Isolation and purification of genomic DNA from prokaryotes

AIM

To isolate and purify genomic DNA from prokaryotic cells using a combination of enzymatic lysis, chemical treatments, and precipitation methods.

PRINCIPLE

The principle of isolating and purifying genomic DNA from prokaryotes relies on breaking open the bacterial cells to release their genomic contents while protecting the DNA from degradation and contamination. This involves enzymatic digestion of the cell wall using lysozyme and disruption of the cell membrane with detergents like SDS or CTAB. Proteins and other cellular components are removed through enzymatic digestion with Proteinase K and phase separation using Phenol:Chloroform:Isoamyl alcohol, which separates DNA into the aqueous phase and proteins into the organic phase. RNA contaminants are eliminated using RNase. DNA is then precipitated from the solution using ethanol in the presence of salts like sodium acetate, which neutralize the negative charges on the DNA backbone, facilitating its aggregation. The DNA pellet is washed with 70% ethanol to remove residual impurities and resuspended in a suitable buffer for downstream applications. This process ensures the isolation of high-quality, intact genomic DNA.

MATERIALS REQUIRED:

Reagents:

- TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0)
- Lysozyme (10 mg/mL)
- SDS (10%) or CTAB
- Proteinase K (20 mg/mL)
- RNase A (10 mg/mL)
- Phenol:Chloroform:Isoamyl alcohol (25:24:1)
- 3M Sodium acetate (pH 5.2)
- Ethanol (100% and 70%)
- Nuclease-free water or TE buffer for resuspension

Equipment:

- Microcentrifuge and sterile centrifuge tubes
- Incubator or water bath
- Pipettes and tips
- Vortex mixer
- Spectrophotometer
- Electrophoresis unit with agarose gel and staining dyes (optional for observation)

PROCEDURE:

- **1.5 mL of bacterial culture was taken** (grown overnight).
- The culture was centrifuged at 6000 rpm for 10 minutes to pellet the cells.

- The supernatant was discarded, and the pellet was resuspended in 500 µL of TE buffer.
- **20 µL of lysozyme (10 mg/mL) was added** to the suspension.
- The mixture was incubated at 37°C for 30 minutes to digest the cell wall.
- **50 µL of SDS (10%) or CTAB was added** to disrupt the membrane, and the mixture was gently mixed.
- **20 µL of Proteinase K (20 mg/mL) was added**, and the sample was incubated at 55°C for 1 hour to digest proteins.
- **10 µL of RNase A (10 mg/mL) was added**, and the sample was incubated at 37°C for 30 minutes to remove RNA.
- An equal volume of Phenol:Chloroform:Isoamyl alcohol (25:24:1) was added to the lysate.
- The lysate was thoroughly mixed and centrifuged at 12,000 rpm for 10 minutes.
- The upper aqueous phase (containing DNA) was transferred to a new tube.
- This extraction step was repeated twice more.
- **1/10th volume of 3M sodium acetate (pH 5.2) and 2.5 volumes of chilled ethanol (100%) were added** to the aqueous phase.
- The solution was gently mixed and incubated at -20°C for 1 hour to precipitate DNA.
- The sample was centrifuged at 12,000 rpm for 15 minutes at 4°C, forming a white pellet of DNA.
- The supernatant was discarded, and the pellet was washed with 70% ethanol.
- The pellet was briefly air-dried and resuspended in 50-100 µL of TE buffer or nuclease-free water.

OBSERVATION:

A visible white or clear pellet at the bottom of the tube after ethanol precipitation indicates successful DNA isolation.

RESULT:

Genomic DNA is successfully isolated from prokaryotic cells. The extracted DNA is pure, as indicated by spectrophotometric analysis and agarose gel electrophoresis. The sample is ready for downstream applications such as PCR, cloning, or sequencing.

2. Isolation and purification of genomic DNA from eukaryotes

AIM:

To isolate and purify genomic DNA from eukaryotic cells by disrupting the cell and nuclear membranes, removing proteins and RNA, and recovering pure DNA through precipitation methods.

PRINCIPLE:

Eukaryotic cells require more complex DNA extraction protocols due to the presence of cellular organelles and a nuclear membrane. The process involves breaking open the cell and nuclear membranes using mechanical and chemical methods, such as detergent (e.g., SDS). Proteins are digested using Proteinase K, while RNA is removed with RNase. Organic solvents like Phenol:Chloroform:Isoamyl alcohol are used for phase separation to eliminate proteins and lipids. The DNA is precipitated using alcohol in the presence of salt, which neutralizes the DNA backbone and promotes aggregation. The final steps involve washing and dissolving the DNA pellet in a suitable buffer. This ensures the isolation of high-quality, intact DNA for downstream applications.

MATERIALS REQUIRED:

Reagents:

- PBS (Phosphate-Buffered Saline)
- SDS (10%)
- Proteinase K (20 mg/mL)
- RNase A (10 mg/mL)
- Phenol:Chloroform:Isoamyl alcohol (25:24:1)
- 3M Sodium acetate (pH 5.2)
- Ethanol (100% and 70%)
- TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0)

Equipment:

- Centrifuge and sterile microcentrifuge tubes
- Pipettes and tips
- Incubator or water bath
- Vortex mixer
- Spectrophotometer
- Agarose gel electrophoresis system

PROCEDURE:

- Cells were detached using trypsin, washed with PBS, and centrifuged to pellet.
- The cell pellet was resuspended in 500 µL of PBS.
- **50 µL of SDS (10%) was added**, and the mixture was gently mixed to lyse the cell membrane.
- **20 µL of Proteinase K (20 mg/mL) was added** to digest cellular proteins, including histones.
- The mixture was incubated at 55°C for 1-2 hours.

- **10 µL of RNase A (10 mg/mL) was added** to the lysate, and the sample was incubated at 37°C for 30 minutes to remove RNA.
- An equal volume of Phenol:Chloroform:Isoamyl alcohol (25:24:1) was added to the lysate.
- The lysate was thoroughly mixed and centrifuged at 12,000 rpm for 10 minutes.
- The upper aqueous layer was transferred to a new tube, ensuring no contamination from the interphase.
- The extraction step was repeated twice more to ensure protein removal.
- **1/10th volume of 3M sodium acetate (pH 5.2) and 2.5 volumes of chilled 100% ethanol were added.**
- The solution was gently mixed by inverting the tube and incubated at -20°C for 1 hour to precipitate DNA.
- The sample was centrifuged at 12,000 rpm for 15 minutes at 4°C to pellet the DNA.
- The pellet was washed with 1 mL of chilled 70% ethanol to remove salts and impurities.
- The pellet was briefly air-dried and resuspended in 50-100 µL of TE buffer or nuclease-free water.

OBSERVATION:

A visible white DNA pellet should form at the bottom of the tube after ethanol precipitation.

RESULT:

Genomic DNA is successfully isolated and purified from eukaryotic cells. The DNA is of high quality and suitable for downstream applications such as PCR, sequencing, and cloning.

Observation of DNA - Agarose gel electrophoresis.

AIM:

To observe the integrity, purity, and size of isolated genomic DNA by performing agarose gel electrophoresis and visualizing the DNA bands under UV light.

PRINCIPLE:

Agarose gel electrophoresis is a technique used to analyze the integrity and quality of isolated DNA. DNA molecules are negatively charged due to their phosphate backbone and migrate through the agarose gel matrix when an electric field is applied. The rate of migration depends on the size of the DNA fragments; larger molecules move slower, while

smaller ones move faster. DNA is visualized under UV light after staining with a dye such as ethidium bromide (EtBr) or SYBR Green, which intercalates with the DNA and fluoresces.

PROCEDURE:

- A 1% agarose gel was prepared by dissolving 1 g of agarose in 100 mL of 1X TAE buffer.
- The solution was heated until the agarose was fully dissolved, then cooled slightly, and a staining dye like ethidium bromide (0.5 µg/mL) was added.
- **5-10 µL of the DNA sample was mixed** with 2 µL of loading dye (bromophenol blue or xylene cyanol).
- The mixture was loaded into the wells of the agarose gel along with a DNA ladder (molecular weight marker) in a separate lane.
- The gel was placed in the electrophoresis chamber filled with 1X TAE buffer.
- The gel was run at 80-120 volts for 30-60 minutes or until the dye front had migrated sufficiently.
- The gel was viewed under a UV transilluminator to observe the DNA bands.

RESULT:

The appearance of an intact, high molecular weight DNA band on the agarose gel confirms successful isolation and good quality of the genomic DNA. The absence of smears or additional bands indicates purity and suitability for downstream applications.

Quantification of nucleic acids – DNA & RNA

AIM

To determine the concentration and purity of DNA and RNA samples using spectrophotometric and fluorometric methods.

PRINCIPLE

DNA and RNA absorb ultraviolet (UV) light at 260 nm due to their nitrogenous bases. The absorbance at 260 nm (A_{260}) is proportional to the concentration of nucleic acids. The purity of the sample can be assessed using the A_{260}/A_{280} ratio, where a ratio of ~1.8 indicates pure DNA, and ~2.0 indicates pure RNA. The A_{260}/A_{230} ratio can also indicate the presence of contaminants like phenols or salts. Alternatively, fluorescent dyes like SYBR Green or Qubit reagents specifically bind to nucleic acids, enabling more sensitive quantification.

Quantification of DNA

Materials Required

- TE buffer or nuclease-free water
- UV-Vis spectrophotometer or NanoDrop
- Quartz cuvettes (if using a conventional spectrophotometer)

Procedure

- The DNA sample was diluted in TE buffer. For NanoDrop, 1-2 µL of undiluted sample was used.
- The spectrophotometer was calibrated using TE buffer.

3. Measurement:
    - Measure absorbance at 260 nm (A_{260}) for DNA concentration.
    - Measure absorbance at 280 nm (A_{280}) for protein contamination.
    - Optionally, measure A_{230} for contaminants like salts or phenols.
4. Calculation:
    - DNA concentration (µg/mL) = $A_{260} \times 50 \times \text{dilution factor}$
    - Purity (A_{260}/A_{280}) = ~1.8 (pure DNA).

Quantification of RNA

Materials Required

- TE buffer or nuclease-free water
- RNase-free environment and reagents
- UV-Vis spectrophotometer or NanoDrop

Procedure

- The RNA sample was diluted in TE buffer. **1-2 µL was used** if measured using NanoDrop.

- The spectrophotometer was calibrated using TE buffer.

3. **Measurement:**
    - Measure absorbance at 260 nm (A_{260}) for RNA concentration.
    - Measure absorbance at 280 nm (A_{280}) for protein contamination.
    - Optionally, measure A_{230} for contaminants like salts or phenols.
4. **Calculation:**
    - RNA concentration (µg/mL) = $A_{260} \times 40 \times \text{dilution factor}$
    - Purity (A_{260}/A_{280}) = ~2.0 (pure RNA).

Calculation of DNA/RNA Concentration

To calculate the approximate volume or concentration of DNA/RNA, we use the formula:

Concentration (µg/mL) = $A_{260} \times \text{Conversion Factor} \times \text{Dilution Factor}$

Conversion Factors:

- DNA: 50 µg/mL per absorbance unit at A_{260}.
- RNA: 40 µg/mL per absorbance unit at A_{260}.

Example for DNA

1. Data:
   - Absorbance at A_{260} = 0.8
   - Dilution factor = 50 (sample diluted 1:50)
2. Calculation:
   DNA concentration = $0.8 \times 50 \times 50$
   DNA concentration = 2000 µg/mL (or 2 µg/µL)
3. Volume for a given DNA amount:
   If 1 µg of DNA is required:

$$\text{Volume} = \frac{\text{Amount Required (µg)}}{\text{Concentration (µg/µL)}}$$

Volume = $\frac{1}{2}$ µL = 0.5 µL

Example for RNA

1. Data:
   - Absorbance at A_{260} = 0.6
   - Dilution factor = 10
2. Calculation:
   RNA concentration = $0.6 \times 40 \times 10$
   RNA concentration = 240 µg/mL (or 0.24 µg/µL)
3. Volume for a given RNA amount:
   If 5 µg of RNA is required:

$$\text{Volume} = \frac{\text{Amount Required (µg)}}{\text{Concentration (µg/µL)}}$$

Volume = $\frac{5}{0.24}$ µL ≈ 20.83 µL

Separation of protein by SDS PAGE

AIM

To separate proteins based on their molecular weight using Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE).

PRINCIPLE

SDS-PAGE is a technique used to separate proteins by molecular weight under denaturing conditions. SDS, an anionic detergent, binds to proteins, imparting a uniform negative charge proportional to their length. This eliminates differences in charge and shape, allowing separation based solely on size during electrophoresis. The polyacrylamide gel acts as a molecular sieve, where smaller proteins migrate faster toward the anode under an electric field, while larger proteins move slower. Protein bands can then be visualized using staining techniques such as Coomassie Brilliant Blue or silver staining.

MATERIALS REQUIRED

- Protein samples
- SDS-PAGE gel apparatus
- 10% resolving gel and 4% stacking gel solutions
- SDS-PAGE running buffer (25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3)
- Protein loading buffer (Laemmli buffer)
- Molecular weight marker (protein ladder)
- Electrophoresis power supply
- Staining solutions (Coomassie Brilliant Blue or silver stain)
- Destaining solution (methanol, acetic acid)

PROCEDURE

- The protein sample was mixed with an equal volume of loading buffer (Laemmli buffer).
- The mixture was heated at 95°C for 5 minutes to denature the proteins.
- The resolving gel (10-12%) was prepared and poured into the gel apparatus and allowed to polymerize.
- A thin layer of isopropanol was added to level the gel surface.
- Once the resolving gel had set, the stacking gel (4%) was poured on top, and a comb was inserted to form wells.
- The comb was removed, and the gel apparatus was assembled. The apparatus was filled with running buffer.

- The protein samples and molecular weight marker were loaded into separate wells.
- The gel was run at 80-100 V through the stacking gel, then at 120-150 V for the resolving gel.
- After electrophoresis, the gel was immersed in Coomassie Brilliant Blue staining solution for 30-60 minutes.
- The gel was destained with a destaining solution until the protein bands became clearly visible.

OBSERVATION

The separated proteins appear as distinct bands on the gel. The migration distance of each band corresponds to its molecular weight, with smaller proteins traveling further.

RESULT

The proteins are successfully separated based on molecular weight using SDS-PAGE, and their relative sizes can be estimated by comparing their migration to the molecular weight marker.

Staining of proteins - Amido black, coomassie brilliant blue & AgNO3

1. Amido Black Staining

Aim

To detect and visualize proteins separated by SDS-PAGE using Amido Black, a general protein stain.

Principle

Amido Black is an anionic dye that binds nonspecifically to proteins via ionic and hydrophobic interactions. Proteins in the gel have a net negative charge due to SDS, and Amido Black interacts with positively charged amino acid residues and hydrophobic regions. The dye stains proteins a dark blue color. This method is less sensitive than other techniques, making it suitable for detecting higher quantities of proteins. Excess stain can be removed with a destaining solution, leaving stained protein bands visible against a light background.

Materials Required

- Amido Black staining solution (0.1% Amido Black, 25% isopropanol, 10% acetic acid)
- Destaining solution (25% isopropanol, 10% acetic acid)

Procedure

- The gel was immersed in Amido Black staining solution and gently agitated for 1-2 hours.
- Excess stain was removed by washing the gel in destaining solution with gentle agitation.
- The destaining was repeated until the protein bands became clearly visible.

Observation and Result

Proteins appear as dark blue bands on a light background.

2. Coomassie Brilliant Blue Staining

Aim

To stain and visualize proteins separated by SDS-PAGE using Coomassie Brilliant Blue, a commonly used and moderately sensitive protein stain.

Principle

Coomassie Brilliant Blue R-250 dye interacts with proteins through ionic and non-ionic interactions. The negatively charged sulfonic acid groups in the dye bind to positively charged amino acid residues (e.g., arginine, lysine, histidine) and hydrophobic regions of proteins. When bound to proteins, the dye undergoes a conformational change, resulting in a deep blue color. Excess dye is removed using a methanol-acetic acid-based destaining solution, enhancing the contrast between stained proteins and the transparent gel background. This method has moderate sensitivity and is widely used in protein biochemistry.

Materials Required

- Coomassie Brilliant Blue staining solution (0.1% Coomassie Brilliant Blue R-250, 50% methanol, 10% acetic acid)
- Destaining solution (40% methanol, 10% acetic acid)

Procedure

- The gel was placed in Coomassie Brilliant Blue staining solution and agitated for 30-60 minutes.
- The gel was washed with destaining solution to remove excess dye, leaving only the protein bands stained.
- The destaining was continued until a clear background was achieved.

Observation

Proteins appear as dark blue bands on a transparent background.

3. Silver Nitrate ($AgNO_3$) Staining

Aim

To detect and visualize proteins separated by SDS-PAGE with high sensitivity using silver nitrate staining.

Principle

Silver nitrate staining relies on the reduction of silver ions (Ag^+) to metallic silver (Ag^0) at protein sites. Proteins in the gel bind silver ions through interactions with amino acid residues, such as cysteine, lysine, and tyrosine. During the development step, a reducing agent like formaldehyde reduces the bound silver ions to metallic silver, producing dark brown or black protein bands. The background remains transparent as unbound silver ions are washed away. Silver staining is extremely sensitive, detecting protein quantities as low as nanograms. However, it requires careful handling of reagents and precise timing for optimal results.

Materials Required

- Fixing solution (40% ethanol, 10% acetic acid)
- Silver nitrate solution (0.1% $AgNO_3$)
- Developing solution (2.5% sodium carbonate, 0.02% formaldehyde)
- Stop solution (5% acetic acid)

Procedure

- The gel was fixed in fixing solution for at least 2 hours.
- The gel was washed with distilled water to remove fixative residues.
- The gel was stained with silver nitrate solution for 30-60 minutes.

- The gel was rinsed with water, and the bands were developed using the developing solution.
- The reaction was stopped with stop solution once clear bands became visible.

Observation and Result

Proteins appear as dark brown or black bands against a clear background.