Practical activity (2)

Blue-white Screening

(Student version)

I. Background

Transformation is a technique that allows us to incorporate a foreign plasmid, i.e. a small circular piece of DNA, into a bacterium. Upon taking up the foreign plasmid, the bacteria are transformed. The bacteria will amplify the plasmid DNA during its replication. Importantly, the plasmid DNA may in turn affect the growth and other cellular properties of the bacteria. In fact, we can even insert a specific DNA fragment into the plasmid before transformation, in order to get the bacteria to multiply the gene of interest. Such artificial gene propagation is known as molecular cloning.

However, plasmids, DNAs and individual microbes are too small to be seen by the naked eye. To distinguish the transformed bacterial colonies, blue-white screening is considered a simple and rapid method for us because we can observe visible colour development on the agar plates.

The principle of blue-white screening is highly attributed to the presence of the *LacZ* gene and the enzymatic activity of beta-galactosidase. In fact, the *LacZ* gene encodes for a protein that is responsible for the production of the enzyme beta-galactosidase. This enzyme is able to metabolise its substrate X-gal and then forms a product with blue pigments. As far as we know, many of the known plasmids carry only part of the *LacZ* gene which encode for the alpha fragment of the enzyme. The gene is denoted as *LacZα*. The alpha fragment is not functional by itself. However, there is a mutant strain of *E.coli* that has the part of the *LacZα* deleted, and this particular *E.coli* strain can only produce the omega fragment of the enzyme. Similar to *LacZα*, the omega fragment is not functional itself. Only when both the alpha fragment and the omega fragment are expressed will a functional beta-galactosidase be formed (Fig. 1). If a plasmid containing the *LacZα* gene is transformed into this mutant strain of *E. coli*, then functional beta-galactosidase can be produced in the bacteria (Fig. 1). Consequently, the enzyme metabolises X-gal in the growth agar and turns the bacterial colonies blue.

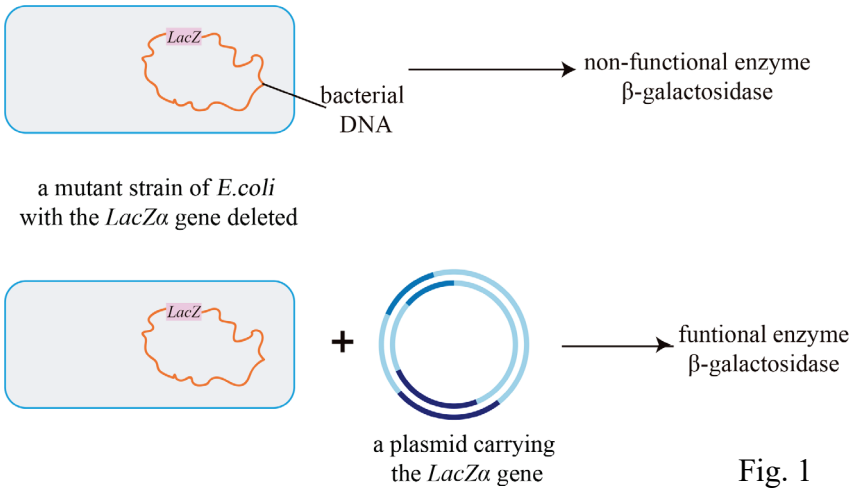


Fig. 1

If a DNA fragment of interest is inserted into the *LacZα* gene region of the plasmid before transformation, no functional beta-galactosidase can be made in the host bacteria, due to the disruption of the *LacZα* gene sequence. Even if X-gal is provided in the growth agar, no enzymatic metabolism occurs. As a result, no products with blue pigments are formed, and the bacterial colonies appear white (Fig. 2). However, if the DNA fragment of interest cannot be inserted into the plasmid, the production of a functional beta-galactosidase will not be affected. In this case, the X-gal provided in the growth medium can be metabolised by the enzyme, and blue colonies should be observed.

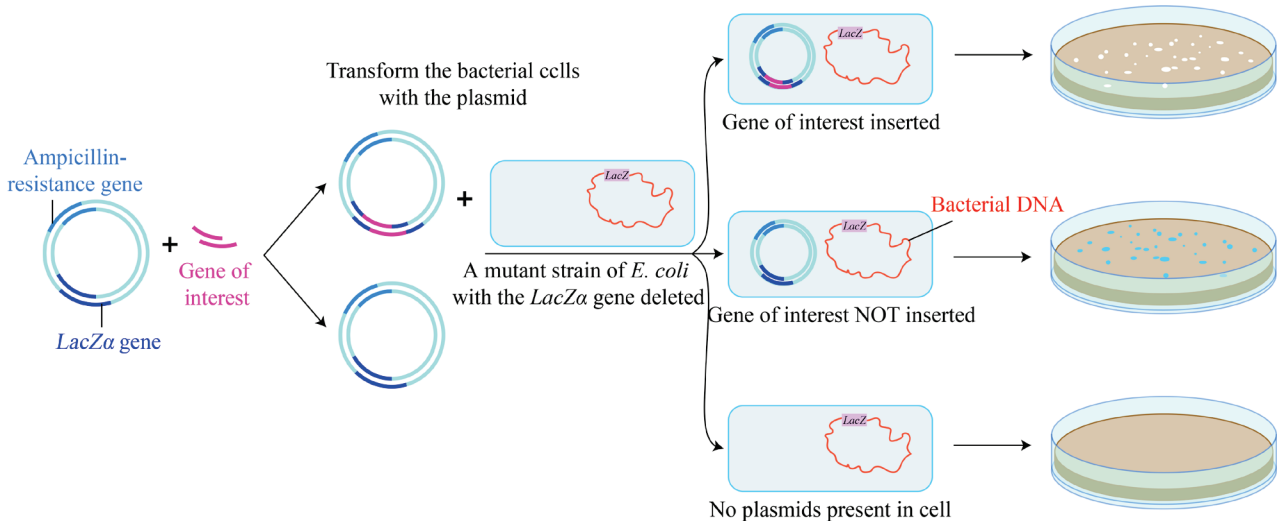


Fig. 2

Overall, the blue-white screening helps us identify the transformed bacteria carrying the DNA of interest.

In this experiment, you are going to clone a particular sequence of DNA by inserting it into a plasmid containing a *LacZα* gene. The given plasmid also contains an antibiotics-resistant gene, which allows the transformed cells to grow in the presence of ampicillin. You are going to perform a blue-white screening test on *E. coli* cells to identify the *E.coli* which have been successfully transformed with the given plasmid carrying the DNA of interest.

II. Guiding questions about the design of the experiment

1. Suggest a molecular technique for introducing external plasmids into *E. coli* cells.

2. Which chemical(s) should be added into the agar plates in order to induce the development of blue colour in the colonies?

III. Objectives of the experiment

1. To perform bacterial transformation using a recombinant plasmid with the *LacZα* gene for the production of β-galactosidase;
2. To perform the blue-white screening test; and
3. To demonstrate the successful transformation in the mutant strain of *E. coli* cells.

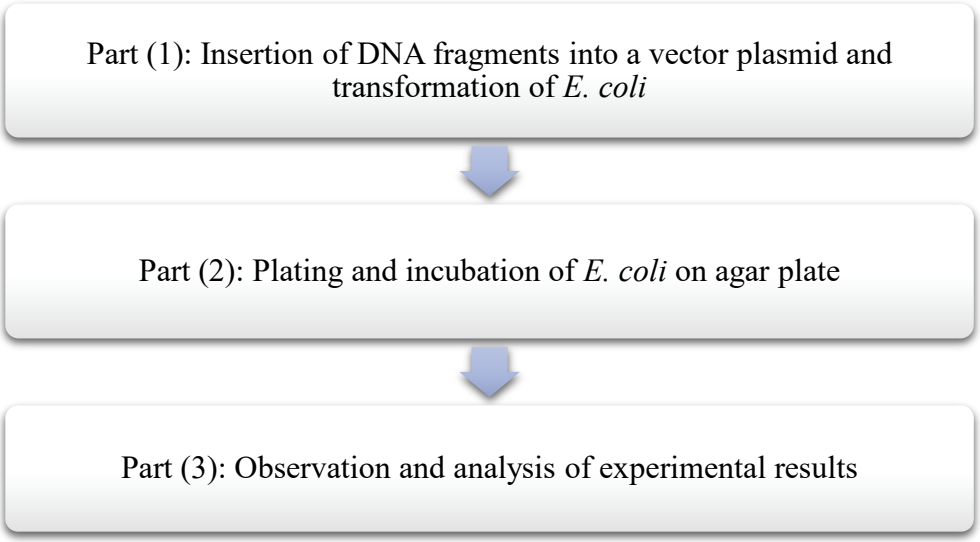
IV. Expected Learning Outcomes

Upon completion of the activities, students should be able to:

1. outline the principle of transformation;
2. apply aseptic techniques in bacterial culture;
3. carry out bacterial transformation;
4. understand the principle of blue-white screening; and
5. perform the blue-white screening test.

V. The experiment

A. Overview



B. Part (1) of the experiment: Insertion of DNA fragments into a vector plasmid and transformation of *E. coli*

a) Equipment and materials (per group)

Equipment

- Water bath (42°C)	× 1 (per class)
- Water bath (37°C)	× 1 (per class)
- Centrifuge	× 1 (per class)
- Bunsen burner	× 1
- Spark lighter	× 1
- Ice bath	× 1
- Timer	× 1
- Micropipettes (P1000, P200 and P20) and sterile tips	

Materials

- Culture plate with *E. coli* cells	× 1
- Sterile micro-centrifuge tubes	× 3
- Micro-centrifuge tube rack	× 1
- Inoculating loop	× 1
- Tube "D" containing DNA and plasmid mixture	× 1
- Tube "P" containing plasmid solution	× 1
- Tube "C" containing sterile distilled water	× 1
- Reaction buffer (60 µl)	× 1
- $CaCl_2$ solution (800 µl)	× 1
- Recovery broth (800 µl)	× 1
- Permanent marker	× 1
- 70% ethanol in spray bottle	× 1
- Paper towel	× 1 box
- Biohazard bag	× 1
- Disposal container with 10% chlorine bleach	× 1

b) Safety precautions

- Wear a laboratory gown.
- Tie up long hair.
- Wear gloves during the experiment.
- Wash hands to remove all possible contamination before and after the experiment.
- Keep ethanol away from fire.
- Make sure ethanol is fully vaporised before approaching any flame.
- Dispose of or disinfect all materials properly after each experiment.

c) Procedure

1. Disinfect the bench top and gloved hands with 70% ethanol*.

***Caution: Keep away from fire! DO NOT light the flame until the ethanol has been completely vaporised!**

2. Label the lid of the 3 micro-centrifuge tubes "D", "P" and "C" with your group number (Fig. 3).

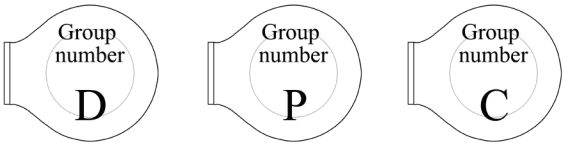


Fig. 3

3. Using the P20 micropipette and sterile tips, add 15 µl reaction buffer to each of the micro-centrifuge tubes (Fig. 4).

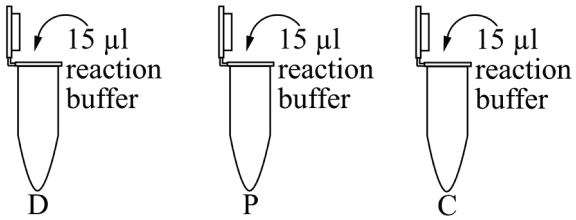


Fig. 4

4. Using the P200 micropipette and a sterile tip, add 20 µl DNA fragment and plasmid mixture to tube "D" (Fig. 5).

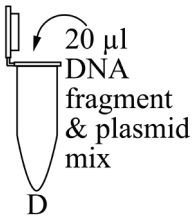


Fig. 5

5. Using the P200 micropipette and a sterile tip, add 20 µl plasmid solution to tube "P" (Fig. 6).

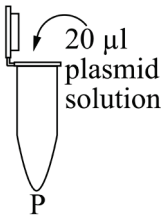


Fig. 6

6. Using the P200 micropipette and a sterile tip, add 20 µl sterile distilled water to tube "C" (Fig. 7).

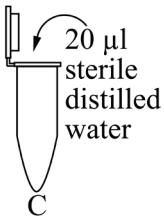


Fig. 7

7. Incubate the 3 tubes at room temperature for 1 hour. Finger-flick the tubes every 10 minutes (Fig. 8).

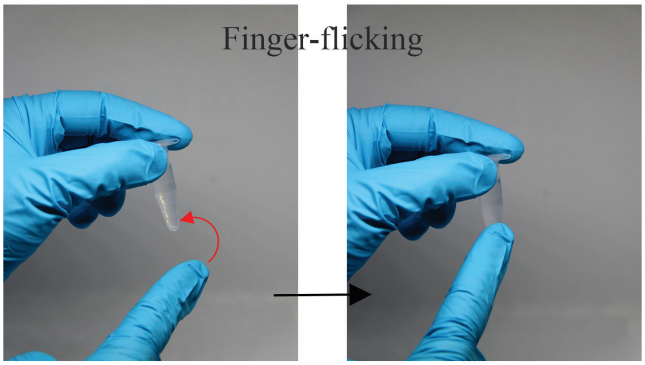


Fig. 8

8. Label the lid of the 3 micro-centrifuge tubes "D1", "P1" and "C1" with your group number (Fig. 9).

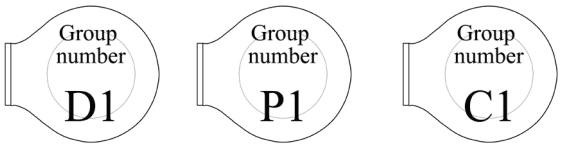


Fig. 9

9. Using the P1000 micropipette and sterile tips, add 250 µl $CaCl_2$ solution* to each of the micro-centrifuge tubes (Fig. 10).

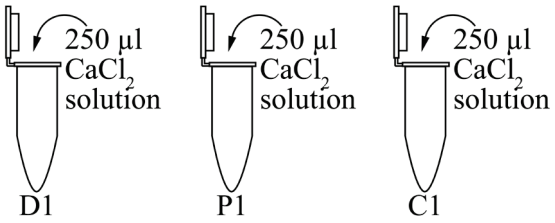


Fig. 10

*Note: Under normal conditions, DNA cannot pass through the cell membrane of bacteria. Calcium ions in the competent buffer help increase the permeability of the bacterial cell membrane, so that the plasmid can be incorporated.

10. Light up a Bunsen burner.

11. Place the inoculating loop at the tip of the inner core of the blue flame, i.e. the hottest part of the flame. Flame the loop to red hot (Fig. 11) and allow it to cool down*.

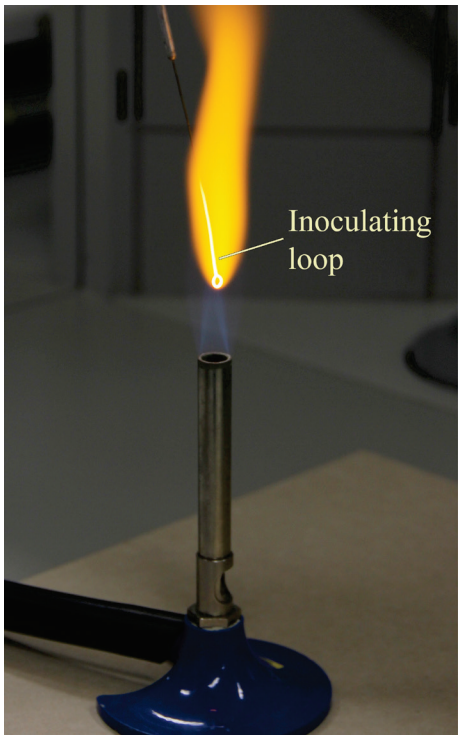


Fig. 11

*It is essential to flame the metal inoculating loop to red hot to eliminate any previous bacterial residue, so as to prevent contamination of the current sample.

It is also important to cool the loop, as insufficient cooling kills the inoculated bacteria.

If a sterile plastic disposable inoculating loop is provided, flaming is not required.

Use a new inoculating loop when handling different samples.

12. Using the sterilised inoculating loop, transfer two *E. coli* colonies to each of the micro-centrifuge tubes (Figs. 12 and 13). Flame-sterilise the loop after each use.

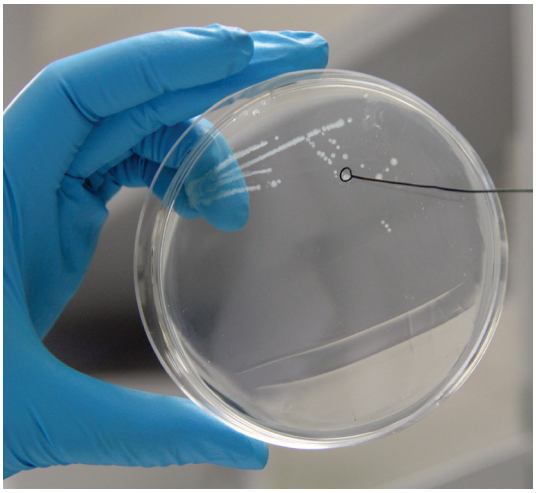


Fig. 12

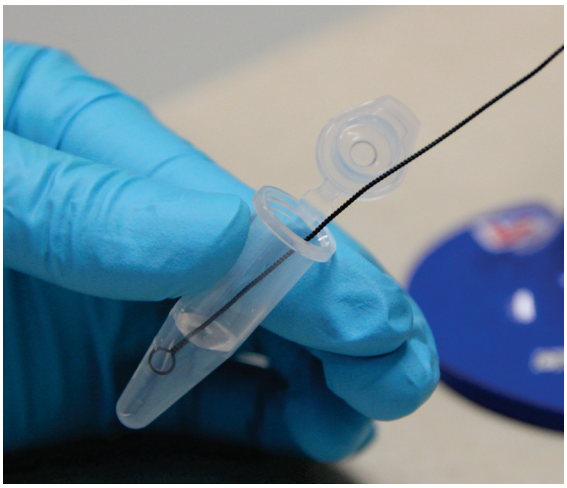


Fig. 13

13. Shut off the Bunsen burner.

14. Using the P20 micropipette and sterile tips, transfer 5 µl solution from tube "D" to tube "D1" (Fig. 14).

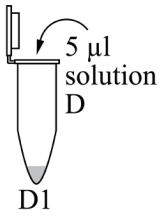


Fig. 14

15. Using the P20 micropipette and sterile tips, transfer 5 µl solution from tube "P" to tube "P1" (Fig. 15).

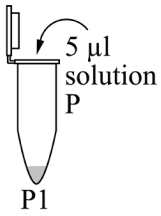


Fig. 15

16. Using the P20 micropipette and sterile tips, transfer 5 µl solution from tube "C" to tube "C1" (Fig. 16).

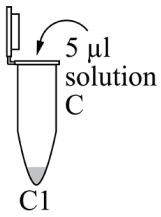


Fig. 16

17. Incubate all the tubes on ice for 15 minutes (Fig. 17).

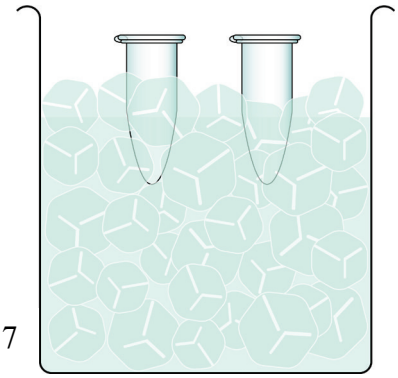


Fig. 17

18. After incubation, move the ice bath containing the 3 tubes next to the hot water bath. Make sure the tubes are tightly closed.

19. As quickly as possible, transfer all the tubes from the ice bath to the 42°C water bath (Fig. 18) for a "heat shock" of exactly 90 seconds*.

*Note: A sudden increase in temperature creates pores on the plasma membrane of *E. coli* and allows plasmid DNA to enter the bacterial cells. Transfer the tubes as quickly as possible, since the more distinct the temperature change in the heat shock, the greater the transformation efficiency.

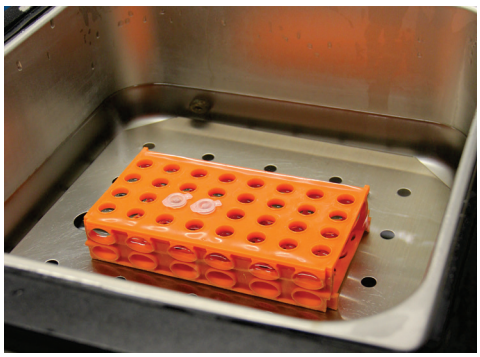


Fig. 18

20. After 90 seconds, quickly return the tubes to ice bath for 2 minutes (Fig. 17).

21. Using the P1000 micropipette and sterile tips, add 250 µl recovery broth to each of the micro-centrifuge tubes (Fig. 19). Gently finger-flick (Fig. 20) or wrist-flick (Fig. 21) the tubes.

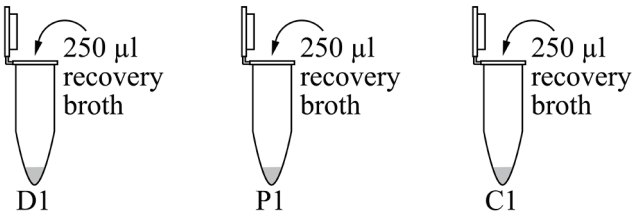


Fig. 19

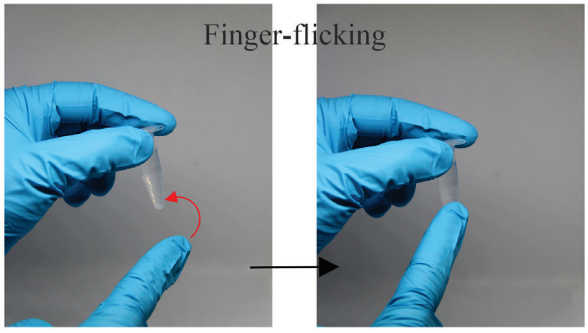


Fig. 20

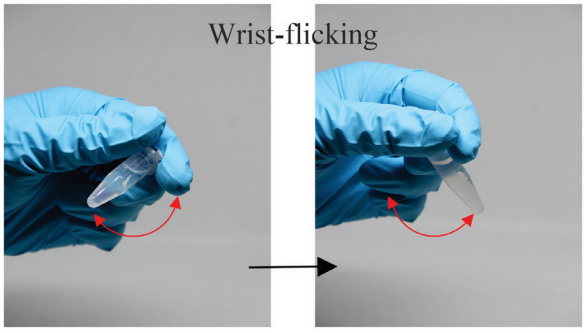


Fig. 21

22. Incubate all the tubes in a 37°C water bath for 30 minutes (Fig. 22).

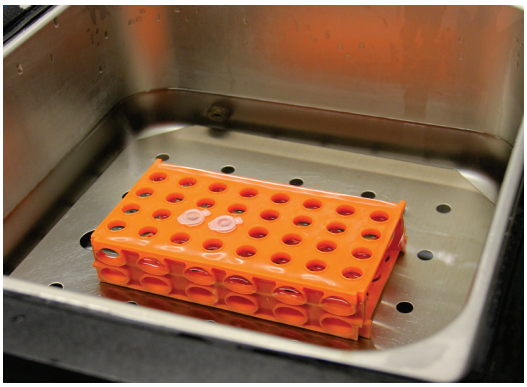


Fig. 22

23. Centrifuge the 3 tubes at 5,000 rpm for 5 minutes.

24. To each of the tubes, using the P1000 micropipette and sterile tips, remove 400 µl supernatant and discard it in a designated disposal container. Re-suspend the *E. coli* cells in the remaining liquid by pipetting it up and down (Fig. 23).

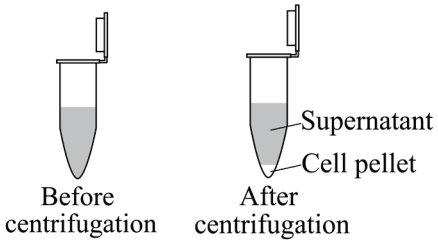


Fig. 23

25. Discard all unwanted tubes and tips in the designated disposal container with 10% chlorine bleach (no autoclave processing required) or in a biohazard bag for autoclave processing afterwards.

C. Part (2) of the experiment: Plating and incubation of *E. coli* on agar plate

a) Equipment and materials (per group)

Equipment

- Bunsen burner × 1
- Spark lighter × 1

Materials

- X-gal/IPTG/ampicillin agar plate × 1
- Inoculating loop × 1
- Permanent marker × 1
- Adhesive tape × 1
- Parafilm

- 70% Ethanol in spray bottle × 1
- Paper towel × 1 box
- Biohazard bag × 1
- Disposal container with 10% chlorine bleach × 1

b) Safety precautions

- Wear a laboratory gown.
- Tie up long hair.
- Wear gloves during the experiment.
- Wash hands to remove all possible contamination before and after the experiment.
- Keep ethanol away from fire.
- Make sure ethanol is fully vaporised before approaching any flame.
- Dispose of or disinfect all materials properly after each experiment.

c) Procedure

1. Obtain 1 X-gal/IPTG/ampicillin-containing agar plate. Label the bottom of it with the date of experiment, your class and group number.

2. To the bottom of the agar plate, draw 3 lines to divide the plate into 3 sections. Label them "D1", "P1" and "C1" (Fig. 24).

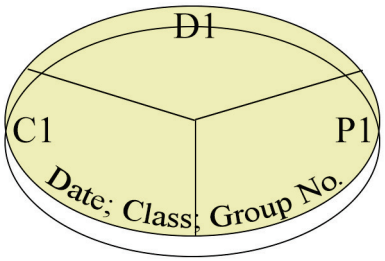


Fig. 24

3. Light up the Bunsen burner.

4. Flame the loop to red hot and allow it to cool down.

5. Raise the lid of the agar plate at 45° (Fig. 25). Using the flame-sterilised inoculating loop, transfer a loopful of microbial culture from tube "D1" onto section "D1" of the agar plate, and spread it evenly across the section (Fig. 26).

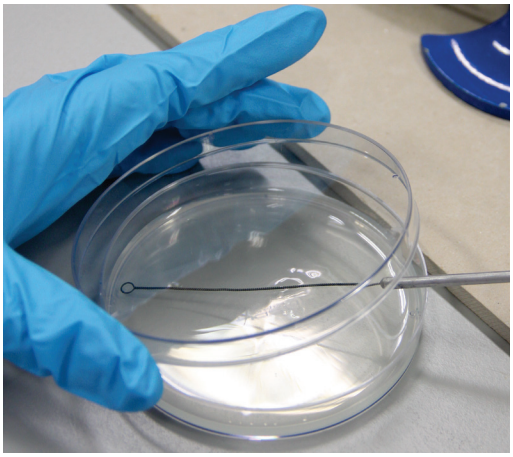


Fig. 25

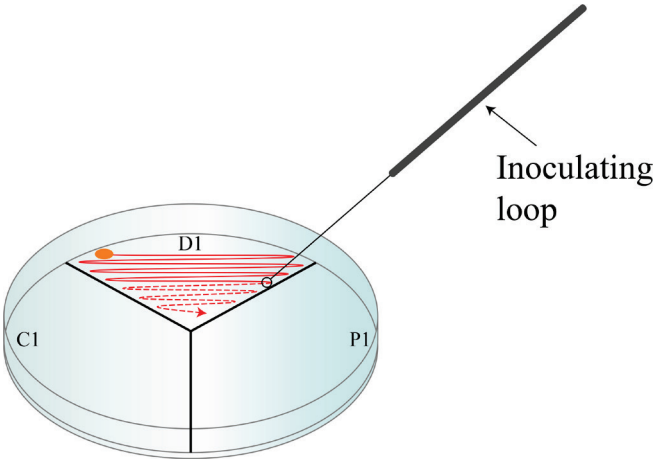


Fig. 26

6. Repeat steps 4–5 for transferring the microbial culture from tube "P1" onto the corresponding section "P1" of the agar plate.

7. Repeat steps 4–5 for transferring the microbial culture from tube "C1" onto the corresponding section "C1" of the agar plate.

8. Flame-sterilise the inoculating loop.

9. Shut off the Bunsen burner.

10. Stick short strips of adhesive tape at opposite edges of the agar plates to hold the lid and base together (Fig. 27). Invert the agar plate and incubate it at room temperature or in an incubator set at 25–30°C for 1–2 days* until observable colonies are found.

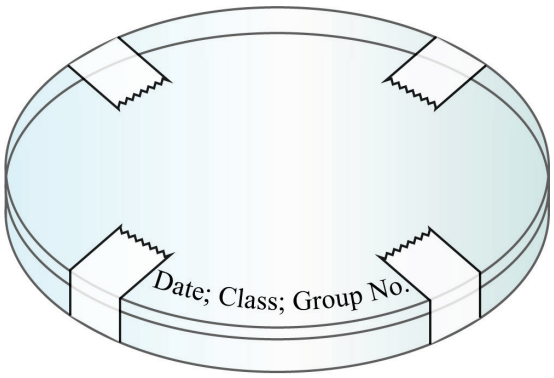


Fig. 27

*Note: Room temperature may vary depending on the ambient conditions of the laboratory environment. Hence, the incubation time may also vary.

11. After the suggested incubation period, wrap the edge of the plate with parafilm and store it at 4°C until lab session 3.

D. Part (3) of the experiment: Observation and analysis of experimental results

a) Equipment and materials (per group)

Equipment

- Mobile device × 1

Materials

- Agar plate from part (2) × 1
- Permanent marker × 1
- 70% ethanol in spray bottle × 1
- Paper towel × 1 box
- Biohazard bag × 1
- Disposal container with 10% chlorine bleach × 1

b) Safety precautions

- Wear a laboratory gown.
- Tie up long hair.
- Wear gloves during the experiment.
- Wash hands to remove all possible contamination before and after the experiment.
- Discard your plates in the designated container.

c) Procedure

1. Collect the agar plate of your group. Using a permanent marker, count the colonies by marking dots on the back of the plates.

2. Record the number of blue and white colony-forming units (CFU)*. Mark TNTC (Too Numerous To Count) if the number of colonies on the plate is higher than 250 CFU.

> *Note: Only plates with a colony number between 25 and 250 can be used for counting.
>
> Use the term "colony-forming unit" (CFU) instead of the term "colony" to reflect the fact that a single colony observed on an agar plate may not be formed by a single bacterium. In fact, such a colony can be formed by several bacteria (more than one bacterium) growing in proximity.
>
> If we present our data using the term "colony", then the actual number of bacteria in the test sample may be underestimated.

3. Take pictures of the plates, using a mobile device.

4. Discard the unwanted agar plate in the designated disposal container with 10% chlorine bleach (no autoclave processing required) or in a biohazard bag for autoclave processing.

VI. Results

1. Paste the photo or draw a picture of your agar plate in the space provided below.

2. Record the number of blue colonies and white colonies in the following table.

   [Hint:
   Blue colonies: pale blue in the centre with a dense blue periphery.
   White colonies: faint blue in the centre but colourless at the periphery.]

Section	Number of blue colonies	Number of white colonies
D1		
P1		
C1		

3. Do you observe any colonies in section C1? Give a brief explanation of your observation.

4. What do you observe in section P1 of the agar plate? Describe and explain your observation.

5. What do you observe in section D1 of the agar plate? Describe and explain your observation.

VII. Discussion

1. After the heat shock [Part (1) of the experiment, Step 21] the tubes were mixed by "finger-flicking" or "wrist-flicking" instead of vortexing. Suggest the reason for this.

2. During the heat shock step, a student accidentally heated the cells at 42^{o}C for 5 minutes. Only a few colonies were found on the agar plates at the end of the experiment. Explain the results.

3. What are the functions of X-gal and IPTG in blue-white screening?