Procedure Manual

GENERAL AND MOLECULAR GENETICS

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BASIC MOLECULAR BIOLOGY TECHNIQUES

INTRODUCTION

Molecular biology techniques are applicable not only to modern medical practice but also to the identification of genetically modified organisms, forensics, and quality assessment of laboratory animals, pharmacogenomics, and other fields.

This procedure manual encompasses most widely used molecular biology techniques namely DNA extraction, end-point and Real-Time PCR.

SAFETY CONSIDERATIONS

- Use personal protective equipment such as disposable gloves, lab coats, disposable masks, etc.
- Handle all sharps with care and dispose of sharps in the sharps disposal containers.
- Handle hazardous chemicals and samples carefully. Blood and other body fluid must be considered potentially hazardous. Biological waste should be disposed of in the designated trash bags that could be incinerated later on.
- Decontaminate the work benches regularly and especially before and after work. For decontamination, wipe the surfaces with 10% bleach followed by water.

MINIMUM STANDARDS AND CONTROLS

During DNA extraction, PCR and other experiments appropriate standards and controls should be used to assure the quality of the results.

- Positive control to check the efficiency of the reagents, procedure and equipment.
- Negative Control to check the contamination

Validation of Critical Reagents and Procedures

All technical procedures and critical reagents should be tested and validated before performing the actual case work or research work experiments.

Calibration of Instruments

All instruments should be calibrated according to required schedule and before performing the validation studies, case work and research experiments.

Practical No. 1: Preparation of different stock solutions used in molecular biology (solution used in PCR, electrophoresis, DNA isolation, RNA isolation and Protein isolation)

PREPARATION OF REAGENTS

The following general instructions are applicable in the preparation of all reagents. Use graduated cylinders or pipettes closest to the volume being measured for preparing liquid reagents. Store all reagents in sterile containers unless otherwise noted. Label all reagents with name of reagent, date prepared , initials of scientist that prepared reagent , lot number , and expiration date . Record each preparation in the lab's reagent logbook.

1M Tris-HCl [Tris (Hydroxymethyl) aminomethane

Tris base	121.1g
H_2O	to 800ml

Adjust to desired pH with concentrated HCl.
Mix and add H_2O to 1 Liter.
Store at room temperature.

0.5 M EDTA (Ehylenediamine Tetraacetic Acid) pH 8.0

$Na_2EDTA.2H2O$	186.1g
H_2O	to 700ml

Adjust pH to 8.0 with 10M NaOH (almost 50ml)
Mix and add H_2O to 1 Liter.
Store at room temperature.

10M NaOH

NaOH	400 g
H_2O	to 1 Liter

Store at room temperature.

10 mg/ml Ethidium Bromide

Ethidium Bromide	0.2 g
H_2O	to 20ml

Mix well and store at 4^oC in dark.

TE (Tris 10 mM-EDTA 2mM) pH 8.0

1M Tris-HCl ph 8.0	10 ml
0.5 M EDTA pH 8.0	4 ml
H_2O	to 1 Liter

Store at room temperature.

Low TE (Tris 10 mM-EDTA 0.2 mM) pH 8.0

1M Tris-HCl ph 8.0	10 ml
0.5 M EDTA pH 8.0	0.4 ml
H_2O	to 1 Liter

Store at room temperature

PROTEINASE K (20mg/ml)

Proteinase K	100 mg lyophilized powder
Ultra-pure H_2O	to 5 ml

Aliquot and store at approximately -20°C.

CAUTION: Powder and solutions of Proteinase K can be irritating to mucous membranes.

SDS 20% w/v

Sodium dodecyl sulfate	200g
H_2O	to 700ml

Heat to approximately 65^{o}C to dissolve.

Bring to a final volume of 1.0 L with ultra pure water.

Store at room temperature.

CAUTION: SDS can be irritating to mucous membranes. Wear safety glasses, mask and gloves when handling.

TEN buffer (10mM Tris, 2mM EDTA, 400 mM NaCl)

1 M Tris-HCl ph 8.0	10 ml
5M NaCl	80 ml
0.5M EDTA	4 ml
H_2O	to 1 Liter

Store at room temperature.

50x TAE (Tris-Acetate-EDTA) Electrophoresis Stock buffer

Tris base	242g
Glaciall acetic acid	57.1 ml
0.5 M EDTA pH 8.0	100ml

H_2O to 1 Liter

Store at room temperature.

50x TAE (Tris 40mM-Acetate 20mM-EDTA 2mM) Electrophoresis working buffer

50x TAE	10 ml
H_2O	to 500 ml

The pH of diluted buffer is 8.3.

Store at room temperature.

10x TBE (Tris 90mM-Borate 90mM-EDTA 2mM) Electrophoresis buffer

Tris base	108g
Boric Acid	55g
0.5M EDTA pH 8.0	40 ml
H_2O	to 1 Liter

Store at room temperature

2x Gel Loading Dye

2% Bromophenol blue	0.25 ml
2% Xylene cyanol	0.25 ml
Glycol	7ml
H_2O	10ml

Store at room temperature.

5M Sodium Chloride

Sodium Chloride	292.2 g
H_2O	to 1 Liter

Store at room temperature.

6M Sodium Chloride

Sodium Chloride	351 g
H_2O	to 1 Liter

Store at room temperature.

Practical No. 2: Isolation of DNA from Human Blood

Principle

The extraction of DNA involves three main steps that are cell lysis, protein separation, and DNA purification. Cell lysis is usually performed by incubation of cell in buffer containing detergent and protease. Cellular proteins are salted out or phase separated using organic solvents. Finally DNA is isolated and purified either by alcohol precipitation or adsorption with silica and elution.

Reagents required

- TE buffer (10mM Tris, 2mM EDTA, pH 8.0)
- TEN buffer (10 mM Tris, 2mM EDTA, 400mM NaCl)
- 10% SDS
- Proteinase-K solution 20mg/ml
- 6M NaCl
- Phenol:choloroform:isoamylalcohol (25:24:1)
- Absolute Ethanol or Isopropanol
- 75% Ethanol
- Low TE buffer (10mM Tris, 0.2mM EDTA)

Consumables required

- Filter barrier tips 200 µl
- Filter barrier tips 1000 µl
- Wide bore tips 1000 µl
- Falcon tubes 15 ml
- Microcentrifuge tubes 1.5 ml

Equipment required

- Centrifuge for 15 ml falcon tubes
- Microcentrifuge for 1.5 ml tubes
- Adjustable micropipettes 1 ml and 200 µl

Procedure

1. Add 1 ml chilled TE buffer to 200 µl blood. Mix by inverting the tube several times.
2. Spin at 4000 rpm for 15 min at room temperature.
3. Discard the supernatant and add 900 µl chilled TE buffer. Re-suspend the pellet by vigorous shaking by hand.
4. Spin at 4000 rpm for 15 min at room temperature.
5. Discard the supernatant and add 800 µl TE buffer. Re-suspend the pellet by vigorous shaking by hand.
6. Spin at 4000 rpm for 15 min at room temperature.

7. Discard the supernatant and add 200 µl TEN buffer, 20 µl SDS (10% solution) and 10 µl Proteinase-K solution. Re-suspend the pellet by shaking and vortex mixing.
8. Incubate the mixture at 56°C overnight.
9. Next day, place the tubes on ice and add 50 µl 6M NaCl. Shake the tube vigorously and place on ice again for 15 min.
10. Spin at 4000 rpm for 15 min to pellet down the salts and proteins.
11. Transfer the supernatant in a fresh properly labeled 1.5-ml centrifuge tube.
12. Add equal volume of chilled isopropanol and invert the tubes gently till DNA is visible.
13. Spin at 8000 rpm for 1 min at room temperature. Discard supernatant.
14. Add 200 ml absolute ethanol and vortex for 15 sec.
15. Spin at 8000 rpm for 1 min at room temperature.
16. Add 200 ml 75% ethanol and vortex for 15 sec.
17. Spin at 8000 rpm for 1 min at room temperature.
18. Discard the supernatant and add 100 µl low TE buffer or sterile distilled water to dissolve the DNA pellet. Incubate at 72°C for 30 min.
19. Store DNA at -20°C.

Practical No. 3: Quantification of DNA and RNA through spectrophotometer

Purpose:

DNA / RNA Concentration

Spectrophotometry is commonly used to measure DNA / RNA concentration. This method can be used to measure microgram quantities of pure Nucleic Acid samples. DNA / RNA concentration in a pure sample is determined by measuring the absorbance at 260 nm (A_{260}) in a spectrophotometer using a quartz cuvette. Samples contaminated by molecules that also absorb light at 260 nm (proteins, phenol, agarose) will result in inaccurate quantification.

For greatest accuracy, readings should be between 0.1 and 1.0. An absorbance of 1.0 unit at 260 nm corresponds to 50 μg of DNA per ml. This relationship is valid only for measurements made at neutral pH, therefore samples should be diluted in a low-salt buffer with neutral pH (e.g. 10mM Tris-Cl, pH 7.0)

Purity of DNA / RNA

The purity of a Nucleic Acid sample, with respect to contaminants that absorb UV light, can be estimated by calculating the A_{260}/A_{280} ratio. However, this ratio is influenced considerably by pH. Since water is not buffered, the pH and the resulting A_{260}/A_{280} ratio can vary greatly. Lower pH results in a lower A_{260}/A_{280} ratio and reduced sensitivity to protein contamination. Pure DNA has an A_{260}/A_{280} ratio of 1.7-1.9. Scanning the absorbance from 220-330 nm will show whether there are contaminants at 260 nm.

I. Spectrophotometric Measurement of DNA / RNA Concentration and Purity

The following protocol outlines the steps involved in measuring Nucleic Acid concentration using 500 μl quartz cuvettes and a 10mM Tris-Cl, pH 7.0 buffer.

1. Turn on the spectrophotometer and set the wavelength to 260 nm.

2. Transfer 495 μl of 10mM Tris-Cl, pH 7.0 to two 500 μl quartz cuvettes.

3. Insert the first cuvette into the spectrophotometer and zero the instrument.

   Note: This cuvette acts as the blank

4. Insert the second cuvette into the spectrophotometer and record the reading.

   Note: This is called matching the cuvettes. The value recorded is the correction factor that will be added or subtracted (depending if it is negative or positive) from the reading recorded for the DNA sample.

5. Remove the second cuvette and add 5 μl of the DNA sample.

6. Mix by placing parafilm over the cuvette and inverting several times.

7. Place cuvette in the spectrophotometer and record the absorbance at 260 nm.

8. Calculate the DNA concentration.

9. To determine the purity of the DNA sample, set the wavelength of the spectrophotometer to 280nm.

10. Place the first cuvette in the spectrophotometer and zero the instrument.

11. Place the second cuvette containing the DNA sample in the spectrophotometer and record the absorbance at 280nm.

II. Example

Calculating DNA Concentration

DNA Concentration = Spectrophotometric Conversion x (A_{260} – Correction Factor) x Dilution Factor

1. Correction Factor = 0.012
2. A_{260} of DNA sample = 0.127
3. Spectrophotometric conversion for dsDNA: 1.0 A_{260} unit = 50 μg/ml
4. Dilution Factor = 100 (5 μl DNA sample + 495 μl Buffer)

DNA Concentration = 50 μg/ml x (0.127 – 0.012) x 100

DNA Concentration = **575 μg/ml or 575 ng/μl**

Calculating DNA Purity

DNA Purity = A_{260}/A_{280}

1. A_{260} of DNA sample = 0.127
2. A_{280} of DNA sample = 0.068

DNA Purity = 0.127/0.068

DNA Purity = **1.87**

Practical No. 4. DNA amplification through polymerase chain reaction

PCR technique can be used to exponentially amplify a specific segment of DNA. The process relies on repeated cycles of denaturation, annealing, and extension using a thermostable DNA polymerase (commonly Taq polymerase) and a pair of oligonucleotide primers that flank the target region.

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2. Materials and Reagents

- **Template DNA:** Purified sample containing the target region.
- **Primers:**
    - Forward Primer (10 µM)
    - Reverse Primer (10 µM)
      Tip: Design primers with similar melting temperatures (Tm) and minimal secondary structure.
- **dNTP Mix:** A balanced mixture of dATP, dCTP, dGTP, and dTTP (typically 10 mM each).
- **10X PCR Buffer:** Often includes $MgCl_2$ (final concentration usually 1.5–2.5 mM in the reaction).
- **Taq DNA Polymerase:** A thermostable enzyme (provided at 5–10 U/µL).
- **Sterile, nuclease-free Water**

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3. Equipment

- **Thermocycler:** For precise thermal cycling.
- **Pipettes and Filter Tips:** To ensure accurate and contamination-free pipetting.
- **PCR Tubes or Plates**
- **Vortex Mixer and Microcentrifuge:** For thorough mixing and quick collection of reagents.

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4. Reaction Setup

a. Master Mix Preparation (for a 50 µL reaction)

Component	Volume (µL)	Final Concentration in Reaction
10X PCR Buffer	5	1X
dNTP Mix (10 mM each)	1	200 µM each
Forward Primer (10 µM)	1	0.2 µM
Reverse Primer (10 µM)	1	0.2 µM
Taq Polymerase	0.25–0.5	Typically 1–2.5 U per reaction
Template DNA	1–5	Depends on concentration
Sterile Water	To 50 µL	–

Setup Steps:

1. **Thaw and Mix Reagents:** Thaw all reagents (except Taq polymerase) on ice and mix by gentle vortexing.
2. **Prepare the Master Mix:** Combine all components *except* the template DNA in a sterile tube.
3. **Aliquot the Master Mix:** Dispense the master mix into individual PCR tubes.
4. **Add Template DNA:** Add the template DNA last to each tube.
5. **Mix and Centrifuge:** Gently vortex and briefly spin down the tubes to ensure a homogeneous mixture.

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5. PCR Cycling Conditions

A typical PCR program includes the following steps:

1. **Initial Denaturation:**
    - 94°C – 95°C for 2–5 minutes
      Purpose: To completely denature the double-stranded DNA.
2. **PCR Cycling (30–35 Cycles):**
    - **Denaturation:**
        - 94°C for 30 seconds
          Purpose: To melt the DNA strands.

- **Annealing:**
  - 50°C – 65°C for 30 seconds
    Tip: Optimize based on primer Tm (usually Tm minus 3–5°C).
- **Extension:**
  - 72°C for 1 minute per kilobase (kb) of target DNA
    Purpose: To synthesize new DNA strands.

3. **Final Extension:**
   - 72°C for 5–10 minutes
     Purpose: To ensure complete extension of all PCR products.
4. **Hold:**
   - 4°C indefinitely until the samples are retrieved.

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6. Post-PCR Analysis and Results

a. Gel Electrophoresis

After PCR, analyze the amplified products using agarose gel electrophoresis:

- **Gel Preparation:**
  - Use a 1–2% agarose gel depending on the size of your expected product.
  - Include a DNA ladder (molecular weight marker) for size reference.
- **Sample Loading:**
  - Mix 5–10 µL of the PCR product with an appropriate loading dye.
  - Load the samples into the wells along with a negative control (no template) and a positive control if available.
- **Electrophoresis Conditions:**
  - Run the gel at 80–120 V until the dye front has migrated an adequate distance.
- **Staining:**

- Stain the gel with ethidium bromide or an alternative nucleic acid stain.

b. Results

- **Positive Sample Lane:**
  - A bright, distinct band corresponding to the expected amplicon size (e.g., a 500 bp fragment) should be visible.
- **Negative Control Lane:**
  - No bands should be present, confirming that there is no contamination.
- **DNA Ladder Lane:**
  - Bands at known sizes, which allow you to verify the size of your PCR product.

Lane 1: DNA Ladder | 100 bp, 200 bp, 300 bp, 400 bp, 500 bp, 600 bp, etc.
Lane 2: Negative Ctrl | No band present
Lane 3: PCR Product | -----[Bright Band at ~500 bp]-----

Practical No. 5: Separation of different sized DNA fragments on agarose gel

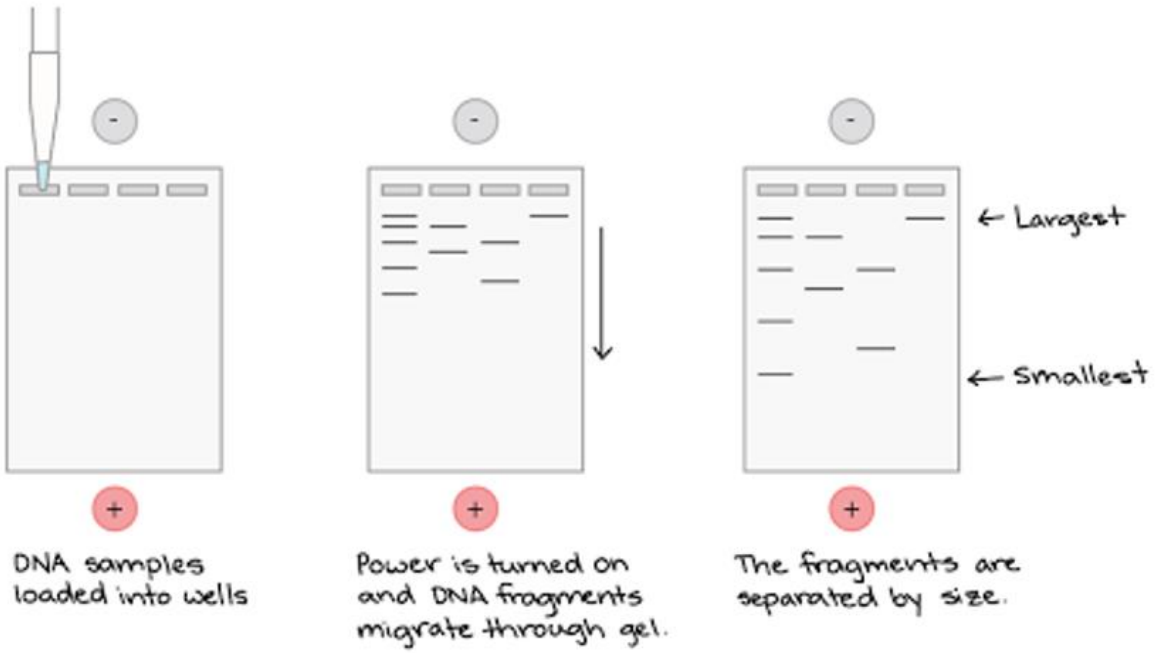
Aim:

- Evaluating the intactness of the extracted DNA by agarose gel electrophoresis.
- To separate and calculate the molecular size of DNA fragment by comparing the separated bands with known standard molecular weight marker.
- To quantify DNA fragment by comparing the separated band with known quantity of DNA.

Introduction:

Agarose gel electrophoresis is method for separation (by size), quantifying, purification of nucleic acids fragments mixture, and analysis of DNA restriction fragments. It is one of the most widely-used techniques in biochemistry and molecular biology. Agarose is a liner polymer composed of alternative residues of D-galactose and 3,6-anhydro-L-galactopyranose joined by α (1→3) and β (1→4) glycosidic linkages. Agarose and acrylamide matrices are used to separate DNA by gel electrophoresis. The choice of gel matrices and gel concentration depends on the size of nuclear acid molecules, as the concentration of the agarose or acrylamide determine the pores size:

w/v % Gel type	Size of DNA fragments (Kb = 1000 bp)
0.5 %	1 kb to 30 kb
0.7 %	800 bp to 12 kb
1.0 %	500 bp to 10 kb
1.2 %	400 bp to 7 kb
1.5 %	200 bp to 3 kb
2.0 %	50 bp to 2 kb



Several buffers are used for agarose gel electrophoresis, but the most common are: Tris-acetate EDTA buffer (TAE) and Tris-borate EDTA buffer (TBE). The DNA mobility in TBE buffer is approximately two times slower than in TAE buffer. This is due to the lower porosity of agarose gel when agarose polymerizes in the presence of borate.

Since DNA is colourless, the loaded sample need to be tracked. This is achieved by using a loading dye solution. Finally, to visualize DNA (result), agarose gels are usually stained with ethidium bromide and illuminated with UV light.

Identifying the size of a DNA sample is one of the common AGE uses and this accomplished through what called: DNA marker (Ladder). A DNA and RNA size markers contain a mixture of DNA (or RNA) fragments of known length, making them suitable for estimating the fragment length of concurrently run samples.

Principle:

Nucleic acids are separated by applying an electric field, so these negatively charged molecules will move through an agarose matrix towards the anode, and the biomolecules are separated by size in the agarose gel matrix, where the distance travelled by a DNA molecule is inversely correlated with its size.

Materials:

Agarose powder, 1X TBE buffer (89 mM Tris-base, 89 mM boric acid and 2 mM EDTA) prepared from 10X TBE, Ethidium Bromide (5 mg/ml), Gel loading dye (Glycerol and orange dye),1 kb and 100 bp DNA ladder, horizontal electrophoresis apparatus and power supply.

Protocol:

1. Measure the desired grams of agarose to make 1% agarose gel.
2. Heat the solution to boiling in the microwave to dissolve the agarose to produce a homogeneous mixture.
3. Add 4 µl of ethidium bromide CARFULLY to the dissolved agarose and mix .
4. Get a gel plate and a comb. Put the two dams into the slots on each side of the gel plate. Make sure that they fit tight. Pour the melted agarose onto the gel plate in the electrophoresis tray.
5. Place the comb in its place. Let the gel cool to room temperature.
6. Place the gel in the electrophoresis chamber.
7. Pour enough electrophoresis buffer (1X TBE) to cover the gel to prevent overheating of the gel.

8. Carefully remove the comb.
9. Prepare the DNA sample by mixing around 300 ng of DNA sample with 3-4 µl of loading dye.
10. Add 3 µl DNA ladder into the first well by using a micropipette.
11. Carefully place the prepared samples into adjacent wells
12. Electrophorese the samples at 95 V for 45 minutes. (Check the gel while it is running).
13. Carefully remove the gel, place it onto the UV light box and take a picture for the gel.

Results:

Picture of the gel.