MOLECULAR BIOLOGY

1. Aim: Preparation of ball and stick model for B-DNA molecule (A=T and G=C base pairs).

Materials Needed:

- Molecular modeling kit or software (e.g., ChemDraw, Avogadro, PyMOL)
- Different-colored balls representing atoms (carbon, nitrogen, oxygen, etc.)
- Stick connectors representing bonds (single, double)

Procedure:

1. Gather Materials: Ensure you have the necessary modeling kit or software along with balls and sticks of appropriate colors.

2. Build the Backbone:
    a. Begin by constructing the backbone of the DNA molecule using balls and sticks.
    b. Use different colors for different atoms in the backbone: black for carbon, red for oxygen, blue for nitrogen, and white for hydrogen.

3. Add Base Pairs:
    a. Represent each base pair (A=T and G=C) with the appropriate atoms and bonds.
    b. Adenine (A) and Thymine (T) form two hydrogen bonds between them.
    c. Guanine (G) and Cytosine (C) form three hydrogen bonds between them.
    d. Ensure the correct placement of hydrogen bonds between the base pairs.

4. Create the Double Helix:
    a. Connect the backbone to the base pairs using appropriate bonds to form the double helix structure.
    b. Ensure the correct distance and angle between base pairs to maintain the typical helical structure of DNA.

5. Check and Refine:
    a. Verify that the model accurately represents the structure of B-DNA, with the major and minor grooves clearly visible.
    b. Make any necessary adjustments to ensure the model is correct and symmetrical.

6. Label the Components (Optional):
    a. Optionally, you can label the different components of the DNA molecule, such as bases (A, T, G, C) and atoms (carbon, nitrogen, oxygen).

7. Display and Present:
    a. If using software, visualize the 3D model on the screen and adjust the view as needed.

b. If using physical models, carefully handle and display the model to demonstrate the structure of B-DNA.

Precutions:

- Refer to structural data or diagrams of B-DNA for accuracy in modeling.
- Ensure proper bonding angles and distances between atoms to maintain structural integrity.
- Double-check base pairing rules (A=T, G=C) to avoid errors in the model.

By following these steps and using appropriate materials, you can create a ball-and-stick model of a B-DNA molecule with A=T and G=C base pairs.

2. Aim: Extraction and isolation of genomic DNA by ethanol precipitation method

Reagents:

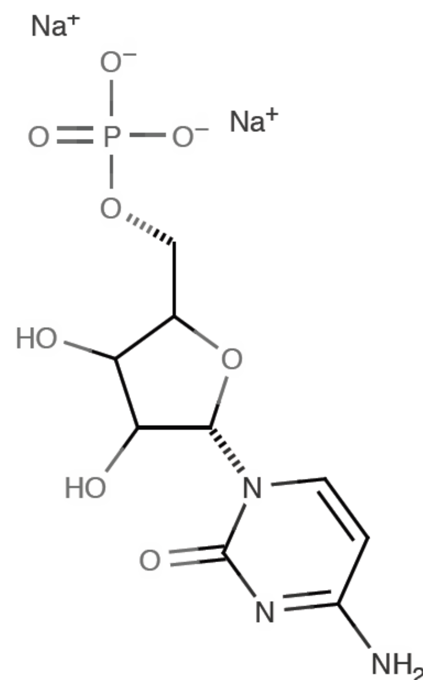
1. DNA sample/tissue sample
2. TRI reagent
3. Ethanol 95%
4. Ethanol 70%
5. Sodium acetate (3 M, pH 5.2)
6. TE buffer, 10× (pH 8.0) [100 mM Tris-Cl, 10 mM EDTA (pH 8.0)]

Principle:

DNA has net negative charge because of its phosphate backbone. sodium acetate (CH3COONa) which is most widely used in the ethanol precipitation is a carboxylate salt, carboxylate salts contain a carboxylate anion and a positively charged metal ion. when salt is added to a solution containing nucleic acids, the salt dissociates to CH3COO- and Na+.

When the cations and negatively charged nucleic acid backbone interact, nucleic acids are neutralized, therefore no longer dissolve in water and precipitate out of solution.

DNA surrounded bv water molecules



Sodium cations neutralizing a nucleotide.

Procedure:

1: First step is the extraction of genomic DNA by crushing the desired tissue in TRI reagent, making 10% W/V homogenate.

2: Centrifuge the homogenate at 10000g for 10-15 min at 4^0C and collect the supernatant containing DNA.

3: Isolation of genomic DNA:

- **i)** Add sodium acetate (3.0 M, pH 5.2) to the DNA solution to a final concentration of 0.3 M.
- **ii)** Add 2 volume of ice cold 95% ethanol with the DNA solution and mix well at room temperature.
- **iii)** Centrifuge the sample immediately for 20–30 min in a microcentrifuge tube at 0-4˚C to precipitate the DNA in pellet form.
- **iv)** Carefully decant the supernatant fluid into a fresh labelled tube without disturbing the DNA pellets.
- **v)** Now fill the centrifuge tube halfway with 70% ethanol and centrifuge at 10000g for 5 min at 4^0C.
- **vi)** Repeat the step v 2-3 times more. After final wash, carefully discard the supernatant with micropipette without disturbing the DNA pellet. As precaution, left some amount of ethanol in the tube and let it be air dry.
- **vii)** Do not allow the DNA to dry completely; otherwise, the pellet will be very difficult to dissolve. When the pellet is 80-90% dried, dissolve it in appropriate volume of 1X TE buffer (pH 8.0) and store at -80^0C for future use.

3. Aim: Isolation of the plasmid DNA from the E. coli culture by alkaline lysis method.

Material Required:

- Microfuge tubes
- Resuspension buffer (50 mM Tris HCl, pH 8, 10 mM EDTA, 100 µg/ml RNase A)
- Lysis buffer (0.2 N NaOH, 1% SDS)
- Neutralization buffer (3/5 M Potassium acetate, pH 6)
- Spin columns (Product Number SSC 100-01)
- Isopropanol
- Wash buffer (70% Ethanol)
- Elution buffer (water or TE buffer- 10 mM Tris, pH 8, 1 mM EDTA)

Equipment:

- Vortexer
- Centrifuge

Principle:

The isolation of plasmid DNA from E. coli using an alkaline lysis is a well-established method. E. coli with plasmid is cultured in media with antibiotics to a high cell density, harvested, and then lysed with a SDS/NaOH solution. Rapid acidification using concentrated potassium acetate causes the precipitation of protein and chromosomal DNA. Plasmid DNA, which is supercoiled, remains in solution and can be captured on a silica spin column. The plasmid DNA is washed with an ethanol solution and then eluted in water or TE buffer.

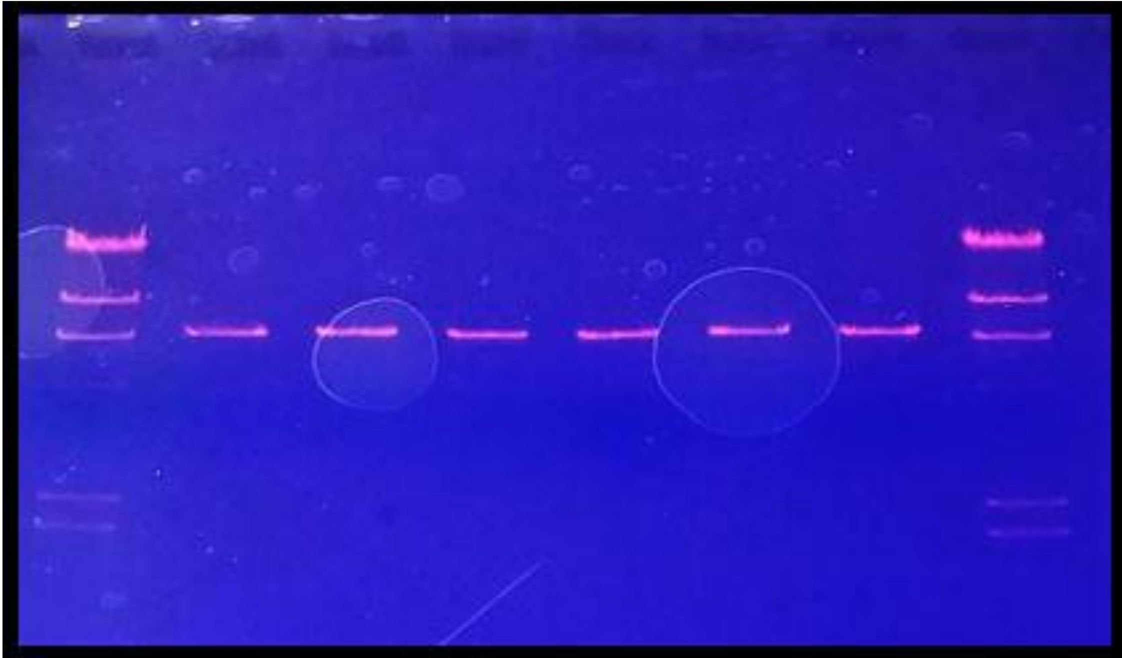
Procedure:

1. Culture E. coli with plasmid in LB media with antibiotic selective pressure, overnight on a shaker at 37°C.
2. Pellet 1.5 ml of bacterial culture in a microfuge tube by centrifuging for 2 minutes at 10,000 rpm.
3. Decant the supernatant and add 200 µl of the resuspension buffer. In order to resuspend the pellet you may have to vortex.
4. Add 250 µl of the lysis buffer, invert the tube 10 times to mix thoroughly. The solution should become clear and viscous.
5. Add 350 µl of the neutralization buffer, invert the tube 10 times or until a precipitate forms. The precipitate is a mixture of protein and chromosomal DNA.
6. Centrifuge the tube for 10 minutes at 10,000 rpm. Transfer the supernatant to a microfuge tube and add 0.7 isopropanol. Incubate at -20°C for 15 minutes.
7. Transfer the solution to a spin column.
8. Centrifuge the spin column for 1 minute at 7,000 rpm. Discard the flow through.
9. Add 400 µl of the wash buffer and centrifuge for 1 minute at 7,000 rpm. Discard the flow through. Repeat this step.
10. Centrifuge for an additional 2 minutes at 10,000 rpm to remove residual wash buffer.
11. Transfer the column to a clean microfuge tube. Add 50 µl of elution buffer and centrifuge for 1 minute at 10,000 rpm.

Results:

Eluted plasmid was read on a denovix spectrophotometer, to determine concentration. The machine was blanked with di water.

Sample Name	Concentration (ng/µl)	260/280	260/230
E. coli DH5a with pSVß	1143.41	2.06	2.38



The isolated plasmid under went an enzymatic digest with HindIII (sourced from NEB) and was run on an Agarose Gel with Ethidium Bromide, along side two lanes of digested Lambda ladders

4. Aim: Agarose gel electrophoresis of the plasmid DNA.

Requirements:

- Mini
- prep plasmid DNA extraction kit
- ddH_2O (deionized, sterile, DNase free)
- DNA ladder (25bp/ 100bp/ 1kb)
- 6x /10x DNA loading dye
- DNA staining dye (SYBR Green/ Red Safe/ Gel Red…DO NOT use Ethidium Bromide)
- Agarose gel (use DNA grade agarose)
- TAE buffer (Tris-Acetate EDTA buffer)
- Gel imager: UV trans illuminator

Principle:

Agarose gel electrophoresis is a gel electrophoresis technique used in biochemistry, molecular biology, genetics, and clinical chemistry to separate a mixed population of macromolecules in an agarose matrix. Agarose gel electrophoresis is the most efficient method for isolating DNA fragments ranging in size from 100 bp to 25 kb. An electric field is used to drive charged molecules across an agarose matrix, and the biomolecules are segregated by size in the agarose gel matrix.

Plasmid DNA can exist in three conformations: supercoiled, open-circular (oc), and linear (supercoiled plasmid DNA is often referred to as covalently closed circular DNA, ccc).

In vivo, plasmid DNA is a tightly supercoiled circle to enable it to fit inside the cell. In the laboratory, following a careful plasmid prep, most of the DNA will remain supercoiled, but a certain amount will sustain single-strand nicks. Given the presence of a break in only one of the strands, the DNA will remain circular, but the break will permit rotation around the phosphodiester backbone and the supercoils will be released.

A small, compact supercoiled knot of ccc-DNA sustains less friction against the agarose matrix than does a large, floppy open circle of oc-DNA. Therefore, for the same over-all size, supercoiled DNA runs faster than open-circular DNA. Linear DNA runs through a gel end first and thus sustains less friction than open-circular DNA, but more than supercoiled. Thus, an uncut plasmid produces two bands on a gel, representing the oc and ccc conformations. If the plasmid is cut once with a restriction enzyme, however, the supercoiled and open-circular conformations are all reduced to a linear conformation.

Following isolation, spontaneous nicks accumulate as a plasmid prep ages. This can clearly be seen on gels as the proportion of the two conformations change over time: plasmids preps that have been thawed and refrozen many times, show more oc DNA than fresh preps.

Procedure:

Agarose gel electrophoresis

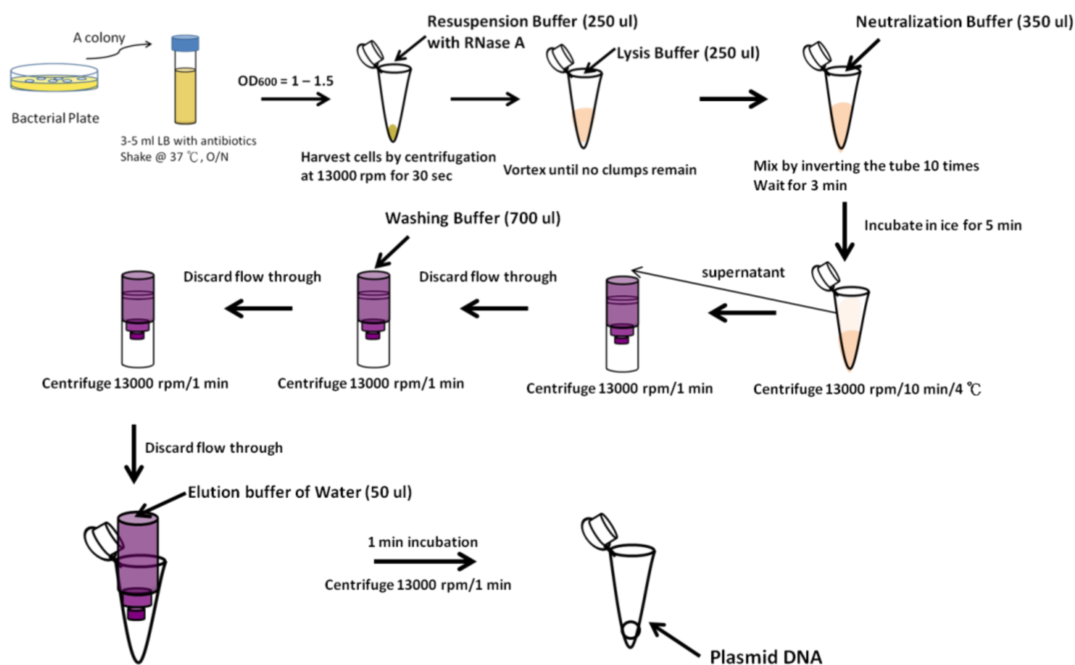
1. Setting up an agarose gel:
    1. For a small gel (the one used in our lab), add 20 ml 1X TAE buffer to a conical flask. (If there is none, dilute the 50X TAE buffer by 50 times.)

2. Then, add 0.2 g agarose (1%) to the conical flask and heat it by microwave oven by 30 45s to dissolve it until it becomes a clear and transparent liquid.
3. Cool it down a little bit by running water for around 15 s.
4. Add about 1 µl (for 20 ml TAE) of DNA staining dye, red safe (20000X).
5. Pour the solution to the white tightened tank with gates to allow it to solidify. Add the gel comb so as to create wells for the gel. Wait >15 30 min until it is gel like and ready to use.

2. Running agarose gel:
   1. Orient the gel with wells (comb removed) facing the BLACK negative electrode.
   2. Check if the gel is covered by TAE buffer in the tank.
   3. Add 6 x /10 x loading dye to the DNA to a total volume of < 25 µl (depended on the well) before adding to the wells. Mix loading dye to DNA to make the solution colored.
   4. Load the sample to the wells (<25 µl/ well)
   5. Add 3 5 µl DNA ladder to a separate well.
   6. Connect the electrodes to the power supply with correct col or, black to black, red to red. Apply power supply with 120 V. Check if there are bubbles on the negative electrodes.
   7. Allow it to run for about ~30 min (the time is variable based on the gel concentration and the size of interested DNA. Be aware the sample s run into the gel by checking if the blue band stays on the gel.
   8. After electrophoresis for 30 min, disconnect power, take the gel to imager, and turn UV on to observe bands.

Precautions:

- Never run a gel with >200V, as the heat so generated can melt the gel
- and also easier to cause electric leakage. Range from 80 160 V is acceptable.
- Usually it needs at least 100 ng DNA for a band to be seen and visualized on the UV
- trans illuminator.



5. Aim: Staining of B-galactosidase activity in the DH5alpha cells with pBluescript plasmid by IPTG+X-Gal as an example of induction of gene expression.

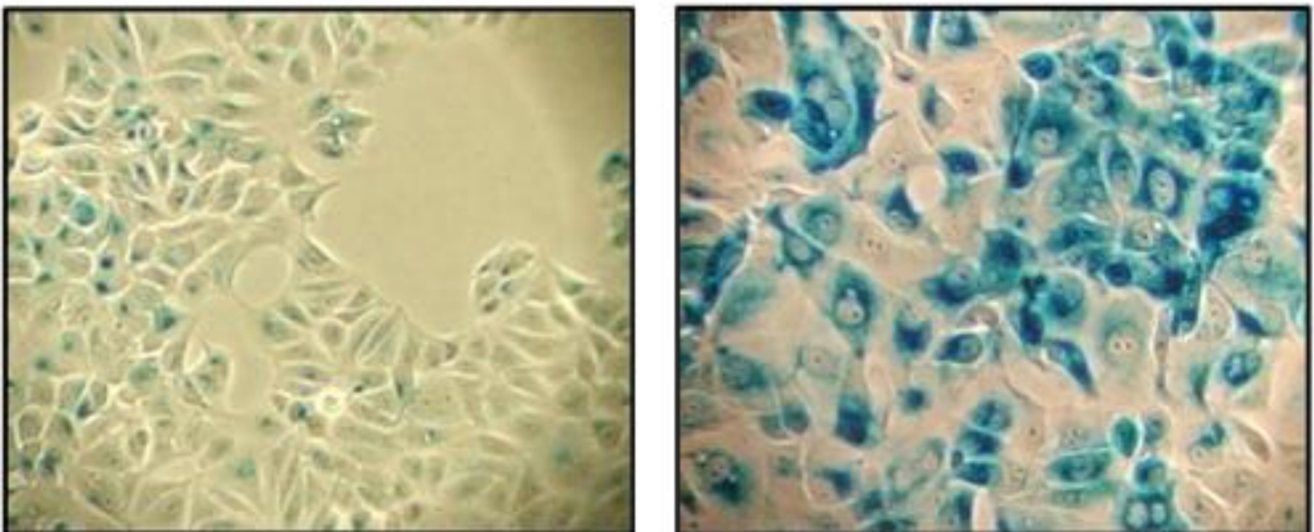
Materials Required:

- PBS
- 0.05% glutaraldehyde: Dilute 25% stock 500x in PBS. Stock should be stored frozen at -20C in aliquots
- X-gal solution: Prepare in PBS 30 mM potassium ferricyanide ($K_3Fe(CN)_6$), 30 mM potassium ferrocyanide ($K_4Fe(CN)_6$-$3H_2O$), 1 mM $MgCl_2$ or $MgSO_4$. Add Xgal to 1 mg/ml final concentration just before use. The solution minus the Xgal can be stored a few months at room temp.

Principle:

Staining for beta-galactosidase activity is based on the enzymatic activity of beta-galactosidase itself. Beta-galactosidase is an enzyme that hydrolyzes lactose into its constituent monosaccharides, galactose, and glucose. In the laboratory, it's commonly used as a reporter gene, meaning that its activity can be used to indicate the presence of certain genetic elements or the expression of specific genes.

The principle behind staining for beta-galactosidase activity involves the use of a substrate that can be cleaved by beta-galactosidase, resulting in the production of a detectable product. One of the most widely used substrates for this purpose is X-gal (5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside).



When X-gal is hydrolyzed by beta-galactosidase, it produces a blue-colored insoluble product. This product precipitates within the cells or tissues where beta-galactosidase is active, resulting in visible blue staining. Importantly, this staining is localized to the specific cells or tissues expressing beta-galactosidase, allowing for the visualization and identification of those cells.

The staining process typically involves incubating the samples with a solution containing X-gal. During this incubation period, beta-galactosidase enzymatically hydrolyzes X-gal, leading to the formation of the blue precipitate. After sufficient incubation time, the reaction is stopped, usually by washing the samples to remove excess substrate and halt further enzymatic activity.

The stained samples can then be visualized under a microscope. Cells or tissues expressing beta-galactosidase will appear blue, indicating the presence of enzymatic activity. This staining technique is widely used in molecular biology and biochemistry research for various applications, including the detection of gene expression, promoter activity, and the analysis of cellular differentiation and development.

Procedure :

1. Discard medium and wash twice with PBS. Add 2 ml fix (0.05% glutaraldehyde) per 60 mm dish and incubate for at least 5 minutes and no longer than 15 minutes.
2. Rinse cells three times in PBS as follows: Wash 1 and 3 are quick washes. Wash 2 is 10 minutes.
3. Add X-gal solution. Use a minimal volume to cover the cells, about 0.5 ml for a 60 mm dish.
4. Incubate from 1 hr to overnight at 37°C. Positive cells will stain blue.
5. Remove stain and overlay with 80% glycerol. The dishes can be stored indefinitely at 4°C

6. Aim: Preparation of LB-agar plates (with and without 100 microgram/ml Ampicillin and 10 microgram/ml Tetracycline), streaking of E. coli DH5alpha strain (normal) and transformed with plasmids [Ampicillin-resistant (pBluescript) and Tetracyclineresistant (pBR322)].

Materials Required:

Chemicals and Reagents:

- **Luria-Bertani (LB) Agar Powder**: LB agar is a complex medium containing tryptone, yeast extract, and agar. It provides essential nutrients for bacterial growth.
- **Ampicillin**: An antibiotic used for selection of bacteria containing ampicillin-resistant plasmids such as pBluescript.
- **Tetracycline**: An antibiotic used for selection of bacteria containing tetracycline-resistant plasmids such as pBR322.
- **Distilled Water**: Used for preparing LB agar solution and diluting reagents.
- **LB Broth**: For overnight culture of E. coli DH5alpha strains.
- **5-Bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-gal)**: If you plan to perform blue/white screening for bacterial colonies containing the pBluescript plasmid.
- **Isopropyl β-D-1-thiogalactopyranoside (IPTG)**: Inducer of beta-galactosidase activity, also used for blue/white screening.
- **Ethanol**: Used for sterilizing equipment like inoculating loops.
- **Petri Dishes**: Sterile plates for pouring and solidifying LB agar.
- **Sterile Inoculating Loop**: Used for streaking bacterial cultures onto agar plates.
- **Sterile Tubes**: For preparing overnight cultures of E. coli DH5alpha.
- **Autoclave Bags**: For autoclaving LB agar.
- **Incubator**: Set to 37°C for bacterial culture incubation.

Theory:

Luria-Bertani (LB) agar is a commonly used growth medium in microbiology for the cultivation of bacteria. It's composed of three main ingredients: tryptone, yeast extract, and agar.

Components of LB Agar:

1. Tryptone: Tryptone is a pancreatic digest of casein, which provides a source of amino acids and peptides. These amino acids are essential for bacterial growth and protein synthesis.

2. Yeast Extract: Yeast extract is derived from autolyzed yeast cells and serves as a source of vitamins, minerals, amino acids, and other growth factors necessary for bacterial metabolism and growth.

3. Agar: Agar is a polysaccharide extracted from marine algae. When added to the LB medium, agar acts as a solidifying agent, transforming the liquid medium into a semi-solid gel. This allows bacteria to grow on the surface of the agar in distinct colonies.

Preparation of LB Agar:

LB agar is typically prepared by dissolving the appropriate amounts of tryptone, yeast extract, and agar in distilled water, adjusting the pH to around 7.0, and autoclaving the mixture to sterilize it. After autoclaving, the molten agar is poured into sterile Petri dishes and allowed to solidify.

Uses of LB Agar:

LB agar is widely used for various applications in microbiology and molecular biology, including:

1. Bacterial Culture and Enumeration: LB agar supports the growth of a wide range of bacterial species, making it suitable for culturing and isolating bacteria from various sources.

2. Plasmid Isolation and Purification: LB agar is often used to grow bacterial cultures for plasmid isolation and purification. Plasmids are small, circular DNA molecules that replicate independently of the bacterial chromosome. LB agar provides a nutrient-rich environment for plasmid-bearing bacterial strains.

3. Transformation and Recombinant DNA Work: LB agar is used in transformation experiments, where foreign DNA is introduced into bacterial cells. It also serves as a medium for the selection of recombinant bacteria expressing specific traits or containing antibiotic resistance genes carried on plasmids.

4. Phage Assays: LB agar can be used for plaque assays to enumerate and characterize bacteriophages (viruses that infect bacteria). Phage particles form visible plaques on the bacterial lawn growing on the LB agar surface.

Procedure:

Preparation of LB-Agar Plates:

1. LB Agar Preparation:

- Dissolve LB agar powder (Luria-Bertani agar) according to the manufacturer's instructions. Typically, you'll dissolve 25 grams of LB agar powder in 1 liter of distilled water.
- Autoclave the LB agar solution to sterilize it.

2. Adding Antibiotics:

- For LB-agar plates with ampicillin (100 µg/ml), add ampicillin to the autoclaved LB agar when it has cooled down to around 50°C. Mix well by swirling.
- For LB-agar plates with tetracycline (10 µg/ml), add tetracycline to the autoclaved LB agar when it has cooled down to around 50°C. Mix well.

3. Pouring Plates:

- Pour the LB-agar mixture into sterile Petri dishes (approximately 20-25 ml per plate).
- Allow the plates to cool and solidify at room temperature with their lids slightly ajar to prevent condensation.

Streaking of E. coli DH5alpha Strain (Normal):

1. Preparation of Inoculum:

- Take a sterile inoculating loop and pick a small colony of E. coli DH5alpha from an LB agar plate.
- Transfer the colony to a sterile tube containing 5 ml of LB broth and incubate at 37°C overnight with shaking.

2. Streaking Procedure:

- Sterilize an inoculating loop by passing it through a flame and let it cool.
- Remove the lid from the LB agar plate and streak the surface of the agar with the inoculating loop containing the E. coli DH5alpha culture. Streak using the quadrant method or zig-zag method.
- Close the plate and incubate it at 37°C overnight.

Streaking of E. coli DH5alpha Transformed with Plasmids:

1. Transformation:

- Transform E. coli DH5alpha with the ampicillin-resistant plasmid (pBluescript) and tetracycline-resistant plasmid (pBR322) using standard molecular biology techniques such as heat shock or electroporation.
- Plate the transformed cells on separate LB-agar plates containing the appropriate antibiotic (ampicillin for pBluescript, tetracycline for pBR322).

2. Streaking Procedure (similar to normal DH5alpha):

- Repeat the streaking procedure as described above, but this time use transformed E. coli DH5alpha cells containing the respective plasmids.

3. Selective Growth:

- Incubate the plates at 37°C overnight.
- Transformed colonies should grow on plates containing the corresponding antibiotic, while non-transformed cells will not grow or will grow poorly on these plates.