**GBLOCKS**

**Material required:**

Gblocks

TE buffer (1x)

**Preparing gblocks for use:**

Suspend the gblocks in TE buffer (1x).

Store it in -20ºC.

Spin the gblock before use.

**PCR**

**Material required:**

Template DNA

Master Mix (Q5)

Forward and reverse primers ( VF2 and VR )

Nuclease free water

**Procedure (for parts) - (20 µl reaction)**

The Nuclease free water was added to the PCR tube placed in ice.

1.2 µl each of forward and reverse primer was added.

The Template DNA (1µl) was added to the mixture.

Master Mix (Q5) 12.5 µl was added and mixed gently.

The PCR tubes were transferred from ice to PCR machine.

The reaction was carried out and visualized on agarose gel.

**PCR CLEAN UP**

**MATERIALS REQUIRED**

Marshell and Nagal kit for PCR clean up and gel extraction.

**PROCEDURE**

200 µl of buffer NTI was added to the eppendorf containing PCR sample

The spin column was placed into the collection tube.

700µl of the mixture was taken from the eppendorf and added into the spin column.

It was centrifuged for 30 seconds at >8000 rpm.

The flow through was discarded and the spin column was placed back into the collection tube.

700 µl buffer NT3 was added to the spin column and centrifuged for 30 seconds at >8000 rpm.

The flow through was discarded and the spin column was placed back into the collection tube.

The spin column was centrifuged for 1 min at >8000 rpm to remove buffer NT3 completely.

Care must be taken to avoid the contact between the spin column with the flow through while removing it from the centrifuge and the collection tube.

The spin column was placed onto the new eppendorf.

15-30 μL buffer NE was added and incubated at room temperature for 1 min.

Then it was centrifuged for 1 min at >8,000rpm.

The flow through in the eppendorf was visualized in agarose gel to measure the concentration.

**RESTRICTION DIGESTION**

**Materials Required:**

Ice container

Eppendorfs

Purified DNA

Double distilled water (nuclease-free)

Restriction enzymes ( ECOR I and Pst I )

Buffer cutsmart(10x)

**Procedure: (15 µl reaction)**

The restriction buffer cutsmart was added to the PCR tube

The template DNA was added to it.

1µL restriction enzymes was added to the mixture carefully

The total volume of the mixture was made to 15 µl with nuclease free water.

It was incubated at 37ºC for 1 hour.

To denature the enzymes present in the reaction mix, the mixture was maintained at 65ᵒC for 20 minutes and the digested fragments were visualized by agarose gel electrophoresis.

**LIGATION TO PLASMID**

**Materials Required:**

Linearized plasmid backbones

Restriction-digested fragments with complementary overhangs

T4 DNA ligase

T4 DNA ligase buffer-10X (usually added after bringing down to room temperature)

Double distilled water (nuclease-free)

**Procedure: (1:3)- (15 µl reaction)**

Equimolar amounts of restricted DNA fragments were added.

The restricted DNA fragments was added thrice the concentration of plasmid backbone (1:3)

6 µl of T4 DNA Ligase Buffer (10X) was added and lastly the T4 DNA ligase of 1 µl volume was added.

The reaction mixture was made up to 15 µl with nuclease free water.

The reaction mix was mixed gently and incubated at 16ᵒC for 1 hour or overnight.

The ligated product was visualized on agarose gel electrophoresis.

**Competent cells and transformation**

**Materials required:**

E.coli DH5α cells- 3hrs culture (OD- 0.3 to 0.6) usually 1-2 ml mother culture was inoculated for 50 ml of fresh LB broth

LB broth

CCMB80 buffer (6.4 pH)

10 mM potassium acetate (pH 7)

80 mM Cacl₂.2H₂O

20 mM MnCl₂.2H₂O

10 mM MgCl₂.2H₂O

10% glycerol

The above chemicals were dissolved in 1000 ml distilled water and the pH was adjusted to 6.4 and stored in cold room

The ccmb80 buffer was heat sterilized and thenfilter sterilized before use.

Eppendorf and centrifuge tubes

**Procedure:**

**Preparation of competent cells**

5 ml of culture was taken in a centrifuge tube.

The cells were pelleted by centrifuging at 11,000rpm for 10 minutes at 4ºC.

The supernatant was decanted and the cells were resuspended in 3-4ml of cold CCMB80 buffer.

The tubes were incubated on ice for 20 minutes.

They were then centrifuged at 11,000rpm for 10 minutes at 4ºC.

The supernatant was decanted and the cells were again resuspended in 1-2ml of cold CCMB80 buffer.

The OD of the cells obtained was measured by taking 200µl of LB and 50µl of resuspended cells in an eppendorf tube. The control was taken to be a mixture of 200µl of LB broth and 50µl of CCMB80 buffer. The total volume was made upto 3ml.

The observed OD should be multiplied by 12(dilution factor) which should be around 1.0 to 1.5.

If the required OD was not observed in the range 1.0 – 1.5, then it can be concentrated or diluted by pelleting and resuspending them again in ccmb80 buffer.

They were then incubated in ice for 15 minutes.

**Transformation procedure:**

50 μl of competent cells was pipetted into eppendorf tubes and 1 μl of plasmids were added to them.

The tubes were mixed well and incubated on ice for 30 minutes.

Heat shock was provided by placing the cells at 42ºC for 2 minute and immediately the tubes were snap chilled in ice and incubated for 10 minutes.

200 μl of LB medium was added to each tube and incubated at 37ºC for 1-2 hours.

After incubation the culture was spread plate onto the respective antibiotic containing plates.

The plates were incubated for 48 hr at 37ºC and checked for the transformation efficiency.

**PLASMID ISOLATION**

**Materials required:**

Transformed culture

Qiagen plasmid isolation kit

**Preparation of culture:**

Pick a single colony from a freshly streaked transformed plate and inoculate a starter culture of 2–5 ml LB medium containing the appropriate selective antibiotic. Incubate for approximately 8 h at 37°C with vigorous shaking (approx. 300 rpm).

**Procedure:**

1 – 1.5ml of the culture was take in eppendorf tube and centrifuged at high speed for 5 minutes.

The supernatant was discarded and the pellet was resuspended using the same culture.

It was centrifuged at high speed for 5 minutes.

The supernatant was discarded and the pellet is resuspended using 250µL of buffer P1 and it was mixed.

250 µL of Buffer P2 was added to the mixture and mixed by inverting the tube.

350 µL of Buffer N3 was added and mixed again by inverting the tube

The mixture was centrifuged for 5 minutes at 8000rpm

Now a spin column was taken and placed onto the collection tube.

800 µL of the supernatant was taken in the spin column and centrifuged for minute at 4000rpm.

The flow through was discarded and this step was repeated for the remaining supernatant.

750 µL of buffer PE was added to the spin column and centrifuged at 4000rpm for 1 minute.

The flow through was discarded & The spin column was centrifuged again to remove the remaining PE buffer

The spin column was placed onto a new eppendorf tube & 50 µL of elution buffer was added and centrifuged at 4000rpm for 1 minute and the flow through was stored.

The plasmid was re-eluted by adding 20 µL of buffer EB to the spin column by placing onto the another new eppendorf.

The plasmid was visualized in agarose gel electrophoresis.

**OPTIMIZATION OF LBK MEDIUM OF DIFFERENT pH**

Potassium modified LB medium

**Materials required per litre:**

Trypton ( casein enzyme hydrolysate ) – 10g

Yeast exract – 5g

Potassium chloride – 7.45g

100mM MOPS – 20.926g

**Preparation**

The LBK medium was prepared with the above mentioned components as per the required volume.

The pH of LBK medium was adjusted using 1M potassium hydroxide(KOH) and .1N Hydrochloric acid(HCl).

Growth and expression studies for E.COLI DH5α was done using the LBK medium in the following pH: 6.5, 7.0, 7.5, 8.0, 8.5, 9.0.