TRANSFORMATION

Preparation of competent cells:-

1. Dilute an overnight culture of E. coli 1:200 with LB broth.
2. Incubate at 37°C with shaking (at 200 rpm) until the cells reach early log phase (OD600 = 0.25-0.4).
3. We already have 1X TSS in 4 deg fridge(old). Use it without dilution or thawing. Keep it inside the icebox just after taking out from the fridge.
   OR
   (if the above 1X TSS is not available)
   While cells are growing, thaw 2X TSS on ice and dilute an appropriate amount 1:1 with sterile distilled water (100µl of diluted TSS will be needed for each ml of cells). Chill on ice.
4. Place 2-ml aliquots of early log-phase cells into sterile 2-ml micro-centrifuge tubes and pellet the cells by centrifugation at 4°C at 3000g for 10 min.(6-8 mins for taking part from iGEM kit)
5. Remove the supernatant and discard. Add 0.2 ml of the ice-cold 1X TSS and place the tubes on ice.
6. Gently suspend the cells by pipetting.
7. Proceed with the transformation protocol below (Step 2), or immediately freeze cells by immersion in liquid nitrogen or a dry ice/ethanol bath. Store the frozen cells at –70°C.

Transformation Protocol:-

1. Thaw frozen TSS-competent cells slowly on ice (if stored at -70°C).
2. Add 100 pg -200 ng (2.5 to 4 ul)(15ul for ligation product)of DNA to each tube of competent cells.
   Note:Addition of more than 10ng of DNA may significantly decrease transformation efficiencies.
3. Flick the tubes to mix the cells and DNA and incubate the cells on ice for 30 minutes.
4. Transfer the tubes to water bath/dry bath(42°C) for 90 seconds.
5. Transfer the tubes to ice and incubate for an additional 10 minutes.
6. Add 800 ul (total 1 mL)of LB broth and incubate the cells at 37°C for up to 1 hour with shaking (at 200 rpm).

7. Centrifuge the cells at 3000g for ~ 6min (10 mins after ligation)at 4deg(in temperature control centrifuge).
8. Aspirate the tubes to leave the pellets with 1/4 broth .(keep ~300ul)
9. Plate the cells on-to the appropriate selective or differential medium and incubate overnight at 37°C.Check the procedure for antibiotic.
    1. For Ampicillin: 12ul Amp + 188 ul MQ. In MCT spread it on the culture plate before adding the DNA.
    2. For Chloramphenicol: 1:1000 volume ratio of antibiotic : culture broth. Directly suspend into the culture broth and spread it on the plate.
    3. For Kanamycin: 1:1000 volume ratio of antibiotic : culture broth. Directly suspend into the culture broth and spread it on the plate.
    - DNA should be added as soon as the last trace of ice in the tube disappears.
    - Incubate on ice for 30 minutes. Expect a 2-fold loss in TE for every 10 minutes you shorten this step.

WASHING 96 WELL PLATES

1. Perform all the steps gently.
2. Wash twice with tap water, jerk it off.
3. Wash twice with distilled water, jerk it off.
4. Put ethanol in plates and stand for 5 min Jerk it off.
5. Put open plate in oven for 20 minutes.
6. Put closed plate in UV for 10 minutes

AGAROSE GEL ELECTROPHORESIS

1. Prepare sufficient electrophoresis buffer (usually 1x TAE) to fill the electrophoresis tank and to cast the gel:
   For example, we take 2ml of TAE stock solution in an Erlenmeyer flask and make the volume to 100ml by adding 98 ml of distilled water. The 1x working solution is 40 mM Tris-acetate/1 mM EDTA.

It is important to use the same batch of electrophoresis buffer in both the electrophoresis tank and the gel preparation.

2. Loosely plug the neck of the Erlenmeyer flask using a paper cap. Heat the slurry in a boiling-water bath or a microwave oven until the agarose dissolves. The agarose solution can boil over very easily so keep checking it. It is good to stop it after 45 seconds and give it a swirl. It can become superheated and NOT boil until you take it out where upon it boils out all over your hands.
3. When the molten gel has cooled to ~50 deg C, add 0.5µg/ml of ethidium bromide. Mix the gel solution thoroughly by gentle swirling.
4. While the agarose solution is cooling, choose an appropriate comb for forming the sample slots in the gel. Apply cellotape tightly onto the sides of comb to form a mold for gel.
5. Pour the warm agarose solution into the mold.(The gel should be between 3 - 5 mm thick. Check that no air bubbles are under or between the teeth of the comb).
6. Allow the gel to set completely (30-45 minutes at room temperature), then pour a small amount of electrophoresis buffer on the top of the gel, and carefully remove the comb. Pour off the electrophoresis buffer. Mount the gel in the electrophoresis tank. Wash the Erlenmeyer flask as quick as possible. (Add just enough electrophoresis buffers to cover the gel to a depth of approx. 1mm).
7. Mix the samples of DNA with 0.20 volumes of the desired 6x gel-loading dye on a clean piece of cello tape spread on an even surface.
8. Slowly load the sample mixture into the slots of the submerged gel using a disposable micropipette or an automatic micropipettor or a drawn-out Pasteur pipette or a glass capillary tube. Load size standards into slots on both the right and left sides of the gel.
9. Close the lid of the gel tank and attach the electrical leads so that the DNA will migrate toward the positive anode (red lead). Apply a voltage of 7-10 V/cm (measured as the distance between the positive and negative electrodes). If the electrodes are 10 cm apart then run the gel at 50V. It is fine to run the gel slower than this but do not run it any faster. Above 5V/cm the agarose may heat up and begin to melt with disastrous effects on your gel's resolution. If the leads have been attached correctly, bubbles should be generated at the anode and cathode.
10. Run the gel until the loading dye has migrated an appropriate distance(~ 2/3 the length of gel) through the gel.
11. Remove the gel tray and place directly on a UV transilluminator or under a gel doc. NOTE:
    - Use TBE 1x for < 1000bp(can't extract DNA) and TAE for large DNA(12-15kb).
    - Percentage of agarose matters Gel thickness should not be more than 3-4mm.
    - Comb should be cleaned well Add buffer before removing the comb.

PLASMID ISOLATION

1. Inoculate 5 ml of rich medium (LB, YT, or Terrific Broth) containing the appropriate antibiotic with a single colony of transformed bacteria. Incubate the culture overnight at 37°C with vigorous shaking at 200 rpm.
2. Pour 2 ml of the culture into a microfuge tube. Centrifuge at maximum speed(13400 rpm) for 4-5 minutes in a microfuge. Store the unused portion(if any) of the original culture at 4°C.
3. Remove the medium by aspiration leaving the bacterial pellet as dry as possible.
4. Resuspend the bacterial pellet in 100 µl of ice-cold Alkaline lysis solution I by vigorous vortexing.
5. Add 200 µl of freshly prepared Alkaline lysis solution II to each bacterial suspension. Close the tube tightly, and mix the contents well by inverting the tube.
   NOTE: Do not vortex! Do not let the reaction proceed for more than 1 minutes.
6. Add 150 µl of ice-cold Alkaline lysis solution III. Close the tube and disperse Alkaline lysis solution III through the viscous bacterial lysate by inverting the tube several times. Store the tube in ice for 3-5 minutes(optional).
7. Centrifuge the bacterial lysate for 10 minutes at maximum speed in a microfuge. Collect the supernatant to a fresh 1.5 ml mctube.
8. (Optional) Add equal volume of phenol: chloroform. Mix the organic and aqueous phases by vortexing and then centrifuge the emulsion at maximum speed for 2 minutes in a microfuge. Transfer the aqueous upper layer to a fresh tube.
9. Precipitate nucleic acids from the supernatant. Now add 2 volumes of ethanol at room temperature to the supernatant in a 1.5 ml MCT. Mix the solution by vortexing and then allow the mixture to stand for 2 minutes at room temperature(optional). Discard the pellet in the original 2 ml MCT.
10. Collect the precipitated nucleic acids by centrifugation at maximum speed for 10 minutes in a microfuge.
11. Discard the supernatant by aspiration. Stand the tube in an inverted position on a paper towel to allow all of the fluid to drain away. Use a Kim wipe or disposable pipette tip to remove any drops of fluid adhering to the walls of the tube. Stand the tube for 10 minutes.
12. (Optional) Add 1 ml of 70% ethanol to the pellet and invert the closed tube several times. Recover the DNA by centrifugation at maximum speed for 2 minutes at in a microfuge.
13. (Optional) Remove all of the supernatant by aspiration. Take care with this step, as the pellet sometimes does not adhere tightly to the tube.
14. (Optional) Remove any beads of ethanol from the tube. Store the open tube at room temperature until the ethanol has evaporated and no fluid is visible in the tube (5-10 minutes).

15. Store the tube for 10 minutes at 37 degrees without shaking.
16. Dissolve the nucleic acids in 20 µl of mQ (1/100 times the initial volume in the MCT) or TE (pH 8.0) [containing 20 µg/ml Dnase-free RNase A (pancreatic RNase)] . Vortex the solution gently for a few seconds and store the DNA at -20°C.

RESTRICTION DIGESTION

1. Transfer the following solutions in a micro centrifuge tube.

COMPONENT	VOLUME (uL)
Deionized water	5
10 X Reaction Buffer	1
Plasmid DNA	3
Enzyme 1	0.5
Enzyme 2	0.5
Total	10

2. Incubate the mixture at 37 o C for 1 h to overnight. Keep the tubes in -4o C freezer or in -20o C freezer, after the incubation.

NOTE:

- 10X buffer should be added in the reaction such that its final concentration is 1x (e.g. - in a 50 ul reaction 5 ul of buffer would be added).
- Amount of DNA to be added depends on concentration and the amount of DNA to be digested.
- Make sure that the restriction enzyme does not exceed more than 10% of the total reaction volume, Otherwise the glycerol and the EDTA in the enzyme storage buffer may inhibit digestion process.
- Enzymes on ice, when not in freezer.
- Mix by pipetting.
- A 50 µl reaction volume is recommended for digestion of 1 µg of substrate Control DNA (DNA with multiple known sites for the enzyme, e.g. lambda or adenovirus-2 DNA) with restriction enzyme to test enzyme viability.
- If the control DNA is cleaved and the experimental DNA resists cleavage, the two DNAs can be mixed to determine if an inhibitor is present in the experimental sample. If an inhibitor (often salt, EDTA or phenol) is present, the control DNA will not cut after mixing.
- Cleanup of the PCR fragment prior to restriction digestion is recommended. PCR components can inhibit enzyme activity. In addition, the polymerase present in the PCR is active during the digestion step, and can modify the newly created ends by blunting them.
- Some enzymes may bind tightly to the substrate DNA. This binding can result in smearing or the presence of unexpectedly high molecular weight bands on a gel. To prevent this, add SDS to a final concentration of approximately 0.1%, or use Gel Loading Dye, Purple (6x), which contains sufficient SDS to dissociate the enzyme from the substrate.

- To prevent star activity, make sure that you use the recommended buffer, that the amount of glycerol in the reaction is no more than 5% of the total reaction volume.

GEL EXTRACTION

1. Excise the DNA fragment from agarose gel with clean, sharp scalpel.
2. Weigh the gel slice. Add 3 volumes buffer QG to 1 volume of gel (100 mg gel = 100 ul). (if we have 18 mg of gel take around 20 ul of buffer QG)
3. Incubate at 50 degree celsius for 10 minutes. Vortex after 5 minutes to dissolve the gel slice. After gel has dissolved the color of mixture is yellow. NOTE: tilt the MCT upside down, to see if the gel has completely dissolved. If it has not dissolved, incubate it for 2-3 minutes more. NOTE: if mixture color is orange or violet, add 10 ul 3M sodium acetate, pH 5.0 and mix. The mixture turns yellow.
4. Add 1 gel volume isopropanol to sample and mix.
5. Take the whole volume in the MCT and transfer it to spin column.
6. Spin it on minispin for 90 sec at maximum speed.
7. Discard the flow through
8. Add 750 ul of PE buffer. in the spin column.
9. Spin it on minispin for 90 sec at maximum speed.
10. Discard the flow through and centrifuge once again for 90 seconds at maximum speed.
11. Place QIAquick column in 1.5 mL fresh MCT.
12. Add EB buffer through the QIA quick column center. (around 20 – 25 uL depending on application)
13. Spin for 2 minutes at maximum speed in minispin.

COLONY PCR

Component	Volume (uL)
TaqMM	10
Deionised water	9.2
Forward Primer	0.4
Reverse Primer	0.4
Colony	A bit
Total	20