GENERAL BIOLOGY LABORATORY II

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Bioassays of major biomolecules: *Nucleic acids*

DNA is the genetic material in all organisms. Scientists work with DNA for a variety of reasons, such as cloning, amplification, sequencing, and other genetic manipulations. In general, the first steps in DNA (or RNA) studies involve DNA isolation and their qualitative, and quantitative determination. DNA or RNA concentration in solution can be determined through the optic properties (max. absorbance) of nucleotides at 260 nm. Once their concentration and purity are determined, nucleic acids can be investigated with more specific and sensitive methods (e.g., agarose gel electrophoresis, etc.).

1 DNA can be extracted and isolated from any cell, tissue, or organ using a variety of methods such as alkali lysis, enzymatic lysis and boiling methods and it can be precipitated from the rest of cell components by ethanol, isopropanol precipitation.

As we have seen for proteins which have specific absorbance maxima at 280 nm, nucleic acids absorb light maximally at 260 nm. However, while one unit A at 280 nm is equal to one unit of protein (as mg ml^{-1}), for DNA and RNA 1 A unit at 260 nm is equal to about 50 and 40 μg ml^{-1}, respectively.

The cell extract is a mixture of all cell components and organelles. Once large particles (e.g., organelles, membrane fragments, etc) are removed by a low speed centrifugation, the solution part (i.e., supernatant) contains cell components such as proteins and nucleic acids dissolved in an aqueous environment. While proteins can be salted out with a salt (e.g., ammonium sulfate) at different concentrations, DNA or RNA can be precipitated using ethanol. Ethanol disrupts the screening of charges by water and thus the electrical attraction between phosphate groups and any positive ions present in solution becomes strong enough to form stable ionic bonds causing DNA precipitation.

The purity of DNA in a mixture of proteins and RNA can be understood from a simple absorbance reading at 260 and 280 nm. If, A_{260}/A_{280} = 1.8-2.0, that means we have an almost pure DNA solution. If this ratio is higher than 2.0, sample contains significant amount of RNA also. A value lower than 1.8 indicates the presence of substantial amount of protein.

Preparation and Analysis of DNA

The ability to prepare and isolate pure DNA from a variety of sources is an important step in many molecular biology protocols. The isolation of genomic, plasmid, or DNA fragments from

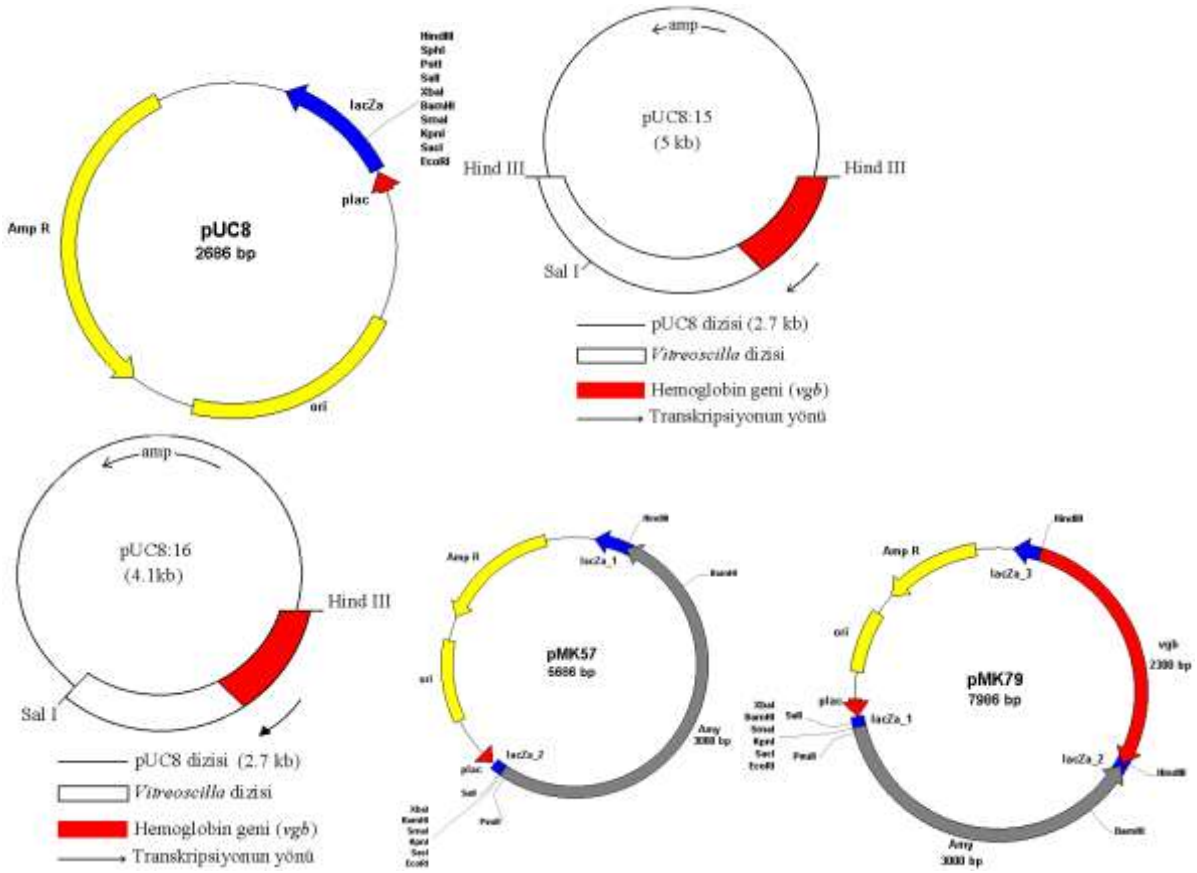
restriction digests and polymerase chain reaction (PCR) products has become a common everyday practice in almost every laboratory.

Day 1

Miniprep of Plasmid DNA

Although there are a large number of protocols for the isolation of small quantities of plasmid DNA from bacterial cells (minipreps), this unit presents the alkaline lysis procedure which is the most commonly used miniprep. Plasmid DNA is prepared from plasmid-containing bacteria. Bacteria are lysed by treatment with a solution containing sodium dodecyl sulfate (SDS) and NaOH (SDS denatures bacterial proteins, and NaOH denatures chromosomal and plasmid DNA). The mixture is neutralized with potassium acetate, causing the covalently closed plasmid DNA to reanneal rapidly. Most of the chromosomal DNA and bacterial proteins precipitate—as does the SDS, which forms a complex with potassium—and are removed by centrifugation.

We will isolate and purify (miniprep) below plasmids from recombinant bacteria that have been constructed in our laboratory.



Materials

- LB medium containing appropriate antibiotic
- Glucose/Tris/EDTA (GTE) solution
- TE buffer
- NaOH/SDS solution
- Potassium acetate solution
- 95% and 70% ethanol
- 1.5-ml disposable microcentrifuge tubes

Protocol

1. Inoculate 5 ml sterile LB medium with a single bacterial colony. Grow to saturation (overnight).

2. Spin 1.5 ml of cells 20 sec in a microcentrifuge at maximum speed to pellet. Remove the supernatant with a Pasteur pipet.

3. Resuspend pellet in 100 µl GTE solution and let sit 5 min at room temperature.

4. Add 200 µl NaOH/SDS solution, mix by tapping tube with finger, and place on ice for 5 min.

5. Add 150 µl potassium acetate solution and vortex at maximum speed for 2 sec to mix. Place on ice for 5 min.

6. Spin 3 min as in step 2 to pellet cell debris and chromosomal DNA.

7. Transfer supernatant to a fresh tube, mix it with 0.8 ml of 95% ethanol, and let sit 2 min at room temperature to precipitate nucleic acids.

8. Spin 1 min at room temperature to pellet plasmid DNA and RNA.

9. Remove supernatant, wash the pellet with 1 ml of 70% ethanol, and dry pellet under vacuum.

10. Resuspend the pellet in 30 µl TE buffer and store as in support protocol. Use 2.5 to 5 µl of the resuspended DNA for a restriction digest.

REAGENTS AND SOLUTIONS

- ***LB Medium, pH 7.0***
  10 g tryptone
  5 g yeast extract
  10 g NaCl
  deionized water to1000 mL

- ***Glucose/Tris/EDTA (GTE) solution***
  50 mM glucose
  25 mM TrisHCl, pH 8.0
  10 mM EDTA
  Autoclave and store at 4°C

- ***NaOH/SDS solution***
  0.2 N NaOH
  1% (wt/vol) sodium dodecyl sulfate (SDS)
  Prepare immediately before use

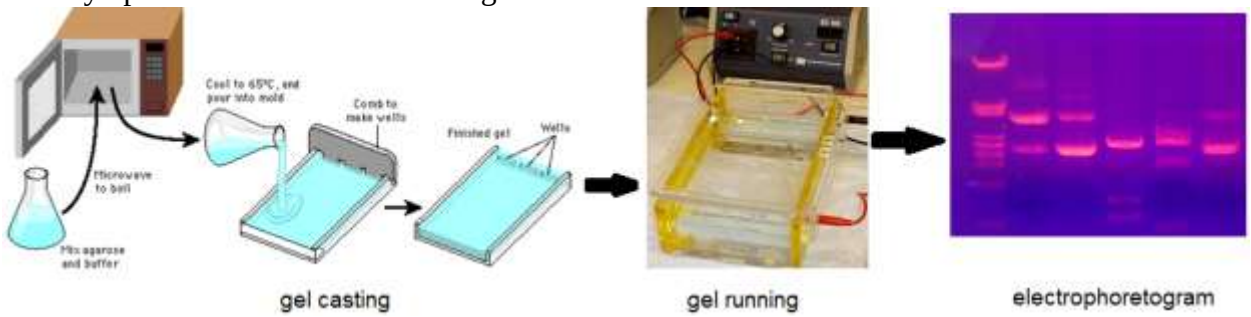
- ***5 M potassium acetate solution, pH 4.8***
  29.5 ml glacial acetic acid
  KOH pellets to pH 4.8 (several)
  H_2O to 100 ml
  Store at room temperature (do not autoclave)

- ***TE (Tris/EDTA) buffer***
  10 mM TrisHCl, pH 7.4, 7.5, or 8.0 (or other pH; see recipe)
  1 mM EDTA, pH 8.0

Day 2

Agarose Gel Electrophoresis

Agarose gel electrophoresis is a simple and highly effective method for separating, identifying, and purifying 0.5- to 25-kb DNA fragments. The protocol can be divided into three stages: (1) a gel is prepared with an agarose concentration appropriate for the size of DNA fragments to be separated; (2) the DNA samples are loaded into the sample wells and the gel is run at a voltage and for a time period that will achieve optimal separation; and (3) the gel is stained or, if ethidiumbromide has been incorporated into the gel and electrophoresis buffer, visualized directly upon illumination with UV light.



Materials

- Electrophoresis buffer
- Ethidium bromide solution
- Electrophoresis-grade agarose
- 10× loading buffer
- DNA molecular weight markers
- Horizontal gel electrophoresis apparatus
- Gel casting platform
- Gel combs
- DC power supply

Preparing the gel

1. Prepare an adequate volume of electrophoresis buffer (TAE or TBE) to fill the electrophoresis tank and prepare thegel.

To facilitate visualization of DNA fragments during the run, ethidium bromide solution can be added to the electrophoresis buffer to a final concentration of 0.5 μg/ml. If buffer is prepared for the electrophoresis tank and the gel separately, be sure to bring both to an identical concentration of ethidium bromide.

CAUTION: *Ethidium bromide is a mutagen and potential carcinogen. Gloves should be worn and care should be taken when handling ethidium bromide solutions.*

2. Add the desired amount of electrophoresis-grade agarose to a volume of electrophoresis buffer sufficient for constructing the gel. Melt the agarose in a microwave oven or autoclave and swirl to ensure even mixing. Gels typically contain 0.8 to 1.5% agarose.

3. Seal the gel casting platform if it is open at the ends. Pour in the melted agarose and insert the gel comb, making sure that no bubbles are trapped underneath the combs and all bubbles on the surface of the agarose are removed before the gel sets.

Most gel platforms are sealed by taping the open ends with adhesive tape. As an added measure to prevent leakage, hot agarose can be applied with a Pasteur pipet to the joints and edges of the gel platform and allowed to harden.

Loading and running the gel

4. After the gel has hardened, remove the tape from the open ends of the gel platform and withdraw the gel comb, taking care not to tear the sample wells.

Most gel platforms are designed so that 0.5 to 1 mm of agarose remains between the bottom of the comb and the base of the gel platform. This is usually sufficient to ensure that the sample wells are completely sealed and to prevent tearing of the agarose upon removal of the comb. Low percentage gels and gels made from low gelling/melting temperature agarose should be cooled at 4°C to gain extra rigidity and prevent tearing.

5. Place the gel casting platform containing the set gel in the electrophoresis tank. Add sufficient electrophoresis buffer to cover the gel to a depth of about 1 mm (or just until the tops of the wells are submerged). Make sure no air pockets are trapped within the wells.

6. DNA samples should be prepared in a volume that will not overflow the gel wells by addition of the appropriate amount of 10× loading b u ffer. Samples a re typically loaded into the wells with a pipettor or micropipet. Care should be taken to prevent mixing of the samples between wells.
Be sure to include appropriate DNA molecular weight markers.

7. Be sure that the leads are attached so that the DNA will migrate into the gel toward the anode or positive lead. Set the voltage to the desired level, typically 1 to 10 V/cm of gel, to begin electrophoresis. The progress of the separation can be monitored by the migration of the dyes in the loading buffer.

CAUTION: *To prevent electrical shocks, the gel apparatus should always be covered and kept away from heavily used work spaces.*

8. Turn off the power supply when the bromphenol blue dye from the loading buffer has migrated a distance judged sufficient for separation of the DNA fragments. If ethidium bromide has been incorporated into the gel, the DNA can be visualized by placing on a UV light source and can be photographed directly.

Gels that have been run in the absence of ethidium bromide can be stained by covering the gel in a dilute solution of ethidium bromide (0.5 μg/ml in water) and gently agitating for 10 to 30 min. If necessary, gels can be destained by shaking in water for an additional 30 min. This serves to remove excess ethidium bromide which causes background fluorescence and makes visualization of small quantities of DNA difficult.

REAGENTS AND SOLUTIONS

Agarose gel

Gels typically contain 1% agarose in 1× TAE or TBE. Electrophoresis-grade agarose powder is added to 1× gel buffer and melted by boiling for several minutes. Be sure all agarose particles are completely melted. To facilitate visualization of DNA fragments during the run, ethidium bromide can be added to 0.5 μg/ml in the gel.

- ***TAE (Tris/acetate/EDTA) electrophoresis buffer***
  50× stock solution:
  12.1 g Tris base
  2.85 ml glacial acetic acid
  1.86 g $Na_2EDTA{\cdot}2H_2O$
  H_2O to 50 ml

  Working solution, pH 8.5:
  40 mM Tris·acetate
  2 mM $Na_2EDTA{\cdot}2H_2O$

- ***Ethidium bromide solution***
  1000× stock solution, 0.5 mg/ml:
  50 mg ethidium bromide
  100 ml H_2O
  Working solution, 0.5 μg/ml:

Dilute stock 1:1000 for gels or stain solution
Protect from light.

- ***10× loading buffer***
  20% glycerol
  0.1 M disodium EDTA, pH 8
  1.0% sodium dodecyl sulfate
  0.25% bromphenol blue

Learning Outcomes and Objectives

- What are the main genetic engineering tools you have used in your experiments? Describe each.
- What is the function of each reagent you have used (e.g., brome phenol blue, ethidium bromide, EDTA, glycerol, SDS, etc.)
- What is the basis of DNA precipitation with ethanol?
- Find out the size of DNA fragments on your agarose gel, extrapolating them against fragments of known size (i.e., lambda DNA digest).
- Try to reconstruct the physical map of plasmids from fragment profile of different digests.