# **Manual of cultivation of bacteria, Gram staining and microscopic observation**

**Materials and equipment**

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| Part A - Preparation of nutrient broth |  |
| 1g powder of nutrient broth (DifcoTM Nutrient Broth) | x1 |
| 125mL purified water | x1 |
| 250mL beaker | x1 |
| Glass stirrer rod and heating plate (or magnetic stirrer) | x1 |
| Autoclave | x1 |

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| Part B - Cultivation of bacteria |  |
| 9mL nutrient broth | x1 |
| 1mL water sample | x1 |
| P5000 auto-pipette | x1 |
| Pipette tips (1 box) | x1 |
| 50mL centrifuge tube | x1 |
| Permanent marker pen | x1 |
| Shaking incubator | x1 |

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| Part C - Gram staining |  |
| 70% ethanol in a spray bottle | x1 |
| Bunsen burner | x1 |
| Heating mat | x1 |
| Glass slide | x2 |
| Wooden slide holder | x1 |
| P20 or P100 auto-pipette | x1 |
| Pipette tips (1 box) | x1 |
| Incubated mixture of water sample and nutrient broth | x1 |
| Inoculation loop | x1 |
| Crystal violet solution (1 mL) | x1 |
| Gram’s iodine solution (1 mL) | x1 |
| 95% ethanol (5 mL) | x1 |
| Safranin solution (1 mL) | x1 |
| Gram staining waste box or a 250mL beaker | x1 |
| Sterile Distilled water in wash bottle | x1 |
| Permanent marker pen | x1 |
| Paper towel (several pieces) | x1 |
| Disposal container with 10% chlorine bleach | x1 |
| Yakult drink (1 small bottle) (optional) | x1 |

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| Part D - Microscopic observation |  |
| Glass slide | x1 |
| Cover slip | x3 |
| Compound microscope | x1 |
| Mobile device with camera | x1 |
| Lugol’s solution (optional) | x1 |

**Steps**

1. **Preparation of nutrient broth**
2. Suspend 1g of the powder of nutrient broth in 125mL of purified water in a 250mL beaker.
3. Mix thoroughly. Heat with frequent agitation using a glass stirrer rod (or magnetic stirrer) and boil for 1 minute to completely dissolve the powder.
4. Autoclave at 121oC for 15 minutes. The cooled nutrient broth is ready for use.
5. **Cultivation of bacteria**
6. Measure 9mL nutrient broth and 1mL of the water sample with an auto-pipette. Mix them in a 50mL centrifuge tube (Figure 1). Label the tube.

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| **Figure 1 Mixture of water sample and nutrient broth in a centrifuge tube (before cultivation)** | **Figure 2 Shaking incubator** |

1. Place the centrifuge tube into a shaking incubator set at 37oC for 24 hours (Figure 2).
2. The mixture will turn cloudier after incubation (Figure 3).

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|  | **Figure 3 Mixture of water sample and nutrient broth in a centrifuge tube (after cultivation)** |

1. **Gram staining**
2. Disinfect the working bench and gloved hands with 70% ethanol. Make sure the ethanol is fully vaporized before using any flame.
3. Set up a Bunsen flame.
4. Clip a glass slide with a test tube holder.
5. Use the auto-pipette to take out 20µL of the solution containing bacteria from the centrifuge tube. Load it onto the glass slide.
6. Put the wire of an [inoculation](https://www.sciencedirect.com/topics/biochemistry-genetics-and-molecular-biology/inoculation) loop in the Bunsen flame until the wire is bright red. Then remove the loop from the flame and allow it to cool down.
7. Spread the mixture in step 10 on the glass slide by the inoculation loop. Allow some time (1-2 minutes) for it to dry.
8. Heat-fix the bacteria to the glass slide by carefully passing the slide with mixture through a Bunsen flame several times.
9. Add 2-3 drops of crystal violet (the primary stain) to the area with heat-fixed bacteria (sample area) and wait for 1 minute. On top of the Gram staining waste box (Figure 4), rinse the sample area with a gentle stream of water (from a wash bottle) for a few seconds to remove excess unbound crystal violet (Figure 5).

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| **Figure 4 Gram staining waste box** | **Figure 5** **Solution used in Gram staining (from left to right: crystal violet, iodine solution, 95% ethanol, and safranin)** |

1. Add 2-3 drops of iodine solution (fixing agent) to the sample area and wait for 1 minute.
2. Rinse the sample area with 95% ethanol for a very short time (i.e. about 3 seconds) and rinse with a gentle stream of water for a few seconds.
3. Add 2-3 drops of safranin (secondary stain) to the sample area and wait for 1 minute. Rinse the sample area with a gentle stream of water for a few seconds.
4. **Microscopic observation**
5. Let the glass slide dry for a while (but still a bit damp) and place a cover lid with an inclination angle of 45° onto the sample area.
6. When using a compound microscope, use a low-power objective to locate and focus on the centre of the sample area.
7. Turn to higher power objective to see the colours and distribution of bacteria on the glass slide (Figure 6).

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|  | **Figure 6** **Bacteria under a microscope (400X) (purple: Gram-positive bacteria; and**  **red: Gram-negative bacteria)** |

1. For your information: Bacteria in a Yakult drink under a microscope (400X) (Figure 7a), phytoplankton in a fresh water sample (Figure 7b) and phytoplankton in the water sample a day after the field trip (Figure 7c). The preservative of phytoplankton, Lugol’s solution can be added to water samples if the phytoplankton cannot be observed in time. (Bu putting a few drops of Lugol’s solution in 100 mL water, the sample can be preserved up to about 1 month)

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|  | **Figure 7a Bacteria in Yakult drink under microscope (400X) (all Gram negative bacteria)** |
|  | **Figure 7b Phytoplankton under microscope (400X)** |
|  | **Figure 7c Phytoplankton under microscope (100X)** |

**Manual of Nitrate and phosphate concentration test**

**Materials and equipment**

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| 2mL water sample | x1 |
| Nitrate and phosphate concentration test kit, which include |  |
| • Sample cup | x1 |
| • Testing tube for nitrate | x2 |
| • Testing tube for phosphate | x2 |
| • Colour chart for nitrate | x1 |
| • Colour chart for phosphate | x1 |

**Steps**

1. Rinse the sample cup with water sample.
2. Half-fill (to the halfway line (Figure 8)) the sample cup with the water sample.

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| **Halfway line** | **Figure 8 Sample cup (with halfway line)** |

1. Remove the yellow pin (Figure 9) from the top of the testing tube to open a hole.

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| **Yellow pin** | **Figure 9 Testing tube with a yellow pin** |

1. Press the sides of the testing tube to expel approximately half of the air inside. Keep it pressed.
2. Insert the testing tube fully in the sample cup (Figure 10). Now release the sides to half fill the testing tube up.

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|  | **Figure 10** **Testing tube is inserted into a sample cup** |

1. Shake the tube gently a few times.
2. After 5 minutes (for phosphate concentration test) or 3 minutes (for nitrate concentration test), place the testing tube in front of the colour chart (Figure 11) to compare with the colours (Figures 12 and 13).

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|  | **Figure 11** **Comparison of the colour in the testing tube with standard colours in the chart** |
|  | **Figure 12 Standard colours of different nitrate concentrations** |
|  | **Figure 13 Standard colours of different phosphate concentrations** |

# **Manual of *E. coli* test (by Petrifilm *E. coli* / coliform count plate)**

**Materials and equipment**

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| Petrifilm *E. coli* / coliform count plate (3M Petrifilm) | x2 |
| Spreader | x1 |
| 1mL water sample | x1 |
| P1000 auto-pipette | x1 |
| Pipette tips (1 box) | x1 |
| Incubator | x1 |
| Permanent marker pen | x1 |
| Refrigerator (optional) | x1 |

**Steps**

1. Take out a piece of petrifilm from the refrigerator. Allow some time for it to reach room temperature, ensuring it is not exposed to moisture (Figure 14).

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|  | **Figure 14 3M Petrifilm *E. coli* / coliform count plate and the spreader** |

1. The petrifilm consists of two layers- top film and bottom film. Lift the top film.
2. Use a P1000 auto-pipette to dispense 1mL of water sample onto the centre of the bottom film (Figure 15).

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|  | **Figure 15 Adding water sample to bottom film by auto-pipette** |

1. Gently roll the top film down onto the water, taking care not to push the sample off the film and to prevent the formation of air bubbles. Make sure not to let the top film drop.
2. With the flat side down, place the spreader on the centre of the petrifilm. Gently press the centre of the spreader in order to distribute the water sample evenly. Make sure not to slide the spreader across the top film (Figure 16).

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|  | **Figure 16 Placing the spreader on the petrifilm** |

1. Remove the spreader and leave the petrifilm undistributed for 1 minute.
2. Label the petrifilm with site name and date of sampling using a marker pen (Figure 17).
3. Place the petrifilm into an incubator at 35-37oC for 24 hours (Figure 18) (incubation longer than 24 hours is not recommended, for storage of results, place the petrifilm in the refrigerator at 4oC instead).

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| **Figure 17 Petrifilms before incubation** | **Figure 18 Petrifilms placed in an incubator** |

1. The number of CFU of *E. coli* in 1 mL of the water sample is shown by the number of blue dots, while the number of red dots represents the number of CFU of other coliform bacteria (Figures 19 and 20).

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|  | **Figure 19 Petrifilms after incubation** |
| ***E.Coli***  **Coliform bacteria** | **Figure 20 Counting number of bacteria (Blue dots: colonies arisen from *E. coli*; Red dots: colonies arisen from other coliform bacteria)** |

**Overall safety remarks for microbiology**

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| You must wear gloves when handling bacteria during experiments. Remember to wash your hands before and after the experiment to remove any contaminants. |  |

It is important to follow aseptic measures and/or autoclave processes to prevent sample contamination.

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| An autoclave | This label indicates that the pipette tips have been autoclaved. |

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| To wash the container or to discard samples containing bacteria, use 10% chlorine bleach.  The making of 10% chlorine bleach: |  |