaClO_4$, solution c contains 50µL of commercial E.coli DNA with a 1:20 dilution factor in 950 µL of 15 mM citrate buffer at pH 7 with 4.75 M $NaClO_4$ and solution d contains 200µL of a preparation of extracted *E.coli* DNA with a 1:5 dilution factor in 800µL of 15 mM citrate buffer at pH 7. T_m is the maximum or inflexion point of the graph for the first derivative (dA/dT). ΔT is difference in the final and initial temperature for each sample. For solution a, T_i= 73.52 ºC and T_f= 87.47 ºC. For solution b, T_i= 77.82 ºC and T_f= 90.73 ºC. For solution c, T_i= 58.07 ºC and T_f= 76.05 ºC. For solution d, Ti= 81.18 ºC and Tf= 87.22ºC. The hyperchromicity is essentially a percent difference between the absorbance of the single-stranded (A_s) and double-stranded DNA (A_d). Solution a has A_s and A_d 0.763 and 0.592. Solution b has A_s and A_d 0.799 and 0.622. Solution c has A_s and A_d 0.751 and 0.576. Solution d has A_s and A_d 0.701 and 0.673.

DNA sample	T_m (ºC)	ΔT (ºC)	h (%)	h/ΔT (ºC^{-1})
a) Commercial (Solution a)	80.17	13.95	28.89	2.07
b) Commercial + 0.03M $NaClO_4$ (Solution b)	82.5	12.91	28.45	2.20
c) Commercial + 4.75M $NaClO_4$ (Solution c)	64.92	17.98	30.38	1.69
d) Extracted DNA (Solution d)	83.42	6.04	4.16	0.689

<u>Sample Calculations</u>:

The calculations below are shown for solution a i.e. the commercial solution.

<u>Calculation for ΔT:</u>

T_i: Initial Temperature= 73.52ºC

T_f: Final Temperature= 87.47ºC

$\Delta T = T_f - T_i$

$\Delta T = 87.47 - 73.52$

$\Delta T = 13.95\ ^oC$

The value above shows the temperature range for the hypochromic shift of DNA in solution a (commercial *E.coli* DNA.

Calculation for hyperchromicity (h):

A_s: Absorbance of the single-stranded DNA= 0.763

A_d: Absorbance of the double-stranded DNA= 0.592

$h\% = [100 * (A_s - A_d)] \div A_d$

$h\% = [100 * (0.763 - 0.592)] \div 0.592$

h%= 28.89%

This value is the hyperchromicity of the DNA in solution a.

Calculation for h /ΔT ratio:

h: Hyperchromicity = 28.89%

ΔT: Temperature range of the hypochromic shift = 13.95°C

Ratio= h/ΔT

Ratio= 28.89/13.95

Ratio= 2.07 $^oC^{-1}$

The above value shows the ratio between the hyperchromicity and the temperature range for the DNA in solution a.

R3:

DNA exists in a double helix structure which is stabilized by hydrogen bonds between complementary nucleotide bases on opposite strands and hydrophobic interactions between the stacked bases of each strand. When DNA is exposed to different concentrations of salts, it denatures and uncoils. This causes an increase in the polarity of the environment of the nucleotide bases and increases the absorbance of the solution (at 260 nm). This increase is called the hyperchromic shift. Another parameter that is used to assess the effect of salt concentrations on DNA is its melting temperature, T_m. This is obtained from the thermal denaturation curve, which is of the slope of the absorbance curve and temperature. T_m corresponds to the inflexion point of the curve, which is the maximum point until which the energy required for the dissociation of the double-stranded DNA decreases. For solution a, a commercial source of *E.coli* DNA was used which was not mixed with any salt. Solution b contained the commercial *E. coli* DNA in 15 mM citrate, pH 7 of 0.03M $NaClO_4$, which is a low concentration of salt. Solution c contained a high concentration of salt i.e. commercial *E. coli* DNA in 15 mM citrate, pH 7 of 4.75M $NaClO_4$. Solution d, which was the extracted DNA sample that we prepared, contained no salt. The T_m values for these solutions were 80.17°C, 82.5°C, 64.92°C and 83.42°C respectively. These values are reliable since we know that salts in the range 0.01 to 1 M will increase the T_m because cations surround the phosphate backbone which is negatively charged and reduce the repulsion between different chains of DNA, which can destabilize the double helix. On the other hand, higher salt concentrations (from 1 to 4 M), decrease in the T_m value since the anions in solution break the hydrogen bonds between the complementary strands causing a destabilization on the double helix. ClO_4^- anions cause an even larger decrease in the T_m value since they are very strongly negatively charged.

If the hyperchromicity of each of the four solutions is considered, then the values for solution a, b, c and d are: 28.89%, 28.45%, 30.38% and 4.16%, respectively. Since all of these values are less than 40%, we can say that the DNA was relatively impure. This could also be because of the fact that the DNA might be partially denatured, shortened or contaminated with UV absorbing materials. The value for the solution c is relatively higher than solution a, b and d which makes sense because as the salt concentration increases, the amount of denatured DNA increases and there is less contamination of the denatured DNA with the double-stranded DNA. Due to this difference in the absorbances A_s and A_d is small. Our extracted DNA sample had a very small hyperchromic shift which tells us that it was contaminated with protein (as determined earlier). Furthermore, ΔT for solution c is much higher (17.98^oC) is a lot higher than all the other solutions since this solution contains a higher concentration of salt and so the more negative solution destabilizes the double-stranded DNA more.

R4:

It was determined earlier (in question R1) that the extracted DNA sample that we prepared was contaminated by protein. This conclusion can be further justified by looking at the values obtained for T_m and hyperchromicity. The T_m value for our extracted DNA is 83.42^oC, which is similar to the T_m values for solutions a and b (80.17^oC and 82.5^oC). This is because all of these solutions either have no salt or only a small concentration of salt added to them. The ΔT value for the extracted DNA is very small i.e. 6.04^oC. This further tells us that our DNA sample was not homogenous and was contaminated with other agents. The $h/\Delta T$ value for the extracted DNA was also very small (0.689 $^oC^{-1}$), inidicating that the DNA was impure and not intact. Lastly, the hyperchromicity of the extracted DNA was only 4.16%. A very small hyperchromic shift indicates relatively impure DNA. This could also mean that the DNA is already partially denatured, shortened or contaminated with UV absorbing materials. The shape of the temperature profile can

also be used as an indication of integrity and homogeneity. Since our graph is not smooth and the T_i value occurs after the T_m value, we can say that our sample was not pure and was denatured. Further trials and multiple DNA samples could be used to improve the results.

Restriction Digestion and Gel Electrophoresis:

R5

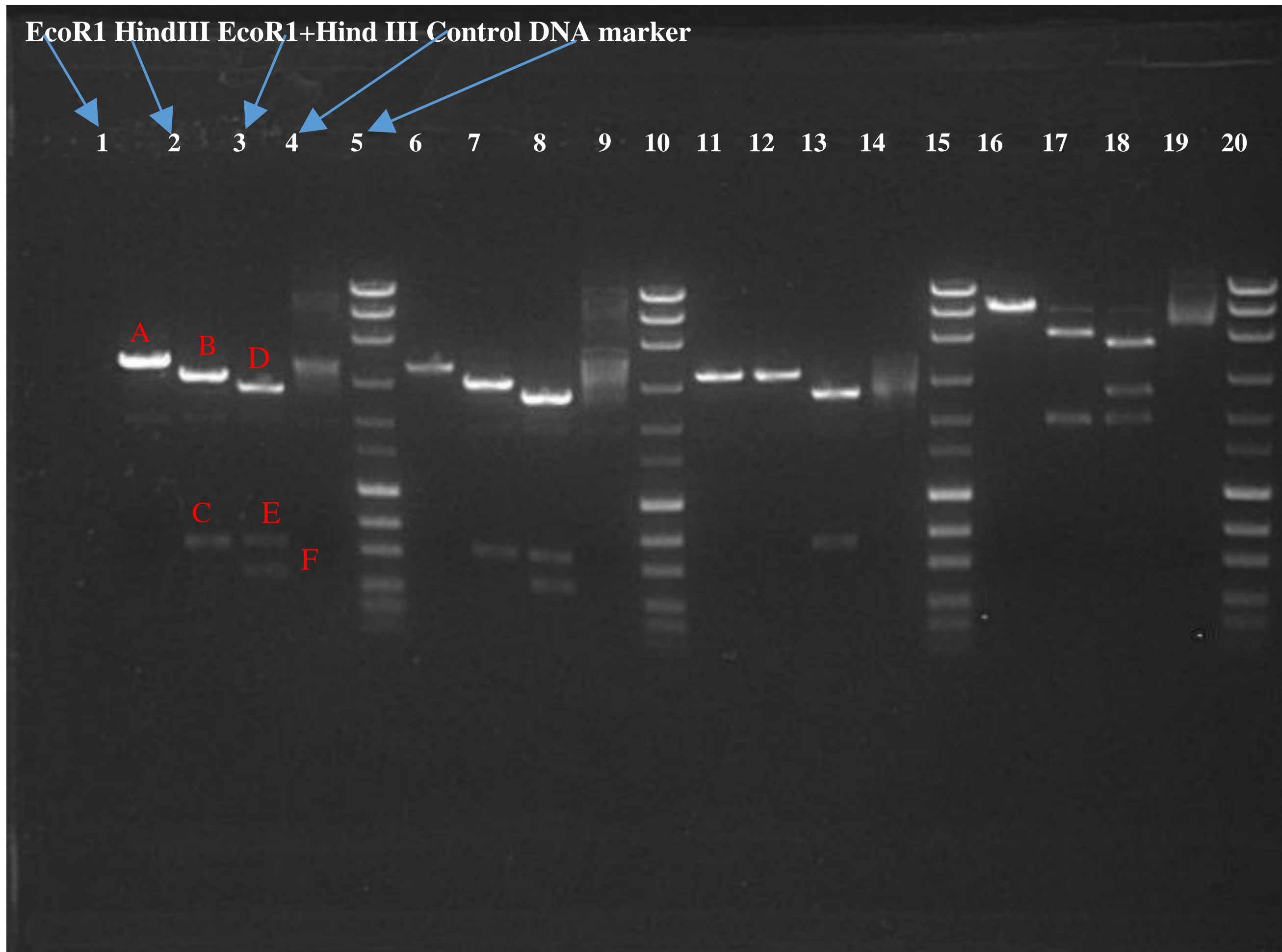


Figure 5: Agarose gel showing the bands obtained when a plasmid sample was exposed to restriction enzymes EcoR1 and Hind III, along with a ladder of DNA marker. Agarose gels are prepared by mixing agarose powder with buffer solution to a final concentration of 1%, followed by heating until a clear solution is obtained. DNA fragments are visualized by staining with 10 µL of SYBR-safe and then viewing the gel under UV-light after samples have been allowed to separate. An unknown plasmid (100 ng/µL) is digested with a combination of two different restriction enzymes: EcoRI (10 U/µL) and HindIII (10 U/µL). Each of the four mixtures (EcoR1, Hind III, EcoR1 + Hind III and the control), contained 2µL of the 10X digestion buffer (Buffer O) and 6µL of the unknown plasmid (Plasmid # 41). In addition to this the EcoRI mixture contained 11µL of H_20, and 1µL of EcoRI, the Hind III mixture contained 11µL of H_20, and 1 µL of

HindIII, The EcoRI + Hind III mixture contained 10µL of H_20 and 1µL each of EcoRI and HindIII, and the control mixture contained 12 µL of H_2O. The total volume of each mixture was 20µL and the four tubes were incubated for an hour at 37ºC. 10 µL of the provided *MassRuler Express* Forward DNA ladder marker (M) and 15 µL of the samples are loaded on to the gel and are allowed to separate at a 100V power for 45 minutes. The gel and the running tray are placed on the UV Gel Doc System. The digested plasmid fragments are also labelled (A, B, C, D and E).

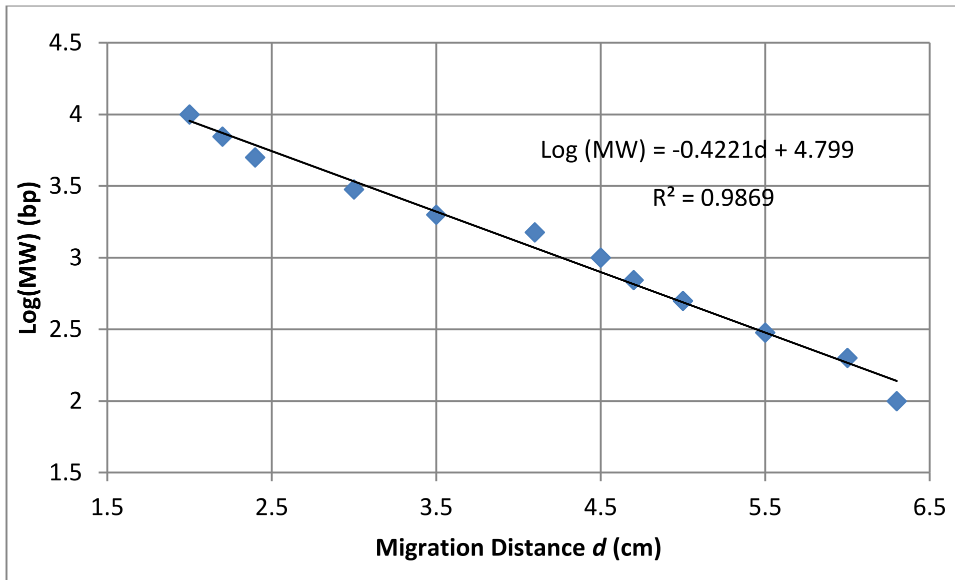


Figure 6: Linear regression of the log of fragment length from the DNA ladder against the migration distance (d). The values for the log (length in bp), and the following information were both taken from appendix C of the lab manual "Introduction to Biochemistry 2333, Winter 2015, Josée Coutu and Luc Poitras, University of Ottawa". The *MassRuler Express Forward DNA ladder* used for the gel electrophoresis is constituted of 12 DNA fragments of sizes varying from 100 base pairs (bp) to 10,000 bp. 10µL of this maker is loaded on to the gel and is allowed to separate along with the sample mixtures at 100V for 45 minutes. The linear DNA fragments migrate through the agarose gel with a mobility that is inversely proportional to the log of their molecular weight. The distance that each fragment used is measured using a standard ruler (in cm). The equation of the graph is shown and it is used to find the length of the plasmid fragments. In order to improve the correlation (R^2) value, the plot points for the last two bands (100bp and 200bp) are removed.

Calculation for length of fragment:

The calculation below is shown for fragment A (EcoR1 digestion).

Y: Log (MW) = Log of Unknown fragment length=?

X: d= Migration distance of fragment in cm= 2.0cm

L: Unknown Length of fragment A=?

Log (MW)= -0.4221d + 4.799

y= -0.4221 (2.85) + 4.799

y= 3.596

Log (L) = 3.596

L= $10^{3.596}$

L= 3944bp

Table 3: Length of each fragment (bp) in the *MassRuler Express Forward DNA ladder* along with its logarithm and its migration distance (in cm).

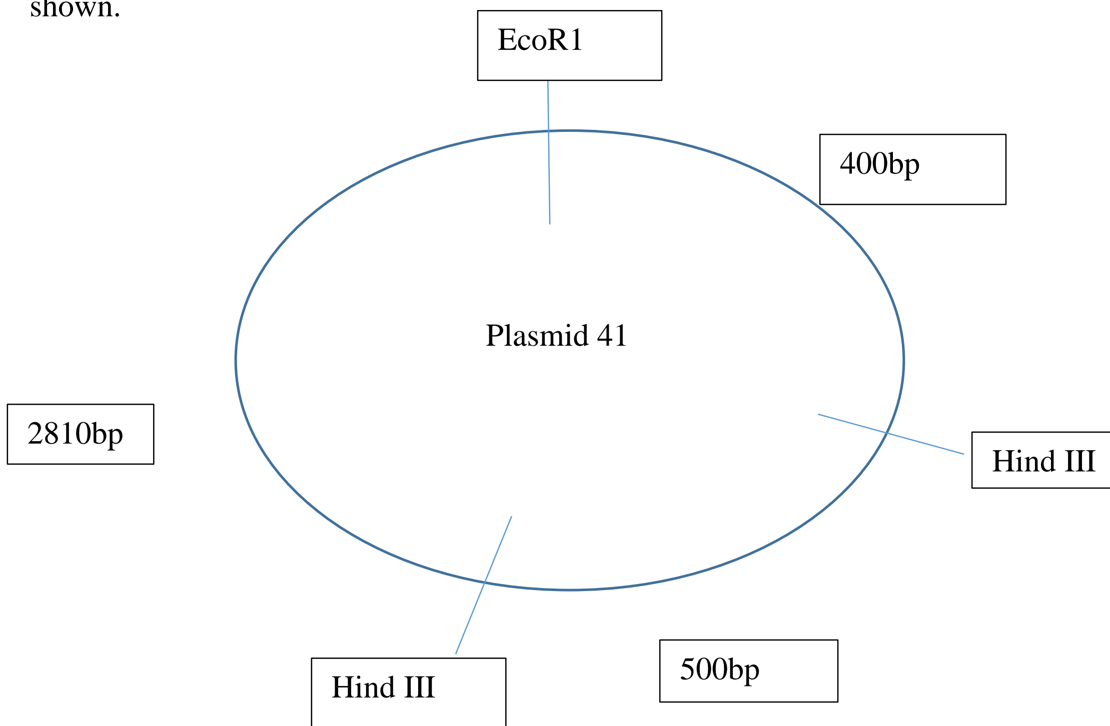
DNA Fragment Length (bp)	Log (MW)	Migration Distance (cm)
10,000	4.0	2.0
7000	3.85	2.2
5000	3.70	2.4
3000	3.48	3.0
2000	3.30	3.5
1500	3.18	4.1
1000	3.00	4.5
700	2.85	4.7
500	2.70	5.0
300	2.48	5.5
200	2.30	6.0
100	2.00	6.3

Table 4: Length of plasmid gel fragments (bp) along with their migration distances (cm). The table below shows the length of the three major plasmid fragments A, B, C, D, E and F which were obtained through agarose gel electrophoresis. Their migration distances were measured using a ruler. Fragment A corresponds to EcoR1 digestion, fragment B and C correspond to Hind III digestion, fragments D, E and F correspond to the digestion fragments obtained with a mixture of EcoR1

Plasmid fragments	Migration Distance (cm)	MW (bp)
A	2.85	3944
B	3.02	3344
C	4.98	497.7
D	3.20	2807
E	4.98	497.7
F	5.21	397.9

R6:

Figure 7: **Plasmid map of unknown plasmid #41**. The data from Table 4 is used to construct a plasmid map which shows the sites at which the EcoRI and the HindIII enzymes cut the plasmid. The length of each fragment and the point at which the enzymes cleave the plasmid are shown.



R7:

The control is undigested since there is no enzyme present and only the plasmid is present. There are two bands in the control lane. The top band is for the nicked or open plasmid strand and it is similar to the linear plasmid strands and travels slower than the second band in this lane. The second band is dark due to a super coiled form of the plasmid. The super coiled form is highly twisted and is in a compact, circular state. This travels down the gel faster because there is less hindrance to its path. The linear strands have a higher surface area and therefore experience greater resistance and travel slower. We know that the digestion was complete because of the dark bands present at the upper end of the gel. These are the fully digested linear fragments, all of similar length. Some light bands are present indicating that there was partial or no digestion of part of the plasmid ring. Light bands tell us that the enzymes did not cut the plasmid fully or properly, resulting in fragments of variable length that travel different distances along the gel.