Chapter 5

An Introduction to DNA: Spectrophotometry, Degradation, and the 'Frankengel' Experiment

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Introduction

The manipulation of DNA in the laboratory, the basis of molecular biology, depends upon a solid understanding of how the properties of DNA affect the way it is handled and analyzed. This laboratory consists of three parts, which when taken together are intended to introduce students to the basic methods used in the handling and analysis of DNA. Two of the most common analytical methods for nucleic acids, gel electrophoresis and UV spectrophotometry, will be used. The practical experience gained here will provide the groundwork necessary to proceed with other molecular experiments involving DNA.

Gel electrophoresis is one of the most common tools of molecular biology. Depending on the size of DNA being analyzed, the correct concentration of agarose in the gel is essential to permit an effective resolution of the DNA under consideration. The first part of this exercise introduces students to the protocols involved in pouring and loading a gel. They will compose a gel ("Frankengel") that consists of three concentrations of agarose. When a 1-kb DNA ladder is run on the Frankengel, students are able to visualize the resolving power of specific concentrations of agarose for different fragment sizes of DNA.

There are many myths surrounding the precautions that need to be taken when handling purified DNA. Part of the exercise is intended to investigate some of this lore. Purified DNA from three different biological sources (covalently-closed circular plasmid DNA, linear bacteriophage DNA, and high molecular weight mammalian genomic DNA) will be subjected to a variety of treatments and the resultant DNA integrity analyzed by agarose gel electrophoresis. The plasmid samples may be tested for biological activity by transformation.

The last section of this exercise introduces students to the power of UV spectrophotometry as a method to estimate DNA purity and concentration. As these are important aspects of the DNA to know before restrictions, ligations, transformation (cloning), etc. can be performed, this exercise

provides students with a basic technique. Students will use a spectrophotometer to determine the absorption spectrum of solutions of DNA and to determine their purities/ concentrations based on simple absorbance ratio determinations.

Materials

Equipment

1 P-10 Pipetman (Gilson) micropipettor, or equivalent
1 Box of tips (P-10)
6 P-20 Pipetman (Gilson) micropipettors, or equivalent
6 P-200 Pipetman (Gilson) micropipettors, or equivalent
6 Boxes of tips (P-20 and P-200)
6 Minigel horizontal electrophoresis apparati (each with a 6, 7, or 8-slot comb; removable-tray type preferable)
6 Mini power supplies with patch cords
6 125 mL glass Erlenmeyer flasks
6 100 mL graduated cylinders
6 Scalpels (or new single-edged razor blades)

Note: If equipment is limited, the laboratory may be performed using 3 of each of the items listed above.

1 Standard-sized horizontal gel electrophoresis apparatus, complete with three 10,12, or 14-slot combs (or equivalent), patch cords, and a power supply
1 500 mL glass Erlenmeyer flask
1 UV transilluminator with several UV face shields or goggles
1 Hand-held (portable) UV transilluminator
1 Autoclave (or steam bath)
1 Small boiling water bath
1 Small amount of dry ice or liquid nitrogen
1 Sonicator with microprobe (optional)
1 Top loading balance
1 37°C incubator
1 Bag of 1.5-mL microcentrifuge tubes

Chemicals:

25 g agarose
1 L 5X TBE
Six 1-mL aliquots 10X Ficoll/Orange G loading buffer
10 mL 0.25N HCl
10 mL 0.25N NaOH
10 mL TE buffer, pH 8.0
12 ccc (covalently closed circular) plasmid DNA aliquots (1 µg/100 µL each)
12 lambda bacteriophage DNA aliquots (1 µg/100 µL each)
12 eukaryotic genomic DNA (high molecular weight) aliquots (1 µg/100 µL each)
20 µg marker DNA (or equivalent) in TE buffer (e.g. '1-kb ladder', Life Technologies, Catalog number 15615-016) with Ficoll/Orange G added to 1X (See Appendix E.)
1 mL aqueous ethidium bromide (10 mg/mL)

Student Outline

A. Pouring, Reconstructing, Loading, and Running of Agarose 'Frankengels'

One of the most powerful tools used in molecular biology is agarose gel electrophoresis. Gel electrophoresis separates DNA molecules based on size. However, the concentration of agarose used in the gel is an important factor that must be taken into consideration when dealing with DNA of specific sizes. The concentration of the gel affects the ability of fragments to be separated and resolved on the gel. In this exercise, you will prepare gels of three concentrations: 0.5%, 1.0%, and 2.0% w/v, construct a Frankengel, run marker DNA on the gel, and examine the ability of each concentration of gel to resolve particular fragment sizes of DNA.

The marker DNA that you will use in this exercise is a broad range commercial product known as a "1-kb ladder" (Life Technologies™). This marker DNA contains 23 double-stranded DNA fragments ranging in size from 75 bp to 12.216 kb, which appear as a ladder when run on an agarose gel (See Figure 5.1). The marker DNA has been prepared for you in a TE buffer supplemented with Ficoll and Orange G dye. Ficoll is added to increase the density of the samples to make "submarine" loading of the sample into the well of the gel much easier. During electrophoresis, Orange G migrates in the same direction as the DNA, but more rapidly so that it does not interfere with or quench the viewing of the DNA bands, and allows you to monitor the progress of electrophoresis.

Procedure

1. Each group of students will pour one minigel of a certain agarose concentration: 0.5, 1.0, or 2.0% (w/v). Weigh out 0.25 g, 0.5 g, or 1.0 g of agarose (for the 0.5%, 1.0%, or 2.0% gel, respectively) into a 125 mL Erlenmeyer flask. Add 50 mL of 1X TBE buffer and swirl *gently* to mix the contents. Heat the mixture in a boiling water bath until completely melted (~5 minutes) or in a microwave oven. However, care must be taken with the microwave oven to avoid boiling over of the agarose. Once melted, remove the flask from the water bath and let cool for 5 minutes.

Note: Ethidium bromide (EtBr) is a mutagen! Handle with care and only while wearing gloves and a lab coat. Do not touch the area around you or any equipment while you are wearing the gloves used when dealing with EtBr. This will prevent contamination of the lab benches and equipment. Dispose of all waste in the designated containers.

2. Add 2 µL of EtBr (10 mg/mL) to the cooled, melted agarose and swirl to mix evenly. EtBr intercalates the DNA double helix and fluoresces under UV light allowing the visualization of small amounts of DNA (down to about 20 ng) in the bands resolved on the gel.

3. Pour the agarose into the gel bed following the directions of your instructor. Place the slot-former in the agarose and let the gel sit (without moving it) for 30 minutes until the gel solidifies.

   ** Part B (Degradation Treatments) can be started while the gel sets.

4. After 30 minutes, remove the slot-former and carefully slice the gel into three equal pieces. For those groups with the 1.0% gel, remove a small portion of the corner of each of the three pieces of the gel in the left-hand corner (See Figure 5.1). For those groups with the 2.0% gel, remove a small portion of the corner of each of the three pieces of the gel in both the left-hand and right-hand corners of the gel. The "corner notches" will help identify the % agarose of the gel sections when the results are photodocumented.

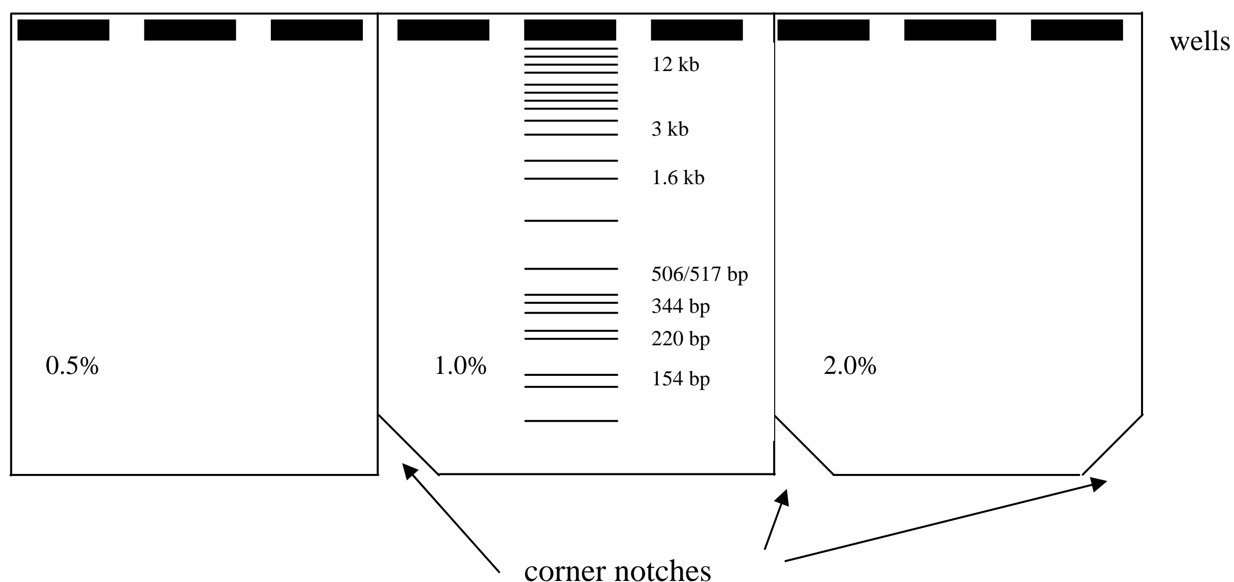

Figure 5.1: Schematic representation of a prepared Frankengel with marker DNA loaded in a central well of the 1.0% agarose section.

5. Trade gel pieces with other lab groups until you have all 3 concentrations. Construct a Frankengel using the three gel pieces with the 0.5% concentration on the left side and the 2.0% concentration on the right side (See Figure 5.1). Be especially careful when handling the 0.5% piece as it will be fragile.

6. Pour 1X TBE running buffer over the gel until it is just completely covered (~1-2 mm over surface of gel). With a micropipettor, load a central well in each of the three sections of the Frankengel with 10 µL of the 1-kb ladder DNA provided.

7. Connect the electrodes from the power supply to the gel box. Under these conditions DNA is negatively charged and will run toward the positive electrode or anode (red).

8. Run the gel at 70 V (constant voltage) for about 90 minutes or until the Orange G dye front is 1 cm away from the bottom of the gel. The resolved DNA fragments will already be stained with EtBr since it was added to the agarose before the gels were poured. Note that free EtBr itself also migrates, but in the opposite direction to that of DNA. Why?

Note! Excessive UV exposure will cause severe sunburns and is harmful to the eyes. Minimize your exposure to UV light. Use goggles, gloves, and your lab coat, and **only have the UV on when actually needed** for viewing and recording the image.

9. With the gel on the UV transilluminator use the photo-documentation system to record the gel image.
10. In your report, comment on both the staining intensity and the distribution of the various DNA fragments in the three sections of Frankengel.
11. Determine the range of linear DNA fragments that can be resolved for each of the three concentrations of agarose. Plot molecular weight (base pairs) versus distance migrated (mm) for marker DNA (e.g. 1-kb ladder) on 4-cycle semilog paper. The range is determined by the areas of the graph that produce a straight line. Do your results agree with the information provided in Table 5.1?

12. What do you expect would happen to the various DNA bands if you were to leave the Frankengel at 4°C until the next lab period? Why? Do it to test your predictions.

Table 5.1: Effective dsDNA Fragment Resolving Power of Agarose Gels *

Agarose % (w/v) in Gel	0.5	0.75	1	1.25	1.5	2
DNA Fragment Sizes (kb) Resolved	25-2	10-0.7	7-0.5	5-0.4	4-0.2	3-0.1

* Adapted from Maniatis, et al. (1982) "Molecular Cloning - A Laboratory Manual"

B. Degradation Treatments of DNA Samples Followed by Electrophoretic Analysis

With the advent of PCR and DNA forensics, and due to the extensive lore surrounding DNA handling and stability, it is informative to experiment with some of the actual conditions to which DNA samples might be exposed in the laboratory. As a class we will test various conditions which traditionally are thought to lead to denaturation and/or degradation of DNA.

Double-stranded DNA's of three different types will be treated:

P plasmid DNA (**c**ovalently-**c**losed **c**ircular, ~3 kb, common cloning vector in *E. coli*).

λ bacteriophage DNA from lambda (about 48 kb with 12 base cohesive ends, linear).

G mammalian genomic DNA (comprised of fragments all greater than 100 kb in length, linear - Note that all genomic DNA from higher organisms will be mechanically sheared to a certain extent just due to the process of extraction).

You will treat aliquots of these DNA's in a number of different ways and then analyze, by agarose gel electrophoresis, how they have been affected by these treatments.

Following treatment, the DNA is run on an agarose gel and the resultant pattern of bands is indicative of the extent of denaturation and degradation. For instance, has the DNA been degraded into small pieces (oligonucleotides), even further to the four deoxyribonucleotides, or have they aggregated, or polymerized, into huge conglomerates?

As a further test of the effect of the treatments on the biological activity of the DNA, treated plasmid samples will be tested for the ability to transform ampicillin-sensitive *E. coli* cells, making the *E. coli* ampicillin resistant.

Procedure

1. Using the supplied aliquots (1 µg/100 µL) of the three sources of DNA, expose the samples to one of the following conditions. Use a new micropipettor tip for each sample. Each group of students will be assigned a treatment.

Control: No treatment. (Reserved for the instructor).

Physical shearing: Pipet the sample up and down 20 times using a micropipettor (Bernoulli principle). Use a new tip for each of the three samples. Let the samples sit at room temperature until the other treatments are done (approximately 1 hour).

Boiling: Suspend the microfuge tubes containing the DNA aliquots in a boiling water bath for five minutes. Let the samples sit at room temperature until the other treatments are done (approximately 1 hour).

Autoclaving: Place the samples in the liquid cycle of an autoclave (121°C) for 3 minutes. Let the samples sit at room temperature until the other treatments are done (approximately 1 hour).

Sonication: Set the sonicator to a high setting. Place the samples in the sonicator for 10 seconds, then turn the sonicator off for 10 seconds. Repeat 3 times. Let the samples sit at room temperature until the other treatments are done (approximately 1 hour).

Skin nucleases: Pipet the DNA samples onto your fingers and let sit for one minute. Replace the samples into the correct microfuge tubes and incubate at 37°C for at least one hour.

Mouth nucleases: Add 2 µL saliva to each DNA sample, mix, and incubate at 37°C for at least one hour.

Freezing: Using liquid N_2, repeatedly freeze (5 seconds) and thaw (30 seconds) the three samples ten times. Let the samples sit at room temperature until the other treatments are done (approximately 1 hour).

Acid treatment: To each of the three DNA samples add 1 µL 0.25N HCl, mix, and incubate the samples at 37°C until the other treatments are done (approximately 1 hour).

Alkali treatment: To each of the three DNA samples add 1 µL 0.25N NaOH, mix, and incubate the samples at 37°C until the other treatments are done (approximately 1 hour).

UV Exposure: Pipet the three DNA samples onto a piece of plastic wrap on the UV transilluminator. Expose the samples to UV for 30 seconds; retrieve the samples. Let the samples sit at room temperature until the other treatments are done (approximately 1 hour). **Remember** that UV presents a safety hazard (See A.9. above) so use all precautions.

EtBr / UV Exposure: To each of the three DNA samples add 0.5 µL 10 mg/mL EtBr solution, pipet the three DNA samples onto a piece of plastic wrap on the UV transilluminator. Expose the samples to UV for 30 seconds; retrieve the samples. Let the samples sit at room temperature until the other treatments are done. (approximately 1 hour). **Remember** that both EtBr (See A.2. above) and UV (See A.9. above) present safety hazards.

** Complete Part A (loading and running of Frankengel) while the treatments incubate / sit.

2. To each microfuge tube containing treated DNA, add 11 µL of 10X Ficoll/Orange G, using a fresh micropipettor tip for each sample. Gently mix the samples and load 40 µL into the group gel (0.8% agarose) in the lane specified by your instructor. The gel will be run at 100V for 2 hours and photographed for you. You will be supplied with the results next week in lab.
3. Give your instructor the unused treated plasmid DNA samples. These samples will be tested for biological activity using a transformation assay of ampicillin-sensitive *E. coli* cells. Since the plasmid DNA used in this exercise carries the gene for ß-lactamase, which confers resistance to ampicillin, successful transformation and plasmid expression will result in the formation of colonies on ampicillin-containing agar plates. The results of this bioassay will be provided to you next week for you to comment upon in your lab report.

C. Spectrophotometric Analysis of DNA

The concentration of DNA in a sample, and its condition, are often estimated by running the sample on an agarose gel. Such concentration estimates are semi-quantitative at best and are time-consuming and confounded when numerous bands or a 'smear' of DNA are observed. For a more accurate determination of the concentration of DNA in a sample, a UV spectrophotometer is commonly used for DNA solutions (or a fluorometer can be used if the solution is known to be pure DNA).

The purity of a solution of DNA can be determined using a comparison of the optical density values of the solution at various wavelengths. For pure DNA, the observed 260/280 nm ratio will be near 1.8. Elevated ratios usually indicate the presence of RNA, which can be tested by running the sample, ~1µg, on an agarose gel. 260/280 ratios below 1.8 often signal the presence of a contaminating protein or phenol. Alternatively, protein or phenol contamination is indicated by 230/260 ratios greater than 0.5. Once you are sure your sample contains pure DNA, an accurate determination of the concentration of DNA can be made.

Procedure

1. You will be provided with four samples of DNA:
   - A pure DNA sample (Sample 1, double-stranded bacteriophage *lambda* DNA) dissolved in TE buffer (pH 8.0) for stability, for which you will determine an optical density (absorbance) spectrum using an UV spectrophotometer.

   - Three impure samples, which you will determine the absorbance readings at three wavelengths (230, 260, and 280 nm). Using the wavelength ratios of these samples you will attempt to determine the possible contaminants of the impure samples.

2. Using the UV spectrophotometer, set the wavelength to 230 nm, measure the absorbance of Sample 1, and record the results in Table 5.2. Repeat for each of the wavelengths in Table 5.2. Use TE buffer as the zeroing reference.

3. Graph the results to obtain an absorbance spectrum for Sample 1 (pure DNA).

4. Determine the absorbance readings for Samples 2, 3, and 4 at 230, 260, and 280 nm. Record the results in Table 5.2.

Table 5.2: DNA Spectrophotometric Spectrum

wavelength (nm)	230	240	250	260	270	280	290	300	310	320
Sample 1 (OD)										
Sample 2 (OD)										
Sample 3 (OD)										
Sample 4 (OD)										

5. Determine the 260/280 and 230/260 ratios for each of the four samples. Fill in Table 5.3 with these values.

Table 5.3: 260/280 and 230/260 Ratio Values for 4 Samples of DNA

Sample	260/280 ratio	230/260 ratio
1		
2		
3		
4		

6. Based on these ratios, how pure are the DNA samples you were given? If a DNA sample is not pure, what are the possible contaminants of the sample? At which point in the extraction procedure would these contaminants appear? What steps of the extraction procedure would you need to change to get rid of these contaminants?

7. For Sample 1, determine the concentration of the DNA in solution assuming that you are dealing with pure dsDNA (see Table 5.4).

Table 5.4: Accepted Spectrophotometric Nucleic Acid Conversion Values	
1.0 A260 Unit of double-stranded DNA	50 µg/mL
1.0 A260 Unit of single-stranded DNA	37 µg/mL
1.0 A260 Unit of RNA	40 µg/mL

Notes for the Instructor

This lab is intended to introduce students to some practical aspects of the basic ways DNA is handled and analyzed in the laboratory. It will provide the opportunity to review the nature of DNA, as discussed in your lectures. This lab relates the characteristics of DNA to its manipulation - the cornerstone of modern molecular biology.

In general, discussion should include:

- The effect that extraction and purification of the DNA from other cellular (or viral) components has on the DNA;
- The stability of extracted DNA under different conditions while in solution; and
- The properties of extracted DNA as revealed by the commonly applied analytical techniques of UV spectrophotometry and agarose gel electrophoresis.

Extraction

DNA, once extracted from cells or viruses, is typically purified such that other molecular components, most notably proteins, are removed. This ensures the stability of the DNA and allows for directed enzymatic *in vitro* manipulation. The extraction/purification process involves physical, as well as chemical procedures that lead to shearing or breakage of very high molecular weight DNA molecules (>50 kb). Gentler procedures tend to result in lower purity DNA, often contaminated with proteins, which are less stable and often resistant to directed enzymatic modification in the test tube (or more commonly, the plastic microcentrifuge tube). Also, the presence of contaminating molecules can interfere with concentration and yield determinations (see below).

Stability, following extraction

Typically, DNA, once extracted and purified, is stable when stored in TE, a Tris (hydroxymethylaminomethane) buffer at pH 8.0 containing 1 mM EDTA as a Mg^{2+} chelator (Mg^{2+} ions are required by DNAses - ubiquitous enzymes which degrade DNA.). Note that under these conditions DNA molecules are negatively charged, which affects their direction of migration during electrophoresis (See the next section.). Storage of DNA is usually at 4°C for short periods of time, or frozen when weeks, months, or years are involved.

Agarose gel electrophoresis of DNA

One dimensional horizontal "submarine" agarose slab gels are the norm for standard non-denaturing DNA electrophoresis. Double stranded (ds) DNA samples should be diluted in TE buffer, not water, prior to electrophoresis, as they require a minimal amount of ionic strength to prevent strand separation (denaturation). Ethidium bromide is often used to stain the DNA molecules for subsequent visualization under UV light. Both UV and EtBr are mutagenic and precautions must be taken when using them. EtBr, if present in solution with DNA, intercalates in the double helix and is thereby locally concentrated by the DNA, allowing visualization of small amounts of DNA. The EtBr concentration is important as it can affect the order of migration of different forms of the same DNA (*e.g.* ccc (covalently closed circular), nicked, and linear plasmid DNA; Johnson and Grossman, 1977). Normally, a final concentration of 0.1-0.5 µg/mL EtBr is used (Maniatis et al., 1982). Free EtBr, if present during electrophoresis, will migrate in the opposite direction of that of the DNA bound dye, and over time will clear from the gel during the electrophoretic run. In Appendix A, the typical Frankengel results demonstrate this unbound EtBr - note the brighter area near the well-end of the gel. Although electrophoresis allows the separation and resolution of DNA of different sizes, the determination of those sizes, and a crude estimate of the amount of DNA present, it does not give much of an indication of contaminating molecules (other than RNA) in the sample.

Spectrophotometry

DNA quantification is generally performed by ultraviolet spectrophotometric measurement of the absorption at 260 nm on a sample of the DNA in solution. The fraction of light passing through the solution is measured. Spectrophotometric analysis of DNA solutions requires an instrument with a deuterium source or other means of determining absorbances in the UV range (below 320 nm). Nucleic acids, depending on base composition, absorb maximally at about 260 nm. Proteins absorb maximally at 280 nm and 230 nm. Typically, quartz cuvettes are used to hold the samples as they do not absorb in the UV range the way most glasses and plastics do. TE buffer is used to zero the instrument ('set reference') and the DNA samples themselves should be appropriately diluted in TE buffer such that the OD_{260} readings obtained are above ~ 0.02 (to avoid statistical error) but below ~ 0.8 (Beer's Law). We typically dilute the DNA such that the OD_{260} readings are 0.15. If these precautions are followed the readings will be reliable. Generally, readings are taken at 230, 260, and 280 nm. Single beam instruments require re-zeroing, with TE as the blank, at each wavelength. The observed 260/280 ratio will be near 1.8 for pure DNA. Elevated ratios usually indicate the presence of RNA which can be confirmed by running a sample, ~ 1 µg, on an agarose gel. 260/280 ratios below 1.8 often signal the presence of contaminating protein, or phenol (from the extraction protocol). Alternatively, 230/260 ratios greater than 0.5 also suggest protein or phenol contamination (Johnson, 1981). Samples with the correct spectrophotometric ratios allow for accurate DNA concentration determinations (see Procedures in Student Outline).

Preparation of "Contaminated DNA" for the Spectrophotometry Lab

Sample 1 is pure lambda DNA diluted to a final absorbance value of approximately 0.15 at 260 nm. It must be diluted in TE buffer, pH 8.0 (for stability). Our source is lambda DNA from Gibco-BRL, catalog number 25250-010. The 260/280 ratio of pure DNA will be ~1.8.

Sample 2 is lambda DNA + RNA. Our source of RNA is yeast tRNA (Gibco-BRL, catalog number 15401-029). We added the RNA to a sample of the pure DNA at a concentration of approximately 10 µg/mL. This was enough to affect the 260/280 ratio. You will have to trouble shoot your DNA and RNA samples such that the 260/280 ratio (with RNA present) are >1.8.

Pure RNA will have a 260/280 ratio of approximately 2.2-2.3. In our experience, we have found that the 230/260 ratio is a less reliable indicator of RNA contamination than the 260/280 ratio. A 260/280 ratio >1.8 is the best indication of RNA contamination. RNA contamination can be verified by running a sample of the DNA in question on an agarose gel. RNA will be observed as a smear and will migrate further than the DNA.

Sample 3 is lambda DNA + protein. Our source of protein is nuclease-free BSA (Roche Biochemicals, catalog number 711454). It is important to use a nuclease-free protein source. We added the protein to a sample of pure DNA at a concentration of approximately 20 µg/mL. We found that 10 µg/mL was not enough protein to affect the ratio values. You will have to trouble shoot your system to obtain a 260/280 ratio (with protein present) of <1.8 and a 230/260 ratio (with protein present) of >0.5.

Sample 4 is lambda DNA + RNA + protein. The concentrations of RNA and protein are the same as above. Again, you will have to trouble shoot your system to obtain a 260/280 ratio (with RNA and protein present) of >1.8 and a 230/260 ratio (with RNA and protein present) of >0.5.

See Appendix C for typical data obtained for DNA with added RNA and/or protein.

How to Dispose of Ethidium Bromide

As ethidium bromide (EtBr) is a potential mutagen, it is essential that it is disposed of properly. The guidelines for disposal will be specific for each province/state so you should check with your institution's safety officer to obtain the proper procedures for your area. However, we have included the guidelines we follow at the University of Alberta.

EtBr-containing gels and gloves that have come in contact with the gel or the running buffer (*i.e.* the TBE buffer the gel is run in) are disposed of in a plastic bag inside an identified plastic bucket. Only EtBr waste is to enter this bag. Once the bag is full it is marked as hazardous and sent for incineration.

EtBr-containing running buffer is filtered through a charcoal filtration system. The filtration system we use is the S&S Extractor sold by VWR. The running buffer is filtered through activated charcoal, the filtrate is disposed of down the drain and the charcoal, once completely used, is sent for incineration.

Gel beds and gel boxes that have housed an EtBr gel are rinsed thoroughly with tap water.

Specific points to keep in mind

- The mass of agarose and volume of 1X TBE buffer used in the Frankengel experiment (A.1) will make 50 mL of agarose, the required amount for a Pharmacia GNA 100 gel apparatus. Depending on the type of gel bed available to you, more or less than 50 mL will be required and the mass and volume will have to be adjusted accordingly.
- The mini-gel apparatus used may be of two types: the gel bed is removable from the gel box or the gel bed and gel box are housed as one unit. Depending on which apparti your institution has, the removal of the "Frankengel" will follow different procedures. For apparati with removable gel beds, once the gel has been cut into thirds, the gel bed is lifted out of the gel box, the gates removed, and the gel pieces gently pushed off the side onto another gel bed. For apparatus with immobile gel beds, removing the gel pieces, especially

the 0.5% gel pieces is a bit more problematic. We have solved this problem by making specialized pancake flippers. Plastic pancake flippers can be trimmed to the width of the gel pieces and the flipper can then be easily slid under the pieces and the pieces removed. We also found that thin pliable plastic rulers may be placed under the pieces to remove them from the gel bed.

- The list of treatments in the student outline is not exhaustive; you may like to have your students perform others not listed here. Conversely, all treatments do not have to be done.
- For the freezing treatment (Part B), if liquid N_2 is unavailable, dry ice can be used. In this case, the sample should be exposed to dry ice for 15 seconds to permit freezing and removed for 30 seconds to permit thawing.
- For the UV exposure treatment (Part B), every transilluminator is different and over time, the transilluminator will become less effective in causing damage to the DNA. The time listed in the Student Outline (30 seconds) may not be long enough to observe a change. In addition, transilluminators may emit a 254 nm, 302 nm, or 310 nm wavelength. These wavelengths cause different amounts of damage, with 254 nm causing the most. For this aspect of the lab, it is important for you to test your transilluminator in advance to find out what works the best for your lab.
- For the transformation portion of Part B, the Ficoll/Orange G added to the DNA samples does not interfere with the transformation of plasmid DNA.
- Ideally, the students themselves could perform the transformations in a subsequent laboratory period as a follow-up exercise. However, if your assay examines the ability of plasmid DNA to confer ampicillin resistance to the transformed cells, concerns about student penicillin allergies must be kept in mind.
- Although more time consuming, some instructors may wish to stain the gel with ethidium bromide after running the DNA, instead of adding the EtBr directly to the melted agarose. The advantage of this technique is that the instructor is the only person to handle EtBr; the students do not come in contact with the mutagen. In order to stain the gel in this manner, the gel is placed in a 0.5 µg/mL EtBr solution in TBE buffer (500 mL for a standard gel, 200 mL for a mini-gel) in a plastic or glass tray for 20 minutes. The gel is then de-stained in an equal volume of TBE buffer for 30 minutes before viewing.

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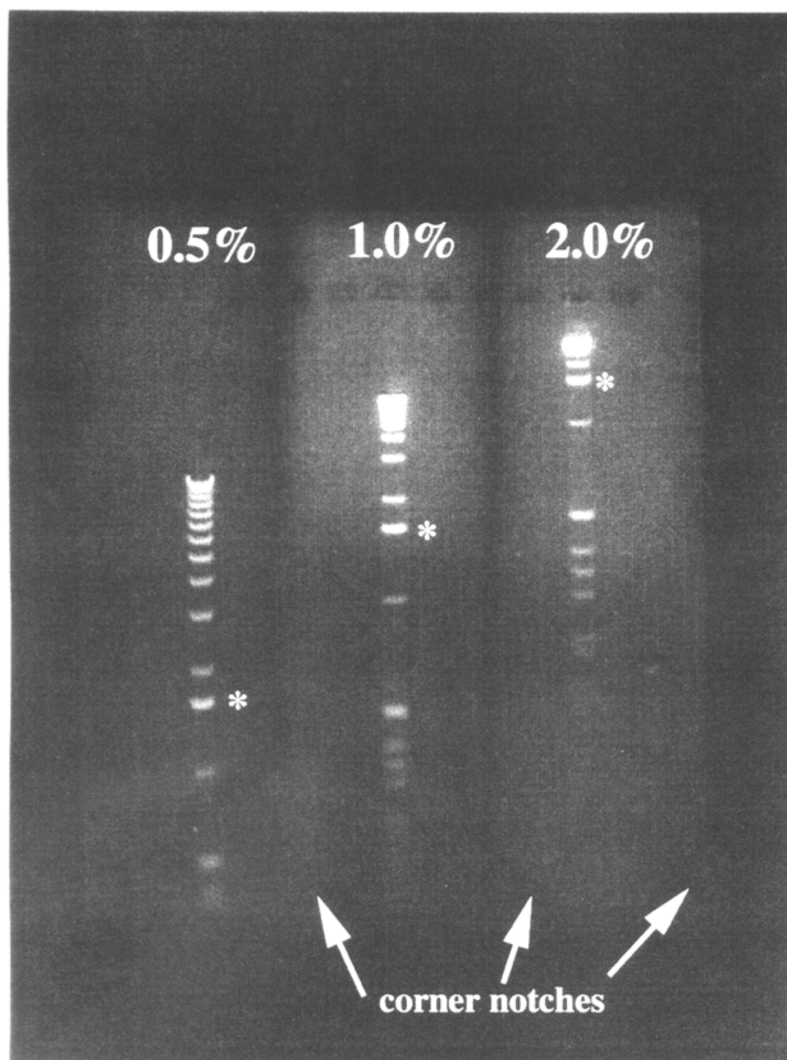
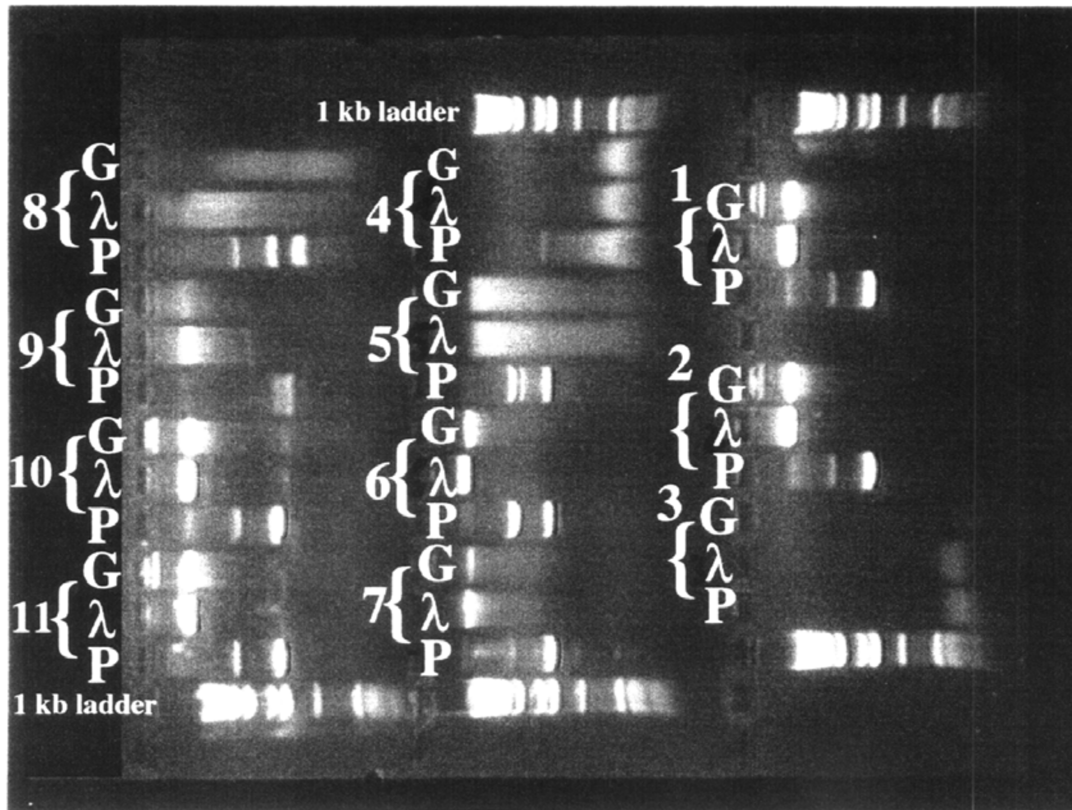
Appendix A: Typical Frankengel Results



* Indicates the 1.6 kb fragments, which always appear bright on the gel in relation to their size. The sizes of the fragments in this 1 kb ladder are as follows (in base pairs): 12 216, 11 198, 10 180, 9 162, 8 144, 7 126, 6 108, 5 090, 4 072, 3 054, 2 036, 1 636, 1 018, 506/516, 394, 344, 298, 220, 200, 154, 142, 75.

Appendix B: Typical DNA Degradation Experiment; Agarose Gel Results (0.8% agarose in TBE)



Numbers correspond to the following treatments:

1. Control, DNA not treated
2. Physical shearing
3. Autoclave
4. Sonication
5. Mouth nucleases
6. Skin nucleases
7. Freezing
8. Boiling
9. Alkali treatment
10. UV exposure
11. EtBr/UV exposure

Main points to consider with each treatment

Control: You should observe one very slow migrating band of genomic DNA since the sample placed on the gel is comprised of fragments all greater than 100 kb in length (none of which are resolved at these gel concentrations). Lambda DNA is a sample of 48 kb linear

fragments and thus a band is observed above the 12 kb molecular weight marker fragment. Circular plasmid DNA has three conformations. These are nicked (one DNA strand is broken - *i.e.* "nicked" - so the DNA cannot assume a supercoiled conformation), linear (both strands of the DNA strand are broken and the DNA molecule is linearized), and covalently closed circular (ccc; circular plasmid DNA can assume a supercoiled conformation when both DNA strands are intact). At the concentration of EtBr used in this gel (0.4 µg/mL), the three conformations of plasmid DNA will run in the following order: nicked, linear, ccc, with ccc migrating the furthest distance from the loading well. The plasmid DNA run on this gel is approximately 3 kb in length, which can be verified by comparing the molecular weight marker run on the gel to the linear form of the plasmid.

Physical shearing: The effect of physical shearing is greater on genomic DNA due to the great length of the molecules. Lambda DNA and plasmid DNA are affected to a lesser extent and any structural changes cannot be observed on the gel.

Autoclave: Autoclaving breaks all DNA types into nucleotides so no bands are observed.

Sonication: The energy of sonication will denature DNA. If the DNA samples are not kept on ice, the increase in temperature due to sonication will also cause DNA to denature. In this case, bands have migrated further through the gel compared to control.

Mouth nucleases and skin nucleases: Nucleases contain a combination of exo- and endo-nucleases, which will degrade genomic and lambda DNA due to their linear conformation. Nicked and linear plasmid DNA will be more affected than ccc because of their conformation. Nuclease activity is individual specific so results will vary depending on the student.

Freezing: The effect of freezing is not unlike that of physical shearing of the genomic DNA. Lambda DNA and plasmid DNA are not affected by this treatment.

Boiling: Boiling denatures linear DNA molecules (genomic, lambda, and non-ccc plasmid); ccc plasmid is denatured but can re-anneal due to the close proximity of the DNA strands in the "coil".

Alkali treatment: Alkali treatment will denature all DNA to single stranded (ss) DNA when the concentration is high (0.25N is high enough).

Acid treatment: Acid treatment causes hydrolysis of the sugar-phosphate backbone. DNA becomes nicked and eventually will breakdown into nucleotides. Note that alkali and acid treatments are often useful to the molecular biologist. Many DNA manipulations (*i.e.* mini plasmid preparations, Southern blotting) take advantage of the effect of alkali and acid on DNA. (The results of acid treatment are not shown but significant degradation is often observed on all three DNA types. This is also reflected in plasmid transformation results - Appendix D):

UV exposure: This has no apparent effect on DNA structure, but function is affected (*i.e.* transformation is significantly decreased, see Typical Transformation Results in Appendix D); UV exposure nicks DNA and causes formation of thymine dimers.

UV exposure + EtBr: This has no apparent effect on DNA structure, but function is affected (*i.e.* transformation is significantly decreased). However, a comparison between the transformation results of UV exposed DNA to UV exposed + EtBr DNA suggests that the presence of EtBr "protects" DNA from UV exposure. The exact mechanism for this "protection" is unknown but it is a reproducible result.

Interpretation of Degradation Treatment Results

1. A shift in the migration distance (compared to the control) means the treatment somehow affected the structure of the DNA.

2. No shift does not necessarily indicate that the treatment does not affect DNA structure. DNA function may be severely altered.

3. Specific bioassays need to be done to confirm an effect on DNA function (*e.g.* transformation, infection, and plaque assays).

Appendix C. Typical Spectrophotometry Results

DNA Spectrophotometric Spectrum										
wavelength (nm)	230	240	250	260	270	280	290	300	310	320
Sample 1 (OD)	0.030	0.080	0.159	0.185	0.158	0.106	0.058	0.024	0.017	0.016
Sample 2 (OD)	0.2			0.347		0.166				
Sample 3 (OD)	0.175			0.147		0.089				
Sample 4 (OD)	0.24			0.344		0.164				

260/280 and 230/260 Ratio Values for 4 Samples of DNA		
Sample	260/280 ratio	230/260 ratio
1 *	1.75	0.162
2 **	2.09	
3 ***	1.65	1.19
4 ****	2.1	0.7

* 260/280 ratio of ~1.8 suggests pure DNA.
** 260/280 ratio of >1.8 suggests RNA contamination.
*** 260/280 and 230/260 ratios of <1.8 and >0.5, respectively, suggest protein and/or phenol contamination. Note that it is quite difficult to determine whether the contamination is protein or phenol or both.
**** 260/280 and 230/260 ratios of >1.8 and >0.5, respectively, suggest RNA and protein/phenol contamination.

Appendix D: Typical DNA Degradation Experiment; Plasmid Transformation Results

Treatment	Number of amp^R colonies / µg of plasmid
Control (untreated)	5×10^6
Physical shearing	3×10^6
Boiling	6×10^5
Autoclave	2×10^2
Sonication	9×10^4
Skin nucleases	2×10^6
Mouth nucleases	3×10^6
Freezing/Thawing	2×10^6
Acid treatment	3×10^5
Alkali treatment	1×10^6
UV exposure	3×10^5
EtBr/UV exposure	9×10^5

Appendix E: Buffer Compositions

TE (DNA storage) buffer

10 mM Tris-Cl
1 mM EDTA
pH 8.0 Autoclaved.

5X TBE (DNA electrophoresis) buffer

Tris base	54 g
boric acid	27.5 g
0.5 M EDTA, pH 8.0	20 mL
H_2O	to 1 liter

10X Ficoll/Orange G (gel loading) buffer:

30% (w/v) Ficoll (400,000 M.W.)	4.35 mL
2% (w/v) aqueous Orange G	1.00 mL
0.5 M EDTA, pH 8.0	0.65 mL