SOPs: MOLECULAR BIOLOGY & IMMUNOLOGY PRACTICAL EXERCISES OF UNDERGRADUATE STUDENTS

(DBT Life Sciences Star College Scheme, Ministry of Science & Technology, Govt. of India)

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SECTION A: Molecular Biology

Experiment No. A.1: Preparation of Molecular Biology Stock and working solutions.

Background: Stock solution: The solution having a concentration many folds higher than actual experimental requisition.

Working solution: The solution with desirable concentration to be used in the experiment.

The mathematical equation used for making working solution from the stock solution is:

$$C_1V_1 = C_2V_2$$

Where C_1 = Conc. of the working solution, V_1 = Volume of the working solution, C_2 = Conc. of the stock solution, V_2 = Volume of the stock solution.

Material and Reagent Requirements: Volumetric flasks (100 ml), Beakers (100 ml, 200 ml), Reagent bottles (250 ml), Falcon tubes (50 ml), measuring cylinder, Whatman filterpaper, Funnel, Pipettes, Sterile double distilled water, Magnetic stirrer, Hot plate, pH meter, Standard pH buffers (pH: 4,7 & 9), magnetic bead, weighing balance, Aluminum foil, CTAB, NaCl, EDTA, Tris-HCl, Sodium acetate, glacial acetic acid, ammonium chloride, ammonium acetate, SDS, glucose, NaOH, Potassium acetate, Conc. HCl, ethidium bromide, lysozyme, xylene cyanol, Bromophenol blue, sucrose.

Methodology:

A. Stock Solutions

1. **Tris-HCl (1M; pH 8.0)**: Dissolve 12.114g of Tris base in 70ml of sterile double distilled water. Adjust pH to 8.0 with 1N HCl and the final volume is thereafter made 100 ml.

2. **Ethylene di-amine tetra acetic acid (EDTA) (0.5M; pH 8.0)**: Dissolve 18.61g of EDTA in 50ml of sterile double distilled water and pH is adjusted to 8.0 with 10M NaOH solution. The final volume is made 100 ml with sterile double distilled water.

3. **Sodium Dodecyl Sulphate (SDS) (10%)**: Dissolve 10g of SDS in 100ml of sterile double distilled water with gentle heating at 65^{o}C.

4. **Ammonium acetate (CH_3COONH_4) (7.5 M**): Dissolve 57.8g of CH_3COONH_4 in 50ml of sterile double distilled water and the final volume is made to 100ml with sterile double distilled water.

5. **Cetyltrimethyl ammonium bromide (CTAB) (10%):** Dissolve 10g of CTAB in 100ml of sterile double distilled water with gentle heating at 65°C.

6. **Sodium Chloride (NaCl) (5M):** Dissolve 29.2 g of NaCl in 80 ml of sterile double distilled water. Adjust the volume to 100 ml with double distilled water.

7. **Sodium acetate ($CH_3CooNa.3H_2O$) (3M, pH 5.2):** Dissolve 40.8 g of CH3CooNa in 80 ml of sterile double distilled water. Adjust the pH to 5.2 with glacial acetic acid. Make the final volume to 100 ml with double distilled water.

8. **Sodium hydroxide (NaOH) (10N):** To 80 ml of sterile double distilled water slowly add 40 g of NaOH pellets with continuous stirring. Adjust the final volume to 100 ml with sterile double distilled water.

9. **Ammonium Chloride (NH_4Cl) (1M)**: Dissolve 5.35 gm of ammonium chloride in 80 ml of sterile distilled water and final volume of the solution is made to 100 ml**.**

10. **Gel Loading Dye (6x):** 0.025 % of bromophenol blue with 40% (w/v) sucrose in water.

11. **Ethidium bromide (10 mg/ml):** 10 mg of ethidium bromide dissolved in 1 ml of sterile double distilled water and stored in a dark bottle at RT.

12. **10x Gel loading dye:** 0.5% (w/v) xylene cyanol dissolved with 40% (w/v) sucrose in water.

13. **Lysozyme (20mg/ml):** Dissolve lysozyme at a concentration of 20mg /ml in 10 µl of 1M Tris, pH 8 and aliquots of 200µl are made and store at -20oC for further use.

B. Working Solutions:

1. **TE buffer (Tris-HCl, 10 mM, EDTA 1mM; pH 8.0)**: 1ml of Tris-HCl (1M; pH 8.0) is mixed with 200µl of EDTA (0.5M, pH 8.0) and the final volume is made to 100 ml with sterile double distilled water.

2. **RBC Lysis buffer (Tris-HCl,10 mM; EDTA ,1mM; NH4Cl,125mM; pH 8.0):** 1ml of Tris-HCl (1M; pH 8.0) is mixed with 200µl of EDTA (0.5M, pH 8.0) and 12.5 ml of NH4Cl (1M). The final volume is made to 100 ml with sterile double distilled water.

3. **Plant Genomic DNA Extraction Buffer (CTAB, 2%; NaCl, 1.4 M; EDTA,20 mM, pH 8; Tris-HCl, 100 mM, pH 8):** Mix 20 ml of CTAB (10%), 28 ml of NaCl (5M), 10 ml of Tris-HCl (1M; pH 8.0) and 4 ml of EDTA (0.5M, pH 8.0). The final volume is made to 100 ml with sterile double distilled water.

4. **TAE buffer (50X):** Add 242 g of Tris-HCl base in 500 ml of sterile double distilled water. Subsequently mix 57.1 ml of glacial acetic acid and 100 ml of 0.5 M EDTA (pH 8). The final volume is made to 1000 ml with sterile double distilled water.

5. **Alkaline Lysis solution I (Glucose, 50 mM; Tris-HCl, 25 mM, pH 8; EDTA, 10 mM, pH 8):** Mix 5 ml of glucose (1M), 2.5 ml of Tris-HCl (1M; pH 8.0) and 2 ml of EDTA (0.5M, pH 8.0). The final volume is made to 100 ml with sterile double distilled water. Store at 4°C after autoclaving.

6. **Alkaline Lysis solution II (NaOH, 0.2N; SDS, 1%):** Mix 2 ml of NaOH (10 N) with 10 ml of 10% SDS. The final volume is made to 100 ml with sterile double distilled water. Prepare solution fresh and use at RT.

7. **Alkaline Lysis solution III (CH_3COOK, 5M; Glacial acetic acid):** Mix 60 ml of CH3COOK (5M) with 11.5 ml of glacial acetic acid and 28.5 ml of sterile double distilled water. Store the solution at 4°C and transfer it to an ice bucket just before use.

Precautions:

1. Use autoclaved / sterilized glassware/plasticware.
2. Autoclave all the reagents except Tris –HCl solutions.
3. Store the reagents in the reagent bottles strictly at the prescribed temperatures.
4. Avoid the direct contact of skin with the ethidium bromide due to its mutagenic and carcinogenic nature.
5. Carefully handle Concentrated HCl.
6. Make sure that the pH of Tris and EDTA solutions is 8. EDTA will dissolved completely only at pH 8.
7. The preparation of sodium hydroxide solution involves a highly exothermic reaction. So make this solution in a plastic beaker.
8. Lysozyme will not work efficiently if the pH of the solution is less than 8.0.

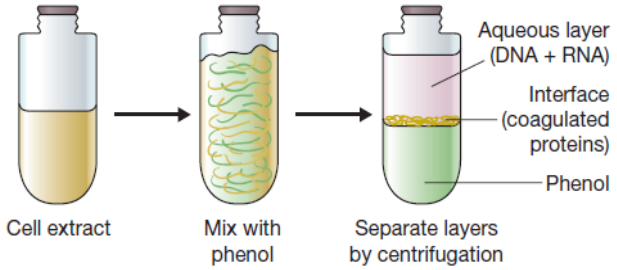
Experiment No. A.2: Extraction of genomic DNA from plant leaves.

Background: The method described in this protocol involves homogenization of plant tissues in hot extraction buffer followed by solubilizing membranes and dissociation of nucleoprotein complex using high conc. of NaCl. The dissociated proteins are further denatured by phenol. Subsequently, nucleic acids are purified by phase extraction with an appropriate combination of organic solvents i.e Phenol, chloroform and isoamyl alcohol (25:24:1). Finally, DNA is precipitated using chilled ethanol.

Material and Reagent Requirements: Centrifuge / Oakridge / Falcon tubes, Centrifuge, Water bath, Vortexer, Mortar and Pestle, young leaves of *Mangifera indica,* measuring cylinder, filterpaper, Pipettes, double distilled water, DNA extraction buffer (2% CTAB; 1.4M NaCl; 20mM EDTA; 100mM Tris-HCl), Tris saturated phenol (pH 8), Chloroform, Isoamylalcohal, Sodium acetate (pH 5.2, 3M), Chilled absolute alcohol, 70% chilled alcohol, TE buffer (10 mM Tris –HCl; 1mM EDTA; pH 8.0).

Methodology:

1. Rinse 1gm of young leaves with double distilled water and make them dry on filter paper.
2. Grind leaves into fine powder in 10 ml of pre warmed DNA extraction buffer by constant crushing using sterilized pestle and mortar.
3. Transfer the homogenate into an oakridge tube and incubate at 60°C in a water bath with constant shaking for 40 minutes.
4. Gently vortex the oakridge tube and add 10 ml of Phenol: Chloroform and isoamylalcohal / PCI (25:24:1). Mix it thoroughly on a vortexer.
5. Spin at 13000 rpm for 10 minutes. The content of the oakridge tube will be segregated into three layers from top to bottom as aqueous phase, protein phase and organic phase. Transfer the top clear aqueous phase to a fresh sterilized falcon tube. If the aqueous layer is not clear, then add the same volume of PCI and repeat the 4^{th} and 5^{th} steps to finally get aproteonic solution.



6. To the separated aqueous phase, add 3M Sodium acetate (pH 5.2) equivalent to the 1/10th the volume of the former.
7. Mix the contents and add equal amount of ice chilled absolute ethanol and gently invert the tube several times to see the whitish floating threads of DNA.
8. Centrifuge it at 13000 rpm for 15 minutes.
9. Wash the DNA pellet with 200 µl of 70% chilled ethanol. Spin it at 13000 rpm for 15 minutes and air dry the pelleted DNA for 15-20 minutes.
10. Dissolve the DNA in 50 µl of TE buffer by incubating at 65°C for 10 minutes**.** Store the purified DNA at 0°C / 4° C / -20° C for downstream applications.

Precautions:

1. Use autoclaved / sterilized glassware/plasticware and reagents except organic solvents.
2. Carefully handle phenol and phenol containing solutions as they cause severe burns. In case if any of skin areas come in contact with phenol, then rinse it with a large volume of water and wash it with a soap. Do not use ethanol.
3. The pH of Tris saturated phenol should be 8.0 to avoid the trapping of DNA at the interface between the organic and aqueous phases. Discard the use of pink or yellow colored phenol.
4. During the preparation of PCI, the phenol should be taken from the bottom to avoid the contamination with Tris traces.
5. Carefully transfer the aqueous phase avoiding the protein contamination. Avoid mechanical shearing of DNA
6. Do not air dry the pelleted DNA for long time otherwise it will be difficult to get dissolved.

Experiment No. A.3: Extraction of genomic DNA from human peripheral blood by using inorganic solvent.

Background: This method involves following three basic steps for the purification of DNA:

(a) Cell Lysis: The denucleated cellular fraction is lysed by using RBC lysis buffer. Subsequently, the white blood cells are disrupted by using WBC lysis buffer.

(b) Removal of contaminants: The lysate is further subjected to treatment with a detergent (SDS) for the disintegration of nucleoprotein complex. The denatured proteins are then precipitated using ammonium acetate solution.

(c) Precipitation of DNA: Finally DNA is precipitated with ice chilled dehydrated ethanol

Material and Reagent Requirements: Micro centrifuge tubes, Cooling Centrifuge, dry bath, Vortexer, Rocker, Micropipettes (2-20 µl; 20-200 µl; 200-1000 µl), Microtips, Anticoagulated blood sample, RBC lysis buffer (10mM Tris-HCl, 125 mM NH_4Cl; 1mM EDTA, pH 8;), WBC lysis buffer ((10 mM Tris –HCl; 1mM EDTA; pH 8.0), Ammonium acetate (7.5M), Chilled absolute alcohol, 70% chilled alcohol, 10% SDS.

Methodology:

1. 300 µl of blood sample is mixed with 900 µl of RBC lysis buffer and incubated at room temperature on a rocker till complete lysis of RBCs which is indicated by the decreased viscosity and shining appearance of the content .
2. Spin at 13,000 rpm for 1 min to pellet down the white blood corpuscles (WBC).
3. Suspend the white pellet in 300 µl of TE buffer and 20 µl of 10% SDS followed by incubation at 56^{o}C for 30 minutes.
4. After a brief vortexing, add 150 µl of 7.5 M ammonium acetate and mix thoroughly at 37^{o}C for 30 min.
5. Centrifuge at 13,000 rpm for 15 min.

6. Transfer the supernatant in a fresh eppendorf tube and add twice the volume of chilled ethyl alcohol. Gently invert the tube several times to allow the precipitation of DNA in the form of white shining threads.
7. Centrifuge it at 13000 rpm for 15 minutes.
8. Wash the DNA pellet with 150 µl of 70% chilled ethanol. Spin it at 13000 rpm for 15 minutes and air dry the pelleted DNA at room temperature for 20-25 minutes.
9. Redissolve the dried pellet of DNA in 30 µl of TE buffer by incubating at 65°C for 10 minutes. Store the purified DNA at 0°C / 4° C / -20° C for further use.

Precautions:

1. Human blood must be collected by a trained phlebotomist under sterile conditions.
2. Use autoclaved / sterilized glassware/plasticware and reagents except organic solvents.
3. If the supernatant obtained after the ammonium acetate precipitation is not clear, then mix the content thoroughly and repeat the centrifugation step (5^{th} step)
4. Avoid mechanical shearing of DNA
5. Do not air dry the pelleted DNA for long time otherwise it will be difficult to get dissolved.
6. Ensure the disposal of blood / components after double autoclaving and following standard procedures.

Experiment No. A.4: Extraction of genomic DNA from human peripheral blood by using organic solvent.

Background: This method involves three basic steps for the purification of DNA as described: (a) Cell Lysis: The denucleated cellular fraction is lysed by using RBC lysis buffer. Subsequently, the white blood cells are disrupted by using WBC lysis buffer. (b) Removal of contaminants: The lysate is further subjected to treatment with a detergent (SDS) for the disintegration of nucleoprotein complex. The denatured proteins are removed with the help of PCI (Phenol, chloroform and isoamylalcohal in 25:24:1). (c) Precipitation of DNA: Finally DNA is precipitated with ice chilled dehydrated ethanol

Material and Reagent Requirements: Micro centrifuge tubes, Cooling Centrifuge, dry bath, Vortexer, Rocker, Micropipettes (2-20 µl; 20-200 µl; 200-1000 µl), Microtips, Anticoagulated blood sample, RBC lysis buffer (10mM Tris-HCl, 125 mM NH_4Cl; 1mM EDTA, pH 8;), WBC lysis buffer ((10 mM Tris –HCl; 1mM EDTA; pH 8.0), PCI, Sodium acetate (pH 5.2, 3M), Chilled absolute alcohol, 70% chilled alcohol, 10% SDS.

Methodology:

1. 300 µl of blood sample is mixed with 900 µl of RBC lysis buffer and incubated at room temperature on a rocker till complete lysis of RBCs which is indicated by the decreased viscosity and shining appearance of the content .
2. Spin at 13,000 rpm for 1 min to pellet down the white blood corpuscles (WBC).
3. Suspend the white pellet in 300 µl of TE buffer and 20 µl of 10% SDS followed by incubation at 56^{o}C for 30 minutes.
4. After a brief vortexing, add 300 µl of PCI and mix thoroughly at RT.
5. Spin at 13000 rpm for 10 minutes. Transfer the top clear aqueous phase to a fresh eppendorf tube. If the aqueous layer is not clear, then add the same volume of PCI and repeat the 4th and 5th steps to finally get aproteonic solution .

6. To the final supernatant, add 3M Sodium acetate (pH 5.2) equivalent to the 1/10th the volume of supernatant.
7. Mix the contents and add twice the volume of ice chilled absolute ethanol and gently invert the tube several times to see the whitish floating threads of DNA.
8. Centrifuge it at 13000 rpm for 15 minutes.
9. Wash the DNA pellet with 150 µl of 70% chilled ethanol. Spin it at 13000 rpm for 15 minutes and air dry the pelleted DNA for 20-25 minutes.
10. Redissolve the dried pellet of DNA in 30 µl of TE buffer by incubating at 65°C for 10 minutes. Store the purified DNA at 0°C / 4° C / -20° C for further use.

Precautions:

1. Use autoclaved / sterilized glassware/plastiware and reagents except organic solvents.
2. Carefully handle phenol and phenol containing solutions as they cause severe burns. In case if any of skin areas come in contact with phenol, then rinse it with a large volume of water and wash it with a soap. Do not use ethanol.
3. The pH of Tris saturated phenol should be 8.0 to avoid the trapping of DNA at the interface between the organic and aqueous phases. Discard the use of pink or yellow colored phenol.
4. During the preparation of PCI, the phenol should be taken from the bottom to avoid the contamination with Tris traces.
5. Carefully transfer the aqueous phase avoiding the protein contamination. Avoid mechanical shearing of DNA.
6. Do not air dry the pelleted DNA for long time otherwise it will be difficult to get dissolved.
7. Ensure the disposal of blood / components after double autoclaving and following standard procedures.

Experiment No. A. 5: Isolation of genomic DNA from *E.Coli*.

Background: The bacterial cells are lysed with the help of SDS and lysozyme. Subsequently, using, ammonium acetate, the lysate is deproteinised. Bacterial DNA is finally precipitated by adding chilled absolute alcohol.

Material and Reagent Requirements: Micro centrifuge tubes, Cooling Centrifuge, dry bath, Vortexer, Rocker, Micropipettes (2-20 µl; 20-200 µl; 200-1000 µl), Microtips, *E.Coli* cell culture suspension, Lysozyme (20mg/ml), TE buffer (10 mM Tris –HCl; 1mM EDTA; pH 8.0), CH_3COONH_4 (7.5M), Chilled absolute alcohol, 70% chilled alcohol, 10% SDS.

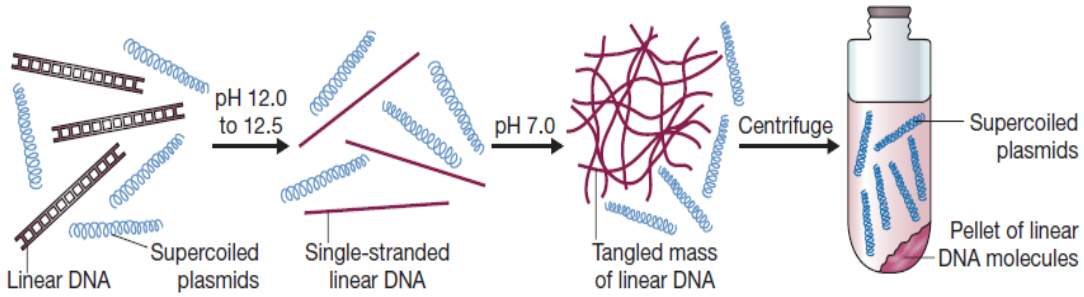
Methodology:

1. Spin 1.5 ml of *E.Coli* culture suspension at 13000 rpm for 2 minutes.
2. Suspend the bacterial cell pellet in 200 µl of TE buffer and add 13 µl of 10% SDS followed by incubation at 56^{o}C for 30 minutes.
3. After a brief vortexing, at 37°C, add 11 µl of 20 mg/ml lysozyme followed by incubation at 37°C for 1 hr.
4. After a brief vortexing, add 100 µl of 7.5M ammonium acetate and mix thoroughly at 37^{o}C for 30 min.
5. Centrifuge at 13,000 rpm for 15 min.
6. Transfer the supernatant in a fresh eppendorf tube and add twice the volume of chilled ethyl alcohol. Gently invert the tube several times to allow the precipitation of DNA in the form of white shining threads.
7. Centrifuge it at 13000 rpm for 15 minutes. Wash the DNA pellet with 150 µl of 70% chilled ethanol. Spin it at 13000 rpm for 15 minutes and air dry the pelleted DNA at room temperature for 20-25 minutes.
8. Redissolve the dried pellet of DNA in 30 µl of TE buffer by incubating at 65°C for 10 minutes. Store the purified DNA at 0°C / 4° C / -20° C for further use.

   Precautions: 1. Use autoclaved / sterilized glassware/ plasticware and reagents except organic solvents. 2. Avoid mechanical shearing of DNA. **3.** Do not air dry the pelleted DNA for long time otherwise it will be difficult to get dissolved.

Experiment No. A.6: Isolation of Plasmid DNA from *E.Coli* by alkaline denaturation method.

Background: The bacterial cells are lysed with the help of SDS and lysozyme. Subsequently, NaOH is added to a cell extract to adjust the pH to 12.0–12.5. This alkaline milieu breaks the hydrogen bonding in non-supercoiled DNA molecules, causing the unwinding and segregation of two polynucleotide chains of double helix. The addition of an acid results in the reaggregation of denatured strands into an insoluble tangled mass which can be pelleted by centrifugation, leaving plasmid DNA in the supernatant.



Material and Reagent Requirements: Micro centrifuge tubes, Cooling Centrifuge, dry bath, Vortexer, Rocker, Micropipettes (2-20 µl; 20-200 µl; 200-1000 µl), Microtips, *E.Coli* cell culture suspension, Alkaline lysis solution I (50mM glucose; 25 mM Tris-HCl pH 8; 10mM EDTA pH 8), Alkaline lysis solution II (0.2N NaOH; 1% SDS), Alkaline solution III (5M Potassium acetate = 60ml; glacial acetic acid = 11.5 ml; DDH_2O = 28.5 ml), TE buffer (10 mM Tris –HCl; 1mM EDTA; pH 8.0), CH_3COONH_4 (7.5M), Chilled absolute alcohol, 70% chilled alcohol.

Methodology:

1. Spin 1.5 ml of *E.Coli* culture suspension at 13000 rpm for 1 minute at 4°C.
2. Suspend the bacterial cell pellet in 100 µl of ice cold alkaline lysis solution I by vigorous vortexing.
3. Add 200 µl of freshly prepared alkaline lysis solution II and mix the contents thoroughly by rapidly inverting the tube.

4. Add 150 µl of ice cold alkaline lysis solution III and invert the tube several times. Keep the tube on ice for 3-5 minutes..
5. Centrifuge at 13,000 rpm for 5 minutes at 4°C.
6. Transfer the supernatant in a fresh eppendorf tube and add 7.5 M ammonium acetate to the final concentration of 3.4 M. Mix thoroughly at 37°C for 30 minutes.
7. Spin at 13000 rpm for 15 minutes.
8. Precipitate the DNA from the supernatant by adding twice the volume of chilled ethyl alcohol.
9. Collect the precipitated nucleic acid by Centrifugation at 13000 rpm for 15 minutes.
10. Wash the DNA pellet with 1 ml of 70% chilled ethanol. Spin it at 13000 rpm for 15 minutes and air dry the pelleted DNA at room temperature for 20-25 minutes.
11. Redissolve the dried pellet of DNA in 50 µl of TE buffer by incubating at 65°C for 10 minutes. Store the purified DNA at -20° C for further use.

Precautions:

1. Use autoclaved / sterilized glassware/plastiware and reagents except organic solvents.

2. Avoid mechanical shearing of DNA.

3. Do not air dry the pelleted DNA for long time otherwise it will be difficult to get dissolved.

4. Prepare alkaline solution II fresh and use at RT. Store alkaline sol I and III at 4°C.

Experiment No. A.7: Quantification of DNA by Ultraviolet (UV) Spectrophotometry.

Background: This is the most commonly used modality for the determination of DNA concentration, purity and yield. The resonance structures of purine and pyrimidine bases have a maximum and minimum absorbance at 260 nm and 234 nm, respectively. The relationship between the concentration of double stranded (ds) DNA and its absorbance at 260 nm (A_{260}) is described by the following mathematical relationship:

DNA conc. (µg/ml) = A_{260} X 50 X dilution factor

Where 50 is constant (A_{260} of 50 µg/ml of DNA conc. = 1.0); Dilution factor = Total volume of sample and diluent in cuvette / Volume of DNA to be diluted

The proteins with aromatic amino acids absorb maximally at 280 nm (**A_{280})** which in turn useful for detection of protein contamination in the isolated DNA samples by determining the **A_{260}** / **A_{280}** ratio. For purity evaluation of ds DNA, this ratio is customarily taken to be 1.8 -1.9.

The DNA yield (ng) can be calculated as: **Conc. of DNA (ng/µl) X Volume of TE buffer (µl) used for redissolving purified DNA**

Material and Reagent Requirements: Quartz cuvette, Vortexer, Palm fuge, Tissue paper, Micropipettes (2-20 µl; 20-200 µl; 200-1000 µl), Microtips, TE buffer (10 mM Tris –HCl; 1mM EDTA; pH 8.0), distilled water

Methodology:

1. Switch on the spectrophotometer and set it for the wavelengths of 260 and 280 nm.
2. Place the reference quartz cuvette containing distilled water in the requisite block of the spectrophotometer and set reading zero at desirable wavelengths.
3. Take 900 µl of TE buffer in the cuvette and record its absorption reading at both the wavelengths. Denote it as ‘a’ at 260nm and ‘b’ at 280 nm.

4. Now after brief vortexing and spinning the DNA sample, thoroughly mix 10 µl of purified DNA sample in 900 µl of TE buffer taken in the cuvette and take the absorbance measurements at both the wavelengths. Record it as: A_{260nm} = x and A_{280nm} = y
5. Calculate the DNA concentration in the solution using the following formula :
   Conc. of DNA (µg/ ml) = A_{260nm} X 50 X dilution factor
   Where A_{260nm} = x – a; dilution factor = 91
6. Check the Purity of isolated DNA solution from the ratio of absorption at 260nm and 280nm.

   Ratio= x – a / Y - b
7. Determine the yield of DNA with the relationship as described in background.

Precautions:

1. Use autoclaved / sterilized glassware/plastiware and reagents except organic solvents.

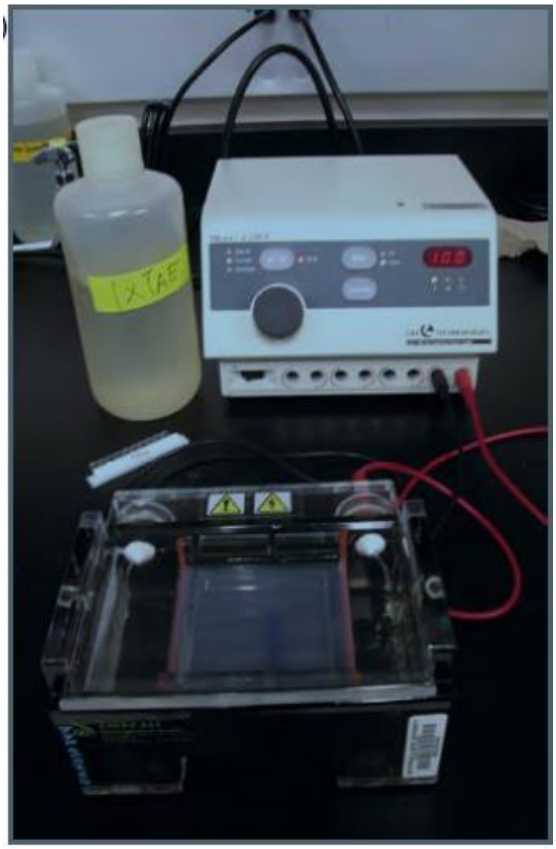
2. Avoid mechanical shearing of DNA.

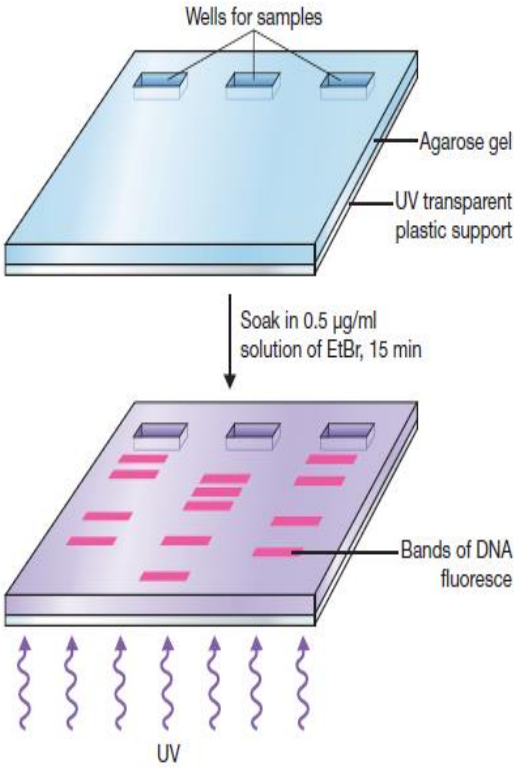
3. Ensure the complete mixing of DNA while diluting it with TE buffer in the cuvette.

4. Carefully handle the quartz cuvette.

Experiment No. A.8: Qualitative analysis of genomic DNA by Agarose gel electrophoresis (AGE).

Background: DNA being negatively charged owing to the phosphate groups migrates towards the positive electrode (Anode) through the pores of the agarose gel under the influence of the applied electric field. The variables affecting the migration of DNA molecules in AGE includes: (a) Size of DNA (b) Shape / confirmation of DNA (c) Concentration of agarose gel (d) Voltage applied (e) Buffer used for electrophoresis. To visualize DNA, usually, the agarose gel stained with 0.5 µg /ml ethidium bromide is illuminated with UV light at 254 to 260 nm.





Material and Reagent Requirements: Vortexer, Palm fuge, Tissue paper, Micropipettes (2-20 µl; 20-200 µl; 200-1000 µl), Microtips, Microwave oven, 100 ml flask with lid, petridish plate, weighing balance, gel casting tray, gel casting platform, combs, leveler, Agarose electrophoretic tank, power supply, Gel documentation system parafilm, Measuring cylinder, distilled water, agarose, 50X TAE Buffer (Dissolve 242 g

of Tris base in 500 ml of sterile distilled water and to this add 57.1 ml of glacial acetic acid and 100 ml of 0.5 M EDTA (pH 8.0) and make the final volume to one liter with sterile distilled water), Ethidium bromide (10 mg/ml) ,Gel Loading Dye (6x :0.025 % bromophenol blue with 40% (w/v) sucrose in water).

Methodology:

1. Set the gel casting tray on a gel casting platform with sealed ends and level it with the help of a leveler.
2. Place the 8 well comb by adjusting it in the requisite slots.
3. Prepare 1% agarose gel by dissolving 0.3 g of agarose in 30 ml of 1X TAE buffer. Melt the agarose to get a homogeneous clear solution by heating / boiling the solution in a microwave oven for approximately 2-3 minutes.
4. Cool the agarose solution to 50-60°C and add1.5 µl of 10 mg /ml ethidium bromide solution.
5. Pour the molten agarose slowly into the gel casting tray without disturbing the casting platform. Allow it to solidify at RT for 30 minutes.
6. Using TE buffer, prepare the serial dilutions of genomic DNA as 1:5, 1:10, 1:15, 1:20 and 1:25.
7. After the gel gets solidify, carefully and gently remove the comb. Submerge the gel tray in a an electrophoretic tank containing 250 ml of 1XTAE buffer.
8. Mix 10 µl of Neat and diluted DNA samples with 1 µl of bromophenol blue dye on a parafilam.
9. With the help of micropipettes, load the samples in the well as:

Well No.	**Content**
1	1 Kb ladder
2	Distilled water as negative control
3.	Neat DNA sample
4.	1:5 DNA sample

5.	1:10 DNA Sample
6.	1:15 DNA Sample
7.	1:20 DNA Sample
8.	1:25 DNA Sample

10. Add 12.5 ml of Ethidium bromide solution in the tank buffer towards the cathode.
11. Place the lid on the electrophoresis tank, connect the electrodes and turn on the power supply. Allow the gel to run at 100V for 1- 2 hours.
12. Examine the gel with a gel documentation system to assess the quality of isolated DNA.

Precautions:

1. Use autoclaved / sterilized glassware/plastiware and reagents except organic solvents.

2. Avoid mechanical shearing of DNA.

3. Swirl the agarose solution after boiling to ensure that agarose is completely dissolved

4. Pour the agarose solution in the gel tray in a slow and uniform go to avoid any air bubble. Remove any air bubble by trapping them with a hand held microtip.

5. Avoid the direct contact of skin with the ethidium bromide due to its mutagenic and carcinogenic nature.

6. Give brief vortexing and spinning to sample before loading.

7. Ensure the sequential loading of samples.

8. Use UV safety goggles while directly examining the gel on an UV transilluminator.

Experiment No. A.9: Restriction enzyme digestion of lambda (λ) DNA with *Hind III* enzyme.

Background: *Hind III* is a sticky end generating type II restriction enzyme that recognizes the following hexanucleotide recognition sequence: 5' – A / AGCTT – 3'. The enzyme has 6 restriction sites in 49 Kb λ genome generating 7 variable fragments after digestion.

Material and Reagent Requirements: Vortexer, Palm fuge, PCR tubes (0.2 ml), Micropipettes (0.5 -10 µl; 2-20 µl; 20-200 µl; 200-1000 µl), Microtips, dry bath, Microwave oven, 100 ml flask with lid, petridish plate, weighing balance, gel casting tray, gel casting platform, combs, leveler, Agarose electrophoretic tank, power supply, Gel documentation system parafilm, Measuring cylinder, distilled water, lambda DNA (200 µg/ µl), *Hind III* (10U/ µl), 10 X RE buffer, Predigested lambda DNA as positive control, 1Kb DNA ladder, agarose, 50X TAE Buffer (Dissolve 242 g of Tris base in 500 ml of sterile distilled water and to this add 5.71 ml of glacial acetic acid and 100 ml of 0.5 M EDTA (pH 8.0) and make the final volume to one liter with sterile distilled water), Ethidium bromide (10 mg/ml) ,Gel Loading Dye (6x :0.025 % bromophenol blue with 40% (w/v) sucrose in water).

Methodology:

1. Prepare the reaction mixture as:

Components (µl)	**Reaction 1 (Negative Control)**	**Reaction 2**	**Reaction 3**	**Reaction 4**
DD H_2O	8.5	6.5	7.5	8.5
10 X buffer	1.5	1.5	1.5	1.5
λ DNA (200 µg/ µl)	5	5	4	3
Hind III (10U/ µl)	0	2	2	2
Total Volume	15	15	15	15

2. Briefly vortex and spin down each reaction aliquot.
3. Incubate the reaction tubes at 37°C in dry bath for overnight.
4. Gently vortex and spin down the reaction mixture and further incubate at 65 °C for 10 minutes.
5. Resolve the fragments on ethidium bromide stained 0.7% agarose gel by electrophoresis according to the methodology described in experiment no. 8 as per the given sequence:

Well No.	**Content**
1	
2	1 Kb ladder
3.	Positive control (Predigested lambda DNA with Hind III)
4.	Negative control (Reaction 1)
5.	Reaction 2
6.	Reaction 3
7.	Reaction 4
8.	

6. Examine the gel with a gel documentation system to assess the result of restriction enzyme digestion.

Precautions:

1. Use autoclaved / sterilized glassware/plastiware and reagents except organic solvents.

2. Avoid mechanical shearing of DNA.

3. Add the reaction ingredients in different tube as per the mentioned sequence.

4. Ensure that the RE should be taken out of deep freezer at the last or just before the use.

5. Follow all the precautions for performing the AGE.

SECTION B: Immunology

Experiment No. B.1: Separation of Serum from blood.

Background: Serum is the fluid component of blood devoid of cells and clotting factors. It can be easily separated from blood by centrifugation. It is an important ingredient of clinical research and diagnostics.

Material and Reagent Requirements: Disposable syringe (3ml), Tonicator, centrifuge machine, Cotton swab, Centrifuge tube, glass rod, 70% ethanol

Methodology:

1. Disinfect the radial part of the forearm using cotton swab dipped with 70% ethanol.
2. Collect 3 ml of blood using tonicator and disposable syringe from the radial vein of forearm in a centrifuge tube without the anticoagulant.
3. Allow the blood to clot for 30 minutes at RT.
4. Retract the clot from the sides of the tube with the help of a glass rod.
5. Centrifuge at 3000 rpm for 15 minutes at RT.
6. Transfer the straw colored supernatant as serum in a fresh storage vial and store it at -20°C for future use.

Precautions:

1. Human blood must be collected by a trained phlebotomist under sterile conditions.

2. Carefully separate the serum without disturbing pelleted cells.

3. Ensure the disposal of blood / components, disposable syringes and swabs after double autoclaving and following standard procedures.

Experiment No. B.2: Separation of Plasma from blood.

Background: Plasma is the cell free fluid portion of blood which contains all the clotting factors. It can be easily separated from anticoagulated blood by centrifugation. It is an important ingredient of clinical research and diagnostics.

Material and Reagent Requirements: Disposable syringe (3ml), Tonicator, centrifuge machine, Cotton swab, Centrifuge tube, glass rod, 0.5 M EDTA, 70% ethanol.

Methodology:

1. Disinfect the radial part of the forearm using cotton swab dipped with 70% ethanol.
2. Collect 3 ml of blood using tonicator and disposable syringe from the radial vein of forearm in a centrifuge tube containing 30 µl of 0.5M EDTA as an anticoagulant.
3. Centrifuge at 3000 rpm for 15 minutes at RT.
4. Transfer the light yellow colored supernatant as plasma in a fresh storage vial and store it at -20°C for future use.

Precautions:

1. Human blood must be collected by a trained phlebotomist under sterile conditions.
2. Carefully separate the plasma without disturbing pelleted cells.
3. Ensure the disposal of blood / components, disposable syringes and swabs after double autoclaving and following standard procedures.

Experiment No. B.3: To determine the self-blood group by direct blood group typing / cell grouping / forward grouping method.

Background: Direct blood group typing method is based on the virtual in vitro agglutination reaction between the agglutinogens / corpuscle factors / antigens (A / B / Rh factor) present on the surface of RBCs and commercially available agglutinins / antibodies (Antiserum A, B and D or Anti Rh antibodies).

Material and Reagent Requirements: Disposable needle, microscopic slide, Cotton swab, Antiserum A, B and D, paper pins, 70% ethanol, Marker.

Methodology:

1. Disinfect the middle finger tip with a cotton swab dipped with 70% ethanol.
2. Prick the middle finger with a disposable lancet and press the finger to exude blood drops equidistantly at 3 different places labeled as A, B and D on the underneath of a clean microscopic slide
3. Add one drop of antiserum A, B and D, respectively to the differentially placed blood drops as per their labeled positions.
4. Mix them separately with separate paper pins.
5. Observe agglutination carefully and interpret the result as follows:

Observation for Agglutination	Antiserum A	Antiserum B	Antiserum D	Blood group
1.	+	-	+	A+
2.	+	-	-	A -
3.	-	+	+	B+
4.	-	+	-	B-
5.	+	+	+	AB+
6.	+	+	_	AB-
7.	-	-	+	O+
8.	-	-	-	O-

Precautions:

1. Human blood must be collected by a trained phlebotomist under sterile conditions.
2. Ensure the disposal of blood / components, slides, needles and cotton after double autoclaving and following standard procedures.

Experiment No. B.4.: To determine the self-blood group by indirect/ reverse blood group typing / serum grouping method.

Background: Reverse blood grouping method is based on the in vitro agglutination reaction performed with the test serum sample containing compatible antibodies and pure RBC suspension with known corpuscle factor as control. However, this method does not tell about the presence or absence of Rh factor status w.r.t the test sample.

Material and Reagent Requirements: Disposable syringe (1 ml), Tonicator, microscopic slide, Micropipettes (20-200 µ), Microtips, Cotton swab, Antiserum A, B and D, paper pins, 70% ethanol, Marker, 1ml each of Control blood samples (A,B), Centrifuge machine, Centrifuge tubes, 0.9% Nacl solution.

Methodology:

1. Centrifuge 1 ml of control blood samples at 3000rpm for 15 minutes.
2. Discard the supernatant and considering cell pellet as 100%, prepare 3% red cell suspension in normal saline. Label the respective cell suspensions as A and B as per their source blood sample.
3. Prepare the test serum sample as described in experiment no. B.1.
4. Equidistantly with the help of a slide marker, mark A and B on a clean microscopic slide.
5. Put 100 µl of the test serum sample just opposite to the each labeled mark.
6. Mix 50 µl of the prepared ‘A’ and ‘B’ cell suspensions to the test serum samples placed on respective marks on the microscopic slide.
7. Thoroughly mix the contents with the help of different microtips.
8. Record agglutination reactions and interpret the results as:

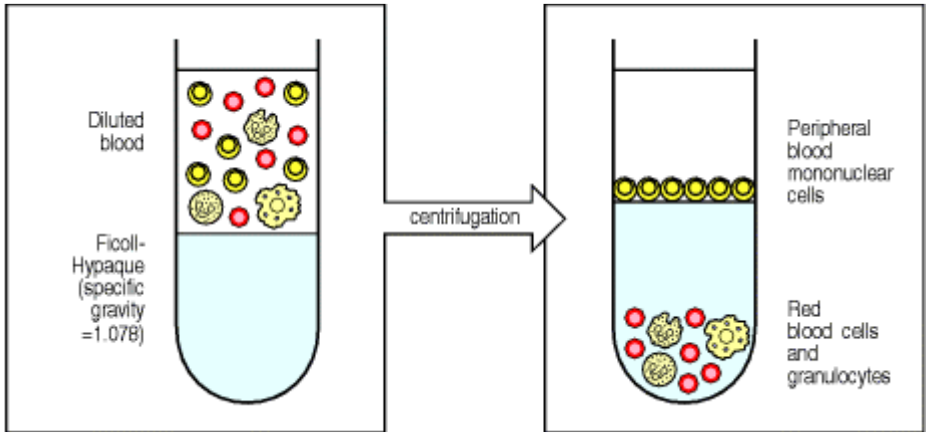
Observation for Agglutination	Cell Suspension A	Cell suspension B	Blood group
1.	+	+	O
2.	-	+	A
3.	+	-	B
4.	-	-	AB

Precautions:

1. Human blood must be collected by a trained phlebotomist under sterile conditions.
2. Ensure the disposal of blood / components, slides, needles and cotton after double autoclaving and following standard procedures.

Experiment No. B.5: Isolation of Peripheral blood mononuclear cells by Ficol – Histopaque gradient and viability test by dye exclusion method .

Background: Ficol Histopaqe (p = 1.077 g/ml^3) facilitates the formation of a density gradient scale under the influence of centrifugal force. Diluted blood sample layered on Ficol histopaque after centrifugation get fractionated into cellular components as per their density. RBCs and granulocytes are denser and centrifuge through the Ficoll-Histopaque, while mononuclear cells consisting of lymphocytes together with some monocytes band over it and can be recovered at the interface. As ficol contamination is toxic for the cells, the viability of the separated cells to make them eligible for culturing can be further determined by the use of 0.1% trypan blue dye. Viable cells don't take up the dye color.



Material and Reagent Requirements: Disposable syringe (5 ml), Tonicator, Cotton swab, Falcon tubes (15 ml, 30 ml / 50 ml), Microscopic slide, Microscope, Centrifuge machine, Micropipettes (20-200 µl; 200-1000 µl), Microtips, Ficol histopaque 1077 (Sigma Inc.), 0.5M EDTA, 0.9% NaCl, 0.1% trypan blue dye, 70% ethanol, Marker.

Methodology:

1. Disinfect the radial part of the forearm using cotton swab dipped with 70% ethanol.
2. Collect 5 ml of blood using tonicator and disposable syringe from the radial vein of forearm in a falcon tube containing 50 µl of 0.5M EDTA as an anticoagulant.
3. Dilute the anticoagulated blood with normal saline in the ratio of 1:2.

4. Add 3 ml room temperature ficol histopaque solution to a fresh 30 / 50 ml falcon tube.
5. Gently overlay 15 ml of diluted blood on the top of the ficol histopaque.
6. Spin at 3000 rpm for 15 minutes. Under the influence of centrifugal force, the blood sample will be fractionated into different layers from top to bottom as: plasma, interface layer, Ficol layer, erythrocytes and granulocytes.
7. Discard the plasma layer. Carefully transfer the buffy coat containing PBMCs (Monocytes and lymphocytes) at the interface to a fresh falcon tube.
8. Wash the separated PBMC with an equal volume of normal saline. Spin at 3000 rpm for 10 minutes.
9. Suspend the washed pellet in equal volume of normal saline and can be stored at -20° C for further use.
10. Mix 1 drop of cell suspension with one drop of 0.1% trypan blue dye and make a thin smear on a clean surface of microscopic slide.
11. Observe the slide under light microscope and try to determine the number of stained (dead) and unstained (viable) cells.

Precautions:

1. Human blood must be collected by a trained phlebotomist under sterile conditions.
2. Ensure the Ficol histopaque solution to be at RT before use.
3. Ensure that the diluted blood is layered over the ficol and does not get mixed with it.
4. While aspirating the buffy coat, avoid the contamination of ficol layer lying immediate below the interface.
5. Ensure the disposal of blood / components, slides, needles and cotton after double autoclaving and following standard procedures.

Experiment No. B.6: Monocyte depletion by plastic adhesion assay.

Background: This protocol exploit the property of monocytes of adhering the plastic surface for their further purification.

Material and Reagent Requirements: Disposable syringe (5 ml), Tonicator, Cotton swab, Falcon tubes (15 ml, 30 ml / 50 ml), Centrifuge machine, Micropipettes (20-200 µl; 200-1000 µl), Microtips, Pertridish (plastic), Ficol histopaque (Sigma Inc.), 0.5M EDTA, 0.9% NaCl, 70% ethanol, Marker.

Methodology:

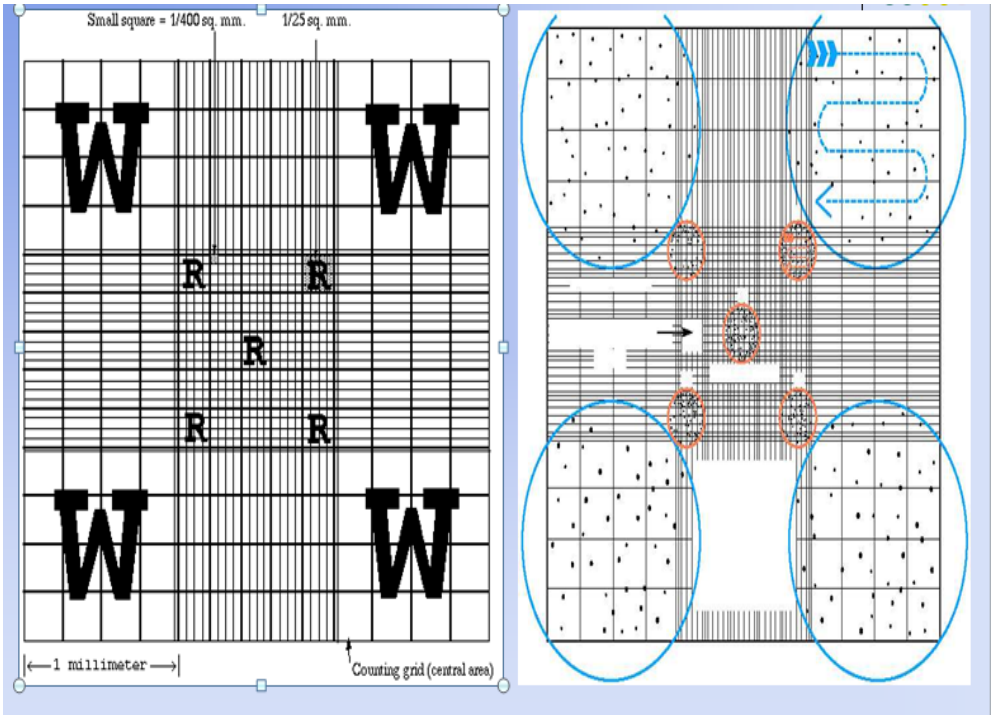
1. Separate the PBMCs by SoP as described in experiment no. B.5.
2. Layer an aliquot of PBMCs, in normal saline, on a plastic petridish for 4 h at 37^{o}C to allow monocyte adhesion.
3. The non adherent cells (lymphocytes) are decanted, pelleted by spinning at 3000 rpm for 10 minutes and store in another tube at -20^{o}C.
4. Gently wash the adherent cells (monocytes) on the petridish with appropriate volume of normal saline. Spin at 3000 rpm for 10 minutes and store at -20^{o}C for future use.

Precautions:

1. Gently and carefully decant the non adherent cells.
2. Follow all the precautions mentioned in SOP B.5.

Experiment No. B.7: To determine the total number of leucocytes per mm^3 of blood sample.

Background: TLC can be determined by employing haemocytometer. It is a specially graduated glass slide of 3 inches long and 5.5 inches wide. For WBC counting, a haemocytometer has 4 corner squares, each with 16 smallest squares. Each of the 4 square has an area of $4mm^2$. WBC diluting fluid is used to dilute blood. It contains 1 ml of 1% gentian violet solution, 2 ml of glacial acetic acid and 97 ml of water. The glacial acetic acid facilitates the haemolysis of red cells while gentian violet stains the WBC nuclei. Normally, a healthy man has 5000-10000 WBC per mm^3 of blood.



Material and Reagent Requirements: Sterilized lancet, Cotton swab, 70% ethanol, Haemocytometer, WBC pipette, coverslip, WBC diluting fluid, light microscope.

Methodology:

1. Disinfect the middle finger tip with a cotton swab dipped with 70% ethanol
2. Prick the middle finger with a disposable lancet.
3. With the help of WBC pipette, suck the blood upto mark 0.5.

4. Carefully suck the WBC diluting fluid upto the mark 11.
5. Ensure thorough mixing by holding the WBC pipette horizontally and rotating several times.
6. Transfer the diluted blood onto the counting chamber and place a coverslip over it.
7. Observe and count the WBCs under the microscope.

General Calculations:

Area of 4 squares = 4 mm^2

Depth of chamber = 0.1 mm

Volume of counting chamber = 0.4 mm^3

No. of WBCs per mm^3 = Y x 10 x 20 / 4

= Y x 50 / mm^3

Where Y is the no. of WBCs counted in 4 squares and 20 is the dilution factor. Division by 4 and multiplication by 10 will give number of cells per mm^3.

Precautions:

1. Wipe the first drop of blood after pricking the finger.
2. Carefully suck the blood and WBC diluting fluid. Avoid the air bubbles
3. Gently and slowly load the diluted blood onto the counting chamber.
4. The WBCs lying on the lower and right sides of the square are to be added only.
5. Ensure the disposal of blood / components, needles and cotton after double autoclaving and following standard procedures.

Experiment No. B.8: To determine the total number of red blood cells per mm^3 of blood sample.

Background: For RBC counting also, haemocytometer is used. The heamocytometer has Central Square which is further divided into 25 smaller squares. Each of these smaller squares is further divided into 16 smallest squares. So there are a total of 400 smallest squares each with an area of 1 / 400 mm^2. RBC diluting fluid / Hayem's solution is used to dilute the blood. It contains 0.5g of mercuric chloride, 1g of NaCl, 5g of Sodium sulphate and 200 ml of water. Mercuric chloride facilitates the fixing of red cells while NaCl and sodium sulphate provide isotonic milieu to the erythrocytes. Normally, a healthy individual has 4.5 – 5 million RBC per mm^3 of blood.

Material and Reagent Requirements: Sterilized lancet, Cotton swab, 70% ethanol, Haemocytometer, RBC pipette, coverslip, RBC diluting fluid, light microscope.

Methodology:

1. Disinfect the middle finger tip with a cotton swab dipped with 70% ethanol Prick the middle finger with a disposable lancet.
2. With the help of RBC pipette, suck the blood upto 0.5 mark.
3. Carefully suck the RBC diluting fluid upto 101 mark.
4. Ensure thorough mixing by holding the RBC pipette horizontally and rotating several times.
5. Transfer the diluted blood onto the counting chamber and place a coverslip over it.
6. Observe and count the RBCs in 5 smaller squares under the microscope.

General Calculations:

Area of 5 squares = 1 / 25 x 5 = 0.2 mm^2

Depth of chamber = 0.1 mm

Volume of counting chamber = 0.02 mm^3

No. of RBCs per mm^3 = Y x 100 x 200 / 2

= Y x 10^4 / mm^3

Where Y is the no. of RBCs counted in 5 squares and 200 is the dilution factor. Division by 2 and multiplication by 100 will give number of cells per mm^3.

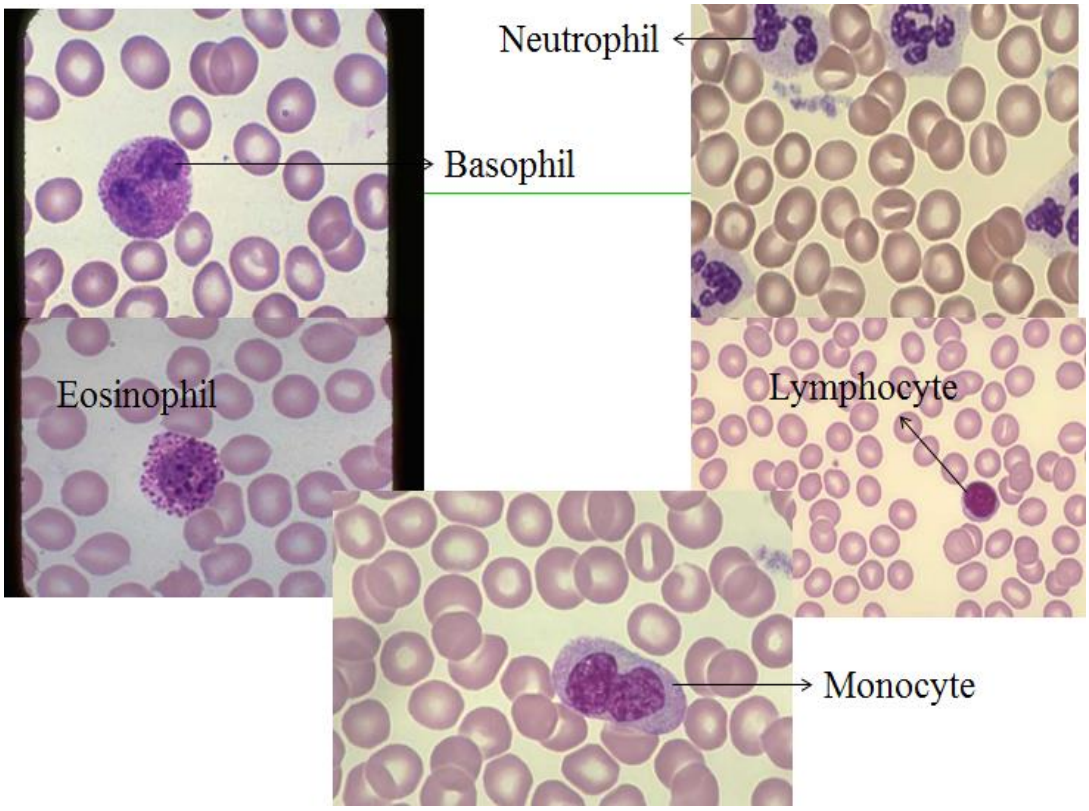
Precautions:

1. Wipe the first drop of blood after pricking the finger.
2. Carefully suck the blood and RBC diluting fluid. Avoid the air bubbles
3. Gently and slowly load the diluted blood onto the counting chamber.
4. The RBCs lying on the lower and right sides of the square are added only.
5. Ensure the disposal of blood / components, needles and cotton after double autoclaving and following standard procedures.

Experiment No. B.9: To determine the differential leucocyte count (DLC) of blood sample.

Background: The shape of nuclei and the chemical nature of granules forms the basis of DLC. Leishman stain, a methanolic mixture of ploychromed methylene blue and eosin dye, is used to determine the relative proportion of white blood corpuscles in the human blood. Chemically, Leishman stain solution consists of 0.15 g of Leishman stain powder in 100 ml of absolute alcohol.

Normal proportion of differential WBCs in a healthy individual is: Neutrophils: 55-70%; Eosinophils: 2-4%; basophils: 0.5 -1%; Lymphocytes: 20-40%, Monocytes: 2-8%



Material and Reagent Requirements: Sterilized lancet, Cotton swab, 70% ethanol, microscopic slide, Leishman stain solution, distilled water, light microscope.

Methodology:

1. Disinfect the middle finger tip with a cotton swab dipped with 70% ethanol
2. Prick the middle finger with a disposable lancet.

3. Put one drop of blood near the end of a clean microscopic slide.
4. Keeping a second slide at the 45° just in front of the blood group, prepare a thin blood smear by the forward movement of the second slide..
5. Airs dry the blood film and stain it with Leishman stain. Keep it at RT for 5 minutes.
6. Gently wash the blood film with distilled water.
7. Dry the slide, observe the different types of WBCs under the 10X and subsequently 45 X power objectives and count them from top edge of the slide to the bottom.

General Calculations:

$$n / N \times 100$$

Where n is the no. of a WBCs type counted and N is the total no. of cells counted

Precautions:

1. Wipe the first drop of blood after pricking the finger.
2. Blood film should be thin.
3. The film should be rose pink color after washing with water, If it is purple, then wash again with water.
4. Ensure the disposal of blood / components, needles, slides and cotton after double autoclaving and following standard procedures.