C H A P T E R 6

Nucleic Acids

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A. Introduction

Use of Radioisotopes in Biological Research

Rapid advances have occurred in biochemistry since compounds containing radioactive isotopes became available as metabolites that could be used to study biological processes. Because a labeled compound is not distinguishable from its normal form, the tracer can be followed *in vivo* as well as *in vitro*, and only a very small fraction of the total amount need be the radioactive form. The use of radioisotopes has facilitated the elucidation of biosynthetic as well as degradative pathways and often made possible the discovery of the steps involved in intermediary metabolism. The most commonly used radioisotopes in the biochemistry laboratory are the following β-particle-emitting radionuclides:

Isotope	*Half-life*
^{14}C	5760 years
^{3}H	12.3 years
^{32}P	14 days

Methods using tracer compounds are sensitive and do not alter the properties of the test compound. These are distinct advantages for conducting experiments in the biological sciences.

Safety Measures and Proper Handling of Radioisotopes

The hazards involved in using weak β-emitting radioisotopes are small, because very small quantities are needed to follow most biological reactions. Nevertheless, precautions when using these materials should be taken, and the work and storage areas as well as the equipment used should be monitored for possible spills. Contamination of ^{32}P or ^{35}S can be measured by using a portable Geiger-Müller counter with a thin mica film attached to a wand, which can be used to scan the surface where possible spillage may have occurred. For ^{14}C and ^{3}H monitoring wipe tests are performed. The test surface is wiped with a swatch of moistened filter paper, which is placed in a scintillation vial to which an appropriate solution is added. The vial is placed in a scintillation counter and counted.

In liquid scintillation counting for assaying radioactivity, the sample is placed in a solvent containing complex aromatic compounds called *primary* and *secondary fluors*. The particles given off by the radioactive sample transfer some of their energy to the solvent molecules, which in turn excite the primary fluor. When this fluor returns to its ground state, a photon is emitted that excites the secondary fluor. The light impulse from the secondary fluor is of a longer wavelength and is detectable by the phototube in the scintillation counter. This fluorescence is converted to electrical current and recorded as counts per minute. The instrument used to detect the radioactive decay in the samples is a scintillation counter, of which there are a number of models. The counter consists of an automatic sample changer that holds 100 or more vials. The activity in the vials will be counted for some preselected time period. Several channels are usually available, but the instrument commonly comes with plug-in modules that are preset for a particular radionuclide.

The data from a Beckman-type scintillation counter are generally recorded on a tape that typically records the following information:

1. *Channel no. 1, 2,* or *3*—selected for radioisotope to be counted, *i.e.*, ^{3}H, ^{14}C, ^{32}P.

2. *Vial no.*—position of sample on the chain (from 1 to 100 or more, depending on sample capacity of the instrument).
3. *Counting time*—period during which the sample was counted. This can be preset (*i.e.*, for 10 min) so that counts are variable, or the counts may be preset (*e.g.*, 10,000), in which case the time it took for that number of counts to accumulate will appear as the variable.
4. *Counts per minute*—calculated on the basis of total counts recorded during the counting period.

It is usual practice to include a blank vial to record background counts, and a standard. The standard is a commercial preparation having a known amount of radioactive material.

The units of measurement of radioactivity recorded by the scintillation counter are counts per minute (cpm), which represent the number of disintegrations that are detected by the instrument but not the actual number of β-particles emitted or disintegrations per minute (dpm). This value is dependent on the efficiency of the counter, which is determined as follows: efficiency = cpm/dpm.

A typical commercial ^{14}C standard is purchased with radioactive carbon emitting 41,500 dpm. If the counter records 36,000 cpm, then the efficiency would be *ca* 87% for this instrument.

Another unit of measurement associated with the use of radionuclides is the *specific activity* of a compound, which is the amount of radioactivity per amount of compound. In biological experiments, specific activity is generally given in μCi/μmol (1 μCi = 2.2×10^6 dpm). One μmol of radioactive compound can go a long way since the counter is quite sensitive, and, furthermore, it is possible to obtain a larger number of counts by counting for a longer period of time. It is usual to make a solution containing mostly unlabeled (or "cold") compound, and only some small fraction of the final amount is represented by the radioactive form.

When working with radioactive substances, a number of **safety measures** must be observed in the laboratory.

1. Cover the work area with absorbent paper with plastic backing.
2. Keep all materials that are to be used in the experiments on this paper.

3. Use latex gloves throughout the experiment.
4. Use automatic pipettors with disposable tips to pipette the radioactive material.
5. Carry out the reactions in disposable test tubes.
6. When finished, empty test tubes, gloves, and contaminated glassware should be wrapped in the absorbent paper, taped into a tight package, and disposed of in a container provided for radioactive waste.
7. Liquid radioactive waste should be discarded in a bottle set aside for that purpose.

B. Role of an Amino Acyl-Transfer RNA Synthetase in Protein Synthesis

An amino acyl-transfer RNA (AA-tRNA) synthetase attaches its cognate amino acid to the 3′ terminal end of its specific RNA. There is at least one specific enzyme and tRNA for each amino acid. The esterification of a given amino acid to its corresponding tRNA is thought to proceed in two steps with the involvement of three substrates. The overall reaction is described by the following equations:

$$\text{AA} + \text{ATP} + \text{E} \rightleftharpoons \text{E}\cdot\text{AA-AMP} + \text{PP}_i \quad (1)$$

$$\text{E}\cdot\text{AA–AMP} + \text{tRNA} \rightleftharpoons \text{AA-tRNA} + \text{AMP} + \text{E} \quad (2)$$

Equation 1 describes an "activation" step that results in the formation of an enzyme-bound aminoacyl–adenylate complex and pyrophosphate. In the second stage of the reaction, the amino acid is transferred to tRNA with the release of AMP and free enzyme. The formation of AA-tRNA is determined by assaying for the esterification of a radioactive amino acid to tRNA. In this series of experiments, partially purified leu-tRNA synthetase will be isolated, and the formation of leu-tRNA^{leu} will be assayed by measuring the amount of [^{14}C]leucine complexed with the tRNA.

The assay used to follow the leu-tRNA^{leu} synthesis depends on the formation of a precipitate in the presence of trichloroacetic acid (TCA). The precipitate is retained by Millipore filter pads when the reaction mixture is filtered. Thus the amount of ^{14}C deposited on the Millipore filters is indicative of the amount of leu-tRNA^{leu} formed. Neither the leucine nor the tRNA is retained

on the pad during filtration. The Millipore filters are then allowed to dry, scintillation fluid is added, and the vials are counted in a scintillation counter.

Isolation of Leu-tRNA Synthetase

Experimental Procedure

1. Growth and harvest of *E. coli* K-12 cells.
   a. (i) Prepare 1 L of minimal salts medium as follows and autoclave in a 2-L Erlenmeyer flask; K_2HPO_4, 10.5 g; KH_2PO_4, 4.5 g; $(NH_4)_2SO_4$, 1.0 g; sodium citrate (dihydrate), 0.97 g; $MgSO_4$ (anhydrous), 0.05 g; and deionized H_2O, 950 mL.

      (ii) Prepare the following solution in a 125-mL Erlenmeyer flask and autoclave separately; glucose, 2 g; and deionized H_2O, 50 mL.

   b. After autoclaving, add the glucose solution to the salts medium and inoculate with 20 mL of an overnight culture that will have been grown in nutrient broth.
   c. Incubate at 37°C with shaking, and allow culture to grow until an $A_{540\,nm}$ value of 0.7 to 0.8 is reached. This should yield 3 to 4 g of cells nearing the end of their logarithmic growth phase.
   d. Transfer the culture into wide-mouthed 250 mL plastic bottles and centrifuge at 8000 rpm for 20 min in a GSA rotor in the refrigerated Sorvall centrifuge.
   e. Resuspend the pellets in 20 mL of 0.05 M Tris buffer at pH 7.5 containing 0.1 mM dithiothreitol (DTT), transfer the suspension to a 50-mL plastic centrifuge tube, and centrifuge at 12,000 rpm for 15 min in a SS-34 rotor. Discard the supernatant.
   f. Obtain the wet weight of the cells. Cover the tubes with parafilm and freeze. Proceed with step 2 or step 3.
2. Preparation of "activating enzyme" and $(NH_4)_2SO_4$ fractionation.

Cell Breakage—Grinding With Alumina

   a. Transfer the weighed cell paste into a chilled mortar, and grind with twice its weight of levigated alumina. Add 0.5 to 1.0 mL of 0.05 M Tris buffer, pH 7.5, containing DTT as needed to obtain a smooth paste.

b. When the mixture has become pasty and viscous and a "popping" sound is heard, take up the extract in about 5 mL of the same buffer per gram of cells. Use another 5 mL of buffer to rinse the mortar and pestle.

c. Centrifuge the suspension at 15,000 rpm for 30 min in 15-mL plastic centrifuge tubes, and save the supernatant. (This is a crude enzyme extract.)

d. Measure the volume of the crude enzyme preparation, and transfer it to a small beaker. Place the beaker in an ice bath on a magnetic stirrer, and slowly add enough solid $(NH_4)_2SO_4$ to achieve a concentration of 75%. (Determine the appropriate amount of $(NH_4)_2SO_4$ to use from Table 6.1, which shows the weight of salt required; *e.g.*, for the 75% concentration, 47.6 g should be added to 100 mL of solution).

e. Centrifuge the ammonium sulfate precipitate at 15,000 rpm for 20 min, discard the supernatant, and resuspend the pellet in 3 mL of 0.05 M Tris buffer, pH 7.5, containing DTT.

3. If a sonifier is available the following method can be used.

Cell Breakage—Sonication

a. Transfer the weighed cell paste to a 30-mL plastic beaker, and add 10 mL of 0.05 M Tris buffer, pH 7.5, containing DTT. Stir gently with a rubber policeman until the cells are evenly suspended. Keep the suspension cold in an ice bucket.

b. Adjust the Branson sonifier to a setting of 3 or 4, and turn the tuning dial until a steady high-pitch sound is obtained (generally occurs at *ca* 3 amp).

   Avoid bubbling air through the solution, and avoid contact between the probe of the sonifier and the walls of the tube.

c. Use 10 mL of water in a small beaker to set the power.

d. Sonicate the cell suspension for a total of 2 min, giving 15 sec bursts. Allow the cell suspension to cool in an ice bucket between treatments. The fine-tuning may need adjustment as cell breakage proceeds.

e. When finished, rinse the probe by allowing the instrument to run for 30 sec while the probe is immersed in a beaker of water.

TABLE 6.1. Fractionation With Solid Ammonium Sulfate[a]

	Solid ammonium sulfate to add to 100 mL of solution																
Initial concentration of ammonium sulfate (% saturation at 0°C)	20	25	30	35	40	45	50	55	60	65	70	75	80	85	90	95	100
0	10.6[b]	13.4	16.4	19.4	22.6	25.8	29.1	32.6	36.1	39.8	43.6	47.6	51.6	55.9	60.3	65.0	69.7
5	7.9	10.8	13.7	16.6	19.7	22.9	26.2	29.6	33.1	36.8	40.5	44.4	48.4	52.6	57.0	61.5	66.2
10	5.3	8.1	10.9	13.9	16.9	20.0	23.3	26.6	30.1	33.7	37.4	41.2	45.2	49.3	53.6	58.1	62.7
15	2.6	5.4	8.2	11.1	14.1	17.2	20.4	23.7	27.1	30.6	34.3	38.1	42.0	46.0	50.3	54.7	59.2
20	0	2.7	5.5	8.3	11.3	14.3	17.5	20.7	24.1	27.6	31.2	34.9	38.7	42.7	46.9	51.2	55.7
25		0	2.7	5.6	8.4	11.5	14.6	17.9	21.1	24.5	28.0	31.7	35.5	39.5	43.6	47.8	52.2
30			0	2.8	5.6	8.6	11.7	14.8	18.1	21.4	24.9	28.5	32.3	36.2	40.2	44.5	48.8
35				0	2.8	5.7	8.7	11.8	15.1	18.4	21.8	25.4	29.1	32.9	36.9	41.0	45.3
40					0	2.9	5.8	8.9	12.0	15.3	18.7	22.2	25.8	29.6	33.5	37.6	41.8
45						0	2.9	5.9	9.0	12.3	15.6	19.0	22.6	26.3	30.2	34.2	38.3
50							0	3.0	6.0	9.2	12.5	15.9	19.4	23.0	26.8	30.8	34.8
55								0	3.0	6.1	9.3	12.7	16.1	19.7	23.5	27.3	31.3
60									0	3.1	6.2	9.5	12.9	16.4	20.1	23.9	27.9
65										0	3.1	6.3	9.7	13.2	16.8	20.5	24.4
70											0	3.2	6.5	9.9	13.4	17.1	20.9
75												0	3.2	6.6	10.1	13.7	17.4
80													0	3.3	6.7	10.3	13.9
85														0	3.4	6.8	10.5
90															0	3.4	7.0
95																0	3.5
100																	0

[a]Reprinted from Segel, p. 400.

[b]Values are final concentrations of ammonium sulfate (% saturation at 0°C).

f. Centrifuge at 15,000 rpm for 30 min in 15-mL plastic centrifuge tubes, and save the supernatant. (This is a crude enzyme extract.)

g. Measure the volume of the crude enzyme preparation, and transfer it to a small beaker. Place the beaker in an ice bath on a magnetic stirrer and slowly add enough solid $(NH_4)_2SO_4$ to achieve a concentration of 75% (see Table 6.1). Add the salt slowly, allowing about 30 min for this procedure, and make sure that all the salt is dissolved.

h. Centrifuge at 15,000 rpm for 20 min, discard the supernatant, and resuspend the pellet in 3 mL of 0.05 M Tris buffer, pH 7.5, containing DTT.

4. Separation on a Sephadex column.

a. Pour a G-25 (coarse) Sephadex column and calibrate it using blue dextran to determine the void volume, V_o. Place glass wool in the bottom of a glass column, and add some Tris buffer. Using a glass rod, gently squeeze out any air bubbles and allow some of the buffer to run out. Add a small amount of sand to achieve a flat surface above the glass wool. With the column about one-third full of buffer, add a 10-mL volume of Sephadex. Be certain that everything is at the same temperature! Once the gel has settled and equilibrated with buffer (about 30 mL), the V_o can be determined by applying 1.5 mL of blue dextran. Collect the eluate in a graduated cylinder, and note when the first blue color appears; this is V_o. Continue to collect eluate until all the blue dextran has eluted, and record the elution volume (V_e).

b. Apply 1.5 mL of the resuspended $(NH_4)_2SO_4$ fraction, and begin collecting 1.0-mL fractions.

c. Take $A_{280\,nm}$ readings, and pool the tubes containing the protein. The volume of the pool should be equal to the volume of blue dextran collected during the calibration. This procedure separates the proteins from the $(NH_4)_2SO_4$ and any endogenous leucine that will be retained on the column. The leucine and the salt must be removed from the enzyme preparation to perform the activity assays and the protein determinations.

d. Freeze this crude "activating enzyme" preparation in 1.0-mL aliquots.

Charging of Leu-tRNA by Leu-tRNA Synthetase

The following experiments are designed to demonstrate one of the important steps in protein synthesis. The charging of an amino acid by its AA-tRNA synthetase will be accomplished in the following two experiments by studying the kinetic properties of the synthetase.

The conditions required to determine the K_m and V_{max} values for an enzyme are studied in some detail in the Enzymology module. Here we have set up the protocols that will enable you to carry out the experiments to obtain the data from which to calculate these parameters for leu-tRNA synthetase.

Experimental Procedure

Enzyme Concentration Curve for Leu-tRNA Synthetase

1. Determination of protein content of the "activating enzyme" preparation.
   a. Using the dye-binding assay, set up a series of six tubes containing from 0 to 20 μg of BSA to establish a standard curve (see Chapter 3 for procedure).
   b. Assay the enzyme as follows: extract diluted 1:9, 0.02, 0.05, and 0.10 mL; and extract diluted 1:99, 0.02, 0.05, and 0.10 mL.
   c. Calculate the average mg/mL for the extract.
2. Carry out the first experiment according to the protocol given in Table 6.2.
   a. Calculate the correct amount of protein to use as "activating enzyme." Make the appropriate dilution in Tris buffer containing DTT.
   b. Start the reaction by addition of the tRNA, and time for *exactly* 10 min. The reaction is stopped by adding 2.0 mL of 8% TCA. Incubate for 20 min in an ice bath.
   c. As is evident in the protocols in Tables 6.2 and 6.3, the precipitate is allowed to form for a period of 20 min following addition of TCA. The leu-tRNA^{leu} is then collected by passing the solution through a Gelman glass fiber filtering apparatus containing a filter 0.45 μm in size. The equipment used is shown in Figure 6.1, in which the proper position of the filter pad is illustrated. The pads to be used are presoaked in 5% TCA and inserted between the funnel and the glass stem as

TABLE 6.2. Enzyme Concentration Curve for Leu-tRNA Synthetase

Additions (mL)	Tube no. 1 (Blank)	2	3	4	5	6	7	8	9	10 (Controls)
"Activating enzyme" (μg protein)	–	5	10	20	40	60	80	100	–	100
Charging "mix"[a]	–	0.2 ⟶								
[^{14}C]leucine (10 μCi/μmol, 0.5 μCi/mL)	–	0.2 ⟶								
H_2O	1.0	⟵ Bring to a final volume of 0.8 mL. ⟶								
Bulk tRNA (1.2 mg/mL)	–	0.2 ⟶								–[b]
	Incubate for exactly 10 min at 37°C.									
8% TCA	2.0 ⟶									
	Incubate in an ice bath for 20 min. Pass through a Millipore filter, and wash pads with 5% TCA.									
cpm/10 min incubation										
Corrected cpm/min incubation										
μmol Leu-tRNA/min										

[a]Charging "mix" contains in 40 mL: 10 mL of 1.0 M Tris, pH 7.4; 10 mL of 0.2 M $MgCl_2$ + 12 mg DTT; 10 mL 0.01 M EDTA; and 10 mL 0.05 M ATP, pH 7.0.
[b]Add an additional 0.2 mL of water to this tube for a final volume of 1.0 mL.

shown; the suction flask is connected to an aspirator to speed up the filtration. After the solution is passed through, the funnel is rinsed three times with 3-mL aliquots of 5% TCA. The funnel is removed with the suction left on, and the pad still resting on the scintered-glass stem is rinsed thoroughly using a wash bottle containing 5% TCA.

Then the filter pad is transferred to a scintillation vial using tweezers and allowed to dry at 110°C for 10 min or until the pads are dry. The vials are cooled, and 8 mL of scintillation fluid is added. Then the caps are fastened on tightly, and the vials are placed on the chain in the

TABLE 6.3. The Effect of Substrate Concentration on Enzyme Velocity

Additions (mL)	Tube no. 1	2	3	4	5	6	7	8	9	10 (Controls)
tRNA-bulk (1.2 mg/mL)	0.01	0.02	0.04	0.08	0.10	0.15	0.20	0.25	–	0.25
H_2O	0.49	0.48	0.46	0.42	0.40	0.35	0.30	0.25	0.50	0.35
[^{14}C]leucine (10 μCi/μmol, 0.5 μCi/mL)	0.2 →									
Charging mix	0.2 →									
Enzyme[a]	0.1 →									–
	Incubate for exactly 10 min at 37°C.									
8% TCA	2.0 →									
	Incubate for 20 min in an ice bath. Pass through a Millipore filter, and wash pads with 5% TCA.									
Bulk-tRNA (mg/aliquot)										
$[S]$[b]										
$1/[S]$										
cpm/10 min incubation										
Corrected cpm/min incubation										
μmol leu-tRNA/min (v)										
$1/v$										

[a]Add an amount of enzyme that is nonlimiting (about 2/3 of the way up the linear portion of the concentration curve).

[b]Assume an average MW of 30,000 for Leu-tRNA. Convert mg tRNA/aliquot to μmol/mL (remember that the assay volume is 1 mL). Calculate the concentration (in mM) of leu-tRNA, $[S]$, in each tube assuming that 10% of the bulk tRNA represents leu-tRNA.

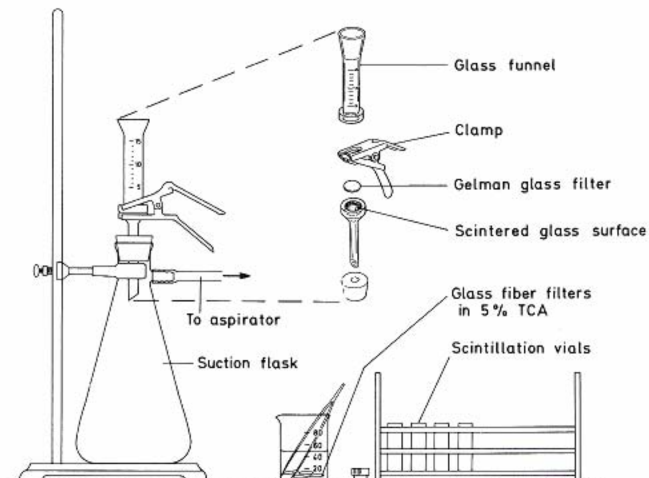

Fig. 6.1. Collection of leu-tRNA^{leu} following incubation with activating enzyme. Filtration apparatus for collecting labeled leu-tRNA^{leu}. A plastic-backed protective pad is used to cover the bench top to collect any radioactive spills, and the filter pads are transferred to scintillation vials arranged in order in the rack; the caps are numbered but are only placed onto their respective vials after drying as they do not tolerate the heat to which the vials are exposed during the drying period.

counter. Each vial is counted for 10 min. The counter records the data on a tape, as described earlier in this chapter.

3. Plot an enzyme-concentration curve.
   a. Correct the cpm/min (counts per min/min of enzyme incubation) obtained by subtracting whichever control tube gives a background value that is higher (tRNA [tube 9] or protein [tube 10]).
   b. Calculate the velocity in "μmol leu-tRNA/min" from the cpm/min incubation data as follows:

$$\underset{(\mu\text{mol leu tRNA per min})}{\text{velocity}} = \frac{\text{cpm/min incubation}}{(\text{counter efficiency})\underset{\text{dpm}}{(2.2 \times 10^6)}\underset{\text{sp. act. of leucine}}{(10\mu\text{Ci}/\mu\text{mol})}}$$

   c. Graph "μmol leu-tRNA/min" *vs.* μg protein.

Michaelis-Menten Kinetics for Leu-tRNA Synthetase

1. Determination of K_m and V_{max} values for leu-tRNA.
   a. Carry out the experiment according to the procedure outlined in the protocol given in Table 6.3 (note that 25 to 50 μg of protein will generally fall about two-thirds of the way up the linear portion of the curve).
   b. The reaction is started by the addition of enzyme and stopped by the addition of 8% TCA.
2. Graph a Michaelis-Menten curve showing velocity as a function of tRNA concentration and calculate values for V_{max} and K_m from a Lineweaver-Burk plot.
3. Use the values of V_{max} and protein concentration to calculate the specific activity of the enzyme.

C. Isolation and Physical Properties of DNA

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The molecular weight of DNA molecules of some cells probably exceeds 10^9g/mol, making them the largest linear covalent structures in nature. Seldom are they isolated intact, however, because of the action of the deoxyribonucleases present during the isolation procedures and because such large molecules are highly sensitive to shear forces. Merely pipetting DNA may result

in breakage. Care should be taken to keep both of these factors to a minimum during isolation of the DNA. Molecules approaching 10^7g/mole can be obtained by conventional purification techniques.

A distinctive property of DNA is its behavior upon "denaturation." The native form of cellular DNA is a helical, double-stranded structure. When the native DNA is disrupted, the molecule loses its highly ordered structure, and single strands as well as random coils result. The disruption is accompanied by significant changes in optical properties and in molecular dimensions. These changes can be followed by absorbance measurements at 260 nm or by viscometric procedures.

The absorbing chromophores in the UV region are the purine and pyrimidine bases. Hypochromicity results from the stacking interactions between these bases, and when DNA is denatured the bases become "unstacked" and the UV absorbance increases, approaching but never equaling the absorbance of a mixture of deoxynucleotides of the same composition as that found in the native DNA. This increase in absorbance is known as a *hyperchromic shift*.

The T_m is the melting temperature of double stranded DNA, that is, the point at which one-half of the DNA molecule is double stranded while the other half of the unwinding molecule is single stranded. T_m is at the midpoint of the hyperchromic shift. It is dependent on the G-C content of the DNA and on the ionic strength of the solution. The G-C content is characteristic of a given species and a plot of T_m *vs*. mol % G+C shows a linear relationship (Fig. 6.2A, B). In the following experiments, DNA will be isolated from calf thymus tissue and T_m determined by following $A_{260\text{ nm}}$ at increasing temperatures.

Isolation of DNA From Calf Thymus

The DNA will be extracted from thymus gland tissue. Caution must be exercised to prevent denaturation during the isolation. For this reason, all glassware that will come in contact with the DNA should be rinsed with 1 mM EDTA, and the preparation should be kept cold (use an ice bucket unless otherwise indicated). The use of a chelating agent, such as EDTA, serves to remove Mg^{++}, which is required for the action of DNase. In the absence of the required ion and in the cold, this degradative enzyme becomes nonfunctional. It is also important to handle the DNA gently to avoid mechanical shearing.

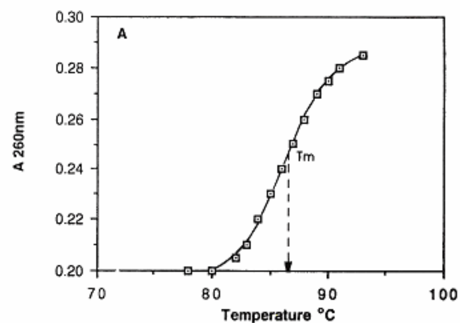

Fig. 6.2A. Typical melting curve for calf thymus DNA. The T_m will vary with the species of DNA examined.

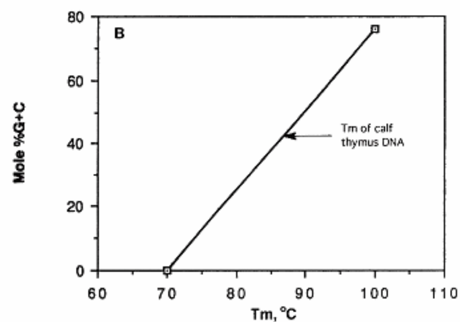

Fig. 6.2B. Relationship between % G+C content of DNA and T_m based on T_m determinations for DNA samples from different sources. (Adapted from Lehninger, p. 815 [1975].)

Experimental Procedure

1. Weigh 15 g of calf thymus (avoid the white fatty tissue), and chop it into small pieces.
2. Place the sample in a chilled Waring blender. Add 50 mL of citrate saline buffer (*CS buffer*: 9 mg/mL NaCl in 0.01 M trisodium citrate), and homogenize for 5 min at high speed at 4°C.
3. Pour the homogenate into 50-mL plastic centrifuge tubes, and centrifuge at 6500 rpm for 15 min.
4. Remove and discard the top lipid layer with a plastic spatula. Pour off and discard the supernatant, remove

pellets, and return to the blender. Add 50 mL of CS buffer, and blend at high speed for 3 min.

5. Resuspend the combined pellets in 90 mL of 0.15 M Na citrate buffer, pH 7.0.
6. Place the suspension in a beaker on a magnetic stirring apparatus and **SLOWLY** add 8 mL of 20% SDS (sodium dodecyl sulfate) solution dropwise with a Pasteur pipette. (The addition should take about 15 min.) Stir the suspension for an additional 5 min. The suspension will become increasingly viscous.
7. Place the suspension in a 55°C water bath and incubate for 15 min, stirring occasionally with a glass rod.
8. Add 8 g of NaCl, keeping the solution at 55°C. Continue to stir for 10 min or until the salt is dissolved.
9. Cool to room temperature in an ice bath, and pour the sample into a 250-mL separatory funnel.
10. Add 100 mL of a chloroform:isoamyl alcohol mixture (24:1), and shake vigorously for 10 min.
11. Pour both layers into a plastic centrifuge bottle, and centrifuge at 10,000 rpm for 15 min. Use a GSA rotor.
12. Carefully remove the top layer to a graduated cylinder, measure the volume of the extract, and transfer it to a 400-mL beaker. Discard the white interface and bottom layers.
13. Slowly add 2 volumes of 95% EtOH while gently stirring with a glass rod.
14. Wind out the gelatinous precipitate onto a glass rod.
15. Add another 20 mL of EtOH and collect any additional precipitate that may wind onto the rod.
16. Gently press out the solvent by turning the rod against the side of the beaker.
17. Rinse the wound DNA with 95% EtOH and then with acetone (AR grade) until the washings are no longer turbid.
18. Remove the DNA from the rod, and dry it in a small beaker in a desiccator overnight.* On the following day, weigh the

---

*Since DNA is a very large molecule, it goes into solution with difficulty. It is therefore advisable to allow 24 hr for a sample to go into solution. To make a *DNA stock* solution, weigh out 300 mg of DNA, crush the sample gently, and transfer the powder to a 50-mL beaker. Add 20 mL of 0.1 M Na phosphate + 1 mM EDTA, pH 7.0, buffer and insert a magnetic stirring bar. Place the beaker on a stirrer in a refrigerator or cold room.

sample and record the dry weight. Calculate the percent crude recovery on a weight basis. After the diphenylamine assay has been performed, calculate the net yield and purity of the product.

Determination of T_m for DNA

The T_m is determined from the midpoint of a melting curve, which is obtained by heating the DNA at various temperatures and observing the increase in absorbance at $A_{260\ nm}$ that results.

Experimental Procedure

1. Centrifuge the stock solution of DNA at 12,000 rpm for 10 min, and discard the pellet. Using the Warburg-Christian method for protein determination, take 260/280 absorbance readings on the supernatant (a dilution of about 1:50 should be within range to read the absorbance) to determine the approximate concentration of DNA in the stock solution. In making any dilution of the stock solution, be sure to mix gently but thoroughly. From these data, calculate also the amount of protein present in the DNA isolated.
2. Dilute an aliquot of the DNA stock solution to obtain a concentration having an $A_{260\ nm}$ of between 0.1 and 0.3 in 0.1 M Na phosphate buffer, pH 7.0. Dilute a solution of standard DNA to the same concentration.
3. Transfer both DNA solutions to quartz cuvettes. Use the buffer as a blank.
4. Place the cuvettes in the carrier of a spectrophotometer, which has a water-jacketed cuvette holder.
5. Set the instrument to zero at a wavelength of 260 nm, and read the absorbance of the samples at room temperature. As the temperature is raised, the cuvettes must be checked for the presence of air bubbles; these must be eliminated prior to making measurements.
6. Set the thermostat of the water bath to 75°C, and allow 5 min for the temperature to equilibrate after the pilot light (which indicates that the bath is heating) shuts itself off. Set the instrument to zero against the buffer, and take readings on the samples.
7. Increase the thermostat setting to 80°C, and proceed as above. Repeat this procedure for the 83°, 86°, 89°, 92°,

95°, and 100°C settings on the thermostat. Reset the instrument to zero against the buffer each time, and determine the *actual* water temperature by reading the thermometer inserted in the water bath alongside the thermostat.

8. Since there is some heat loss during the transfer of the water from the bath to the jacket surrounding the cuvettes, it is necessary to correct the water bath temperature measurements to the cuvette housing temperatures. For an *LKB ultraspec II* spectrophotometer, corrections for these temperature differences can be made by using a temperature conversion graph (Fig. 6.3) generated from the following data:

Water bath temperature (°C)	*Cuvette temperature (°C)*
76.5	70.5
81.0	75.0
86.0	78.5
91.0	82.5
96.0	89.5
100.0	94.0

9. Plot temperature *vs.* absorbance to determine the T_m of the DNA and of the standard. Use the following equation to determine the mole percentage G+C (Marmur, J., and

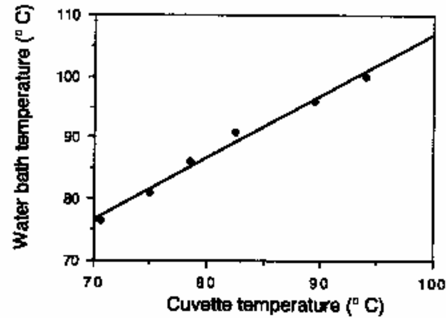

Fig. 6.3. Temperature correction curve for solutions in a spectrophotometer with circulating water-jacket adapters for the cuvettes.

Doty, P. Determination of the base composition of DNA from its thermal denaturation temperature. *J. Mol. Biol* 4:109–118 [1962]):

$$T_m = 69.3 + 0.41\ (\%\ G{+}C)$$

Determination of Base Composition and Polysaccharide Content of the Calf Thymus DNA

When DNA is subjected to mild acid hydrolysis, the sugar–purine bonds are cleaved, leading to the formation of apurinic acids and the release of adenine and guanine. This procedure will be employed to analyze the DNA for contamination with polysaccharides and to determine the G+C ratio of calf thymus DNA. The same sample of hydrolyzed DNA can be used in both studies.

Experimental Procedure

Acid Hydrolysis and Determination of Polysaccharide Content

1. Dilute an aliquot of stock DNA (15 mg/mL) with water to a concentration of 3 mg/mL. Prepare 10 mL of this solution.
2. To 3 mL (*measure carefully*) of the DNA solution, add 0.33 N HCl dropwise until the pH is between 2 and 3 (pH paper may be used for this test).
3. Place a marble on top of the tube to avoid evaporation, and heat the acidified DNA for 40 min in a boiling water bath.
4. Add 3.6 M KOH until the pH is about 7.0. *Measure and record the new volume.* Note the resultant change in the concentration of the DNA.
5. Follow the protocol on Table 6.4 to assay for reducing sugars, including 0.20-, 0.50-, and 1.0-mL samples of hydrolyzed DNA and untreated DNA. See Table 5.1 for the Nelson's reducing sugar assay.
6. Calculate the percent reducing sugar in the hydrolyzed DNA.
7. To the remaining DNA solution, continue adding 3.6 M KOH until the pH reaches 11 to 12 (test again with pH paper).

TABLE 6.4. Polysaccharide Content of DNA

Additions (mL)	Tube no. 1	2	3	4	5	6	7	8 (Blank)
Hydrolyzed DNA[a]	0.2	0.5	1.0	–	–	–	–	–
Untreated DNA (3mg/mL)	–	–	–	0.2	0.5	1.0	–	–
Glucose (4 mM)	–	–	–	–	–	–	0.2	–
H_2O	0.8	0.5	–	0.8	0.5	–	0.8	1.0
Nelson's reagent	1.0 →							
	Mix. Boil all the tubes for 20 min. Cool to room temperature.							
Arsenomolybdate reagent	1.0 →							
	Mix. Incubate for 5 min at room temperature.							
H_2O	7.0 →							
$\%T_{510\ nm}$								
$A_{510\ nm}$								
μmol RS[b]/aliquot of DNA:								
μmol RS/mL of DNA:								
μmol RS/mg of DNA:								
mg RS/mg of DNA[c]:								

[a]See text for concentration of this solution.
[b]RS-reducing sugar.
[c]Assume that the MW of the reducing sugar is 180g/mol.

This sample is now ready for base-ratio analysis by paper chromatography. An untreated sample of DNA will be analyzed for comparison.

Chromatography of DNA Bases

1. Cut a 20 × 30 cm sheet of chromatography paper. Begin to apply spots 1.5 cm from the left edge of the paper and 2.5 cm from the bottom. Allow about 1.5 cm between spots, and be sure to spot the hydrolyzed sample near the center of the paper. (See TLC section in Chapter 4 for spotting technique.)
2. Spot 0.10 mL of the hydrolyzed DNA sample in a band about 1.5 cm wide. (Use a 10-μL microcap, and deposit a series of droplets across the designated area).

3. Spot 0.05 mL of unhydrolyzed DNA (3mg/mL) adjusted to pH 11.
4. Apply 2 μL each of adenine, cytosine, and thymine (5 mg/mL in 0.02 M KOH) and 6 μL of guanine as standards. Include a spot that contains a mixture containing 2 μL of each of the four bases.
5. Develop the chromatogram overnight in a tank containing 145 mL of water-saturated butanol. Allow the chromatogram to dry in a fume hood, and view the spots under a UV lamp in the dark. Circle with a soft lead pencil the UV-absorbing spots, which will be deep purple in color.
6. Cut out these spots, and elute them from the paper with 2 mL of 0.05 M HCl. Also cut out and elute a clear region of paper of about the same size to provide a spectrophotometric blank. (Cut the designated areas into fine strips, and transfer the strips to test tubes with tweezers.)
7. Warm the tubes in a hot water bath, but avoid boiling. Periodically mix the suspensions on a Vortex mixer and return the tubes to the water bath. Continue this procedure for *ca* 15 min; then filter out the paper particles by passing the suspensions through Pasteur pipettes containing some glass wool.
8. Presentation of data and calculation of percent G+C.
   a. Construct absorption spectra of the solutions obtained above for the four standards and for the spots obtained from the hydrolyzed DNA. These spectra are obtained by reading the eluates at 5-nm intervals from a wavelength of 220 to 300 nm. These data should confirm the identity of the bases released from the DNA sample. Verify this by comparing the peaks with the absorption maxima listed below.

Base	*Absorption maximum (nm)*	*Extinction coefficient*
Adenine	263	13,100
Cytosine	274	10,200
Guanine	275	7,350
Thymine	264	7,890

   b. Using the absorbance values for the hydrolyzed DNA at 263 and 275 nm for A and G, respectively, calculate the percent G+C as follows:

i. Determine the concentrations of A and G extracted from the spots according to the Beer-Lambert law. (Refer to the list above for the extinction coefficients of the bases.)

ii. % G+C = (G/G + A) × 100 (since the amount of G is equal to that of C and the amounts of A and T are likewise equal.)

Determination of DNA and RNA Contents of the Isolated DNA Product

Diphenylamine Assay for DNA: Experimental Procedure

1. Construct a standard curve by preparing a series of tubes containing from 0.015 to 0.30 mg of DNA per tube using a 0.15 mg/mL standard DNA solution. Bring the final volumes in the tubes to 2 mL with H_2O. Include two tubes respectively containing 50 and 100 μg of RNA as controls.
2. Add 2.0 mL of diphenylamine reagent to each tube and mix. Heat the marble-topped tubes in a boiling water bath for 15 min. The diphenylamine reagent must be prepared shortly before use. (Dissolve 0.75 g of diphenylamine in 50 mL of glacial acetic acid. Add 0.75 mL of conc. H_2SO_4. Just prior to use, add 0.25 mL of cold 1.6% acetaldehyde. [Prepare in a fume hood.])
3. Allow the tubes to come to room temperature and read % transmittance at 600 nm. If cloudiness develops, warm the tubes for a few minutes at 50°C.
4. Plot $A_{600\ nm}$ *vs.* mg DNA.
5. To determine the DNA concentration in the preparation, estimate the proper amount of sample that would fall on the linear assay range. (Use the 260/280 measurements made earlier to approximate this value.) Assay three tubes at different concentrations of unknown, assuming that the nucleic acid content is about 75% DNA. Average the values that fall within the limits of the standard curve.

Orcinol Assay for RNA: Experimental Procedure

1. Construct a standard curve by preparing a series of tubes containing from 10 to 100 μg of RNA per tube using a 50 μg/mL standard RNA solution. Bring the final volumes in the tubes to 2.0 mL with 5% TCA. Include two tubes respectively containing 150 and 300 μg of DNA as controls.

2. Add 2.0 mL of orcinol reagent and mix. Heat the marble-topped tubes in a boiling water bath for 15 min. (The orcinol reagent deteriorates after 30 min and should be prepared just prior to use. Dissolve 0.5 g of orcinol in 50 mL of 0.1% $FeCl_3$ in conc. HCl. [Prepare in a fume hood.])
3. Remove the tubes and allow them to cool. Read % transmittance at 640 nm.
4. Plot $A_{640\ nm}$ *vs*. µg RNA.
5. To estimate the unknown amounts of RNA in the preparation, calculate the proper amount of sample to assay again by making use of the Warburg-Christian nomograph and the 260/280 measurement. Assume that about 25% of the DNA preparation is RNA. Assay the sample again at three different concentrations, and average the values that fit on the linear portion of the standard curve. Note that this will give a high estimate because of the DNA interference in this assay.

Calculation of Yield and Purity of Calf Thymus DNA

1. From the diphenylamine, orcinol, and Nelson's assays and from the 260/280 absorbance readings (Warburg-Christian method), the percentage DNA, RNA, polysaccharide, and protein, respectively, can be calculated for the DNA isolated.
   a. Calculate the amount of nucleic acids in the thymus sample. Express the values in mg/g wet weight of tissue and in percent yield of crude DNA.
   b. Set up a purification table.

Component	*Method*	*Concentration (mg/ml)*	%[a]
DNA	1. 260/280 2. Diphenylamine assay		
Protein	260/280		
RNA	Orcinol assay		
Glycogen	Nelson's assay		
"Other"			

[a]Based on a solution prepared from crude DNA (15 mg/mL).

2. Evaluate the effectiveness of the purification procedure. Discuss possible further steps that could be taken to achieve greater purity.

D. A Brief Introduction to Recombinant DNA Methodology

A discussion of recombinant DNA methodology and some applications can be found in Chapter 7, in which is a series of experiments on this topic. In this chapter, a brief two-laboratory sequence of experiments is presented to learn to isolate a plasmid (pBR322) from *Escherichia coli* strain HB 101 and to use restriction enzymes and agarose gel electrophoresis to characterize the resulting DNA fragments.

Small-Scale Isolation of Plasmid pBR322 DNA

Plasmid pBR322 carries two antibiotic-resistance genes (see restriction map, Fig. 6.4) for ampicillin and tetracycline. When *E. coli* HB 101 is grown in a medium containing one of these antibiotics, the only cells that will be able to grow are those with plasmids that confer resistance to the bacteria.

Experimental Procedure—Boiling "Miniprep" Method

1. Inoculate 3 mL of sterile LB medium* containing 100 μg/mL of ampicillin. Incubate at 37°C overnight in a shaking water bath. Three milliliters of such a plasmid-carrying culture grown overnight typically yields 4 to 6 μg of plasmid DNA.
2. Pour the grown culture into two plastic microcentrifuge tubes (do not use glass, as DNA will adhere to the walls of the tube). Spin for 2 min in a microcentrifuge.
3. Decant the supernatant completely, and resuspend the cell pellets in 0.5 mL of sucrose solution containing 8% sucrose, 5% Triton X-100 (a detergent), 50 mM EDTA, and 50 mM Tris, pH 8.0.
4. Add 25 μL of a freshly prepared solution of lysozyme (10 mg/mL in 10 mM Tris, pH 8.0). Mix by shaking gently.
5. Place the tube in a boiling water bath for 60 sec. Cool on ice.
6. Centrifuge for 15 min in a microcentrifuge

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*LB medium contains: bacto tryptone, 10 g; bacto yeast extract, 5 g; and NaCl, 10 g; pH 7.5.

Restriction Map of pBR322 DNA

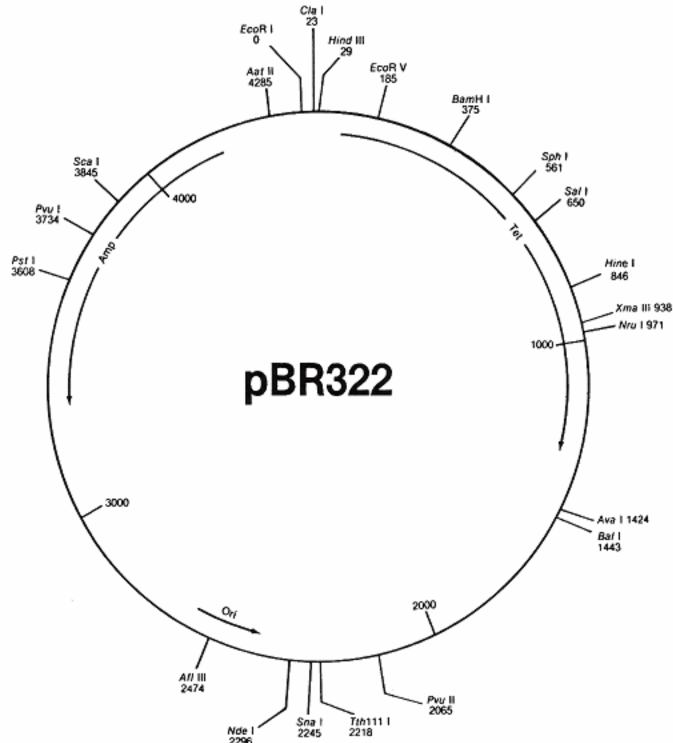


Fig. 6.4. Map of the sites of first bases within the recognition sequences are expressed in nucleotide numbers counting from the origin, which is the center of *Eco*R1. This is also the terminal restriction enzyme recognition sequence at 4360. (From *BRL Catalogue and Reference Guide,* p. 86 [1983], Bethesda Research Laboratories, Inc., Gaithersburg, MD; reprinted with permission of Bethesda Research Laboratories.)

7. Remove the pellets by gently teasing them out of the tube with a toothpick, leaving the clear supernatant behind.
8. Add 25μL of 2.5 M NaCl and 0.6 mL of cold isopropanol to the supernatants. Mix thoroughly and incubate for 15 min in a dry-ice/EtOH bath (This should permit the plasmid DNA to precipitate out of solution.)
9. Centrifuge again in the microcentrifuge for 15 min.
10. Decant and discard the supernatants. Resuspend the plasmid pellets in 50 μL of sterile TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0). Add 6 μL of 2.5 M NaCl and 125μL of cold EtOH. Incubate in a dry-ice/EtOH bath for 10 min, and centrifuge for 10 min. Decant and discard the supernatants.
11. Cover the tubes with parafilm. Punch several holes through the coverings. Place the tubes in a small beaker in a desiccator containing silica gel. (This desiccant has a bright blue color when dry; as it absorbs water, it becomes a faint pink. It can be reactivated by heating at 110°C until it returns to its original color.) Pull a vacuum by attaching the desiccator to an aspirator, and allow the pellet to dry in this fashion for 10 to 15 min.

Digestion of Plasmids With Restriction Enzymes

The enzymes used in molecular cloning are known as *restriction enzymes*. They are endonucleases that have been isolated from prokaryotes, and they recognize specific sequences of nucleotides within double-stranded DNA.

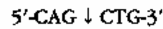
There are three types of restriction enzymes, which are classified according to their mode of action. Among the commonly used enzymes are those that recognize sequences that are four to six nucleotides in length with a twofold axis of symmetry. *Hind* III (purchased from Bethesda Research Laboratories, Gaithersburg, MD), which will be used here, is one such enzyme. It recognizes the hexanucleotide sequence shown below and cuts the plasmid at positions that are four nucleotides apart in the two strands, as indicated by the arrows:

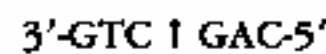
5′-A ↓ AGCT T-3′

3′-T TCGA ↑ A-5′

The resulting DNA fragments thus have protruding "sticky" five-prime (5′) ends, which means that they can form base pairs with other single-stranded DNA termini. Thus new recombinant DNA molecules can be formed from fragments that have been cut by a given enzyme and have been rejoined by base-pairing at another site.

The other enzyme chosen for this experiment is *Pvu* II (also purchased from Bethesda Research Laboratories) which does not generate DNA fragments with protruding 5′ tails but cuts at the axis of symmetry and yields "blunt-end" fragments:


5′-CAG ↓ CTG-3′


3′-GTC ↑ GAC-5′

Information on the optimum conditions required for each enzyme is supplied by the manufacturer. In general, the conditions that vary are the composition of the buffer and the temperature of incubation. For convenience in conducting the experiment done here, two enzymes with similar requirements for temperature and buffer concentration were chosen.

Experimental Procedure

1. Resuspend the dry pellet in 50 μL of sterile TE buffer, pH 8.0. *Use sterile pipettor tips* to prevent contamination of restriction enzymes. Proceed with one of the two DNA plasmid fractions.
2. Set up a series of four sterile microcentrifuge tubes and follow the protocol given in Table 6.5.
3. Incubate the four tubes in a 37°C H_2O bath for 30 min.
4. Add 12 μL of 5× tracking dye to stop the reaction (50% glycerol + 0.25% bromphenol blue).
5. Store these tubes at 4°C until the next laboratory period, when agarose gel electrophoresis will be performed.

Agarose Gel Electrophoresis of Plasmid DNA Fragments

Agarose gel electrophoresis is used to separate DNA fragments, which can then be identified by comparing their electrophoretic patterns with the patterns obtained with λ *Hind* III–cut DNA fragments of known composition. The DNA fragments band within the gel and are stained with ethidium bromide,

TABLE 6.5. Protocol for Digestion of Plasmid pBR322 by Restriction Enzymes

Additions (μL)	Tube no. 1	2	3	4
DNA plasmid solution	5	5	5	5
10× Enzyme buffer[a]	5 →			
Sterile deionized H_2O	40	36	36	34
RNase (1 mg/mL)	–	2	2	2
Hind III (*ca.* 10 to 20 Units)[b]	–	2	–	2
Pvu II (*ca.* 10 to 20 Units)[b]	–	–	2	2

[a]10× Buffer contains 0.6 M NaCl; 60 mM Tris, pH 7.5; 60 mM $MgCl_2$; and 10 mM DTT.

[b]One unit of enzyme is that amount required to digest 1 μg of λ DNA in 1 hr under the appropriate conditions in a volume of 50 μL. Note that enzyme is added in excess to achieve complete digestion during a shorter incubation period.

which is incorporated into the gel. Low concentrations of this fluorescent dye permit detection of small amounts of DNA (down to 1 ng), which can be visualized directly under UV light.

The migration rate of the fragments depends on their size, the concentration of agarose, the conformation of the molecules undergoing electrophoresis, and the current applied. As in disc gel electrophoresis, the distances migrated, as measured in R_m values, are inversely proportional to the log of their molecular weights. Thus, it will be possible to construct a standard curve using λ DNA fragments of known size and to determine the sizes of the pBR322 digests by comparison.

Experimental Procedure

1. Prepare the 0.8% agarose solution by adding 2.4 g of powder to 300 mL of TBE buffer. (See Appendix I for composition.) Dissolve the agarose by gently heating either over a Bunsen burner or in a microwave oven; cool to 50°C.
2. Add 15 μL of ethidium bromide from a stock solution (10 mg/mL) to obtain a concentration of 0.5 μg/mL in the gel. **(WEAR GLOVES WHEN HANDLING; IT IS A POTENT MUTAGEN).**
3. A horizontal slab gel will be used. The plastic form, which is open at both ends, must be taped securely to provide the mold into which the agarose is poured. Insert the comb,

which will form the sample wells. (Be sure that the teeth do not cut through the bottom of the gel. There must be at least a 1-mm layer of agarose between the bottom of the teeth and the base of the gel.) Pour the cool agarose solution carefully to avoid air bubbles.

4. Allow the gel to set completely, about 30 min; *carefully* remove the comb and the two strips of tape.
5. Transfer the slab gel to the electrophoresis tank, and add enough TBE buffer to cover the gel to a depth of at least 1 cm.
6. Load the entire samples from the four microfuge tubes into each of the wells formed by the comb, using an automatic micropipettor and a disposable tip for each sample. The glycerol in the solutions will prevent the liquid from rising, but be careful to inject the sample slowly and avoid contaminating neighboring wells.
7. To a fifth well add 10 μL of commercial λ DNA marker in a tracking dye solution.
8. When all the samples have been loaded, connect the electrophoretor to a power supply (be sure to connect the + [red] lead *to the bottom* of the apparatus) and apply 150 V for about 3 hr.
9. Turn off the power supply, and disconnect the apparatus carefully. Then, wearing gloves to avoid contact with ethidium bromide, move the gel to a UV viewing box to note the fluorescing bands.
10. Photograph the gels under UV light, and make measurements of the bands from the photographs.

Visualization and Calculation of Fragment Sizes

The λ DNA *Hind* III fragments should show six bands clearly:

DNA fragment	*Kilobase pairs (kb)*
1	23.13
2	9.42
3	6.56
4	4.36
5	2.32
6	2.03

1. Measure the bands, and calculate R_m values for the known λ DNA and for the digests of pBR322.
2. Construct a standard curve by plotting R_m *vs.* log kb, and determine the exact sizes of the fragments obtained. Compare these values with those expected on the basis of the restriction map of pBR322 and the known loci for *Hind* III and *Pvu* II.