Plasmid isolation : Alkaline SDS Lysis Method

1- Inoculation of a single colony in (5ml) LB media with required antibiotic, and incubation at 37 degrees celsius overnight.

2- Pellet down the cell.

3- Resuspend the cells in 150 ul of solution I (25 mM Tris-HCl (pH 8.0), 50 mM Glucose, 10 mM EDTA (pH 8)), vortex and be sure that pellets are fully resuspended.

4- Add 300 ul of Solution II (0.2 N NaOH and 1% SDS (prepare fresh and use at RT), and invert the tube 5 times slowly.

5- Add 230 Solution III. (5 M Potassium Acetate (pH 5.2, adjusted with glacial acetic acid) – the Final Conc in solution to be achieved for use should be 3 M), and invert the tube 5 times slowly.

6- Keep in ice for 15 minutes.

7- Spin at 13K 4^{o}C for 15 minutes.

8- Transfer the supernatant into a new fresh Eppendorf tube.

9- Add equal volume of 500 mM Tris-HCl pH 8 saturated phenol and vortex for 10 seconds and then spin at 13K for 5 minutes at room temperature.

10- Transfer the supernatant into a new fresh Eppendorf tube.

11- Add an equal volume of chloroform, vortex for 10 seconds, and then spin at 13K for 5 minutes at room temperature.

12- Transfer the supernatant into a new fresh Eppendorf tube.

13- Add 1/10 volume of 3M sodium acetate (pH 5.2).

14- Add 2-2.5 volumes of ice-cold 100% ethanol to your DNA sample and store the samples at -20^{o}C for 2hrs to overnight.

15- Spin sample at 13K for 20 minutes at 4^{o}C.

16- Discard the supernatant and wash the DNA pellet with 80% ethanol.

17- Spin sample at 13K for 20 minutes at 4^{o}C.

18- Discard the supernatant and dry the DNA pellet at room temperature or 37^{o}C incubator till the white pellet becomes transparent.

19- Add 18 ul MQ water + 2 ul of RNAse A , and incubate at 37^{o}C for 2 to 4 hrs.

20- Quantify the DNA concentration using a Nanodrop machine.

Plasmid digestion

1- For 20 ul reaction, add: (4 to 5 ul of isolated plasmid) + (reaction buffer) + (0.3 ul restriction enzymes) + (MQ water) and incubate at 37^{o}C overnight.

2- Prepare 1% agarose gel in 1X TAE buffer with Ethidium bromide (EtBr).

3- Mix the digested samples with 1X DNA dye and load the samples into the agarose gel.

4- Cut the specific DNA band and elute it using the Qiagen DNA elution KIT.

Ligation

1- Ensure elution is complete.

2- Mix the following total reaction: 10 ul (100 ng of vector, 300 ng of insert, 1 ul reaction buffer, 1 ul 10 mM ATP, 1 ul T4 DNA ligase and up to 10 ul MQ water)

3- Incubate the sample at 16^{o}C overnight

Transformation

1- Add the 10 ul ligation reaction to the prepared TG1 competent cells and keep in ice for 20 minutes in the laminar airflow chamber.

2- Perform heat shock by incubating (the competent cell + ligation reaction) at 42^{o}C for 45 seconds.

3- Immediately keep the samples in ice for 5 minutes.

4- Add 1 ml of LB media to the samples and incubate at 37^{o}C for 1hr while shaking at 180 RPM.

5- Pellet down the cells , discard 1 ml and resuspend the reaming 50 to 100 ul culture and spread them in LB plate containing the specific antibiotics (hood).

Preparation of cultures for optimal growth testing

1. Design compositions of media based on literature.
2. Autoclave media at 121 degrees celsius and 15 atm for 15 minutes.
3. Inoculate bacteria and allow growth while shaking in incubator for stipulated time periods.

Qualitative assessment of ammonium via Indophenol blue test

1. Place the specimen containing 0.5 to 6 µg of ammonia nitrogen, into each test tube.
2. Using an automatic pipet, add 5.0 ml of the phenol plus nitroprusside solution to each tube.
3. Cover the tubes with parafilm and shake vigorously to mix well.
4. Add 5.0 ml of the alkaline hypochlorite solution to each tube.
5. Cover the tubes with parafilm again and mix well.
6. Prepare a reagent blank by mixing 5.0 ml of the phenol plus nitroprusside solution with 5.0 ml of alkaline hypochlorite.
7. Place the tubes in a rack for color development using one of the following methods:

    Method A: Incubate in a water bath at 37°C for 15 minutes.

    Method B: Let stand at room temperature (20°C or more) for 30 minutes.

8. Measure the absorbance at 625 mµ.

9. Subtract the absorbance of the blank from your readings and calculate the concentration based on the absorbance of the standard.

[Adapted from: M. W. Weatherburn. *Phenol-Hypochlorite Reaction for Determination of Ammonia.* Laboratory of Hygiene, National Health and Welfare, Ottawa, Canada.]

Biofilm assays

- Add 3 ml of LB to each well of all the seven 12-well plates.
- For temperature and Antibiotic Stress,

1. Take five plates from the previous step.
2. To the second column, add Amp (concentration, as indicated in diagram) and third column, add Strep (concentration, as indicated in diagram).
3. Now, to the first three columns, add 30 uL of bacterial inoculum, leave the last column empty for control.
4. Place these five plates in different temperatures for incubation for 48 hours, as listed (15°C, 20°C, 25°C, 30°C, 35°C)

- For variable Antibiotic stress,

1. Take two 12-well plates from the initial step.
2. Prepare serial dilutions of the antibiotics (one plate for amp, another for strep) in a 2x manner and add to the wells as indicated in the diagram.
3. To the second and third row, add 30 uL of bacterial inoculum for replicates and leave the first row as control.
4. Put at 30°C for incubation for 48 hours.

- After this, throw the methanol out and add 3 ml of 0.1 or 1% crystal violet stain and incubate for 20 minutes at room temperature.
- Rinse the plate with 1x PBS twice.
- Add 3 ml of 70% ethanol to each well and measure the OD at 570 nm.

[Adapted from: Halocleen, iGEM IISc 2021]