OBJECTIVE: Determining the mechanism of plasmid replication

Chemical required: Glucose, Tris-Cl, EDTA, potassium acetate, glacial acetic acid, NaOH, Sodium dodecyl sulfate, Iso-propanol, RNase (10mg/ml, Qiagen) ddH_2O/MilliQ. Lysozyme (50mg/ml) LE Agarose (EEO: 0.09-0.13), Acridine orange

Plastic ware/ glassware: Measuring cylinder**,** autoclaved micro centrifuge tubes, 1.0 ml tips, 0.02ml-0.2 ml tips, enzyme tips, 100ml/250 ml flasks, reagent bottle, 0.2 µ syringe filter.

Equipment required: Horizontal electrophoresis unit, power supply, casting tray, combs, minispin (centrifuge), pH meter, centrifuge.

Recipe for solutions:

1. Solution I (100 ml)

50 mM glucose	1.8 ml of 50 % glucose
25 mM Tris HCl (pH 8.0)	2.5 ml of 1M Tris-HCl (pH 8.0)
10 mM EDTA (pH 8.0)	2.0 ml of 0.5 M EDTA (pH 8.0)
	93.7 ml of H_2O (autoclaved)

2. Solution II (100 ml)

(This should be freshly prepared)

0.2 N NaOH	10 ml 2N NaOH
1.0 % SDS	10 ml 10% SDS
	80 ml of H_2O (autoclaved)

3. Solution III (100 ml)

Potassium acetate (5M, pH 4.8)	60 ml 5M Potassium Acetate
Glacial acetic acid	11.5 ml Glacial acetic acid
Sterile distilled water	28.5 ml of H_2O (autoclaved)

4. TE (100 ml)

10 mM Tris- HCL (pH 8.0)	1 ml 1 M Tris-HCl (pH 8.0)
1 mM EDTA-sodium salt	0.5 ml 0.5 M EDTA (pH 8.0)
	98.5 ml of H_2O (autoclaved)

PROTOCOL

Inoculate a single colony of *Corynebacterium glutamicum* in 50 ml LB and incubate at 30°C, 180 rpm overnight.

Harvest cells from an exponentially grown culture (~$O.D_{600nm}$ 0.5-1.0) at 12000 rpm for 2min.

Wash the pellet with MilliQ.

Resuspend pellet in 200 µl of Solution I, add lysozyme at a final concentration of 50 µg/ml and keep at 37°C in a shaking water bath for 60 minutes.

Add 400 µl solution II and thoroughly mix by inverting tube 4-5 times.

Add 300 µl ice-cold solution III and mix by inverting tube 4-5 times, incubate for 5- 10 min on ice.

Centrifuge mixture at 12000 rpm for 15 min. Collect supernatant in separate tube and add 0.8 volume of isopropanol followed by incubation on ice for 10-15 min.

To get plasmid DNA centrifuge mixture at 12000 rpm for 5 min.

Wash the pellet with 1 ml 70% ethanol followed by centrifugation at 12000 rpm for 2 min.

Air dry the pellet and dissolve in 40 µl of TE buffer.

Add RNase 100 µg/ml to final concentration and incubate for 1 hr at 37°C.

Load the plasmid DNA (20 µl) on a 1 % agarose gel.

Stain the gel with acridine orange (30 g/ml) for 30 minutes and visualize at two different wavelengths 530 nm and 640 nm.