M.Sc. in MEDICAL LABORATORY TECHNOLOGY LAB MANUAL

4th Semester

Prepared By
Paramedical & Allied Health Science Dept.
MMLT

MIDNAPORE CITY COLLEGE

Conjugation

Principle: Conjugation is the mode of gene transfer in many species of bacteria. In 1950 William Hayes, Francis Jacob and Elie L. Wollman established that conjugating bacteria are of two mating types. Certain "male" types (designated as F^+) donate their DNA and other "female types" (designated as F-) receive the DNA. F cells become F^+ when they acquire a small amount of DNA. Hence the F factor is called as the Fertility factor. In contemporary microbiology, the donor's F factors are known to be plasmids which are the extrachromosomal elements. The factors (plasmids) contain about 20-30 genes, most of which are associated with conjugation. These genes encode enzymes that replicate DNA during conjugation and structural proteins needed to synthesize special pili at the cell surface. Known as F pili or sex pili, these hair-like fibres contact the recipient bacteria, and then retract so that the surfaces of donor and recipient are very close or touching one another. At the area of contact, a channel or conjugation bridge is formed. Once contact via sex pili has been made, the F factor (plasmid) begins replicating by the rolling circle mechanism. A single strand of the factor then passes over or through the channel to the recipient. When it arrives, enzymes synthesize a complementary strand, and a double helix is formed. The double helix bends to a loop and reforms an F factor (plasmid), thereby completing the conversion of recipient from F^- cell to F^+ cell. Meanwhile, back in the donor cell a new strand of DNA forms, to complement the leftover strand of the F plasmid. The transfer of F factors involves no activity of the bacterial chromosome; therefore, the recipient does not acquire new genes other than those on the F factor. On rare occasions an F-plasmid may become integrated in the chromosome of its bacterial host, generating what is known as an Hfr (high frequency of recombination) cell. Such a cell can also direct the synthesis of a sex pilus. As the chromosome of the Hfr cell replicates it may begin to cross the pilus so that plasmid and chromosomal DNA transfers to the recipient cell. Such DNA may recombine with that of its new host, introducing new gene variants. Plasmids encoding antibiotic-resistance genes are passed throughout populations of bacteria, and between multiple species of bacteria by conjugation.

Materials: Donor Strain A, Recipient Strain B, Streptomycin sulphate, Tetracycline hydrochloride, Luria Bertani Broth, Agar, Distilled water, Conical flask, Measuring cylinder, Sterile test tubes, Petriplates, Incubator, Shaker, Spectrometer, Micropipettes, Tips, Sterile Loops and spreader.

Procedure:

1. Tetracycline solution preparation: Dissolve 45 mg of tetracycline in 1.5 ml of 70% ethanol, mix by gentle pipetting to give a final concentration of 30mg/ml. Cover with aluminium foil and store in refrigerator. Use this solution within a month.

2. Streptomycin solution preparation: Dissolve 150 mg of streptomycin in 1.5 ml of sterile distilled water to give a final concentration of 100mg/ml. Cover with aluminium foil and store in refrigerator. Use this solution within a month.

3. Preparation of LB (Luria Bertani) broth (100 ml): Dissolve 2.5 g of Luria Bertani broth in 100 ml of distilled water and autoclave.

4. Preparation of LB (Luria Bertani) agar plates with Streptomycin (100 ml): Dissolve 2.5 g of LB media and 1.5 g of agar in 100 ml of sterile distilled water. Sterilize by autoclaving and pour on sterile petriplates. Sterilize by autoclaving and allow the media to cool down to 40-

45°C. Add 100 µl of streptomycin in 100 ml of autoclaved LB agar media and pour on sterile petriplates.

5. Preparation of LB (Luria Bertani) agar plates with Tetracycline (100 ml): Dissolve 2.5 g of LB media and 1.5 g of agar in 100 ml of sterile distilled water. Sterilize by autoclaving and pour on sterile petriplates. Sterilize by autoclaving and allow the media to cool down to 40-45°C. Add 100 µl of tetracycline to 100 ml of autoclaved LB agar media and pour on sterile petriplates.

6. Preparation of LB agar plates with Tetracycline + Streptomycin (100 ml): Dissolve 2.5 g of LB media and 1.5 g of agar in 100 ml of sterile distilled water. Sterilize by autoclaving and pour on sterile petriplates. Sterilize by autoclaving and allow the media to cool down to 40-45°C. Add 100 µl of tetracycline and 100 µl of Streptomycin to 100 ml of autoclaved LB agar media and pour on sterile petriplates.

7. **Day 1:**

i. Using sterile flexi loop, streak a loopful of *E. coli* Donor Strain from the stab onto two LB plates with Tetracycline (30 µg/ml) and *E. coli* Recipient Strain onto two LB plates with Streptomycin (100 µg/ml). Incubate at 37°C for 18-24 hours

8. **Day 2:**

i. Pick up a single colony from Donor and Recipient Strain grown overnight on LB plates and inoculate in 6 ml of LB broth having respective antibiotics.

ii. Incubate the test tubes overnight at 37°C.

9. **Day 3:**

i. Take 25 ml of LB broth and add 25 µl of tetracycline into it and inoculate 1 ml of overnight grown culture into it. Incubate at 37°C in a shaker.

ii. Take 25 ml of LB broth with streptomycin at a concentration of 100 µg/ml and inoculate 3 ml of overnight grown culture in it. Incubate at 37°C in a shaker.

iii. Grow the cultures till O.D of the donor culture reaches 0.8-0.9 at A_{600}.

iv. Add 0.2 ml of each donor and recipient cultures in a sterile test tube labeled as conjugated sample. Mix by gentle pipetting and incubate at 37°C for 1-1.5 hours.

v. Take 2 sterile test tubes and label them as donor and recipient. Add 0.2 ml of respective cultures to the test tubes and incubate at 37°C for 1-1.5 hours.

NOTE: Do not place the tubes in shaker for conjugation and further incubation period.

vi. Add 2 ml of LB broth into each tube after incubation. Incubate the tubes at 37°C for 1.5 hours.

vii. Plate 0.1 ml of each culture on the antibiotic plates as indicated in Table 2.

viii. Incubate the plates overnight at 37°C overnight.

Table 2: Samples to be spreaded on respective plates as follows:

	LB + Streptomycin	LB + Tetracycline	LB + Streptomycin, Tetracycline
Donor Strain A	0.1 ml	0.1 ml	0.1 ml
Recipient Strain B	0.1 ml	0.1 ml	0.1 ml
Conjugated Sample	0.1 ml	0.1 ml	0.1 ml

Observation and Result: Note down the observations in the following table. Indicate bacterial growth with positive symbol and absence of growth with negative symbol.

	LB + (Streptomycin)	LB + (Tetracycline)	LB + (Streptomycin, Tetracycline)
Donor Strain A			
Recipient Strain B			
Conjugated Sample			

Interpretation: On observing colonies on different plates the following interpretation can be made:

1. Donor strains will grow only on tetracycline plates; similarly, recipient strains will grow only on streptomycin plates.

2. Donor strain is sensitive to streptomycin and recipient strain is sensitive to tetracycline, hence no growth will be seen in these plates.

3. The conjugated sample will grow on tetracycline and streptomycin plate. The reason being, transfer of gene has occurred by means of conjugation.

4. The donor and recipient strain will not grow on tetracycline + streptomycin plate since each of the strain is sensitive to one antibiotic in the plate.

Transformation

Principle: For the incorporation of plasmid into a cell, bacteria must first be made "competent". This process includes the treatment of cells with bivalent calcium ions in ice-cold condition. As a result, small pores are formed on the cell membrane, which makes it permeable. The plasmid DNA may adhere to the surface of the cell and uptake is mediated by a pulsed heat shock at 42°C. A rapid chilling step on ice ensures the closure of the pores. These cells are allowed to propagate and selection of transformants can be done by growing the cells on a selective media which will allow only the plasmid containing cells to grow. Plasmids are extrachromosomal DNA element capable of independent replication inside a suitable host.

Plasmids encode a wide variety of genes, including those required for antimicrobial resistance. These genes act as selective markers when a transformation experiment is carried out. The *E. coli* plasmid pUC19 encodes a gene that can be used as a selectable marker during a transformation experiment. pUC19 has ampicillin resistance marker that enables only transformed cells to grow on LB –Ampicillin plates. Transformants, thus having the ability to grow on ampicillin plates can be selected. This process of direct selection of recombinants is called insertional-inactivation. pUC^{19} also carries the N-terminal coding sequence for β-galactosidase of the lac operon. The E. coli host strain has a deletion at the amino terminal end of the LacZ gene, which codes for β-galactosidase. When pUC^{19} is transformed into the competent host cells, the truncated products from both complement each other and as a result enzymatically active β-galactosidase is produced. This is called α-complementation. The tranformants turn blue on X-gal and IPTG containing plates due to the production of β-galactosidase. X-gal is the chromogenic substrate of β-galactosidase and IPTG acts as the inducer for the expression of this enzyme.

Materials: Ampicillin, Luria Bertani Broth, Agar, *E. coli* Host, Plasmid DNA, Calcium chloride (1M), X-gal, IPTG, Collection Tubes (2 ml), Conical flask, Measuring cylinder, Beaker, Micropipettes, Tips, 50 ml Centrifuge Tubes, Water bath (42°C), 37°C Incubator, 37°C Shaker, Centrifuge, UV Transilluminator, Crushed ice, Sterile double distilled water, Sterile loop and spreader.

Procedure:

1. Preparation of 0.1M Calcium chloride (sterile) (400 ml): To prepare 400 ml of 0.1M Calcium chloride, take 40 ml of 1M sterile Calcium chloride and add 360 ml of sterile distilled water. Store this solution at 2-8°C.

2. Prior to the preparation of competent cells, pre-chill the tubes, Calcium chloride solution (0.1M) and centrifuge tubes. Set the centrifuge at 4°C and water bath at 42°C.

3. Transformation should be carried out as soon as possible after the competent cells are prepared. Storage of competent cells leads to poor or no transformants.

4. Concentration of plasmid is 50 ng/µl.

5. Preparation of LB (Luria Bertani) broth (55 ml): Dissolve 1.38 g of LB media in 55 ml of distilled water. Sterilize by autoclaving.

6. Preparation of LB (Luria Bertani) agar plates (20 ml): Dissolve 0.5 g of LB media and 0.3 g

of agar in 20 ml of sterile distilled water. Sterilize by autoclaving and pour on sterile petriplate.

7. Preparation of Ampicillin: Dissolve 30 mg of ampicillin powder in 600 µl of sterile double distilled water to give a concentration of 50 mg/ml.

8. Preparation of LB (Luria Bertani) Agar plates containing Ampicillin, X-Gal and IPTG (100 ml): Dissolve 2.5 g of LB media and 1.5 g of agar in 100 ml of distilled water. Sterilize by autoclaving and allow the media to cool down to 40-45°C. Add 100 µl of ampicillin, 200 µl of X-Gal and of 100 µl IPTG to 100 ml of autoclaved LB agar media, mix well and pour on sterile petriplates.

9. **Day 1:**

i. Open the bottle containing culture and resuspend the pellet with 0.25 ml of LB broth.

ii. Pick up a loopful of culture and streak onto LB agar plate.

iii. Incubate overnight at 37°C.

10. **Day 2:**

i. Inoculate a single colony from the revived plate in 1 ml LB broth.

ii. Incubate at 37°C overnight.

11. **Day 3:**

i. Take 50 ml of LB broth in a sterile flask. Transfer 1 ml of overnight grown culture into this flask.

ii. Incubate at 37°C shaker at 300 rpm for 3-4 hours till the O. D_{600} reaches ~ 0.6.

A) Preparation of Competent Cells:

Note: Prepare competent cells within 3 days of reviving the strain.

1. Transfer the above culture into a prechilled 50 ml polypropylene tube (not provided).

2. Allow the culture to cool down to 4°C by storing on ice for 10 minutes.

3. Centrifuge at 5000 rpm for 10 minutes at 4°C.

4. Decant the medium completely. No traces of medium should be left.

5. Resuspend the cell pellet in 30 ml prechilled sterile 0.1 M Calcium chloride solution.

6. Incubate on ice for 30 minutes.

7. Centrifuge at 5000 rpm for 10 minutes at 4°C.

8. Decant the calcium chloride solution completely. No traces of solution should be left.

9. Resuspend the pellet in 2 ml prechilled sterile 0.1M Calcium chloride solution.

10. This cell suspension contains competent cells and can be used for transformation.

B) Transformation of cells:

1. Take 200 µl of the above cell suspension in two 2.0 ml collection tubes and label them as ‘control’ and ‘transformed’. Add 2 µl of plasmid DNA to the tube labelled as transformed and mix well.

2. Incubate both the tubes on ice for 30 minutes.

3. Transfer them to a preheated water bath set at a temperature of 42°C for 2 minutes (heat shock).

4. Rapidly transfer the tubes on ice-bath. Allow the cells to chill for 5 minutes.

5. Add 800 µl of LB Broth to both the tubes. Incubate the tubes for 1 hour at 37°C to allow the bacteria to recover and to express the antibiotic resistance marker encoded by the plasmid.

6. Take four LB agar plates containing ampicillin, X-Gal, IPTG and label them as control, A, B and C. Plate 200 µl of culture from the ‘control’ tube and plate it on the corresponding plate with a sterile spreader. Plate 50 µl, 100 µl and 200 µl of cell cultures from the ‘transformed’ tube on the plates labelled as A, B and C, respectively.

7. Store at room temperature till the plates are dry.

8. Incubate the plates overnight at 37°C.

Observation and Result: After incubation observe the plates for the bacterial growth and count the number of visible colonies. Calculate the efficiency of transformation.

Sr. No.	Plate	Growth	Number of colonies	Transformation Efficiency
1	Control plate			
2	Transformed plate (A)			
3	Transformed plate (B)			
4	Transformed plate (C)			

Calculation of transformation efficiency: Transformation efficiency is defined as the number of cells transformed per microgram of supercoiled plasmid DNA in a transformation reaction.

Transformation Efficiency = Number of colonies x 1000 ng/ Amount of DNA plated (ng)

= cells/µg

Concentration of plasmid DNA provided-80-100 ng/µl

Interpretation: On transformation of cells with pUC19 plasmid, antibiotic resistance is conferred on the host as this plasmid carries gene for ampicillin resistance. As a result, those cells that grow in presence of ampicillin are transformed cells. The transformed colonies are blue on X-Gal, IPTG plates due to α-complementation.

Transduction

Principle: In this process DNA is incorporated to a bacterial cell via a bacteriophage intermediate. It does not require cell-to-cell contact and it is DNase resistant. In all these cases the source cells of the DNA are called 'donors' and the cells that receive the DNA are called the 'recipients'. In each case the donor DNA is incorporated into the recipient cell's DNA by recombination exchange. If the exchange involves an allele of the recipient's gene, the recipient's genome and phenotype will change. The ability of a virus (bacteriophage) to carry bacterial DNA between bacteria was discovered in 1952 and named as transduction. The scientists found that, when a donor cell is lysed by P1 (bacteriophage), the bacterial chromosome is broken up into small pieces and sometimes the forming phage particles mistakenly incorporate a piece of the bacterial DNA into a phage head in place of phage DNA. The bacteriophage goes through either the lytic cycle or the lysogenic cycle. During the lysogenic cycle the phage chromosome is integrated into the bacterial chromosome and can remain dormant for several generations. If the lysogen is induced the phage genome is excised from the bacterial chromosome and initiates the lytic cycle, which culminates in lysis of the cell and the release of phage particles. The lytic cycle leads to the production of new phage particles which are released by lysis of the host. The entire process of transduction involves the following steps:

- First, the phage infects a susceptible bacterium (donor) and injects its DNA into the host.
- The phage DNA utilizes the host's machinery and synthesizes phage components including phage DNA. During this process parts of bacterial chromosome is integrated into the phage DNA.
- As a final step in the phage life-cycle, all the phage components in the cytoplasm are assembled into complete phage and the cell is lysed to release the newly made phage particles.
- When this newly made virus-particle infects the 'recipient' bacteria, the phage DNA (containing parts of 'donor's DNA) is injected into it and the transduced bacterial genes can be incorporated by recombination.

The transduction process is different from the usual gene recombination process. The most striking difference is the transfer of genetic material from cell to cell by viruses. The second feature is the fact that only a small part of the total genetic material of one bacterial cell is carried by the bacteriophage. There are two types of transduction:

i) Generalized Transduction - During this process any part of bacterial gene may be transferred to another bacterium via a bacteriophage and carries only bacterial DNA and no viral DNA. If bacteriophages undertake the lytic cycle of infection upon entering a bacterium, the virus will take control of the cell's machinery for use in replicating its own viral DNA. If by chance bacterial chromosomal DNA is inserted into the viral capsid which is usually used to encapsulate the viral DNA, the mistake will lead to generalized transduction.

ii) Specialized Transduction - In this process specific part of bacterial genes that are near the bacteriophage genome may be transferred to another bacterium via a bacteriophage. The genes that get transferred always depend on where the phage genome is located on the chromosome. Specialized transduction leads to three possible outcomes:

- DNA can be absorbed and recycled
- The bacterial DNA can match up with a homologous DNA in the recipient cell and exchange it. The recipient cell has DNA from both itself and the other bacterial cell.
- DNA can insert itself into the genome of the recipient cell like a virus resulting in a double copy of the bacterial genes.

Materials: Donor Strain, Recipient Strain, Susceptible Host, Phage Lysate, Ampicillin, Chloramphenicol, LB Broth, Agar, Collection Tubes (2.0 ml), Calcium chloride (1.0 ml), Conical flask, Measuring cylinder, Sterile tubes (15 and 50 ml), Petri plates, Distilled water, Centrifuge, Incubator, Shaker, water bath (set at 600C), Micropipettes, Tips, Sterile loops and spreaders, 0.45µ filters.

Procedure:

1. Chloramphenicol solution preparation: Dissolve 20 mg of Chloramphenicol in 1 ml of 70% ethanol, mix by gentle pipetting to give a final concentration of 20 mg/ml. Cover with aluminum foil and store in refrigerator. Use this solution within a month.

2. Ampicillin solution preparation: Dissolve 100 mg of Ampicillin 1 ml of sterile distilled water to give a final concentration of 100mg/ml. Cover with aluminum foil and store in refrigerator. Use this solution within a month.

3. Preparation of LB (Luria Bertani) broth (100 ml): Dissolve 5 g of Luria Bertani broth in 200 ml of distilled water and autoclave.

4. Preparation of LB (Luria Bertani) agar plates: Dissolve 2.5 g of LB media and 1.5 g of agar in 100 ml of sterile distilled water. Sterilize by autoclaving and allow the media to cool down to 40-45°C and pour on sterile petriplates.

5. Preparation of LB (Luria Bertani) agar plates with Ampicillin (100 ml): Dissolve 2.5 g of LB media and 1.5 g of agar in 100 ml of sterile distilled water. Sterilize by autoclaving and allow the media to cool down to 40-45°C. Add 100 µl of Ampicillin in 100 ml of autoclaved LB agar media and pour on sterile petriplates.

6. Preparation of LB (Luria Bertani) agar plates with Chloramphenicol (100 ml): Dissolve 2.5 g of LB media and 1.5 g of agar in 100 ml of sterile distilled water. Sterilize by autoclaving and allow the media to cool down to 40-45°C. Add 100 µl of Chloramphenicol to 100 ml of autoclaved LB agar media and pour on sterile petriplates.

7. Preparation of LB agar plates with Ampicillin + Chloramphenicol (100 ml): Dissolve 2.5 g of LB media and 1.5 g of agar in 100 ml of sterile distilled water. Sterilize by autoclaving and allow the media to cool down to 40-45°C. Add 100 µl of Ampicillin and 100 µl of Chloramphenicol to 100 ml of autoclaved LB agar media and pour on sterile petriplates.

8. Preparation of 0.1M $CaCl_2$ (1 ml): Mix 0.1ml of given 1 M $CaCl_2$ with 0.9 ml of sterile distilled water to get 0.1M $CaCl_2$ solution.

9. Preparation of Soft agar: To prepare soft agar, dissolve 2.5 g of LB Media and 0.8 g of Agar powder in 100 ml of sterile distilled water and autoclave.

10. **Day 1:**

i. Streak a loopful of Donor culture on LB C_{20} plate, Recipient on LB A_{100} plate and susceptible host on LB plate.

ii. Along with streaking, inoculate loopful of culture in 5 ml LB broth with respective antibiotics.

iii. Incubate the plates overnight at 37°C and Culture tubes at 37°C shaker for overnight with a speed of 300 rpm.

11. **Day 2:**

i. Store the 5 ml culture tubes at 40C for inoculation on Day 3.

ii. Inoculate 10-15 colonies from revived donor plate into 5 ml LB C_{20} and label as Donor tube.

iii. Incubate at 30°C in shaker for 2 hours.

iv. Keep a 5ml aliquot of sterile LB broth in water bath, set at 60-65°C.

v. Add 100 µl of given phage lysate to above labelled donor tube, continue incubation for 30 mins at 30°C.

vi. Add 2 ml of preheated sterile LB broth to donor tube mix well and incubate this tube at 42°C for another 20 minutes.

vii. Transfer this tube to 37°C and incubate for 3 hours.

viii. After incubation, spin this culture at 5000 rpm for 10 mins. Take the supernatant, filter it through 0.45 µm filter, label as Phage lysate 2. Store at 4°C for further steps.

ix. Inoculate single colony from recipient plate in 5 ml of LB broth with ampicillin (100 µg/ml). This is recipient tube; incubate this tube overnight at 37°C shaker.

12. **Day 3:**

i. Inoculate 100 µl of overnight grown recipient culture in 5 ml of fresh LB broth with ampicillin (100 µg/ml), incubate on shaker at 37°C for 2 hours.

ii. After incubation take 50 µl of this culture in 2 ml collection tube, add 50 µl 0.1M $CaCl_2$ along with 250 µl of Phage lysate 2 obtained and stored at 4°C on Day 2.

iii. Mix well and incubate further at 300C for 2 hours. (Do Not keep on Shaker.)

iv. After 2 hours, take each 50 µl of this culture and plate on LB C_{20}, LB A_{100} and LB C_{20} A_{100} plates.

v. Along with it, take 50 µl of overnight grown cultures of recipient strain and donor strain which are revived on Day 1, plate on LB C_{20}, LB A_{100} and LB C_{20} A_{100} plates.

vi. Incubate all plates at 37°C for overnight.

vii. On next day store these plates at 4°C for observation and results.

13. **Day 4:**

i. Inoculate 20-25 colonies from revived plate of susceptible host (Day 1) in 15 ml of fresh LB broth.

ii. Incubate this culture on shaker at 37°C for 2 hours.

iii. After incubation spin down 1.5 ml of this culture at 8000 rpm for 10 mins. In 7 different collection tubes (2ml) at RT. Then resuspend each pellet in 100 µl of fresh sterile LB broth. Use this as plating cells for titration.

iv. Before starting Titration protocol, keep 5 ml of LB broth at 60°C.

v. To confirm presence of phages in lysogenized colonies of infected recipient culture, inoculate 10-15 colonies from LB C_{20} A_{100} plate (of recipient infected with phage) in 5 ml LB C_{20} A_{100} broth. Label this as Lysogenized culture.

vi. Incubate this tube at 300C for 3 hours. After incubation add 2 ml of hot LB broth (kept at 60°C) to this lysogenized culture tube.

vii. Further incubate this tube at 42°C for 20 mins, again transfer this tube to 37°C and incubate for 2 hours.

viii. After 2 hours of incubation centrifuge the culture tube at 5000 rpm for 10 mins, filter the supernatant through 0.45 µm filter and label this as concentrated lysate.

Phage Titration:

1. Take 7 collection tubes (2 ml) label them as concentrated lysate, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}.

2. Take 900 µl of sterile LB broth from tube 10^{-1} to 10^{-6}; add 100 µl of concentrated lysate to 10^{-1} tube. Perform serial dilution up to 10^{-6} tube. Change tip every time while preparing dilution.

3. In 100 µl of plating cell tubes add 20 µl of concentrated and 20 µl phage lysate dilutions respectively and incubate all tubes at 37°C for 15 mins.

4. Meanwhile, melt the soft agar; dispense 5 ml of melted soft agar in 15 ml tubes. Add 0.1 ml of 10% Dextrose, 0.125 ml of 1M $MgCl_2$ and 0.025ml of 1M $CaCl_2$. Mix well and keep at 45°C.

5. Pipette out the mixture of plating cells and concentrated lysate in Soft agar tube mix well and immediately pour on labeled LB hard agar plate (Concentrated lysate) Let the agar solidify.

6. Repeat step 4-5 for phage dilutions from 10^{-1} to 10^{-6}.

7. Close the lids of Petri plates and incubate these plates at 37°C.

8. Note down the results and observation next day.

Observation and Result:

A. Screening of Transductants (from Day 3) - Look for transductant colonies which are growing on LB plates containing ampicillin and chloramphenicol.

	LB + Chloramphenicol	LB + ampicillin	LB + Chloramphenicol + Ampicillin
Donor Strain			
Recipient Strain			
Transduced/Lysogenized Strain			

B. Phage Titration (from Day 4) - Check the plates for clear and distinct plaques.

Tube No.	Dilutions	Number of plaques	Phage titre value
1			
2			
3			
4			
5			

Interpretation:

A. Screening of Transductants: During this experiment the chloramphenicol resistance gene is transferred from donor bacterial cell to recipient through a bacteriophage by the process known as transduction as only the lysogenized or transduced bacterial cells grow on LB plates containing ampicillin and chloroform. When bacteriophage infects the ampicillin sensitive and chloramphenicol resistant donor strain, the phage DNA enters DNA enters the donor cell and integrates into the bacterial chromosome. Upon induction of lysogen (by heating), the phage DNA is excised from the bacterial chromosome and new phage particles are released by lysing the host cell. When these new phage particles infect the recipient strain lysogenization occurs and as a result the chloramphenicol resistant gene is transferred to the recipient strain which is indicated by the growth on LB plates containing ampicillin and chloramphenicol.

B. Phage Titration: When the bacteriophage is induced and titrated against the given susceptible host, the clear plaques confirm the presence of phage particles in the lysogenized recipient strain and clear plaques.

Mutation

Principles: A mutation is the result of a stable, heritable change in the nucleotide sequence of DNA. By manipulating the chemical or physical environment of a bacterium, one can increase the frequency of mutations. If a mutant bacterium, by virtue of the change that it has undergone, is suited to the environment in which it is formed, its growth can be greater than the parent bacterium. In such a case, the mutant will quickly become the dominant bacterium in a culture. Mutations occur in one of two ways. (1) Spontaneous mutations arise occasionally in all bacteria and develop in the absence of any added agent. (2) Induced mutations are the result of the bacterium's exposure to a mutagen, which is a physical or chemical agent. Induced mutations resistant to physical mutagen (UV light) or chemical mutagen (ethidium bromide) are easily detected because they grow in the presence of such induced agent that inhibit the growth of normal bacteria.

Materials: Nutrient Agar, Petri-plates, 24 h *Escherichia coli* culture, Ethidium bromide, Autoclave, Incubator, and Laminar Air Flow.

Procedure:

1. Prepare 180 ml of nutrient agar medium, sterilized, and poured in 9 plates, each containing 20 ml each. Among these, four plates containing different concentration of ethidium bromide (5-20 µg/ml) in order to isolate chemical agent induced mutants, and inoculated with supplied sample.

2. To isolate physical agent induced mutation, 4 plates will be inoculated with supplied sample and subjected to different period of UV light exposure (30, 60, 90, and 120 Sec).

3. A control plate will also be made (without any chemical or physical mutagen), inoculated with the supplied sample and all the plates will be incubated for 24-48 h.

Result: Any colony appeared on the physical or chemical mutagen induced plates will be treated as mutant and documented after comparing with the control plates.

Control (No. of colonies)	Physically Induced		Chemically Induced	
	Time (Second)	No. of colonies	Concentration (µg/ml)	No. of colonies
	30		5	
	60		10	
	90		15	
	120		20	

Lac operon

Principle: Although bacteria possess a single chromosome, each cell is capable of synthesizing hundreds of different enzymes. Studies have shown that these enzymes are not present within the cells in equal concentrations. Some enzymes, called constitutive enzymes, are synthesized at a constant rate regardless of conditions in the cell's environment. Synthesis of other enzymes, called adaptive enzymes, occurs only when necessary, and it is subject to regulatory mechanisms that are dependent on the environment. One such mechanism, induction, requires the presence of a substrate, the inducer, in the environment to initiate synthesis of its specific enzyme, called an inducible enzyme. An extensively studied inducible enzyme in E. coli is β-galactosidase, which acts on the disaccharide lactose to yield the monosaccharides glucose and galactose. The gene for β-galactosidase is a member of a cluster of genes, called an operon, which is involved in the metabolism of lactose. The member genes of the lactose (lac) operon function as a unit, all being transcribed only when the inducer, lactose, is present in the surrounding medium. To illustrate β-galactosidase induction, two test strains of *E. coli* will be used: a prototrophic (wild type) strain (lactose-positive) and an auxotrophic (mutant) strain (lactose-negative), which carries a mutation in the gene for β-galactosidase as well as a mutation in the lactose operon regulatory gene. Both test strains will grow in the following media:

1. Inorganic synthetic medium lacking an organic carbon and energy source that is required by the heterotrophic *E. coli*.

2. Inorganic synthetic medium plus glucose, which can be utilized by both strains as a carbon and energy source.

3. Inorganic synthetic medium plus lactose, which can be utilized only by the prototrophic strain.

Orthonitrophenyl-β-d-galactoside (ONPG), a colorless analog of lactose, can serve as the substrate for the induction of β-galactosidase synthesis. As the inducer, it is hydrolyzed to galactose and a yellow nitrophenolate ion. Following a short incubation period, growth in all the cultures will be determined by spectrophotometry. Induction of β-galactosidase synthesis and activity will be indicated by the appearance of a yellow color in the medium following addition of ONPG, which occurs only in the presence of the nitrophenolate ion. Absence of this macroscopically visible color change indicates that enzyme induction in the lactose-negative strain did not occur.

Materials:

Cultures-25-ml inorganic synthetic broth suspensions of 12-hour nutrient agar cultures (adjusted to an absorbance of 0.1 at 600 nm) of-Lactose-positive *E. coli* strain (ATCC e 23725), and Lactose-negative *E. coli* strain (ATCC e 23735).

Media-Dropper bottles of sterile 10% glucose, 10% lactose, and Water.

Reagents-Dropper bottles of Toluene, and Orthonitrophenyl -β-D-galactoside (ONPG).

Equipment-1-ml and 5-ml sterile pipettes, Mechanical pipetting device, Six sterile 13-×100-mm test tubes, Test tube racks, Six sterile 25-ml Erlenmeyer flasks, Spectrophotometer, Shaking water-bath incubator, and Glassware marking pencil.

Procedure:

1. Label three sterile test tubes and three sterile 25-ml Erlenmeyer flasks as “Lac^{+}” (lactosepositive), with the name of the substrate to be added (glucose, lactose, or water). Similarly label three sterile tubes and flasks “Lac^{-}” (lactose-negative) for each test organism.

2. Using sterile 5-ml pipettes, aseptically transfer 5 ml of the Lac^{+} and Lac^{-} inorganic synthetic broth cultures to their respectively labelled test tubes.

3. Using a sterile 1-ml pipette, aseptically add 0.5 ml of the glucose and lactose solutions and 0.5 ml of sterile distilled water to the appropriately labelled tubes.

4. Determine the absorbance of all cultures at a wavelength of 600 nm. Record your results in the Lab Report.

5. Aseptically transfer each culture to its appropriately labelled flask. (Note: If side-arm flasks are available, additions and absorbance readings may be made directly.)

6. Incubate all flasks for 2 hours in a shaking water bath at 37°C and 100 strokes per minute.

7. Following incubation, transfer all cultures back to their appropriately labelled test tubes.

8. Determine and record in the Lab Report the absorbance for each culture at a wavelength of 600 nm.

9. To each culture, add 5 drops of toluene and shake vigorously. (Toluene ruptures the cells, releasing intact enzymes.)

10. To each culture, add 5 drops of ONPG solution.

11. Incubate all cultures for 40 minutes at 37°C.

12. Following the addition of ONPG, observe the cultures for the presence of yellow coloration indicative of β-galactosidase synthesis and activity. In the Lab Report, record the colors of your cultures and the presence (+) or absence (-) of the β-galactosidase activity.

Observations and Results:

Cultures	ABSORBANCE AT 600 NM				
	Prior to Incubation	Following Incubation	Growth (+) or (–)	Color of Culture with ONPG	*β*-Galactosidase (+) or (–)
Lac⁺ E. coli Glucose					
Lactose					
Water					
Lac⁻ E. coli Glucose					
Lactose					
Water					

FASTA

Aim: To retrieve the nucleotide sequence of interest from the National Center for Biotechnology Information (NCBI) database.

NCBI Database:

The National Center for Biotechnology Information (NCBI) advances science and health by providing access to biomedical and genomic information. NCBI creates public databases, conducts research in computational biology, develops software tools for analyzing genome data, disseminates biomedical information for the better understanding of diseases and molecular processes affecting human health. This database groups biomedical literature, small molecules, and sequence data in terms of biological relationships. It helps in retrieving DNA & peptide sequences, abstracts to scientific articles, and structural coordinates to visualize the 3D structure of resolved molecules.

Procedure:

1. Open the web browser and type the database address www.ncbi.nlm.nih.gov in the address bar.

2. Type the query, AF375082 (i.e., the accession number of a gene for which the nucleotide sequence has to be retrieved) in the search bar.

3. Select the particular sequence which is required.

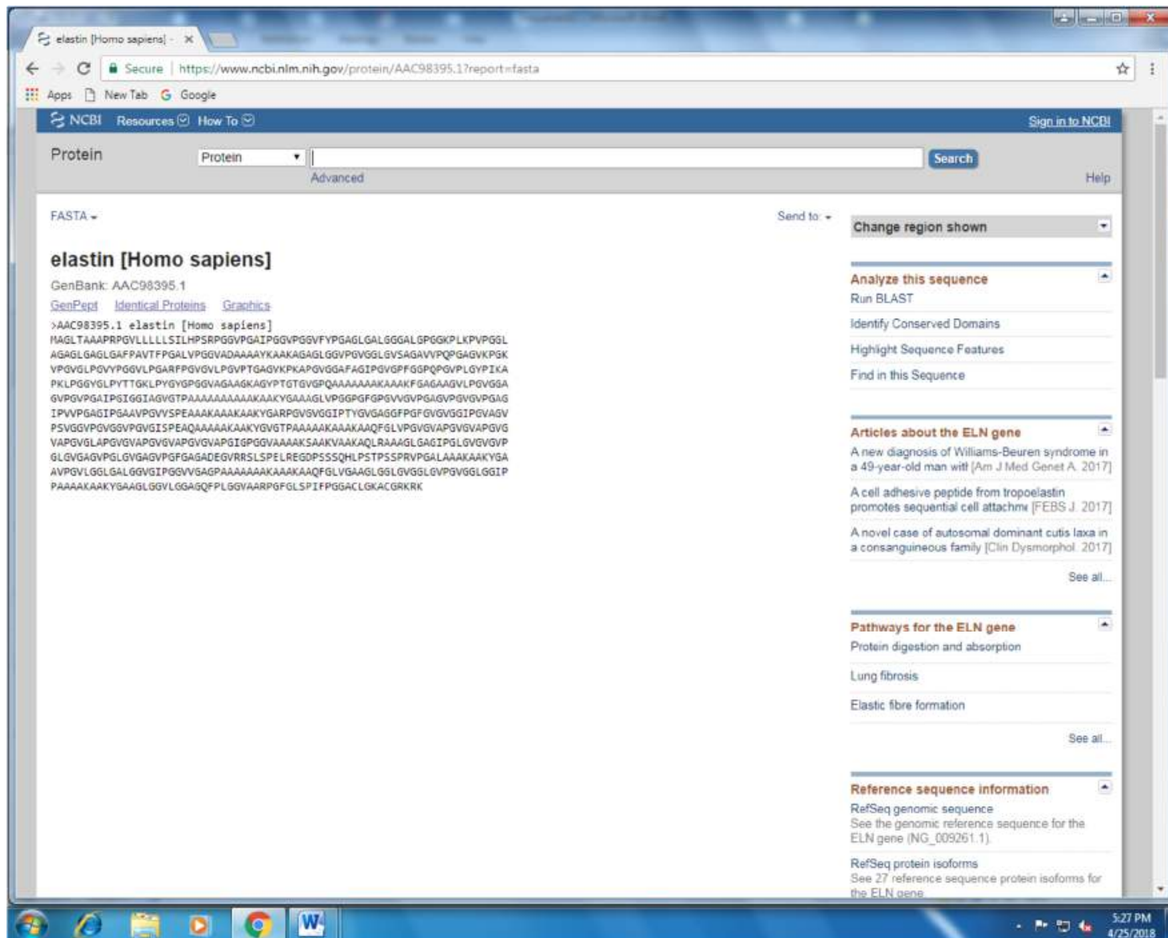
4. Select FASTA format in the display settings and click apply.

5. Copy the sequence data and paste it in the notepad.

6. Save the notepad file in FASTA format.

7. Report the result.

Observations:



FASTA format of elastin.

Result: Thus, the nucleotide sequence of AF375082 was retrieved from NCBI Database.

BLAST

Aim: To perform pair wise and multiple sequence alignment using BLAST tool.

Sequence Similarity: Database Similarity Searches have become a mainstay of Bioinformatics. Sequence database searches can also be remarkably useful for finding the function of genes whose sequences have been determined in the laboratory. The sequence of the gene of interest is compared to every sequence in a sequence database, and the similar ones are identified. Alignments with the best-matching sequences are shown and scored. If a query sequence can be readily aligned to a database sequence of known function, structure, or biochemical activity, the query sequence is predicted to have the same function, structure, or biochemical activity. Pairwise sequence alignment methods are concerned with finding the best matching piecewise local or global alignments of protein (amino acid) or DNA (nucleic acid) sequences. Multiple alignment is an extension of pairwise alignment to incorporate more than two sequences into an alignment. Multiple alignment methods try to align all of the sequences in a specified set.

BLAST is an acronym for Basic Local Alignment Search Tool. BLAST is a sophisticated software package that has become the single most important piece of software in the field of Bioinformatics. The following tools are different type of blast programs used with respect to the aim of the user.

Blast programs:

BLASTp – Compares an amino acid query sequence against a protein sequence database.

BLASTn – Compares a nucleotide query sequence against a nucleotide sequence database.

BLASTx – Compares a nucleotide query sequence translated in all reading frames against a protein sequence database.

tBLASTn – Compares a protein query sequence against a nucleotide sequence database dynamically translated in all reading frames.

tBLASTx – Compares the six-frame translation of a nucleotide query sequence against the 6-frame translation of a nucleotide sequence database.

Megablast – For highly similar sequences.

Procedure:

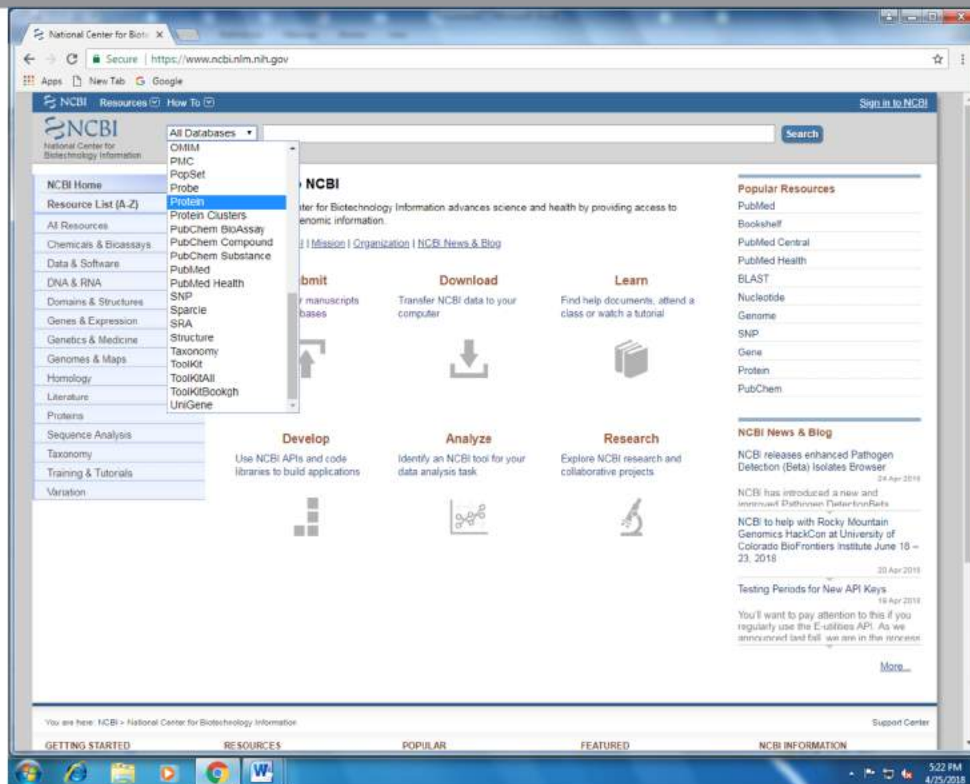
1. Open the web browser and type http://blast.ncbi.nlm.nih.gov/ Blast.cgi

2. Click either nucleotide blast or protein blast icon according to the requirement

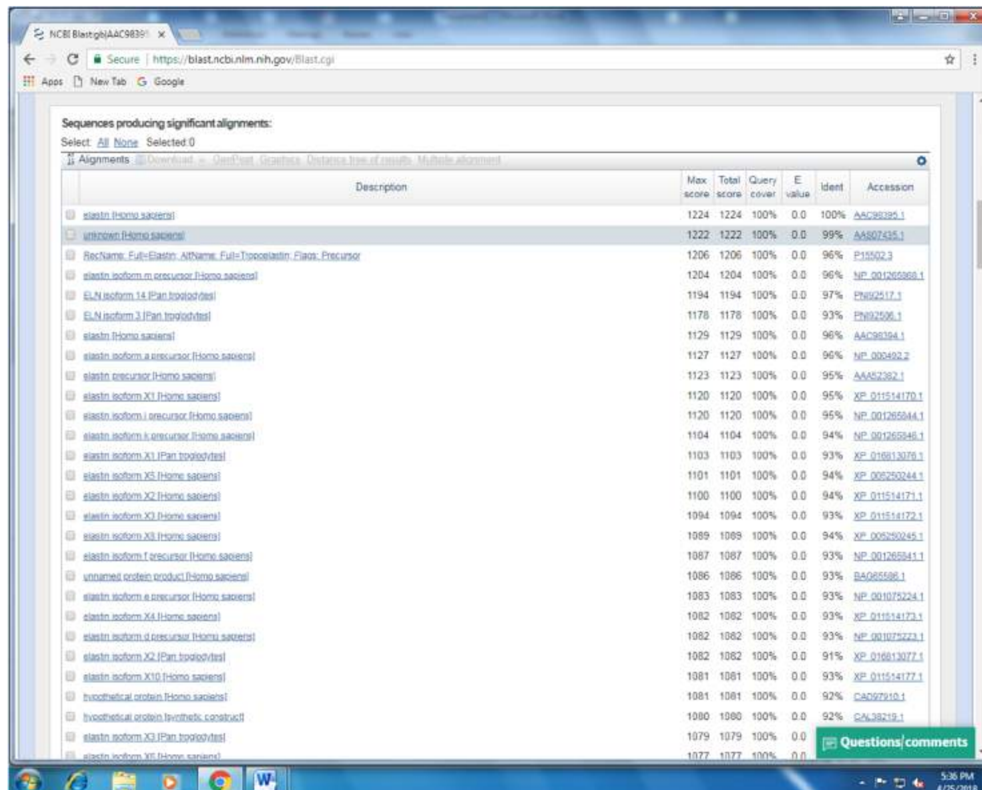
3. Select "Align two or more sequences" check box for opting multiple sequence alignment or deselect for pair wise alignment

4. Upload or paste a query sequence (in FASTA format) in the query box and execute BLAST for pair wise alignment. This will be identifying most similar sequences from the databank.

5. Upload or paste a query sequence (in FASTA format) in the query box and upload more than one sequences (in FASTA format) in the subject box and then execute BLAST for multiple sequence alignment. This will be identifying the similarity /dissimilarity among the sequences.



Observation:



Multiple sequence alignment using BLAST identified similarity between query sequence and other sequences.

Result: Thus, the given sequences are aligned using BLAST.

CLUSTAL

Aim: To perform pair wise and multiple sequence alignment using clustal for given sequences.

ClustalW or Clustal Omega: ClustalW like the other Clustal tools is used for aligning multiple nucleotide or protein sequences in an efficient manner. It uses progressive alignment methods, which align the most similar sequences first and work their way down to the least similar sequences until a global alignment is created. ClustalW is a matrix-based algorithm, whereas tools like T-Coffee and Dialign are consistency-based. ClustalW has a fairly efficient algorithm that competes well against other software. This program requires three or more sequences in order to calculate a global alignment, for pairwise sequence alignment (2 sequences) use tools similar to EMBOSS.

Procedure:

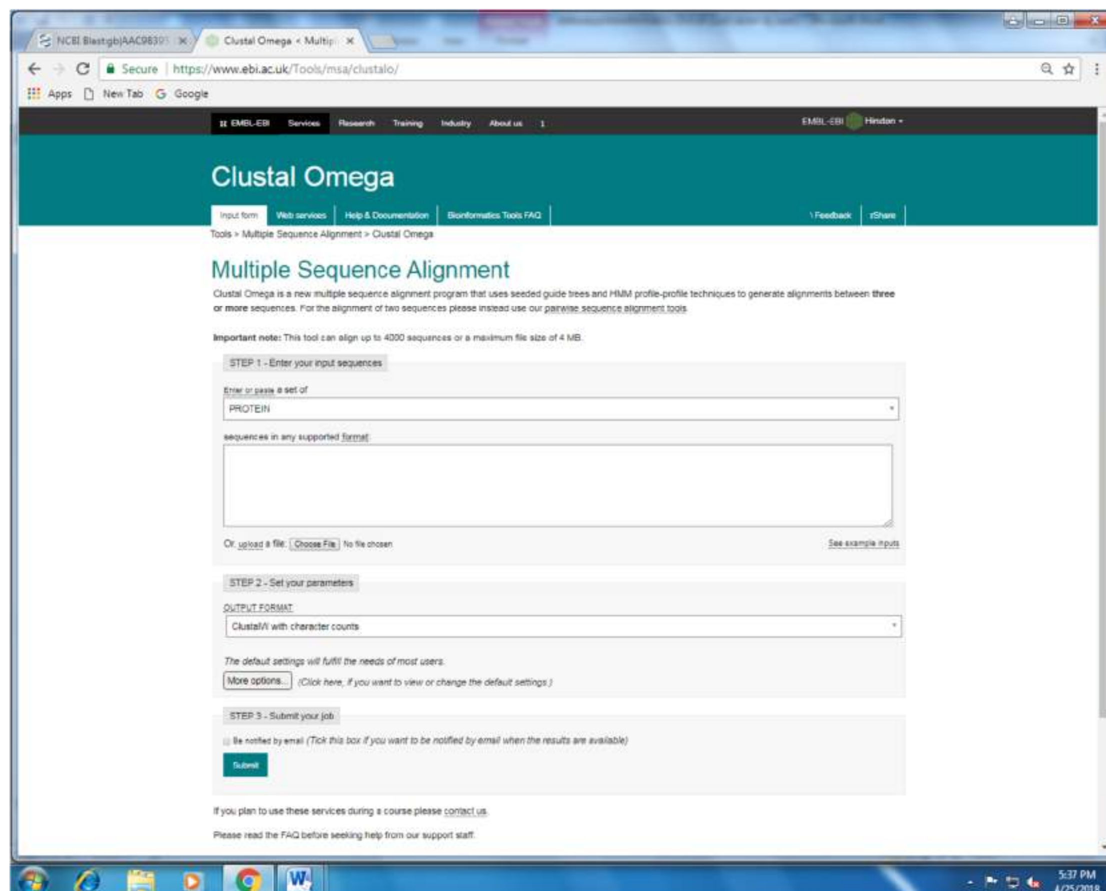
1. Open the web browser and type www.ebi.ac.uk/Tools/msa/ clustalw.

2. Upload the sequences from the Notepad or paste the sequences in FASTA format.

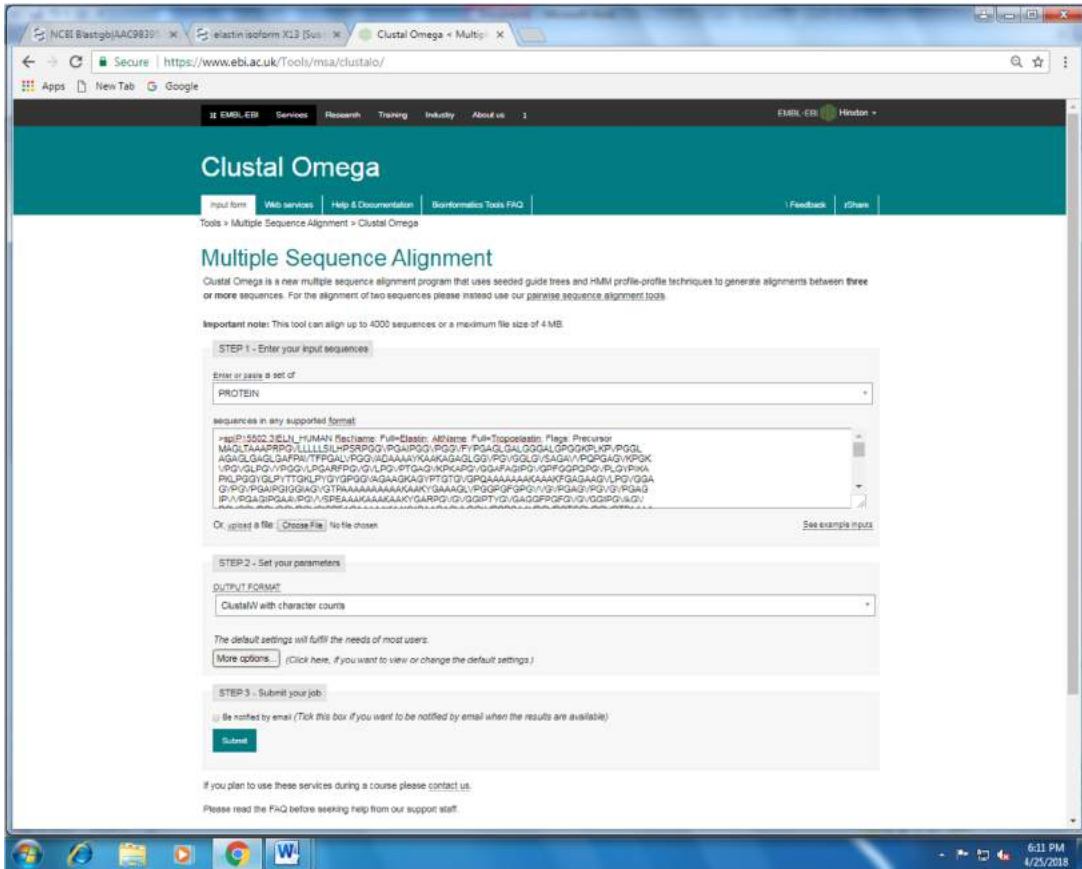
3. Upload two sequences for pair-wise alignment or more than two sequences for multiple sequence alignment After uploading, choose the “Execute Multiple Alignment” option in the alignment icon.

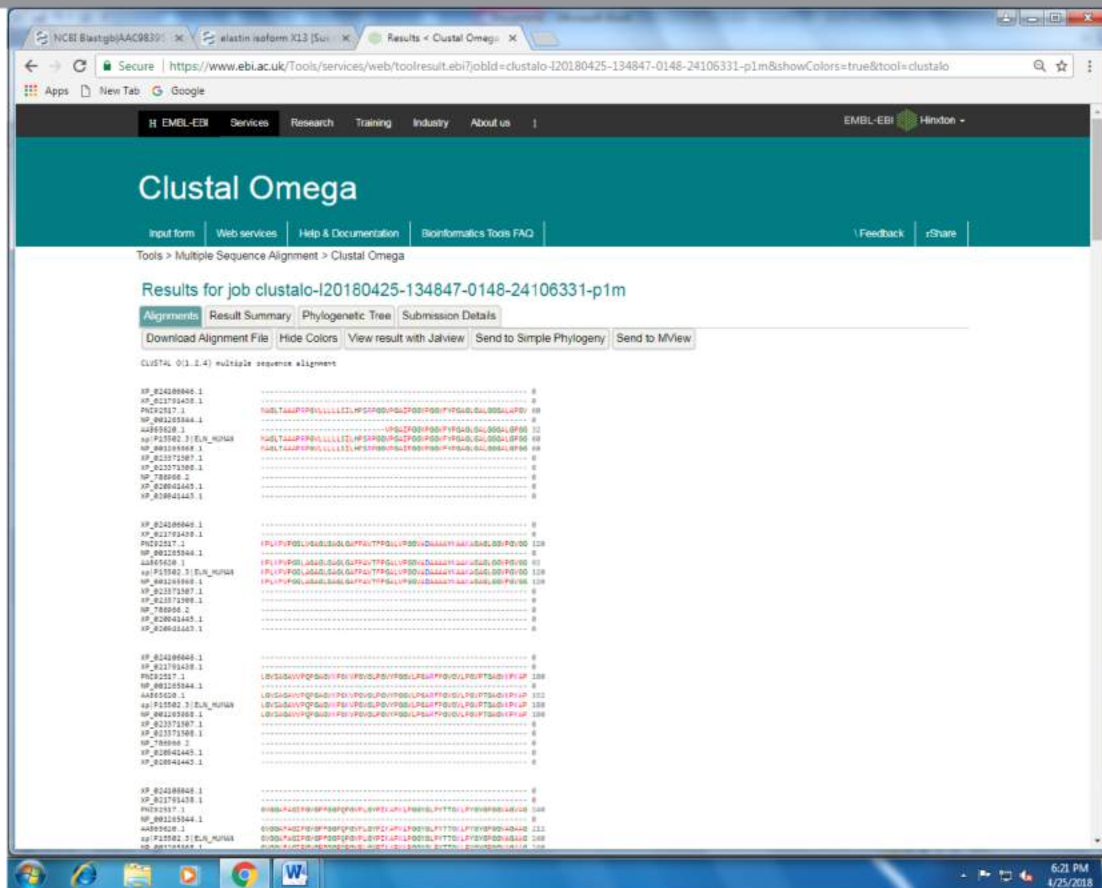
4. Sequence alignment results will be appeared within few seconds after execution.

5. Report the result.



Observation:





Alignment of retrieved sequences using CLUSTALW tool.

Result: Thus, the given sequences are aligned using ClustalW.

Phylogenetic Tree

Aim: To identify the 10-homologues sequences of elastin of various origins. Find the conserved region existing between them comment on the same. Comment on the evolutionary relationship between the sequences.

Phylogenetic Tree: A phylogenetic tree or evolutionary tree is a branching diagram or "tree" showing the inferred evolutionary relationships among various biological species or other entities based upon similarities and differences in their physical and/or genetic characteristics. The taxa joined together in the tree are implied to have descended from a common ancestor.

Method:

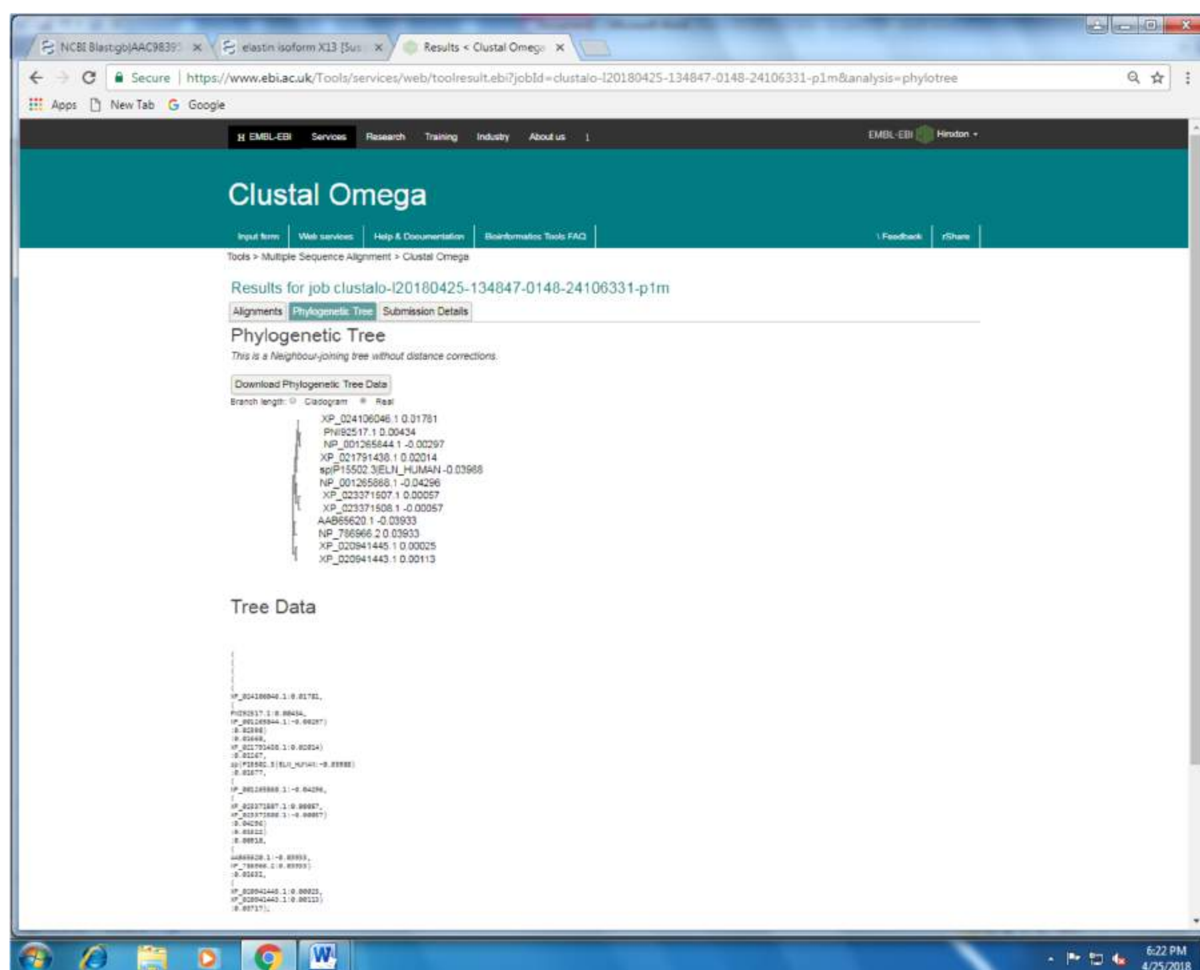
1. Run a blastp for the protein Id.

2. Choose 10 homologous proteins and save in .txt format

3. Input these sequences in Clustalw. Determine conserved reagions.

4. Access the "Phylogenetic Tree" mode

Observation:



Result: Construction of a phylogenetic tree based on the relatedness or distinctness of homologous sequences.

HAEMOGLOBINOPATHIES AND SEROLOGY LAB - II

MLT / VU / 4TH SEM

Code: MLT 494C

1. Estimation of Periodic Acid Phosphatase [PAS] BY Kit Method

ACID PHOSPHATASE

Insert

Ref.: 39

Intended use. System for acid phosphatase determination in blood sample by kinetic method of fixed time and measurement of end point.

Test principle. Acid phosphatase of serum acts on the monophosphate thymolphthalein substrate. The addiction of alkali inhibits the enzymatic action and converts the released thymolphthalein in it blue form that is measured by colorimetry. The final product of reaction consists of a mixture of blue and the substrate color.

Summary. During the selection of a substrate for determination of acid phosphatase, Labtest have focused on monophosphate thymolphthalein research, that posses more specificity to prostatic isoenzymes than the phenyl phosphate and p-nitro phenyl phosphate.

Thymolphthalein specificity to the prostatic fraction is not absolute. Therefore, there is a little activity in serum of healthy women.

This method provides lower values than the obtained using p-nitrophenyl or phenyl phosphate substrate that are also hydrolyzed by phosphatase from hematic and platelets origin.

The technical procedure performed in 30 minutes is extremely simple and has a proportional response to the enzymatic activity up to 200 folds the values of reference with only one colorimetric measurement.
In spite of measuring the total acid phosphatase, increased results may be considered as being correspondent to prostatic fraction because the sensibility of the substrate to other fractions is considerably decreased and the values found to the non-prostatic phosphatase activity are so reduced that are not clinically significant.

Methodology. Roy modified

Reagents

1. R 1 - Substrate - Store at 2 - 8 ºC.
Reagent label bears expiration date. After dissolved, it contains monophosphate thymolphthalein (1.5 mmol/L).

2. R 2 - Color Reagent - Store at 15 - 25 ºC.
Reagent label bears expiration date. Sodium carbonate (50 mmol/L) and sodium hydroxide (50 mmol/L).

3. CAL - Standard 3.0 U/L - Store at 2 - 8 ºC.
Reagent label bears expiration date. In order to avoid evaporation of the Standard, keep the bottle tightly closed. Thymolphthalein (0.09 mmol/L).

4. R 4 - Buffer - Store at 15 - 25 ºC.
Buffer (100 mol/L), pH 5.95 and sodium azide (7.7 mmol/L).

Precautions and warnings

For in vitro diagnostic use.

Disposal of all waste material should be in accordance with local guidelines.

The usual security cares should be applied on the reagent handling.

Buffer contains sodium azide, what is toxic, as preservative. Avoid ingestion. In case of eyes contact, immediately flush eyes with plenty of water and get medical assistance.
Sodium azide may react with lead and copper plumbing to form highly explosive metal azides. On disposal, flush with a large volume of water to prevent azide accumulation.

Cares regarding reaction time, temperature of incubation and pipetting are extremely important in order to obtain correct results. The difference of one minute in the incubation time induces an error of 3.3% in the results.

Storage and stability. Unopened reagents, when stored at indicated temperature, are stable up to expiration date shown on the label.

Deterioration. Microbial or chemical contamination may decrease reagents stability.

Specimen collection and preparation

Use serum or plasma (heparine). The enzymatic activity is sensible to temperature and pH effects. Therefore, separate the serum or plasma within 30 minutes after collecting and add 0.01 mL of acetic acid 20% (v/v) for each 1.0 mL of sample. The acidified sample is reportedly stable in for about 2 days at 2 - 8 ºC and 1 week at -10 ºC.

No known test method can offer complete assurance that human blood samples will not transmit infectious diseases. Therefore, all blood derivatives should be considered potentially infectious.

Interference

Bilirubin up to 5 mg/dL, hemoglobin up to 180 mg/dL and triglycerides up to 750 mg/dL do not interfere significantly.

Bilirubin values over 5 mg/dL and triglycerides values over 750 mg/dL provide false increased results.

Materials required not provided

1. A constant temperature water bath (37 ºC).
2. Photometer capable of measuring absorbance at 570 - 610 nm.
3. Pipettes to measure reagents and samples.
4. Timer.

Manual procedure

See notes 1, 2 and 3.

Preparing the working reagent . Dissolving the substrate: Transfer all the contents of Buffer (20 mL) to substrate bottle and mix gently by inversion until complete dissolution. It is stable 6 months at 2 - 8 ºC.

Set up three tubes and proceed as follows:

	Control	Unknown	Standard
Substrate (nº 1)	0.5 mL	0.5 mL	----
Distilled or deionized water	----	----	0.5 mL
Standard (nº 3)	----	----	0.1 mL

Mix and incubate in a water bath at 37 ºC during 2 minutes.

Sample	----	0.1 mL	----

Mix and incubate in a water bath at 37 ºC during 30 minutes.

Color reagent (nº 2)	2.0 mL	2.0 mL	2.0 mL
Sample	0.1 mL	----	----

Mix and measure the absorbance of the Control, Unknown and Standard against distilled water at 590 nm or orange filter (570 - 610). The color is stable during 120 minutes.
The suggested measurement procedure is appropriated to photometer of which the minimal volume of solution for reading is equal or lower than 2.5 mL. It should be done a verification of the necessity of volume adjustment for the photometer to be used. Sample and reagent volume may be modified proportionally without affecting the test performance and the calculation procedure. In case of volume reduction is important to observe the minimum volume needed to the photometric reading. Volume of sample lower than 0.01 mL is critical in manual applications and should be avoided because it increases the measurement imprecision.

Quality control . For quality control use Qualitrol Level 1 and Qualitrol Level 2 or other suitable control material. The limits and control interval must be adapted to the laboratory requirements. Each laboratory should establish corrective actions to be taken if values fall outside the control limits.

Calculations

$$\text{Acid Phosphatase (U/L)} = \frac{A_{Unknown} - A_{Control}}{A_{Standard}} \times 3$$

Due the great reproductive results of the assays system, it is possible to use the factor method:

Calibration factor = $3 / A_{Standard}$

Acid Phosphatase (U/L) = $(A_{Unkown} - A_{Control}) \times$ Factor

Measurement/reportable range

Up to 110 U/L

If acid phosphatase activity exceeds 110 U/L, the unknown and control must be diluted with the Color Reagent. Multiply the result by the appropriate dilution factor. If afterwards the result is equal to or more than 110 U/L, repeat the measurement reducing to 10 minutes the incubation time after adding the sample. Multiply the result by 3.

Expected values . Each laboratory should evaluate the transferability of the expected values to its own patient population and, if necessary, estimate its own reference interval.

0.15 to 0.56 U/L

Unit definition: a Unit is the amount of enzyme that yields, by hydrolysis, 1 µmol of thymolphthalein per minute, per liter of serum, in the test conditions.

Performance characteristics

Recovery studies . In two samples with acid phosphatase values of 0.6 and 1.5 U/L were added different quantities of the enzyme. Subsequent analyses provided recoveries ranging from 96 to 106%. The mean proportional systematic error at 0.5 U/L was 0.005 U/L or 1.0%.

Method comparison . A group of 80 sera were assayed by the proposed method and a technique using p-nitrophenil phosphate. Serum acid phosphatase values ranged from 0.14 - 11.0 U/L. The comparisons yielded a correlation coefficient of 0.99 and regression equation was y = 0.044 + 0.339x. There is a positive correlation among both methods, observing a 56% systematic difference when the decision level is 0.5 U/L, what is explained by the difference of the substrates used and the methodology.

Imprecision - Within run

	N	Mean (U/L)	SD (U/L)	%CV
Sample 1	20	0.64	0.02	3.4
Sample 2	20	1.52	0.02	1.1
Sample 3	20	2.71	0.02	0.6

Imprecision - Run-to-run

	N	Mean (U/L)	SD (U/L)	%CV
Sample 1	20	0.58	0.04	7.4
Sample 2	20	1.46	0.05	3.2
Sample 3	20	2.69	0.03	1.1

Analytical sensitivity . Detection limit: 0.02 U/L. The detection limit represents the lowest measurable acid phosphatase activity that can be distinguished from zero. Using the standard absorbance as parameter, it was verified that the detection limit is 0.02 U/L, corresponding to a absorbance of 0.001.

Matrix dilution effects . Two sample with values equal of 85 and 113 U/L were used to evaluate the system response on the reduction of incubation time. Recoveries were found a range of 96 and 112 %, using incubation time that varies from 10 to 20 minutes.

Notes

1. The material cleaning and drying are fundamental factors to the reagent stability and to obtain correct results.

2. The deionized or distilled water in the laboratory to prepare reagents, use in the measurements and for final glass washing must have resistivity ≥1 megaohm.cm, or conductivity ≤1 microsiems/cm and silicates concentration must be <0.1mg/L.

3. Cares regarding reaction time, temperature of incubation and pipetting are extremely important in order to obtain correct results. The difference of one minute during incubation time of this measurement induces an error of 3.3% in the result.

4. It is suggested to consult "Young DS. Effects of Drugs on Clinical Laboratory Tests, 3rd Edition, Washington: AACC Press, 1990." in order to review physiopathological source and drugs interference in results and methodology.

References

1. Coleman CM, Stroje RC.: Clin Chim Acta 1966:13:401.
2. Bergmeyer HU. Methods of Enzymatic Analisys, 3 ed. Vol 4, Deerfiled Beach: Verlag Chemie, 1984:92-100.
3. Roy AV, Brower ME, Hayden JE. Clin Chem 1971;17:1093.
4. Tonks DB Quality Control in Clinical Laboratories, Warner Chilcot laboratories, Diagnostic Reagents Division, Scarborough, Canada, 1972.
5. Westgard JO, Groth T. Clin Chem 1981; 27:493-501.
6. Labtest: data on file.

Presentation

Product	Reference	Contents		
Acid Phosphatase	39	R 1	1 X 30 µmL	LYOPH
		R 2	1 X 100 mL	
		CAL	1 X 3 mL	
		R 4	1 X 20 mL	

Consumer information

[Warranty conditions]

Labtest Diagnóstica warrants the performance of this product under the specifications until the expiration date shown in the label since the application procedures and storage conditions, indicated on the label and in this insert, have been followed correctly.

Labtest Diagnóstica S.A.
CNPJ: 16.516.296 / 0001 - 38
Av. Paulo Ferreira da Costa, 600 - Vista Alegre - CEP 33400-000
Lagoa Santa . Minas Gerais Brasil - **www.labtest.com.br**
Consumer Service | e-mail: sac@labtest.com.br

Revision: May, 2009
Ref.: 280113

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2.Sudan Black B staining

Sudan Black B (SBB) is a fat soluble dye which has very high affinity for **neutral fats and lipids**. SBB staining is useful for for the differentiation of Acute myeloid leukemia (AML) from Acute lymphoid leukemia (ALL). It is similar to that of **Myeloperoxidase (MPO) staining** pattern of leukocytes and monocytes. It has few advantages over MPO:

- SBB can be used for staining smears more than 2 weeks old.
- SBB stains both azurophilic and specific granules in neutrophils, whereas MPO stains azurophilic granules only.
- There is only a little fading of the SBB stain over time.

Principle of Sudan Black B Stain

As SBB is a fat soluble dye, it stains lipids such as sterols, neutral fats and phospholipids. These are present in azurophilic and secondary granules of myelocytic and lysosomal granules of monocytic cells. During staining, the dye leaves the solvent because of its high solubility in lipids than solvent. On microscopic examination, varying degree of black colored pigments are seen in the positive reaction.

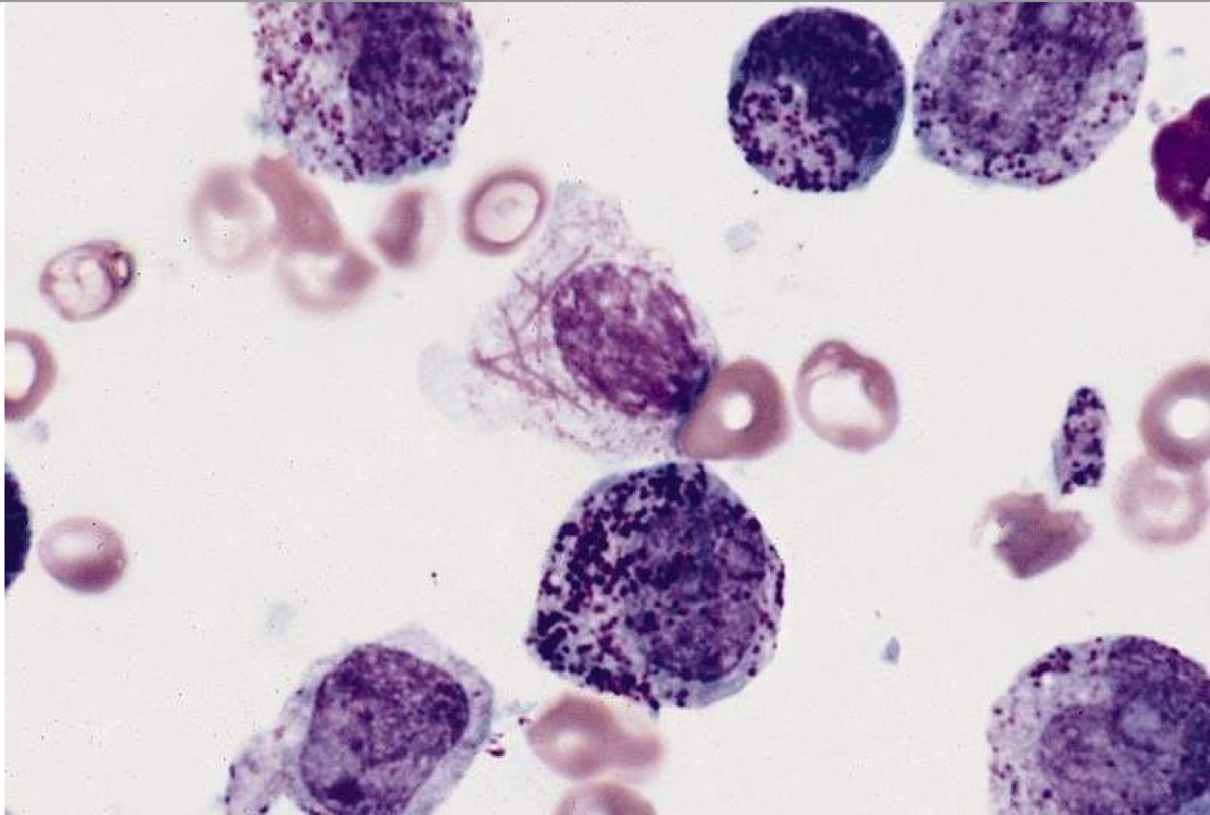
Requirements

1. **Sample**: Fresh anticoagulated whole blood or bone marrow smear may be used. The slides must be fixed as soon as possible.
2. **Fixative**: 40% formaldehyde solution vapor
3. **Stain**: SBB 0.3 g in 100 ml absolute ethanol
4. **Phenol buffer**: Dissolve 16 g crystalline phenol in 30 ml absolute ethanol. Add to 100 ml distilled water in which 0.3 g $Na_2HPO_4.12H_2O$ has been dissolved
5. **Working SBB stain solution**: Add 40 ml buffer to 60 ml SBB solution (*The composition of working stain may slightly vary upon different products.*)
6. **Counterstain**: May–Grunwald–Giemsa or Leishman stain.

Procedure of Sudan Black B Stain

1. Fix air dried smears in formalin vapour, formaldehyde or formalin-ethanol fixative for 10 minutes.
2. Wash gently in water for 5-10 minutes.
3. Place the slides in the working stain solution for 1 hour in a Coplin jar with a lid on.
4. Remove and flood the slides with 70% alcohol for 30 seconds. Tip the 70% alcohol off and flood again. Repeat this three times.
5. Rinse in running tap water and air dry.
6. Counterstain without further fixation with Leishman stain or May–Grunwald–Giemsa stain.
7. Air dry and examine microscopically.

Results and Interpretation



Production of black and granular pigment indicates positive reaction.

- Lipids are present in azurophilic and secondary granules of **myelocytic cells**. Hence, these are SBB positive. The staining becomes more intense as cells mature from myeloblast to mature forms. Basophils are generally not positive but may show bright red/purple metachromatic staining of the granules.
- Lipids are present in lysosomal granules of **monocytic cells**. Monocytic cells show variable reactions, from negative to weakly positive.
- **Lymphoid cells** are SBB negative. However, in ALL, less than 3% of blast cells show positive reaction.

3. Estimation of Leucocyte alkaline Phosphatase by Kit Method

SIGMA-ALDRICH® **ALKALINE PHOSPHATASE**
(Procedure No. 85)

INTENDED USE

Sigma-Aldrich Alkaline Phosphatase kits are intended for the histochemical semi-quantitative demonstration of alkaline phosphatase activity in leukocytes. Alkaline Phosphatase reagents are for "In Vitro Diagnostic Use".

In 1929, Kay first suggested the presence of alkaline phosphatase in leukocytes.[1] However, not until many years later did Kaplow[2] introduce a practical staining method for demonstrating the leukocyte enzyme. Kaplow[2] used sodium α-naphthyl phosphate as substrate, fast blue RR as the diazonium salt and recommended a scoring method for semiquantitative comparisons. Also suggested were improvements resulting in good preservation of cellular morphology. Subsequently, α-naphthyl phosphate was replaced by naphthol AS phosphate. It is noteworthy that a good correlation has been found between cytochemical and biochemical techniques for assaying leukocyte alkaline phosphate activity (LAPA).[3]

With the Sigma-Aldrich method, which is essentially that of Ackerman,[4] leukocyte alkaline phosphatase is determined in blood or bone marrow films after they are gently fixed to slides. The fixed films are then incubated in a solution containing naphthol AS-MX phosphate. As a result of phosphatase activity, naphthol AS-MX is liberated and immediately coupled with a diazonium salt, forming an insoluble, visible pigment at sites of phosphatase activity.

REAGENTS

FAST BLUE RR SALT, Catalog No. FBS25-10 CAP
Preweighed capsules. Actual weight per capsule will vary with dye lot and has been optimized by assay.

FAST VIOLET B SALT, Catalog No. 851-10 CAP
Capsule contains 12 mg.

NAPHTHOL AS-MX PHOSPHATE ALKALINE SOLUTION, Catalog No. 855-20 ml
Naphthol AS-MX phosphate, 0.25% (w/v), buffered at pH 8.6, 25°C

MAYER'S HEMATOXYLIN SOLUTION, Catalog No. MHS1-100 ml
Hematoxylin, certified, 1 g/l, sodium iodate, 0.2 g/l, aluminum ammonium hydroxide, 5 g/l, and stabilizers.

CITRATE CONCENTRATED SOLUTION, Catalog No. 854C-20 ml
Contains citric acid-sodium citrate, 1.5 mol/l.

STORAGE AND STABILITY:

Store Fast Blue RR Salt and Fast Violet B Salt below 0°C. Store Naphthol AS-MX Phosphate Alkaline Solution in refrigerator (2–8°C). Reagents are stable until expiration date.

Store Mayer's Hematoxylin Solution tightly capped at room temperature (18–26°C) protected from light. Do not return solution to original container after use in Coplin jar. Discard when the time required for suitable staining exceeds the time recommended in the procedure by more than 5 minutes.

Store Citrate Concentrated Solution at room temperature (18–26°C). Store Citrate Working Solution in refrigerator (2–8°C). Citrate solutions are suitable for use in the absence of microbial growth.

PREPARATION:

Fast Blue RR Salt, Fast Violet B Salt, Naphthol AS-MX Phosphate Alkaline Solution and Mayer's Hematoxylin Solution are provided ready for use in the procedure.

Prepare Citrate Working Solution by diluting 2 ml Citrate Concentrate Solution to 100 ml with deionized water.

To prepare Fixative Solution (citrate buffered acetone, 60%), warm Citrate Working Solution to room temperature (18–26°C). With constant stirring, add 2 volumes of Citrate Working Solution to 3 volumes of acetone. Discard after use.

Scott's Tap Water Substitute Working solution is prepared by diluting 1 volume of Scott's Tap Water Substitute Concentrate with 9 volumes deionized water.

PRECAUTIONS:

Normal precautions exercised in handling laboratory reagents should be followed. Dispose of waste observing all local, state, provincial or national regulations. Refer to Material Safety Data Sheet and product labeling for any updated risk, hazard or safety information.

PROCEDURE

SPECIMEN COLLECTION:

It is recommended that specimen collection be carried out in accordance with CLSI document M29-A3. No known test method can offer complete assurance that blood samples or tissue will not transmit infection. Therefore, all blood derivatives or tissue specimens should be considered potentially infectious.

Fresh blood or bone marrow films or samples anticoagulated with heparin may be used.[5] AVOID EDTA.[5] Blood smears should be stained for enzyme activity within 8 hours after preparation. However, if this is not possible, gradual loss of alkaline phosphatase activity may be delayed by fixation and storage overnight in freezer.[5] Films should be dried at least 1 hour prior to fixation and 3 hours post-fixation before freezing.

SPECIAL MATERIALS REQUIRED BUT NOT PROVIDED:
Scott's tap water substitute concentrate, Catalog No. S5134
Acetone, ACS reagent.

NOTES:

There is a scarcity of data concerning compounds which may interfere with leukocyte alkaline phosphatase activity (LAPA). Certain drugs and other substances are known to influence circulating alkaline phosphatase activity.[6] Oral contraceptives, cortisol and stress may result in elevated leukocyte alkaline phosphatase scores.[7]

Perform procedure using positive controls. These can be obtained from patients with pyogenic leukocytosis, or women in the third trimester of pregnancy or during the first several days postpartum. Leukocyte alkaline phosphatase scores from these persons usually exceed 100. A negative control can be prepared from a normal fixed smear by immersing it in boiling water for 1 minute to inactivate the enzyme. Control films can be preserved up to 1 year if stored fixed, wrapped in Parafilm® at –70°C. These films should be air dried at least 1 hour prior to fixation and 3 hours post-fixation before freezing.

It is strongly recommended that each laboratory establish it's own expected range, characteristic for the local population.

The procedure depends upon subjective rating of stained cells. This can result in a wide variation of ratings obtained. The temperature of the reaction mixture must be kept between 18–26°C. Lower temperatures will result in significantly lower scores. Above 30°C, marked increases in activity will occur. Eosinophils do not stain but can be recognized by bilobate nuclei and large refractile granules.

The data obtained from this procedure serves only as an aid to diagnosis and should be reviewed in conjunction with other clinical diagnostic tests or information.

PROCEDURE:

1. Measure 48 ml distilled water into a suitable container and adjust temperature to 18–26°C
2. Prepare diazonium salt solution:
   Dissolve contents of one Fast Blue RR Salt capsule or one Fast Violet B capsule in distilled water (from step 1). A magnetic stirrer may be helpful.
3. Add 2 ml Naphthol AS-MX Phosphate Alkaline Solution to diluted diazonium salt solution (from step 2). Mix.
4. Bring Fixative Solution to room temperature (18–26°C). Fix slides by immersing in Fixative Solution for 30 seconds. Rinse gently in deionized water for 45seconds. Do not allow slides to dry.
5. Add slides to alkaline-dye mixture (from step 3) and incubate at 18–26°C for 30 minutes. Protect immersed slides from direct light. Discard alkaline-dye mixture after use.
6. After 30 minutes, remove slides and rinse thoroughly in deionized water for 2 minutes. Do not allow slides to dry.
7. Place slides in Mayer's Hematoxylin Solution for 10 minutes.
   NOTE: If using Fast Blue RR Salt, rinse counterstained slides for 3 minutes in deionized water. This will result in red violet nuclear staining. If using Fast Violet B Salt, rinse counterstained slides in tap water (if alkaline) or immerse in Scott's Tap Water Substitute for 2 minutes. This will result in blue nuclear stain.
8. Evaluate microscopically. If coverslipping is required use only an aqueous mounting media.

PERFORMANCE CHARACTERISTICS

METHOD OF SCORING:

Scan the film (900X) and select a thin area where erythrocytes are barely touching. Sites of phosphatase activity will appear as blue or red granules, depending upon dye used. Select 100 consecutive segmented and band form neutrophilic granulocytes. Rate from 0 to 4 + on the basis of quantity and intensity of precipitated dye within the cytoplasm of these cells. For characteristics of scoring, refer to Table 1. The sum of the ratings of 100 cells is regarded as the score.

TABLE 1. CHARACTERISTICS OF SCORING*

Precipitated Azo Dye in Cytoplasm				
Cell Rating	Amount** (%)	Size of Granule	Intensity of Staining	Background of Cytoplasma
0+	None	—	None	None
1+	50	Small	Faint to Moderate	Colorless to very pale pink or blue
2+	50–80	Small	Moderate to Strong	Colorless to pale pink or blue
3+	80–100	Medium to Large	Strong	Colorless to pink or blue
4+	100	Medium and Large	Brilliant	Not visible

* Table 1 represents modification of observations made by Kaplow.[2,3]
** Percentage of volume of cytoplasm occupied by azo dye precipitate.

To obtain the leukocyte alkaline phosphatase activity (LAPA) score, the number of cells counted is multiplied by the value for cell rating. These figures are added to obtain the LAPA score as shown in the following examples:

Cell Rating	Number Counted	LAPA Score
0	60	0
1+	20	20
2+	14	28
3+	5	15
4+	1	4
Total	100	67

EXPECTED VALUES:

The following scores were obtained at Sigma-Aldrich for 20 normal individuals. Blood was drawn into heparinized tubes and films were prepared within one hour.

	Fast Blue RR	Fast Violet B
LAPA Mean ± 1 SD	96±44	72±50
LAPA Range	52–140	22–122

The range of normal scores is wide, varying from 12 to 182. The following range of scores were obtained from those 20 normal individuals:

Fast Blue RR	Fast Violet B
32–182	12–180

If observed results vary from expected results, please contact Sigma-Aldrich Technical Service for assistance.

EXPECTED OBSERVATIONS:

In humans, alkaline phosphatase activity is restricted to mature and band-form granulocytes. Occasionally weak staining may be observed in lymphocytes. Bone marrow osteoblasts and endothelial cells stain strongly. Marked increase in peripheral blood leukocyte alkaline phosphatase is observed in multiple myeloma, Hodgkin's disease, polycythemia vera and infectious leukocytosis. Low or absence of activity is seen in chronic myelocytic leukemia, hereditary hypophosphatasia and paroxysmal noncturnal hemoglobinuria.

A series of slides was prepared using blood obtained from normal subjects, females in the last trimester of pregnancy, early postpartum patients, and individuals exhibiting a leukemoid reaction. Negative controls were prepared by heat inactivation as described. Various combinations (Fast Blue RR and Fast Violet B) and counterstains (Methylene Blue Solution and Mayer's Hematoxylin Solution) were performed in replicate and the LAPA scored. Values obtained on multiple slides prepared on each subject were in close agreement and did not appear influenced by the staining/counterstaining technique employed. For example, 7 smears made using blood from a prenatal patient yielded scores ranging from 235–269 with a mean, standard deviation and coefficient of variation of 255, 11.3 and 4.4% respectively. Negative controls revealed a complete absence of stained material.

REFERENCES

1. Kay HD: Plasma phosphatase in osteitis deformans and in other diseases of the bone. Br J Exp Pathol 10:253, 1929
2. Kaplow LS: A histochemical procedure for localizing and evaluating leukocyte alkaline phosphtase activity in smears of blood and marrow. Blood 10:1023, 1955
3. Kaplow LS: Leukocyte alkaline phosphatase cytochemistry: Applications and methods. Ann NY Acad Sci 155:911, 1968
4. Ackerman GA: Substituted naphthol AS phosphate derivatives for the localization of leukocyte alkaline phosphatase activity. Lab Invest 11:563, 1962
5. Proposed Standard: Histochemical Method for Leukocyte Alkaline Phosphatase H22-P, Vol 4, No 14, National Comittee for Clinical Laboratory Standards
6. Young DS: Effects of drugs on clinical laboratory tests. AACC Press, Washington D.C., 1990; Supplement No. 1, 1991
7. Elias JM: Principles and Techniques in Diagnostic Histopathology, Noyes Publications, New Jersey, 1982, pp 248–250

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Previous Revision: 2003-09
Revised: 2014-09

MDSS GmbH
Schiffgraben 41
30175 Hannover, Germany

SIGMA-ALDRICH, INC.
3050 Spruce Street, St. Louis, MO 63103 USA
314-771-5765
Technical Service: 800-325-0250
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2-E

4. Serum Protein Electrophoresis

Serum Protein Electrophoresis Procedure

The Serum Protein Electrophoresis procedure is intended for the separation and quantitation of serum proteins using cellulose acetate electrophoresis.

SUMMARY

Serum contains over one hundred individual proteins, each with a specific set of functions and subject to specific variation in concentration under different pathologic conditions.[1]

Since the introduction of moving-boundary electrophoresis by Tiselius[2] and the subsequent use of zone electrophoresis, serum proteins have been fractionated on the basis of their electrical charge into five classical fractions: albumin, $alpha_1$, $alpha_2$, beta, and gamma proteins. Each of these classical electrophoretic zones (with the exception of albumin) normally contains two or more components. Approximately fifteen serum proteins have been studied extensively because they may be measured easily.[3-5]

PRINCIPLE

Proteins are large molecules composed of covalently linked amino acids. Depending on electron distributions resulting from covalent or ionic bonding of structural subgroups, proteins have different electrical charges at a given pH. In the Helena Serum Protein procedure, the proteins are separated according to their respective electrical charges at pH 8.8 on a cellulose acetate plate using both the electrophoretic and electroendosmotic forces present in the system. After the proteins are separated, the plate is placed in a solution of sulfosalicylic acid and Ponceau S (to stain the protein bands). The staining intensity is related to protein concentration.[6] After dehydration in methanol, the plate background is then rendered transparent by treatment with a clearing solution.

REAGENTS

1. Ponceau S Stain (Cat. No. 5526)

Ingredients: After dissolution, each bottle of stain contains 0.5% (w/v) Ponceau S in an aqueous solution of 10% (w/v) Sulfosalicylic Acid.

WARNING: FOR IN-VITRO DIAGNOSTIC USE. DO NOT INGEST. HARMFUL IF SWALLOWED.

Preparation for Use: One vial of Ponceau S Stain is dissolved in 1 L of deionized water. Mix until thoroughly dissolved.

Storage and Stability: The stain may be stored as packaged or in a tightly closed staining dish at 15-30°C. The unopened stain is stable until the expiration date on the bottle.

Signs of Deterioration: The stain should be a homogeneous mixture free of precipitate. Do not use if excessive evaporation occurs or if large amounts of precipitate occur.

2. Electra® HR Buffer (Cat. No. 5805)

Ingredients: HR Buffer is a Tris-barbital-Sodium Barbital buffer.

WARNING: FOR IN-VITRO DIAGNOSTIC USE. DO NOT INGEST. The buffer contains barbital which, in sufficient quantity, can be toxic.

Preparation for Use: Dissolve one package of dry buffer in 750 mL of deionized water. The buffer is ready for use when all material is dissolved and completely mixed.

Storage and Stability: The packaged buffer is stable until the expiration date on the package. The diluted buffer is stable for 2 months at 15-30°C when stored tightly closed.

Signs of Deterioration: Discard packaged buffer if the material shows signs of dampness or discoloration. Discard unused diluted buffer if it becomes turbid.

3. Titan® III Cellulose Acetate Plate (Cat. No. 3013, 3023, 3024, 3033)

Ingredients: Cellulose acetate plates.

WARNING: FOR IN-VITRO DIAGNOSTIC USE

Preparation for Use: The plates are ready for use as packaged.

Storage and Stability: The plates should be stored at 15 to 30°C and are stable indefinitely.

4. Clear Aid (Cat. No. 5005)

Ingredients: Clear Aid contains polyethylene glycol.

WARNING: FOR IN-VITRO DIAGNOSTIC USE ONLY. DO NOT INGEST.

Preparation for Use: When preparing the clearing solution, mix 30 parts glacial acetic acid, 70 parts absolute methanol, and 4 parts Clear Aid. Stir until well mixed.

Storage and Stability: The prepared Clear Aid should be stored in a tightly closed container at 15 to 30°C to prevent evaporation of the methanol. When evaporation occurs, the plates may delaminate. Water contamination, from over-use of the clearing solution, will cause the plate to be cloudy. The Clear Aid is stable until the expiration date on the bottle label.

Signs of Deterioration: Clear Aid should be a clear, colorless liquid, although it may appear cloudy when cold. Do not use the material upon evidence of gross contamination or discoloration. Discard the prepared Clear Aid solution if the plates appear cloudy after the clearing procedure.

5. PermaClear Solution (Cat. No. 4950)-Optional

Ingredients: N-methyl pyrrolidinone and PEG.

WARNING: FOR IN-VITRO DIAGNOSTIC USE - IRRITANT DO NOT PIPETTE BY MOUTH. VAPOR HARMFUL. In case of contact, flush affected areas with copious amounts of water. Get immediate attention for eyes.

Preparation for Use: Add 55 mL PermaClear to 45 mL deionized water to make working clearing solution. Mix well.

Storage and Stability: PermaClear should be stored at 15 to 30°C and is stable until the expiration date on the bottle.

Signs of Deterioration: Discard the PermaClear Solution if the plates turn white and do not clear as expected.

INSTRUMENTS

Any high quality scanning densitometer with visible transmittance capability may be used. Recommended is the Helena QuickScan Touch/2000.

SPECIMEN COLLECTION AND HANDLING

Specimen: Fresh serum is the preferred specimen. The use of plasma should be avoided, as fibrinogen will appear as a distinct narrow band between the beta and gamma fractions. Cerebrospinal fluid may be used if concentrated approximately 100 times; urine may be used if concentrated up to 300 times, depending on original protein concentration.

Interfering Factors:

1. Hemolysis may cause false elevation in the $alpha_2$ and beta fractions.

2. Inaccurate results may be obtained on specimens left uncovered, due to evaporation.

Storage and Stability: If storage is necessary, samples may be stored covered at 2 to 8°C for 48 hours. Cerebrospinal fluid and urine specimens may be used after proper concentration with a concentrator.

PROCEDURE

Materials provided but not contained in a kit: The following materials are needed for the Protein Electrophoresis procedure. All items are available on an individual basis.

HARDWARE	Cat. No.
Super Z Applicator	4084
Super Z Sample Well Plate (2)	4085
Super Z Aligning Base	4086
Super Z-12 Applicator	4090
Super Z-12 Sample Well Plate (2)	4096
Super CPK Aligning Base	4094
TITAN GEL Electrophoresis Chamber	4063
1000 Staining Set	5122
5 µL Microdispenser and Tubes	6008
Bufferizer	5093

CONSUMABLES	
Zip Zone® Prep	5090
Titan Gel III Cellulose Acetate Plates	
25 x 76 mm	3013
60 x 76 mm	3023
76 x 76 mm	3033
94 x 76 mm	3024
Glue Stick	5002
Blotter Pads (76 x 102 mm)	5034
Titan Plastic Envelopes (63 x 120 mm)	5052
Blotter Pads (102 x 108 mm)	5037
Titan Plastic Envelopes (102 x 120 mm)	5053
Helena Marker	5000
Titan Identification Labels	5006
Ponceau S Stain	5526
Electra® HR Buffer	5805
Clear Aid	5005
PermaClear	4950
Zip Zone® Chamber Wicks	5081
SPE Normal Control	3424
SPE Abnormal Control	3425
Titan Plus Power Supply	1504

Materials needed, but not provided:

5% acetic acid (v/v): Add 50 mL of glacial acetic acid to 950 mL of deionized water.

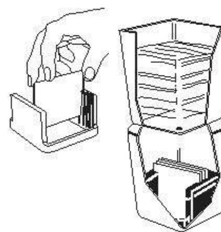
Absolute methanol, reagent grade

SUMMARY OF CONDITIONS

Plate	Titan III Cellulose Acetate Plate
Plate Soaking Time	20 minutes
Buffer Dilution	750 mL
Sample Volume	3 µL
Chamber Voltage	180 volts
Electrophoresis Time	15 minutes
Stain Time	6 minutes
Destain Time in 5% Acetic Acid	3 times/2 minutes each
Dehydration Time in Methanol	2 times/2 minutes each
Clearing Time (Clear Aid)	5-10 minutes
Drying Temperature	50-60°C
Drying Time	15 minutes
Scanning Wavelength	525 nm

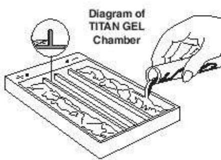
STEP-BY-STEP METHOD

A. Titan III Plate Preparation



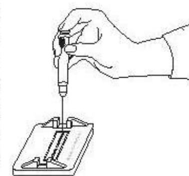
1. Properly code the required number of Titan III Plates by marking on the glossy, hard side with a Helena marker. It is suggested that the identification mark be placed in one corner so that it is always aligned with sample No. 1.
2. Soak the plates for 20 minutes in diluted Electra HR buffer. The plates should be soaked in the Bufferizer according to the instructions for use included with the Bufferizer. Alternately, the plates may be wetted by slowly and uniformly lowering a rack of plates into the HR Buffer such that air is not trapped in the plates. The same soaking buffer may be used for soaking up to 12 plates or for approximately one week if stored tightly closed. If used for a more prolonged period residual solvents from the plate may build up in the buffer or evaporation may alter buffer concentration.

B. Electrophoresis Chamber Preparation

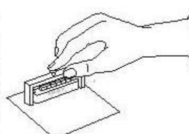


1. Pour approximately 100 mL of diluted HR Buffer into each of the outer sections of the electrophoresis chamber. Do not use the same buffer in which the plates were soaked for electrophoresis.
2. Wet two disposable wicks in the buffer. Stand them lengthwise (on edge) in the buffer compartments. Fold the top edge of each wick over each support bridge, making sure the bottom edge is in the buffer and touching the bottom of the chamber. Press the top edge down over the bridge until the wick makes contact with the buffer, and there are no air bubbles under the wicks.
3. Cover the chamber to saturate the air with buffer. Discard electrophoresis buffer after use.

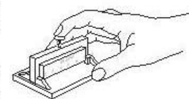
C. Sample Application



1. Fill each well in the sample plate with 3 µL of sample using the microdispenser. Expel the samples as a bead on the tip of the glass tube; then touch this bead to the well. Cover the samples with a glass slide if they are not used within 2 minutes.

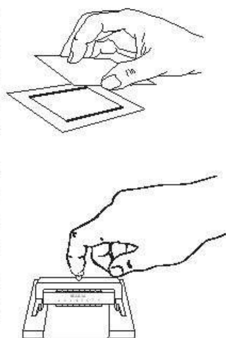


2. Prime the applicator by depressing the tips into the sample wells 3 or 4 times. Apply this loading to a piece of blotter paper. Priming the applicator makes the second loading much more uniform. Do not load applicator again at this point, but proceed quickly to the next step.

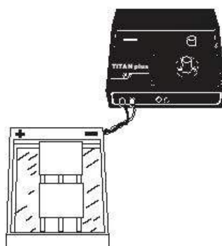


3. Remove the wetted Titan III Plate from the buffer with the fingertips and blot once firmly with a blotter. Before placing the plate in the aligning base, place a drop of water or buffer on the center of the aligning base. This prevents the plate from shifting during the sample application. Place

the plate in the aligning base, cellulose acetate side up, aligning the bottom edge of the plate with the black scribe line marked "CENTER APPLICATION". The identification mark should be aligned with sample No. 1.



4. Apply the sample to the plate by gently depressing the applicator tips into the sample well 3 or 4 times and promptly transferring the applicator to the aligning base. Press the button down and hold it 5 seconds.



D. Electrophoresis

1. Quickly place the plate(s) cellulose acetate side down, in the electrophoresis chamber. Place a weight (glass slide, coin, etc.) on the plate(s) to insure contact with the wicks. Cover the chamber securely and wait 30 seconds for the plate(s) to equilibrate.
2. Electrophorese the plate(s) for 15 minutes at 180 volts. Power must be applied within 5 minutes after the plate(s) has been placed in the chamber.

E. Visualization of the Protein Bands

1. At the end of the electrophoresis time, remove the plate(s) from the chamber. Place them in 40-50 mL of Ponceau S stain (sufficient volume to cover the plate(s) for 6 minutes. When staining 2 or more plates, carry out the protocol vertically in a rack. The stain may be reused until the plate background contains stain precipitate.
2. Destain in 3 successive 2 minute washes of 5% acetic acid or until the plate background is white. The plates may be dried and stored as a permanent record at this point if stored in a plastic envelope to protect the surface. If a transparent background is desired (i.e. for densitometry), proceed to the next step.

If using Clear Aid Solution:

3. Dehydrate by rinsing the plate in two absolute methanol washes for two minutes each wash. Allow the plate to drain for 5-10 seconds before placing in the next solution.
4. Place the plate into the clearing solution for 5-10 minutes.
5. Drain off excess solution. Then place the plate, acetate side up, onto a blotter, and into a drying oven at 50-60°C for 15 minutes or until dry.

If using PermaClear Solution:

3. Place the plate(s) into the diluted PermaClear clearing-solution for 2 minutes.
4. Drain off excess solution by holding plate(s) vertically for 1 minute. Then place the plate, acetate side up, onto a blotter, and into a drying oven at 50-60°C for 15 minutes or until dry.

F. Evaluation of the Protein Bands

Scan the plates on QuickScan using a slit size of 5.

Stability of End Product

The completed, dried serum protein plate is stable for an indefinite period of time and may be stored in Titan Plastic Envelopes.

Calibration

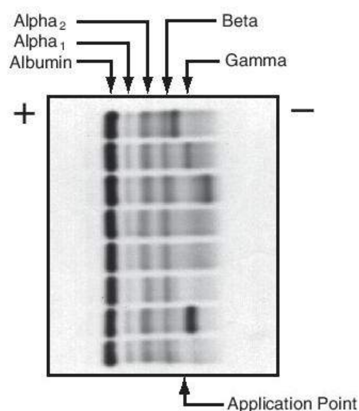
The Optical Density Step Tablet (Cat. No. 1047) should be used to insure the linearity of the instrument and a Neutral Density Densitometer Control (Cat. No. 1031) should be used to validate the zero adjustment and quantitation by the instrument.

QUALITY CONTROL

SPE Normal (Cat. No. 3424) and SPE Abnormal (Cat. No. 3425) Control may be used to verify all phases of the procedure and should be used on each run. Refer to the package insert provided with the control for assay values.

RESULTS

The fastest moving band, and normally the most prominent, is the albumin band found closest to the anodic edge of the plate. The faint band next to this is alpha$_1$ globulin, followed by alpha$_2$ globulin, beta, and gamma globulins. Prealbumin is seldom visible with this system.



Calculation of the Unknown

The QuickScan Touch/2000 will automatically print the relative percent and the absolute values for each band. Alternately, the relative percent of each band can be calculated manually by referring to the Operator's Manual provided with the densitometer. The relative percent of each band is calculated by the following formula:

$$\frac{\text{No. Integration Units of the Band}}{\text{Total Integration Units}} \times 100 = \text{Relative Percent of the Band}$$

$$\text{Relative Percent of the Band} \times \text{Total Serum Protein} = \text{Absolute Value of Protein per band}$$

REFERENCE VALUES

The reference values for serum protein electrophoresis on cellulose acetate stained with Ponceau S were determined from a study of 51 normal subjects. These values are for illustrative purposes only. Each laboratory should establish its own range.

Protein Fraction	Concentration
Albumin	3.63 - 4.91 g/dL
Alpha$_1$	0.11 - 0.35
Alpha$_2$	0.65 - 1.17
Beta	0.74 - 1.26
Gamma	0.58 - 1.74

Variations of Expected Values[4]

Studies show that values are the same for both males and nonpregnant females. (Some differences are seen in pregnant females at term and in women on oral contraceptives.)

Age has some effect on normal levels. Cord blood has decreased total protein, albumin, alpha$_2$ and beta fractions, slightly increased alpha$_1$, and normal or increased gamma fractions (largely of

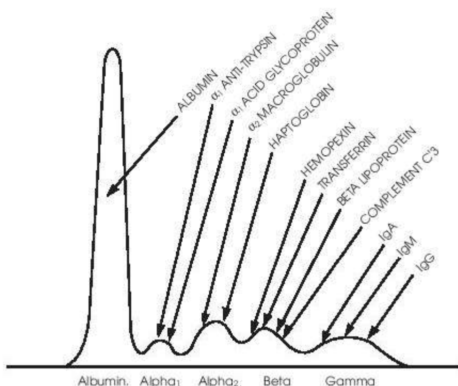
maternal origin). The gamma drops rapidly until about 3 months of age, while the other fractions have reached adult levels by this time. Adult levels of the gamma globulins are not reached until 10-16 years of age. The albumin decreases and beta globulins increase after the age of 40.

Further Testing Required

The serum protein electropherogram or densitometric tracing should be evaluated for abnormalities. If abnormalities are observed, appropriate follow-up studies should be initiated. These may include immunoelectrophoresis, immunofixation, quantitation of individual component immunoglobulins, bone marrow examination, and other appropriate tests.

INTERPRETATION OF RESULTS[5, 7]

Results on normal individuals will cover age and sex-related variations and day-to-day biologic variations. Disease states in which abnormal patterns are observed include inflammatory response, rheumatic disease, liver diseases, protein-loss disorders, plasma cell dyscrasias, and genetic deficiencies. Variant patterns have also been observed during pregnancy. Below is a normal serum protein pattern showing the locations of some of the more commonly known proteins.



SPECIFIC PERFORMANCE CHARACTERISTICS

Precision: A normal serum was run 26 consecutive times. The following data were obtained:

	Mean (Relative %)	S.D.	C.V.(%)
Albumin	55.7	1.4	2.5
$Alpha_1$	3.1	0.4	12.5
$Alpha_2$	11.3	0.4	3.8
Beta	11.8	0.5	4.0
Gamma	18.1	0.6	3.5

Linearity: Since the stain uptake is different for each band, the serum protein procedure is not linear. Do not dilute specimens which have a high total protein concentration. Use only 3 µL of specimen in the sample well plate.

BIBLIOGRAPHY

1. Alper, C.A., Plasma Protein Measurements as a Diagnostic Aid, N Eng J Med, 291:287-290, 1974.
2. Tiselius, A., A New Approach for Electrophoretic Analysis of Colloidal Mixtures, Trans Faraday Soc, 33:524-531, 1937.
3. Ritzmann, S.E. and Daniels, J.C., Diagnostic Proteinology: Separation and Characterization of Proteins, Qualitative and Quantitative Assays in Laboratory Medicine, Harper and Row Inc., Hagerstown, 1979.
4. Ritzmann, S.E. and Daniels, J.C., Serum Protein Abnormalities: Diagnostic and Clinical Aspects, Little, Alan R. Liss Inc., 1982.
5. Killingsworth, L.M. et al., Protein Analysis, Diag Med, Jan/Feb., 47-58, 1980.
6. Henry, R.J., Cannon, D.C., and Winkelman, J.W., ed., Clinical Chemistry: Principals and Techniques, 437, Harper and Row, Hagerstown, 1974.
7. Killingsworth, L.M., Plasma Protein Patterns in Health and Disease, CRC Crit Rev in Clin Lab Sci, 1-30, August, 1979.

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5. Determination platelet count.

Despite their small size (2–4 µm) and being non-nucleated fragments of cytoplasm, the platelets contain a wide variety of chemical substances that play an important role in vasoconstriction, hemostatic plug formation, activation of factor X, conversion of prothrombin to thrombin, and in clot retraction that results in permanent sealing of a ruptured vessel. Thus they take part in almost all stages of hemostasis.

Materials

-Hemocytometer counting chamber.

-1% Ammoium oxalate to hemolyzed erythrocytes.

-EDTA anticoagulated venous blood.

-Capillary blood should not be used because platelets clump as the blood is being collected.

Procedure

1. Pipette 990µl of 1% ammonium oxalate into a clean, dry test tube.

2. Add 10µl well mixed venous blood.

3. Leave at room temperature for 10 minutes to allow the platelets to settle down.

4. Use micropipette tip to take 20 µl from the prepared solution and the tip is touched to the side of the hemocytometer chamber (without any hesitation) till a fluid will run under the cover glass.

5. Mount the hemacytometer on the microscope and total number of cells in 1 large square ona corner (25 small squares) is determined under the high dry objective power (40X).

6. To avoid duplicate counting of a single cell, you must count only those cells that touch the lower and left boundaries. Platelets appear greenish, not refractile.

Calculation

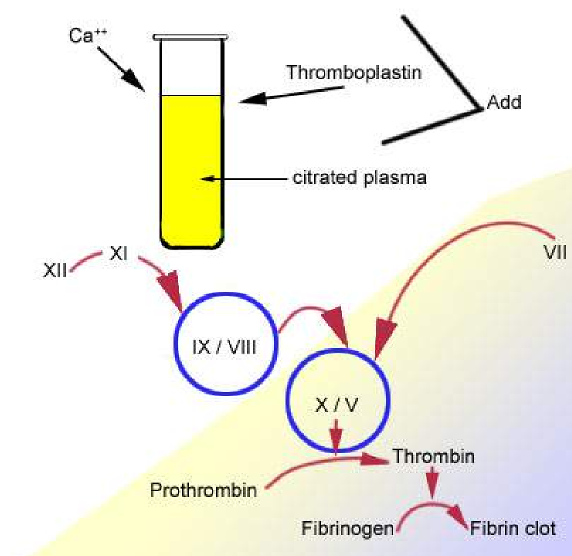
$$\text{Cells/mm}^3 = \frac{\text{Total number of cells counted x Reciprocal of dilution}}{\text{Number of squares counted x Area of each square x Depth of the solution}}$$

Normal Value

The normal number of platelets in the blood is 150,000 - 400,000 platelets/μl.

6. Determination of prothrombin time

The patient's blood is quickly oxalated (or citrated) to remove calcium ions so that prothrombin cannot be converted to thrombin. The sample is then centrifuged. Then to the oxalated plasma, a large excess of calcium ions (as calcium chloride solution) and rabbit brain suspension (to provide tissue thromboplastin; tissue factor, TF) is added. The excess calcium neutralizes the effect of oxalate and the TF converts prothrombin to thrombin via the extrinsic clotting pathway (i.e. factor VII).
The time required for clotting to occur is called the prothrombin time (PT).
Normal PT = 15–20 seconds.
Clinical Significance. Since the potency of tissue thromboplastin (TF) may vary, blood from a normal person is used as a control when the test is used for controlling anticoagulant dose, or in a hemorrhagic disease. Bleeding tendency is present when the prothrombin level falls below 20% of normal (normal plasma prothrombin = 30–40 mg/dl). Prolonged PT suggests the possibility of deficiency of factors II (prothrombin), V, VII and X. Prothrombin level is low in vitamin K deficiency and various liver and biliary diseases.



7. Determination of *Bleeding Time (BT) & Clotting time (CT)*

Bleeding Time (BT) is the time interval between the skin puncture and spontaneous, unassisted (i.e. without pressure) stoppage of bleeding. The BT test is an *in vitro* test of platelet function.

Clotting time (CT) is the time interval between the entry of blood into the glass capillary tube, or a syringe, and formation of fibrin threads.

[I] "Duke" Bleeding time (finger-tip; ear-lobe)

• Since the skin of the fingertip is quite thick in some persons, a small cut in the skin of the earlobe with the corner edge of a sterile blade gives better results. The earlobe method is the original "Duke" method for BT.

• Ask your partner to fill the capillary tube with blood from the same skin puncture from where you are doing the BT (see below for CT).

• **Materials** •Equipment for sterile finger-prick. • Clean filter papers. • Chemically clean, 10–12 cm long, glass capillary tubes with a uniform bore diameter of 1–2 mm. • Stopwatch.

PROCEDURES

1. Get a deep finger-prick under aseptic conditions to get free-flowing blood. Start the stop watch and note the time.

2. Absorb/remove the blood drops every 30 seconds by touching the puncture site with the filter paper along its edges, without pressing or squeezing the wound. Number the blood spots 1 onwards.

3. Note the time when bleeding stops, i.e. when there is no trace of blood spot on the filter paper. Encircle this spot and number it as well. This is the end point. (Do not keep the filter paper on the table and then press your wound on it).

4. Count the number of blood spots and express your result in minutes and seconds.

Normal bleeding time = 1–5 minutes.

• The test is simple and quite reliable in spite of the fact that the depth of the wound cannot be controlled.

• The BT is prolonged in purpura (platelet deficiency, or vessel wall defects) while it is usually normal in hemophilia.

• Lack of several clotting factors may prolong BT, though it is especially prolonged by lack of platelets.

PRECAUTIONS

1. The skin site chosen for BT should be scrubbed well with alcohol to increase the blood flow.

2. The skin should be dry and the puncture should be 3–4 mm deep to give free-flowing blood. Do not squeeze.

3. Do not press the filter paper on the puncture site.

4. If bleeding continues for more than 10–12 minutes, stop the test and press a sterile gauze on the wound. Inform your teacher about the bleeding.

[II] Another method is to get a finger-prick and dip the finger in a beaker containing normal saline at 37°C. The blood drops will be seen falling to the bottom in a continuous stream. Note the time when bleeding stops.

[III] "Ivy" Bleeding Time (Hemostasis Bleeding Time).

This method is more reliable than the "Duke" method. However, it requires some practice to apply the BP cuff and maintain the pressure.

Procedure

1. Clean the skin over the front of the forearm with 70% alcohol.

2. Apply a blood pressure cuff on the upper arm, raise the pressure to 40 mm Hg and maintain it there till the end of the experiment.

3. Clean the skin area once again. Grasp the underside of the forearm tightly, make a 1–3 mm deep skin puncture, about 5–6 cm below the cubital fossa. Note the time.
4.Remove the blood every 30 seconds by absorbing it along the edges of a clean filter paper by gently touching the wound with it, till the bleeding stops. This is the end-point.

Normal bleeding time with this method is upto 9 minutes.

CAPILLARY BLOOD CLOTTING TIME (WRIGHT'S CAPILLARY GLASS TUBE METHOD)

(While your partner is doing BT on your finger prick, you can proceed with your CT.

1. Absorb the first 2 drops of blood on a separate filter paper and allow a large drop to form. Now dip one end of the capillary tube in the blood; the blood rises into the tube by capillary action. This can be enhanced by keeping its open end at a lower level.

2. Note the time when blood starts to enter the tube. This is the zero time.

3. Hold the capillary tube between the palms of your hands to keep the blood near body temperature (in winter, you may blow on it).

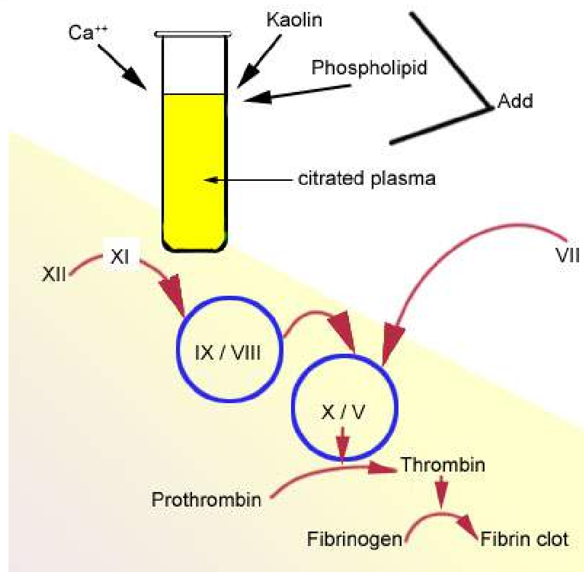
4. Gently break off 1 cm bits of glass tube from one end, at intervals of 30 seconds, and look for the formation of fibrin threads between the broken ends. The end-point is reached when fibrin threads span a gap of 5 mm between the broken ends ("rope formation"). Note the time.

Normal clotting time = 3–6 minutes.

8. Activated partial thromboplastin time

The activated partial thromboplastin time (aPTT) is a test performed to investigate bleeding disorders and to monitor patients taking an anticlotting drug such as heparin which inhibits factors X and thrombin, while activating anti-thrombin.

The aPTT test uses blood which is decalcified to prevent clotting before the test begins. The plasma is separated by centrifugation. (Ionized) Calcium and activating substances are added to the plasma to start the intrinsic pathway of the coagulation cascade. The substances are: kaolin (hydrated aluminum silicate) and cephalin. Kaolin serves to activate the contact-dependent Factor XII, and cephalin substitutes for platelet phospholipids. The partial thromboplastin time is the time it takes for a clot to form, measured in seconds. Normally, the sample will clot in 35 seconds.



PTT measures the integrity of the intrinsic system (Factors XII, XI, VIII, IX) and common clotting pathways. Increased levels in a person with a bleeding disorder indicate a clotting factor may be missing or defective. At this point, further investigation is needed and warrants the use of sensitive assays for specific coagulation factors. Liver disease decreases production of factors, increasing the PTT.

9. Determination of Thrombin time

Der Inhalt dieses Dokuments dient lediglich als Beispiel, die aktuellste Version senden wir gerne zu: info@coachrom.com. The content of this document is an example only. You may request the latest version at: info@coachrom.com

HEMOCLOT™ Thrombin Time (T.T.)

CE REF CK011K R 6 x 2 mL
REF CK011L R 6 x 8 mL
IVD

Clotting method for the determination of Thrombin Time.

Sales and Support:
CoaChrom Diagnostica GmbH
www.coachrom.com | info@coachrom.com
Tel: +43-1-236 222 1 | Fax: +43-1-236 222 111
Toll-free contact for Germany:
Tel: 0800-24 66 33-0 | Fax: 0800-24 66 33-3

English, last revision: 08-2019

INTENDED USE:

HEMOCLOT™ Thrombin Time (T.T.) kit is a clotting method for the *in vitro* qualitative determination of Thrombin Time on human citrated plasma, using manual or automated method.

SUMMARY AND EXPLANATION:

Technical:
The Thrombin Time is a coagulation assay measuring the time to convert fibrinogen to fibrin.

Clinical:[1]
The Thrombin Time is a screening test to assess abnormalities of fibrinogen and to detect inhibitors against thrombin or fibrin. It can be useful for the evaluation of Disseminated Intravascular Coagulation (DIC) and liver disease.
A prolonged Thrombin Time can result from:

- Presence of antithrombin activity induced by therapy (eg Heparin, hirudin, argatroban, dabigatran).
- Presence of high concentrations of Fibrin/Fibrinogen degradation products.
- Qualitative (dysfibrinogenemia) or quantitative abnormalities of Fibrinogen (deficiency, DIC, fibrinolysis, hepatic disorders including cirrhosis).

The Thrombin Time is normal in presence of a Factor XIII deficiency.

PRINCIPLE:

The HEMOCLOT™ Thrombin Time (T.T.) kit is a reagent for Thrombin Time (TT). It measures the clotting time (CT) induced by a controlled and constant amount of bovine thrombin, in presence of calcium, on citrated plasma. The time required for the formation of a stable clot is measured in seconds.

REAGENTS:

R **Bovine Thrombin**, highly purified, and calcium, lyophilized. Contains BSA and stabilizers.

REF **CK011K** ➔ **6 vials of 2 mL.**
REF **CK011L** ➔ **6 vials of 8 mL.**

The bovine Thrombin concentration (about 1.0 NIH/mL) can vary from lot to lot and is adjusted for each lot in order to offer a high sensitivity of Thrombin Time assay to low concentrations of Unfractionated Heparin (UFH) and Low Molecular Weight Heparin (LMWH).

WARNINGS AND PRECAUTIONS:

- Some reagents provided in these kits contain materials of animal origin. Users of reagents of these types must exercise extreme care in full compliance with safety precautions in the manipulation of these biological materials as if they were infectious.
- Waste should be disposed of in accordance with applicable local regulations.
- Use only the reagents from the same batch of kits.
- Aging studies show that the reagents can be shipped at room temperature without degradation.
- This device of *in vitro* diagnostic use is intended for professional use in the laboratory.

REAGENT PREPARATION:

Gently remove the freeze-drying stopper, to avoid any product loss when opening the vial.

R Reconstitute the contents of each vial with exactly:
REF **CK011K ➔ 2 mL of distilled water.**
REF **CK011L ➔ 8 mL of distilled water.**

Shake vigorously until complete dissolution while avoiding formation of foam and load it directly on the analyzer following application guide instruction.
For manual method, allow to stabilize for 15 minutes at room temperature (18-25°C), homogenize before use.

STORAGE AND STABILITY:

Unopened reagents should be stored at 2-8°C in their original packaging. Under these conditions, they can be used until the expiry date printed on the kit.

R Reagent stability after reconstitution, free from any contamination or evaporation, and stored closed, is of:

- **7 days** at 2-8°C.
- **48 hours** at room temperature (18-25°C).
- **Do not freeze**
- **Stability on board of the analyzer: see the specific application.**

REAGENTS AND MATERIALS REQUIRED BUT NOT PROVIDED:

Reagents:

- Distilled water.
- Specific controls such as:

Product name	Reference
BIOPHEN™ Normal Control Plasma	223201
EASYPLASMA™ Control Set	225601

Also refer to the specific application guide of the analyzer used.

Materials:

- Water-bath, semi-automatic or automatic instrument for clotting assays.
- Stopwatch; Calibrated pipettes; silicon glass or plastic test tubes.

SPECIMEN COLLECTION AND PREPARATION:

The blood (9 volumes) should be carefully collected onto the trisodium citrate anticoagulant (1 volume) (0.109 M, 3.2%) by clean venipuncture. Discard the first tube.
Specimens should be prepared and stored in accordance with applicable local guidelines (for the United States, see the CLSI H21-A5[2] guideline for further information concerning specimen collection, handling and storage).
For plasma storage, please refer to references[2,3].

PROCEDURE:

The kit can be used in manual or automated method. Perform the test at **37°C** and the clotting time, triggered by addition of thrombin, is measured.

For an automated method, application guides are available on request. See specific application guide and specific precautions for each analyzer.

<u>Assay method:</u>
1. Reconstitute, if necessary, the controls as indicated in the specific instructions.
2. Plasma should be tested **undiluted.**

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D750-02/CK/011K-011L/v1

3. Introduce into a reaction cuvette, silicon glass or plastic test tube incubated at **37°C:**

Reagents	Volume
Specimens and controls non-diluted	100 µL
Incubate at **37°C** for **1 minute**, then introduce (starting the stop-watch) :	
R **Bovine Thrombin**, preincubated at **37°C**	100 µL
Record the exact clotting time (CT, sec).	

If a reaction volume other than that specified above is required for the method used, the ratio of volumes must be strictly observed to guarantee assay performance. The user is responsible for validating any changes and their impact on all results.

QUALITY CONTROL:

The use of quality controls serves to validate method compliance, along with between-test assay homogeneity for a given batch of reagents.

Include the quality controls with each series, as per good laboratory practice, in order to validate the test. Each laboratory must define its acceptable ranges and verify the expected performance in its analytical system.

RESULTS:

- The obtained CT for the sample must be compared with that of the reference normal range for the laboratory (refer to current local recommendations).
- Results can be reported as a ratio:
  TT ratio = Sample (CT, sec) / Mean of normals (CT, sec).
- The results should be interpreted according to the patient's clinical and biological condition.

LIMITATIONS:

- To ensure optimum test performance and to meet the specifications, the technical instructions validated by HYPHEN BioMed should be followed carefully.
- Any reagent presenting an unusual appearance or showing signs of contamination must be rejected.
- Any suspicious samples or those showing signs of activation must be rejected.
- Various drugs or treatments can affect TT results. An additional investigation should be realized to determine the origin of each unexpected abnormal result.
- The obtained CT for a same sample and a same reagent lot can vary according to the instrument used and the clot detection mode.
  In the same way, many variables (eg: different sources of heparin) can affect the obtained results: each laboratory should consequently establish its own heparin therapeutic range.
- The assay is sensitive to low concentrations of heparin provided the tested plasma is collected without activation and release of platelet alpha granules, which contain PF4, a heparin inhibitor.[1,2]

EXPECTED VALUES:

Thrombin Time is usually expected < 25 sec.

As an example, for one lot, the mean value from healthy adults (n=120) on Sysmex CS-5100 was 18.5 seconds with SD = 0.8 seconds. Each laboratory should determine its own usual ranges (normal range, heparin sensitivity…) for each combination of lot and instrument used.

PERFORMANCES:

- The reagent assay is sensitive to low concentrations of plasmatic heparin (from 0.05 to 0.10 IU/mL of UFH, and from > 0.20 IU/mL LMWH in plasma).
- Performance studies were conducted internally on Sysmex CS-5100. Performance was assessed using laboratory controls over a 5-day period, 2 series per day and 3 repetitions within each series for a control level. The following results were obtained:

Control	Intra assay				Inter assays			
	n	Mean	CV%	SD	n	Mean	CV%	SD
Control 1	40	23.9	2.2	0.5	30	23.9	1.7	0.4
Control 2	40	39.4	1.1	0.4	30	40.0	1.2	0.5

- Correlation with reference method (Test Thrombin Reagent (Siemens) on Sysmex CS-5100, on T.T. seconds) :
  n = 104 y = 1.64x -11.64 r = 0.883
- Interferences:
  No interference, on the analyzer Sysmex CS-5100 was observed with the molecules and up to following concentrations:

Intralipids	Hemoglobin	Bilirubin (F/C)
1000 mg/dL	1000 mg/dL	F : 30 / C : 60 mg/dL
Apixaban	**Rivaroxaban**	**Edoxaban**
400 ng/mL	400 ng/mL	400 ng/mL

Also refer to the specific application guide of the analyzer used.

REFERENCES:

1. Appel I.M. *et al.* Age dependency of coagulation parameters during childhood and puberty. Journal of Thrombosis and Haemostasis. 2012.
2. CLSI Document H21-A5: "Collection, transport, and processing of blood specimens for testing plasma -based coagulation assays and molecular hemostasis assays; approved guideline". 2008
3. Woodhams B. *et al.* Stability of coagulation proteins in frozen plasma. Blood coagulation and Fibrinolysis. 2001.

SYMBOLS:

Symbols used and signs listed in the ISO 15223-1 standard, see Symbol definitions document.

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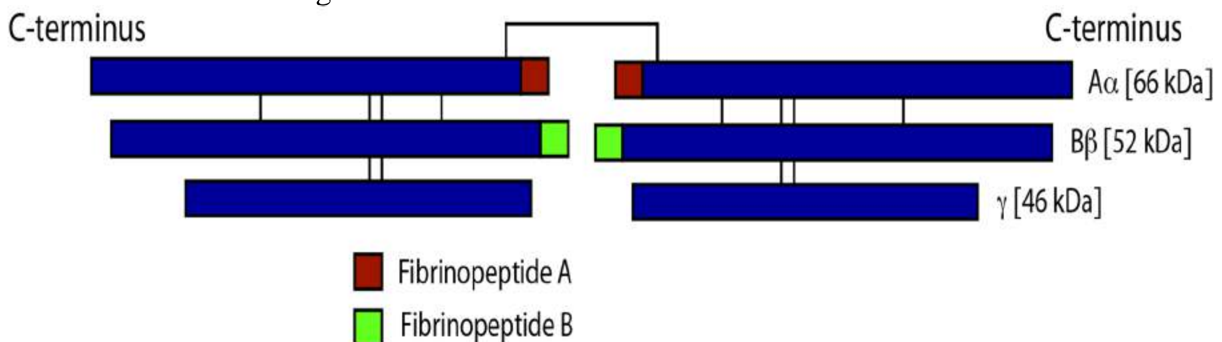
10. Clauss fibrinogen assay

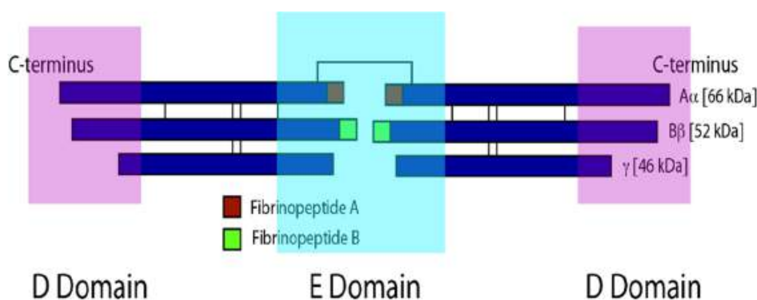
Introduction

Fibrinogen defects may be quantitative (hypo- or hyper-fibrinogenaemia) or qualitative (dysfibrinogenaemia). Inherited dysfibrinogenaemia is rare but an acquired defect of Fibrinogen function is more common, especially in liver disease when the Fibrinogen molecule is excessively glycosylated impairing its activity. Fibrinogen levels may also be reduced in liver disease due to reduced synthesis. Elevated levels of Fibrin Degradation Products (FDPs) also impair the conversion of Fibrinogen to Fibrin. Fibrinogen levels are an important part of the investigation of a bleeding tendency or an unexplained prolongation of the APTT or PT. Elevated Fibrinogen levels may correlate with an increased risk of thrombosis in epidemiological studies although the significance in individual patients is unclear.

The structure of Fibrinogen is shown below:



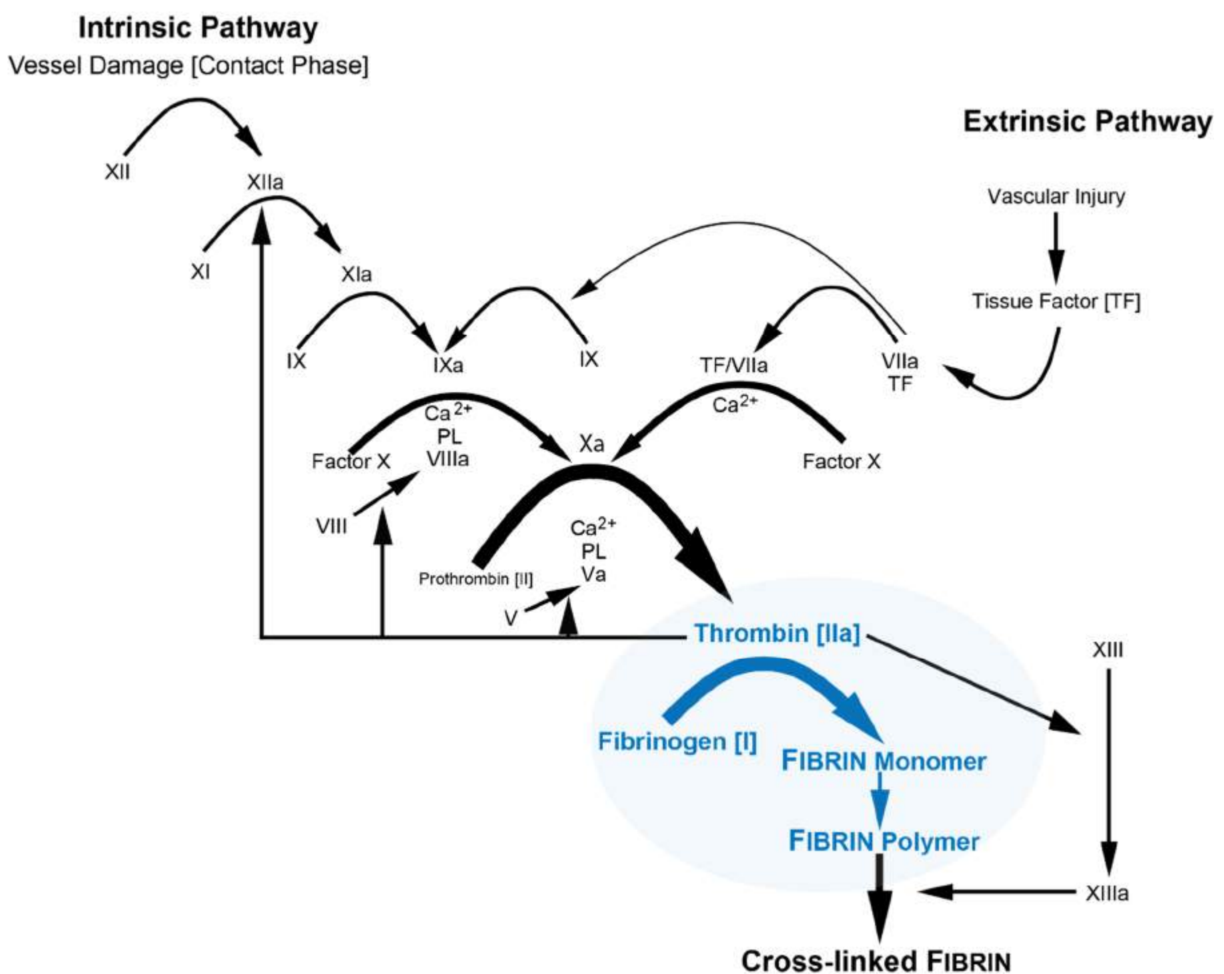
Fibrinogen consists of three pairs of polypeptide chains: two Aα, two Bβ and two γ. These are linked together by 29 disulphide bonds in such a way that N-terminal regions of the 6-polypeptide chains meet to form a central E-domain. The C-terminal regions [Aα, Bβ and γ] form the D-domain and these are joined by α-helical ropes to the central E-domain to give the characteristic Fibrinogen structure.



Activation of Fibrinogen by Thrombin [Factor IIa] cleaves two short peptides from the N-terminal regions of the Aα and Bβ chains - these peptides are known as Fibrinopeptide A [FpA] and Fibrinopeptide B [FpB] respectively. Removal of the N-terminal sequences from Aα and Bβ chains chains reveals new N-terminal sequences in the Aα and Bβ chains located within the E domain, known as 'knobs.' Thrombin cleaves FpA from the Fibrinogen E domain at Arg16-Gly17. These knobs can interact spontaneously with the D-dimer regions to form Fibrin polymers. Under the influence of Factor XIIIa, cross-linking of these Fibrin polymers then occurs to form cross-linked Fibrin polymers.

Principles

There are a number of assay for measuring Fibrinogen levels in plasma although in practice most laboratories use the Clauss method.



Procedure:

Diluted plasma is clotted with a high concentration of Thrombin [~100 U/mL].

1. The test plasma is diluted (usually 1:10 but this may vary if the Fibrinogen concentration is very low or very high) to minimise the effect of 'inhibitory substances' within the plasma e.g. heparin, elevated levels of FDPs.
The use of a high concentration of Thrombin (typically 100 U/ml) ensures that the clotting times are independent of Thrombin concentration over a wide range of Fibrinogen levels.

2. The test requires a reference plasma with a known Fibrinogen concentration and that has been calibrated against a known international reference standard. A calibration curve is constructed using this reference plasma by preparing a series of dilutions (1:5 –1:40) in buffer to give a range of Fibrinogen concentrations. The clotting time of each of these dilutions is established (using duplicate samples) and the results (clotting time(s)/Fibrinogen concentration (g/L) are plotted on Log-Log graph paper. The 1:10 concentration is considered to be 100% i.e. normal. There should be a linear correlation between clotting times in the region of 10-50s.

3. The test platelet poor diluted plasma (diluted 1:10 in buffer) is incubated at 37°C, Thrombin added (all pre-warmed to 37°C). The time taken for the clot to form is compared to the calibration curve and the Fibrinogen concentration deduced. Test samples who clotting times fall outwith the linear part of the calibration curve should be re-tested using different dilutions.

Reference Ranges

The reference range for Fibrinogen is generally between 1.5-4.0g/L

11. Bone Marrow Examination

Red blood cells, most white blood cells, and platelets are produced in the bone marrow, the soft fatty tissue inside bone cavities. Sometimes a sample of bone marrow must be examined to determine why blood cells are abnormal or why there are too few or too many of a specific kind of blood cell. A doctor can take two different types of bone marrow samples:

- Bone marrow aspirate: Removes fluid and cells by inserting a needle into the bone marrow and sucking out (aspirating) fluid and cells

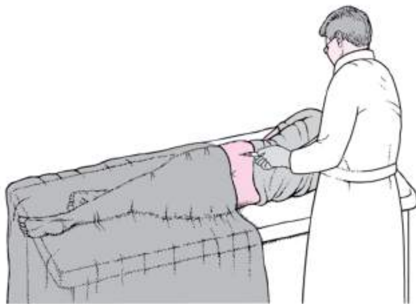
- Bone marrow core biopsy: Removes an intact piece of bone marrow using a coring device (similar to a large diameter needle)

The bone marrow aspirate shows what cells, normal and abnormal, are present in the bone marrow and provides information about their size, volume, and other characteristics. Special tests, such as cultures for bacteria, fungi, or viruses, chromosomal analysis, and analysis of cell surface proteins can be done on the sample.

The core biopsy removes an entire piece of bone marrow and shows not only what cells are present but also how full the bone marrow is with cells and where the cells are located within the marrow.

Taking a Bone Marrow Sample

Bone marrow samples are usually taken from the hipbone (iliac crest). The person may lie on one side, facing away from the doctor, with the knee of the top leg bent. After disinfecting the skin and numbing the area over the bone with a local anesthetic, the doctor inserts a needle into the bone and withdraws the marrow.



Although the aspirate often provides enough information for a diagnosis to be made, the process of drawing the marrow into the syringe breaks up the fragile bone marrow. As a result, determining the original arrangement of the cells is difficult.

When the exact anatomic relationships of cells must be determined and the structure of the tissues evaluated, the doctor also does a core biopsy. A small core of intact bone marrow is removed with a special bone marrow biopsy needle and sliced into thin sections that are examined under a microscope.

Both types of samples are usually taken from the hipbone (iliac crest), often during a single procedure. Aspirates are rarely taken from the breastbone (sternum). In very young children, bone marrow samples are occasionally taken from one of the bones in the lower leg (tibia).

A bone marrow sampling begins with cleaning, sterilizing, and anesthetizing the skin over the bone. The procedure generally involves a slight jolt of pain, followed by minimal discomfort. The procedure takes a few minutes and causes no lasting damage to the bone.

Preparing for a Bone Marrow Examination

Obtaining Patient Medical Information

A successful bone marrow evaluation requires knowledge of the patient and the reason(s) the study was requested. The following information should be obtained when the laboratory is first contacted to schedule the marrow study:

Patient name

Patient age and gender

Patient location/requested time of examination

Primary diagnosis

Clinical indication(s) for examination

Allergies (especially to povidone iodine and lidocaine)

Recent chemotherapy, radiation therapy, bone marrow transplantation, or blood transfusions

Dietary, racial, and family history

Medications (iron, B12/folate, G-CSF, aspirin, coumadin, heparin, antibiotics, etc.)

Special studies requested (immunophenotypic analysis, cytogenetic analysis, culture, etc.)

Special medical problems that may preclude procurement or written consent or complicate the procedure (i.e., unresponsive or mentally incompetent patient, adversity to medical procedures, anxiety, pain intolerance, disease or recent surgery involving the pelvic bone, hemophilia or other bleeding disorder, severe cardiac or pulmonary disease, morbid obesity etc.)

The request for a bone marrow procedure should be validated by reviewing pertinent laboratory data, the patient’s medical record, and a recent peripheral blood smear. It is not unusual for junior doctors to overlook a less invasive diagnostic procedure before requesting a bone marrow examination. For example, a CBC, serum iron studies, and a serum ferritin assay should be performed on a patient with suspected iron deficiency anemia, but a bone marrow examination would be an unusual request. If the request seems inappropriate, the requesting physician should be contacted for verification. Some hospitals require a formal consultation from the Hematology/Oncology Service to justify a request from another department, since the bone marrow examination is an invasive, expensive, and labor intense procedure. If the procedure is requested as part of a research protocol, a detailed list of the required specimens, specimen preparation directives, and transportation directions should be obtained. Arrangements for pain medication of other special patient needs should be made by the requesting physician prior to the arrival of the marrow procurement team. At a minimum, this should include an “as needed” (i.e., “PRN”) medication order placed on the patient’s chart.

Supplies and Equipment

Since bone marrow procedures are usually performed in the clinic or at the bedside, appropriate supplies and equipment must be carried to the site. A compartmentalized plastic or wooden tray is usually used for this propose, or the equipment may be carried in a wheeled cart with a flat work surface for preparing the marrow slides. Adequate routine supplies to perform several bone marrow examinations should be carried in the tray, as well as any special tubes, preservative solutions, etc.

Reviewing the Patient's Medical Record

The patient's chart should be reviewed upon arriving at the location of the marrow procedure to verify the information previously provided to the laboratory. Do not assume that this information is complete or correct. The following facts should be verified:

Is the patient identification correct? Use hospital numbers in addition to names.

Is the request for a marrow procedure justified?

Can the patient give written consent for the procedure? If not, obtain the name and telephone number of the person giving consent Does the patient have special medical problems which may complicate the procedure? These may include disease or recent surgery involving the pelvic bone, bleeding, severe cardiac or pulmonary disease, unusual sensitivity to pain, adversity to medical procedures, allergies to iodine or lidocaine, extreme obesity, etc.

Once the chart review is completed, the nurse caring for the patient should be notified of the procedure and necessary assistance requested.

Meeting the Patient

The identification of the patient must be absolutely confirmed, preferably by verifying the hospital number and name from a wrist band or identification card. If such is not available, the

patient should be asked to state their name and asked whether they were expecting to have a marrow performed. The marrow team should be introduced to the patient. The procedure must be explained to the patient, all questions answered to the satisfaction of the patient and family members, and written consent obtained from the patient. If the patient cannot provide written consent, it should be obtained from the next-of-kin. In the rare circumstance of an incapitated patient without a family, a court order must be obtained. Under no circumstances should a bone marrow be obtained without written permission. Individuals performing a bone marrow procedure must also be thoroughly familiar with and follow all institutional policies regarding consent for medical procedures.

All questions should be answered completely and the patient should then be given the opportunity to sign the written consent form. Some patients are reluctant at first to grant consent and require further persuasion or time to consult with their family or attending physician. The attending physician should be notified if the patient refuses to grant written consent. Although the vast majority of patients do not require pharmacologic intervention other than local anesthesia, the procedure may need to be delayed until the proper type of sedation can be arranged.

Bedside Preparation

General Considerations

Hematopoietically active bone marrow is distributed throughout the skeleton in children, but it is restricted to the axial bones of adults. Of the potential sites to obtain the bone marrow, the posterior iliac crest is optimal for reasons of safety and ease of performance. Alternative sites should be considered if the posterior iliac crest is diseased or inaccessible because of morbid obesity or inability to position the patient correctly. These alternative sites include the tibia (infants only), anterior iliac crest (children and adults), and sternum (adults only, aspiration only). Sternal marrow examination should be considered only if other sites are unacceptable, and is completely contraindicated in patients with diseases associated with bone resorption, including multiple myeloma (Foucar, 1995).

There is a continuing debate about adequate marrow sampling for various purposes. Most studies of multiple marrow sites have revealed marrow cellular content, cellular composition, and pathologic lesions to be rather uniformly distributed through the bone marrow. Therefore, most hematopathologists today consider an adequate sample from a single site acceptable in most patients.

The best sequence to obtain marrow specimens is also controversial. The biopsy may be altered by needle artifact if the aspirate is obtained first, while the aspirate specimen may clot if the biopsy is performed first and locally activates the coagulation system. However, Foucar (1995) feels that the sequence is unimportant, as long as different areas along the posterior iliac crest are sampled.

Positioning the Patient

The patient is positioned as follows, depending on the location of the procedure:

Posterior iliac crest (PIC) – The patient is placed in a right or left lateral decubitus position with their knees flexed, a pillow under their head, and their eyes away. The posterior iliac crest may be used in patients over one year of age.

Anterior iliac crest (AIC) - The patient is placed in a supine position, with their hips and knees flexed, and eyes averted away. This site is appropriate only in adults when the posterior iliac crest is inaccessible because of obesity, infection, injury, or inability to position the patient in the lateral decubitus position. The thick, hard cortical layer of the anterior iliac crest makes satisfactory specimens more difficult to obtain and the needle can enter the peritoneal cavity. In addition, needle biopsy of anterior superior iliac spine has been reported to be more painful, and to produce samples of smaller length and area than biopsies of the posterior superior iliac spine.

Sternum - Supine position, head and eyes away, light towel over face "to keep things sterile" and cover eyes. Sternal aspiration should be performed only if the posterior and anterior iliac crests are inaccessible or unsuitable for the procedure. Furthermore, sternal aspiration should be attempted only in adolescents and adults, since there is a higher incidence of serious complications in infants and children.

Tibia – Marrow aspiration from the anteromedial surface of the tibia is performed only in children less than 18 months of age. The tibia is an unsatisfactory site in older individuals because of variable cellularity and the hardeness of the cortical bone.

A continuing conversation should be began with the patient and continued throughout the entire procedure. This is necessary to inform the patient about anticipated discomfort from the procedure, to assess the patients feeling of pain, and to obtain early warning of complications such as a vasovagal reaction.

Nonsterile latex "examination" gloves and a plastic procedure gown or other protective clothing should be worn. The patient's back should be carefully palpated to identify anatomical landmarks and the appropriate anatomic site for marrow procurement. To identify the chosen site after the area is cleaned with povidone-iodine soap, it can first be highlighted with an indelible pen or by making a shallow impression in the skin with the tip of a plastic ear speculum. One of the following locations is chosen.

PIC - Center of posterior superior iliac spine

AIC - Center of prominence of anterior superior iliac spine, just under lip of crest

Sternum - Second intercostal space in midline The skin surrounding the procedure site should be cleaned as follows:

Use three sterile, disposable swabs soaked with 10% povidone-iodine solution (Betadine Solution, Purdue Frederick Company). For individuals allergic to iodine, chlorhexidine gluconate, 4% (Betasept Surgical Scrub, Purdue Frederick Company) may be utilized.

With each of the three swabs, wash the skin in a circular motion beginning with the marked site and working outward approximately four inches.

Remove the povidone-iodine in the center of the washed area with a single swipe of a sterile isopropyl-soaked swab.

Most patients who are anxious at first are adapting well to the experience by this time, but the anxiety level actually increases in a few patients. occasional patients may require conscious sedation to permit proper marrow procurement.

Anxious patients who have an intravenous (IV) line in place can be given diazepam (“Valium”) by the assisting nurse or physician. This should be slowly hand-pushed (1 mg/min) into a rapidly running IV until the patient’s speech is slurred (keep the patient talking!). This may require 5-20 mg of diazepam over 5-10 minutes. The patient usually falls asleep and snores, but can be aroused. This sedation lasts 20 min to 2 hours and usually produces desirable amnesia for the procedure. Be sure to have Ambu-Bag nearby, just in case! ... But it wouldn’t be needed.

Place a sterile drape with a fenestrated opening over the area to be sampled.

Administering Local Anesthesia

Once a sterile site has been achieved, a local anesthetic is utilized to “numb” the skin and periosteum over the chosen area of the posterior iliac crest. Lidocaine or a similar local anesthetic can be used, providing the patient has no history of an allergic reaction to this medication (BE SURE TO ASK!). During this process, local anesthetic is first infiltrated into the skin and subcutaneous tissue to anesthetize an area approximately 1 cm. in diameter.

* Dosages vary with weight, age, etc. and should be adjusted to the individual patient and desired level of sedation.

** Midazolam may cause severe respiratory depression and should only be used in situations where heavy sedation is required. Consult PDR for current dosing recommendations.

After the skin is numb, lidocaine is infiltrated directly over the periosteum to numb an area approximately 2-3 cm in diameter. Discomfort can be avoided during the remainder of the procedure if adequate time is taken to assure good anesthesia. Local anesthesia is administered as follows:

Aspirate 2 mL of 1% sterile sodium bicarbonate solution (to reduce the burning effect of the acidic lidocaine solution) and 8 mL 1% lidocaine hydrochloride (Xylocaine, Astra Pharmaceuticals) into a 10 mL syringe.

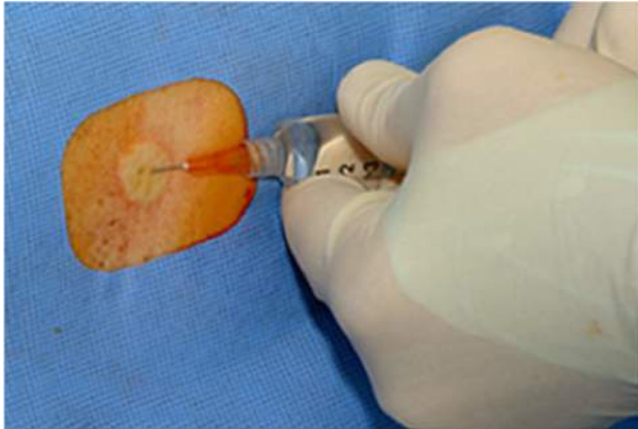
Slowly infiltrate the skin with the buffered lidocaine, raising a “dime-sized” intradermal wheal with 26-gauge needle.

Infiltrate the subcutaneous tissue and periosteum, using a 22-gauge needle (or spinal needle for overweight patients). This usually requires 2-5 mL of buffered lidocaine. Assess the thickness of the subcutaneous tissue and the depth to the periosteum for later reference.

Determine the adequacy of local anesthesia after several minutes by gently tapping the periosteum with the sharp point of the numbing needle. If sharp pain is stilled experienced, the injection of additional lidocaine is required. Unbuffered lidocaine is used for this purpose.

Caution! - Adverse reactions of a neurologic, cardiovascular, and allergic nature can occur to lidocaine. The maximum recommended dose of lidocaine with epinephrine for healthy adults is approximately 7 mg/kg or 500 mg total dose (50 mL of 1% lidocaine).

Alternative local anesthetics can be used in patients who have a known hypersensitivity to lidocaine. These include chloroprocaine (Nesacaine, Astra Pharmaceutical) and bupivacaine hydrochloride (Sensorcaine, Astra Pharmaceuticals). Another alternative in patients who are allergic to lidocaine is administer methylprednisone (40 mg) and benadryl (50 mg) intravenously immediately prior to the injection of lidocaine, followed by oral prednisone (1 mg/kg) in two divided doses over 24 hours after the procedure (Saul Yanovich, M.D., personal communication). Since anaphylactic reactions can occur in patients without previous history of an allergic reaction, an emergency kit with an airway and injectable epinephrine and hydrocortisone should be available for immediate use.



Bone Marrow Aspiration Technique

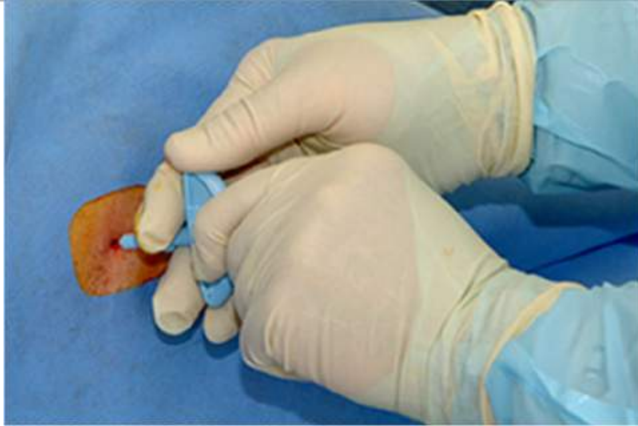
Marrow aspiration from the posterior or anterior iliac crest is performed as follows:

Fill the necessary number of 10 mL syringes with heparin solution or other anticoagulant as required. Regardless of the suspected diagnosis or purpose of the study, it is best to obtain at least one heparin-anticoagulated tube of marrow aspirate, “just in case” it is needed for special studies (i.e., microbiologic culture, immunophenotypic analysis, cytogenetic analysis, molecular biology studies, etc.).

Obtain the desired marrow aspirate needle from the assistant and inspect for signs of manufacturing defects. Remove the plastic guard from the needle (if one is present). Loosen and remove the obturator to make certain that it can be removed with ease. Insert obturator and relock. Hold the needle with index finger near needle tip to control the depth of penetration.

Hold needle horizontally (for a patient lying on their side) or vertically (if supine) to puncture the anesthetized skin. If the skin is tough, make a small incision with a sterile scalpel.

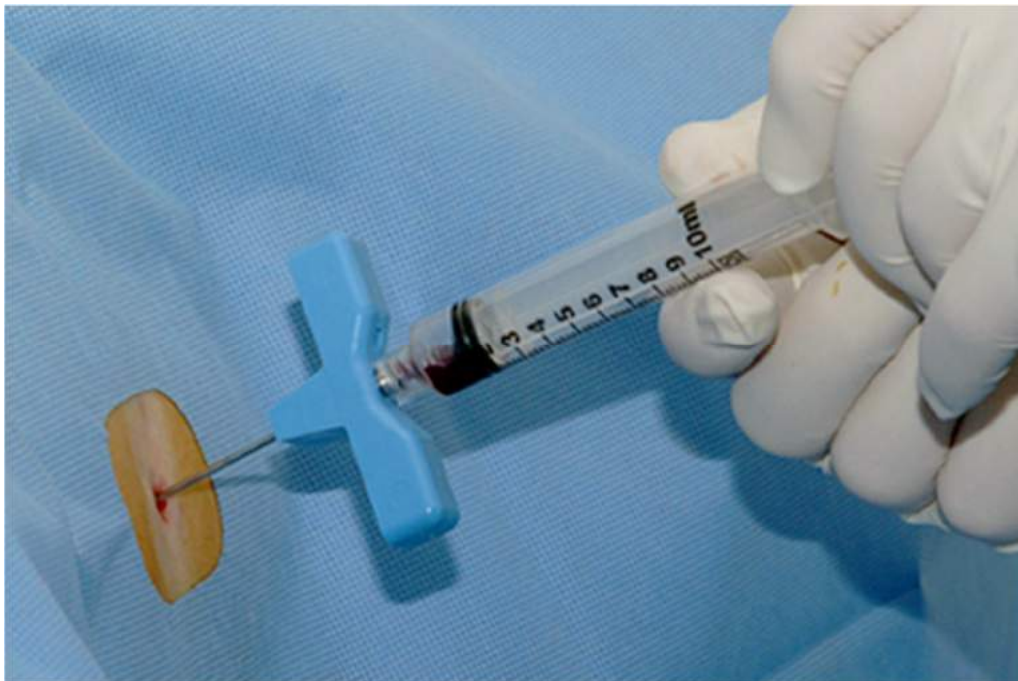
Advance the needle with steady pressure and a slight twisting motion to the center of the posterior iliac prominence (PIC) or to the bone (AIC). Angle the needle 15 degrees caudad (PIC) or cephalad (AIC).

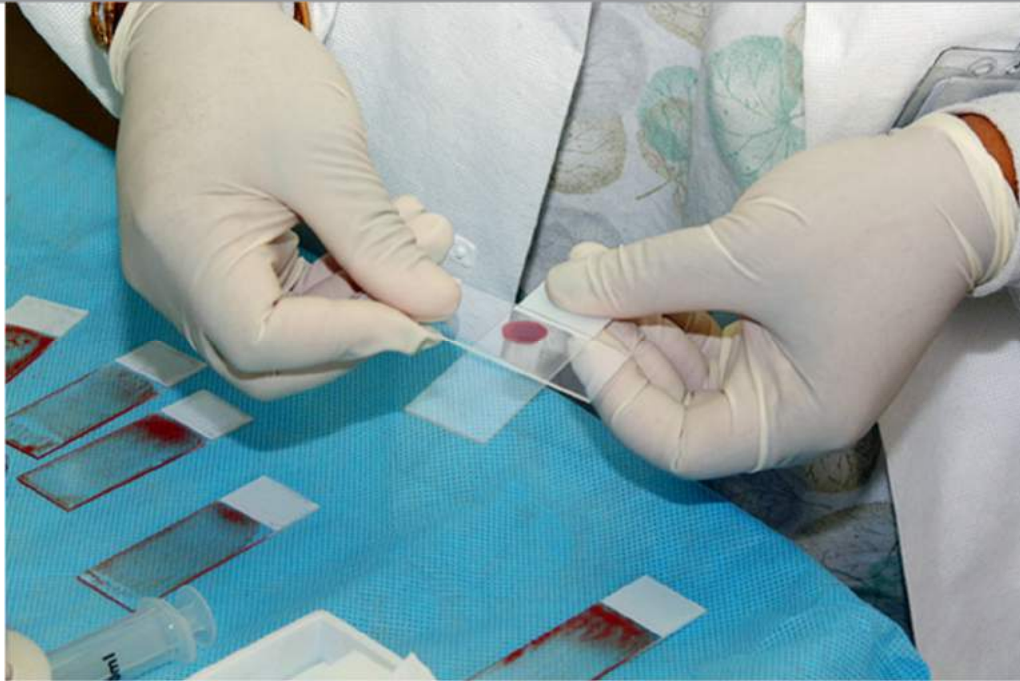


Rotate the needle back and forth (90o-180o) and carefully apply pressure to advance the needle through the cortical bone. The consistency of the bone varies considerably from patient to patient, but may have significance as follows: Soft (“Swiss cheese”) consistency = osteoporotic bone (elderly patient, multiple myeloma, renal failure, some post-chemotherapy patients), firm (“pine board”) consistency = Normal for young athletic individuals, very hard (“oak board”) consistency = possible hyperostosis.

Decreased resistance (Usually!) indicates penetration of cortex and entry into the marrow cavity.

Advance needle about 1 cm into the marrow cavity. Unlock and slowly remove the obturator. Some patients may notice pain if the obturator is not removed carefully.





Attach a 10 ml syringe to the aspirate needle. Quickly (< 5 seconds) aspirate 1.0 mL marrow into the 10 mL syringe (more than this dilutes the specimen with peripheral blood). BEWARE! The sudden sharp pain may cause the patient to shout, move suddenly, or even try the strike you! Remain alert, try to maintain sterility, and calm the patient quickly if this happens.

Quickly give the syringe to the technical assistant to prepare specimen slides. Hold a finger over needle opening to prevent blood flow while the technician prepares slides and evaluates for the presence of spicules.

If spicules are present, extra marrow specimen(s) for special studies can be obtained. Aspirate approximately 2 mL of marrow into a syringe containing 1 mL of heparin solution.

If a “dry tap” (no fluid, no sharp pain) occurs, then reposition needle (depth, angle or location) and try again. As a “last resort” touch preparations can be prepared from the core biopsy. Try the opposite side if necessary.

Remove aspiration needle and apply pressure with a sterile sponge until bleeding ceases.

Perform a bone marrow biopsy or place a folded piece of gauze over the site, apply a pressure bandage, and have patient lie supine for at least 30 minutes (see “Finishing Up” below).

Marrow Aspiration: Sternum

Marrow aspiration from the sternum is usually performed only when the posterior and anterior iliac crests are severely diseases or inaccessible as a result of massive obesity. In addition to the rare, but very serious complication complication of entering the mediastinum during the procedure, the sternum is an unsuitable site for biopsy procurement. If a sternal aspiration is necessary, the following procedure is used.

Use “Illinois” needle with guard.

Assess subcutaneous thickness during local anesthetic.

Adjust guard to 5-10 mm depth.

Hold needle perpendicular to skin; insert down to bone.

Rotate needle and advance.

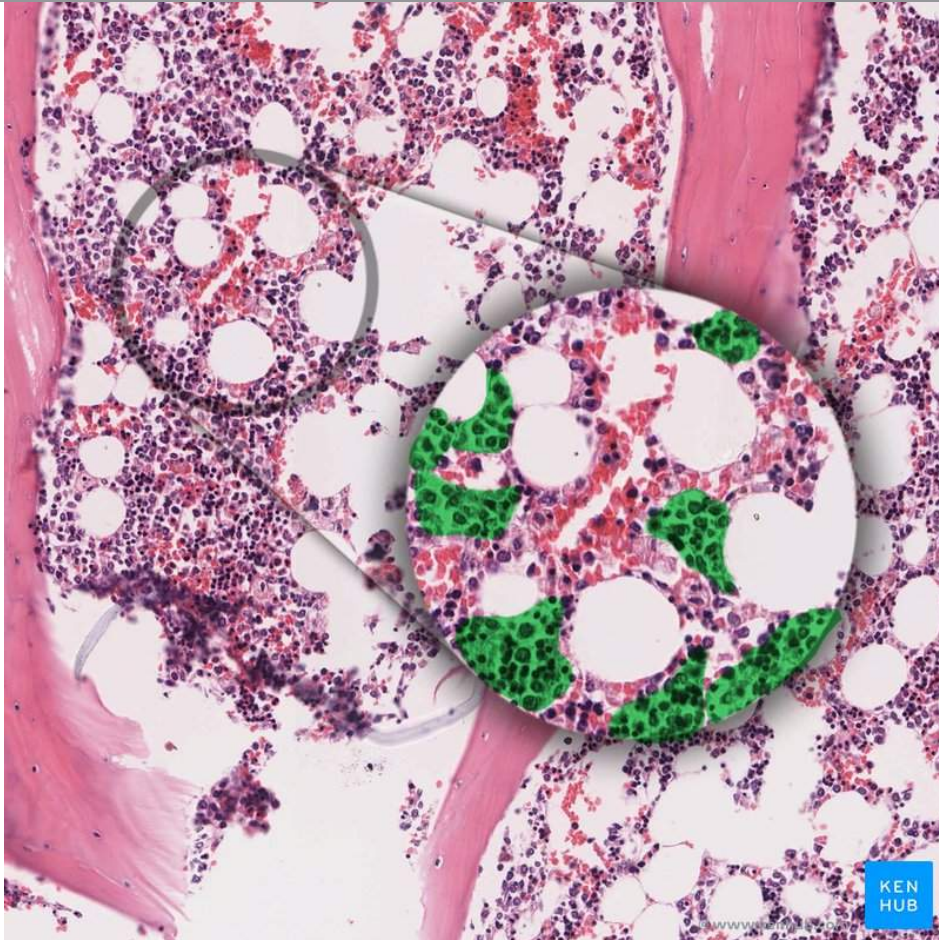
Decrease in resistance (usually!) indicates entry into marrow cavity.

Avoid penetrating the posterior table of the sternum (if the needle enters the mediastinum, you may see fluid bubbles as the patient breathes when the stylus is removed).

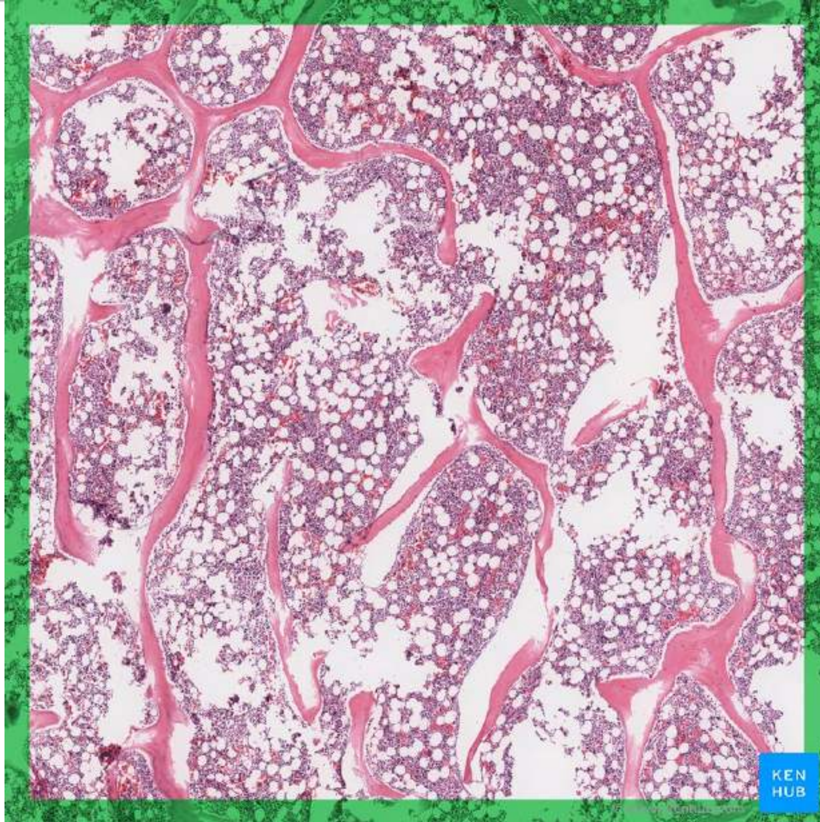
Evaluation technique:

Red bone marrow

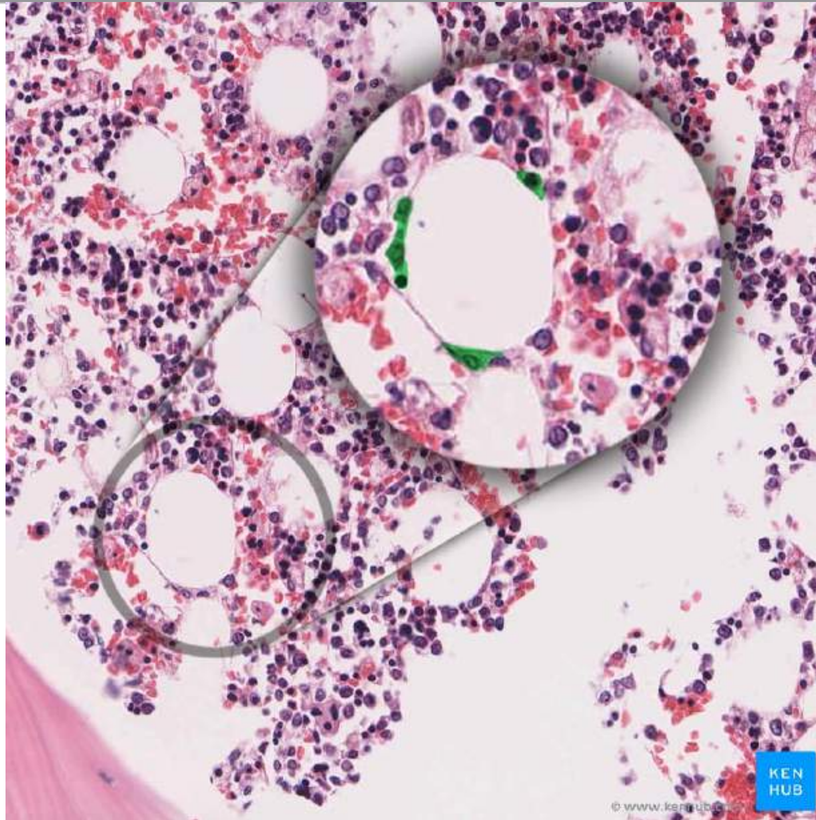
Clusters of haematopoietic cells known as **haematopoietic islands** are widely distributed throughout the loose connective tissue network observed in red marrow. These islands are found next to relatively large, yet thin walled, sinusoids that also communicate with **nutrient vessels** of the bone. The **sinusoids** are situated at a central part of a roundabout circulation such that the nutrient arteries that leave the **nutrient canals** to supply the bones anastomose in the bone marrow and subsequently terminate in arterioles that coalesce to form the sinusoids. The sinusoids then drain to significantly larger veins that form **nutrient veins**, which then leave the bone via the same nutrient canals that the arteries enter by.



Red marrow is most abundant in all skeletal structures from intrauterine life up until around the 5th year of life. As time progresses, red marrow is restricted to the central flat bones (i.e. cranial bones, clavicle, sternum, ribs, scapula, vertebrae, and pelvis) and the proximal ends of the proximal long bones of the upper and lower limbs.

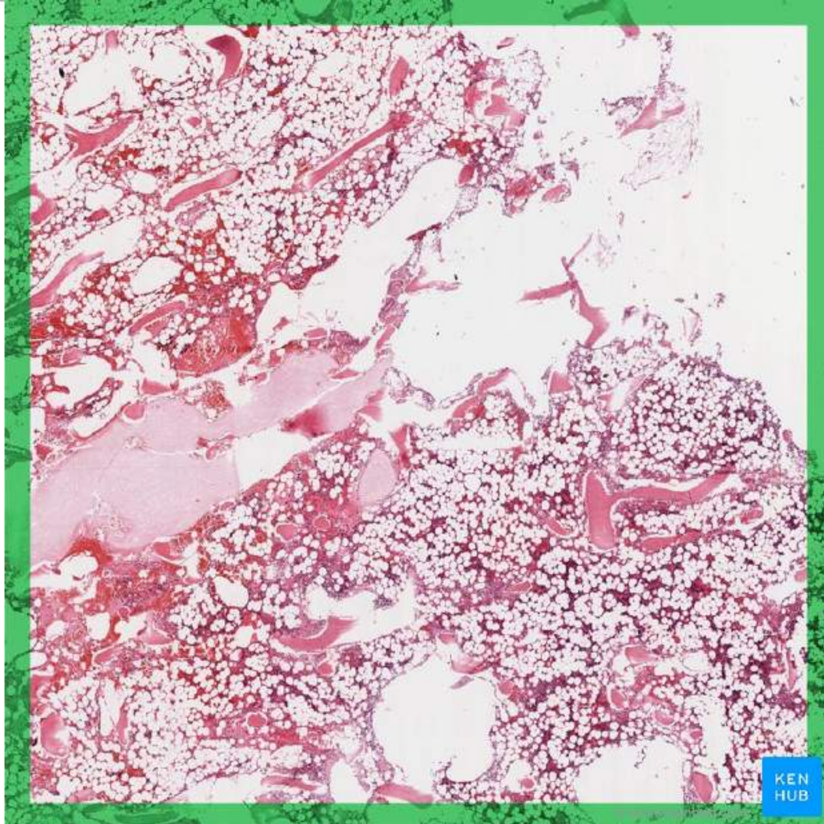


The supporting substance that supports the haematopoietic and adipocyte cells in the marrow is made up of **reticulin**. This is a fine **type III collagen** that is produced by mesenchyme derived **reticular cells** (fibroblast-like cells). Other housekeeping cells like macrophages exist in the stroma and facilitate haematopoiesis by phagocytosing cellular debris generated from the process.



Yellow bone marrow

Depending on the age and haematological demand of an individual, the reticular cells become swollen as a result of increased lipid uptake. Subsequently, yellow marrow is formed. It contains mainly supportive connective tissue that provides scaffolding for the neurovascular structures that traverse the cavitation. There are also numerous **adipocytes** in addition to very few dormant haematopoietic clusters. These latent **haematopoietic centres** can be reactivated in the event of an increase demand for red blood cells.



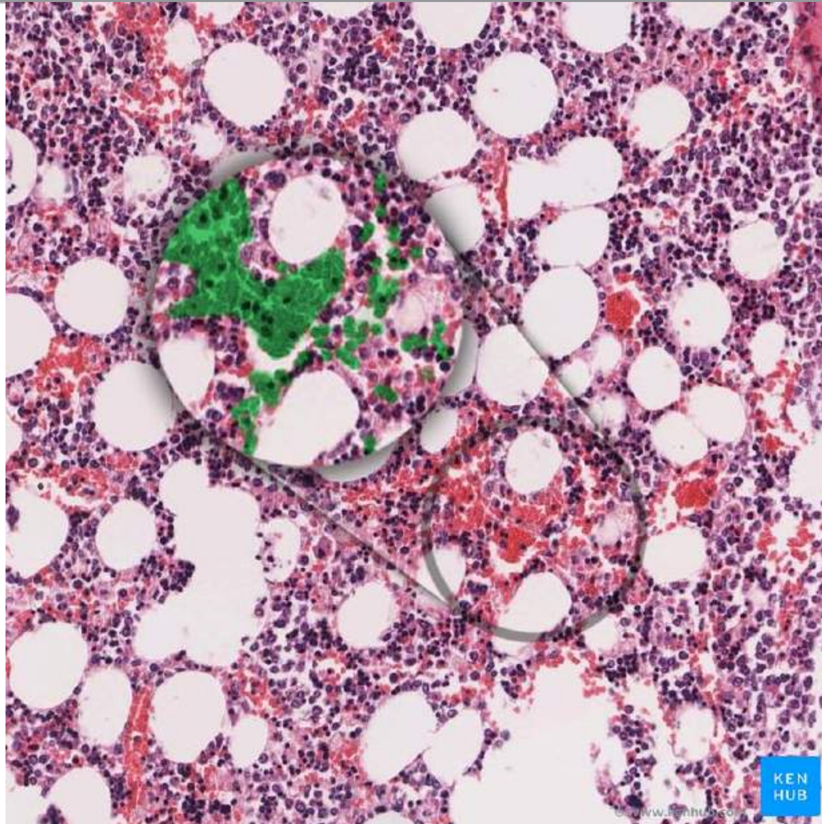
Cell types

Histological analysis of the bone marrow will reveal an abundance of **progenitor cells** and their derivatives at different stages of development. Typically, the progenitor cells are larger than their end products. The suffix "-blast" is often used to denote that the cell line being referenced are the stem cells for that series (i.e. erythroblasts are the precursor cells for red blood cells [erythrocytes]). The following is a list of the cell lines found in the bone marrow:

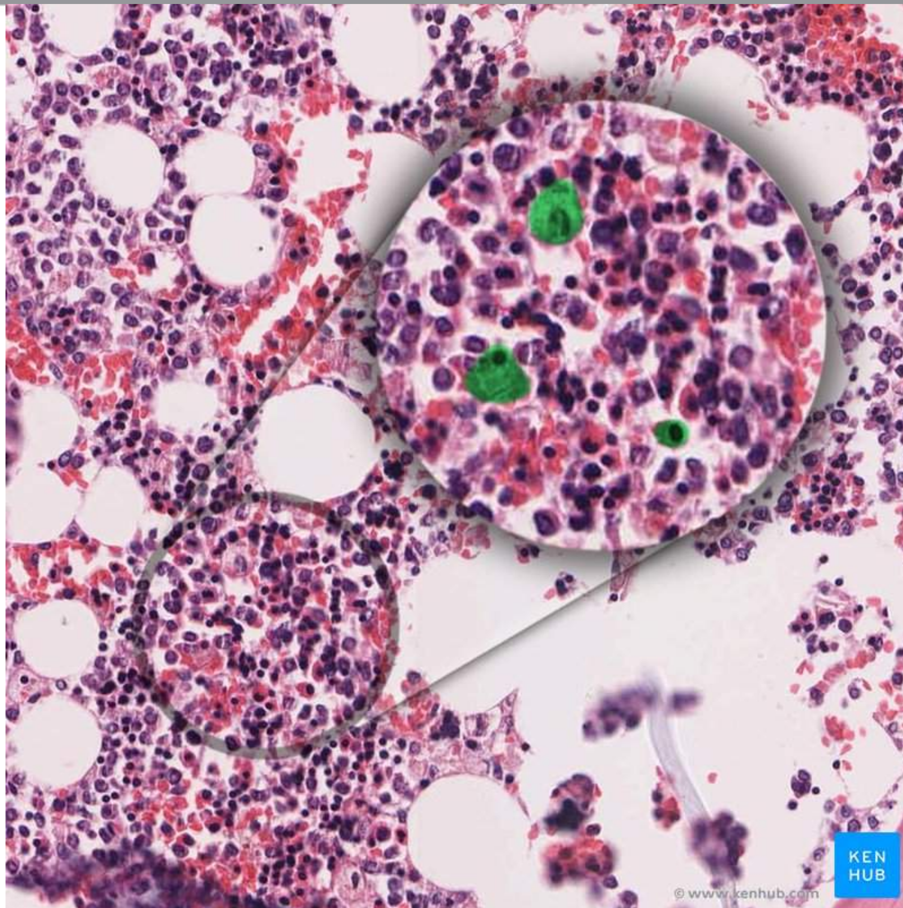
- **Granulocytes** – are a special line of white blood cells that possess secretory granules in their cytoplasm. There are three granulocytes; these are **eosinophils**, **basophils** and **neutrophils**.

- Monocytes – are leukocytes that differentiate into macrophages. Recall that there are different subtypes of macrophages depending on the region of the body that they are found in (i.e. Kupffer cells of the liver).

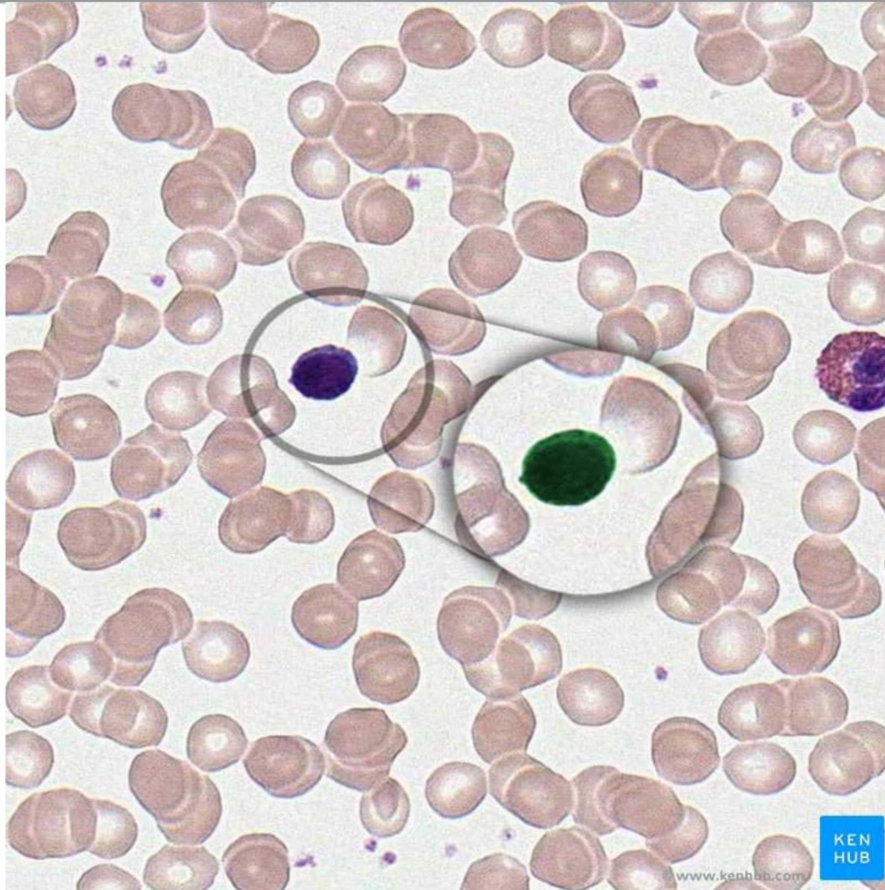
- Erythrocytes – are the anucleate, biconcave, oxygen-carrying species.



- Megakaryocytes – is another large species that is responsible for thrombocytogenesis (i.e. platelet production).



Lymphocytes – are all produced in the bone marrow. However, education and maturation of one subset of lymphocytes occurs in the thymus (i.e. T-lymphocytes)



The stroma also contains a myriad of stem cells of mesenchymal origin. These include **multipotent cell lines** that are capable of differentiating into cartilaginous cell lines (chondrocytes), bone cells (osteoblasts and osteoclasts) in addition to **adipocytes**, **myocytes** (muscle) and **endothelial cells**.

12. Serological Diagnosis of HIV Infection

Laboratory diagnosis by HIV testing is the only method of determining the HIV status of an infected individual's infected blood, blood products, organs, and tissues. HIV diagnosis at ICTCs and other laboratories is based on the demonstration of antibodies. Antibody detection can be done using an ELISA test, rapid test, and western blot test. These tests are used as screening tests and/or confirmatory tests. All tests should be performed and interpreted as per test instruction manuals that are supplied with the kit. HIV testing should be based on testing strategy and algorithm.

A number of moral, legal, ethical, and psychological issues are related to a positive HIV status; hence, any laboratory attempting to assess the HIV status of an individual should be conversant with these issues. Testing laboratories should ensure pre and post-test counselling for every individual and confidentiality to be maintained.

Objectives of Testing

Transfusion and transplant safety

Diagnosis of HIV infection in symptomatic and asymptomatic individuals

Prevention of parent to child transmission

For Post-Exposure Prophylaxis (PEP)

Epidemiological surveillance using unlinked anonymous HIV testing

Research

Pre-test Counselling

HIV testing when undertaken for assessing the status of an individual, should always be done after the pre-test counselling and after an informed consent by client. Testing without informed and explicit consent has proven to be counterproductive and has driven HIV positive individuals underground. Pre-test counselling along with post-test counselling prepares the individual to cope with the HIV test results. It is the responsibility of all blood collection centres to ensure that pre-test counselling is done before collection.

Confidentiality

The confidentiality of HIV test results should be maintained for both positive and negative reports. This is essential for ensuring respect for the privacy and rights of an individual and to protect them from victimization, discrimination, and stigmatization. The results should be handed over directly to the person concerned, to a person authorized by the patient, or in a sealed envelope to the clinician requesting for the test. No results, under any circumstances, should be communicated via telephone, fax, email, etc. The records must be kept secure.

Detection of Anti-HIV Antibodies

The central component in the diagnosis of HIV infection is the detection of anti-HIV antibodies in serum, plasma, or whole blood. Urine and saliva may be tested using specific kits. HIV antibody assays are commercially available in various formats.

Some of these assays can differentiate between HIV-1 and HIV-2 infections. However, the occurrence of antibody-cross reactivity makes differentiation difficult between HIV-1 and HIV-
2. Differentiation between HIV-1 and HIV-2 is required since the treatment varies for the two types.

Technical errors and interference from other medical conditions may compromise the accuracy of HIV tests. Antigens used in HIV diagnostic tests must be appropriately specific and are usually purified antigens from viral lysates or antigens produced through recombinant, or synthetic, peptide technology. Such antigens helps to improve the sensitivity (true positives) and specificity (true negatives) of HIV assays.

Along with the testing process, there is the requirement for a dedicated quality system in the laboratory to ensure accuracy and reproducibility of test result.

Screening Tests

Serological tests for the detection of HIV are classified as first to fourth generation tests based

on the type of antigens used and principle of the assays (Table 3.1). NACO recommends the use of rapid test kits, which detect >99.5% of all HIV-infected individuals and have false-positive results in <2% of all those who are tested.

Commonly used screening tests are:

Enzyme Linked Immunosorbent Assay (ELISA)

Rapid tests

Immunoconcentration/Dot Blot assay (vertical flow)

Agglutination assay

Immunochromatographic assay (lateral flow)

Dipstick and comb assay based on Enzyme Immune Assay (EIA)

Enzyme Linked Immunosorbent Assay (ELISA)

All ELISAs consist of either HIV antigens or antibodies (depending upon the principle), attached to a solid phase (matrix or support), and incorporated with a conjugate and substrate detection system. Viral antigens may be whole viral lysates, recombinant, or synthetic peptides. The matrix can be "wells" or "strips" of a microplate, plastic beads, or nitrocellulose paper. Conjugates are most often antibodies (lgG, sometimes lgM and IgA) coupled to enzymes (alkaline phosphatase or horseradish peroxidase), fluorochromes, or other reagents that will subsequently bring about a reaction that can be detected. In case of enzyme conjugates, the signal generated is a colour reaction and in case of fluorochrome, it is fluorescence. The substrates used are 4-nitrophenylphosphate – for alkaline phosphatase and ophenylenediamine dihydrochloride (OPD) and TMB – for horseradish peroxidase, which produce colour on being acted upon by the respective enzymes. The colour can be measured on an ELISA Reader as optical density (OD) values. ELISAs are suitable for use in laboratories where the specimen load is high.

Indirect ELISA

This is the most commonly used principle. HIV antigens are attached covalently to the solid phase support. This allows HIV antibodies present in the specimen to bind. These bound antibodies are subsequently detected by enzyme labelled anti-human immunoglobulin and a specific substrate system. If the test specimen contains anti-HIV antibodies, a colour reaction will take place.

Procedure: The instructions in the kit insert are to be carefully followed. All specified controls should be included with each test run to validate the test result. Appropriately dilute the specimen, add to the solid phase, and incubate for a specified time and temperature

Solid phase is washed to remove unbound antibodies. Appropriately diluted enzyme conjugate is added and incubated as specified. Solid phase is washed to remove excess conjugate

Substrate is added, Colour change produced is measured after the specified time has passed using an ELISA reader at the specified wavelength

The result is interpreted as detailed in the kit insert from the various OD values obtained.

13. Hepatitis B Rapid Tests

HBcAb

Product Number: RAPG-HBcAb-001

Pack size

20 tests

Product Description

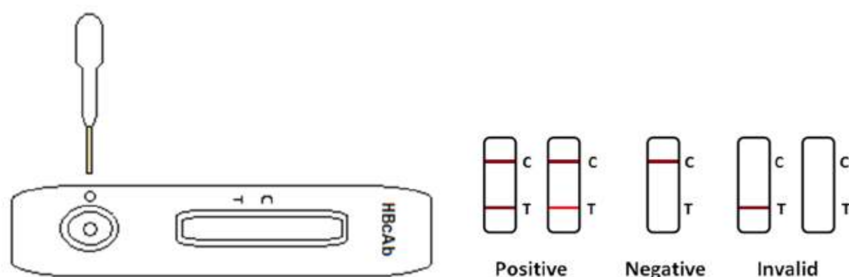
The Biopanda HBcAb Rapid Test qualitatively detects Hepatitis B Core Antibodies (HBcAb) in human serum or plasma samples.

Test Attributes

- Serum or plasma samples may be used for testing
- Results available in only 15 minutes
- Easy to use
- Results can be read visually
- No need for an analyser
- Cost effective method for assisting in the detection of Hepatitis B core antibodies

Test Procedure

1. Ensure specimen and test kits are brought to room temperature before testing.
2. Open the foil wrapped pouch and remove the cassette. Place the cassette on a flat, clean surface. Use test immediately after opening.
3. Using a dropper provided transfer 3 drops of the serum or plasma (approx. 75 µl) to the sample well on the cassette. Avoid trapping air bubbles.
4. Read results at 15 minutes.
5. Results read after 20 minutes are considered invalid. Dispose of the cassette safely after testing.



Principle

The Biopanda HBcAb test is an immunoassay based on the principle of competitive binding. During testing, the mixture migrates laterally on the membrane chromatographically by capillary action. The membrane is pre-coated with Hepatitis B core Antigen (HBcAg) on the test line region of the strip. During testing, if HBcAb is present in the specimen, it will compete with the particle coated HBcAg antibody for limited amount of HBcAg on the membrane. No

line will form in the test region. And a visible coloured line will form in the test region if there is no HBcAb in the specimen because all the antibody coated particles will be captured by the antigen coated in the test line region. To serve as a procedural control, a coloured line will always appear in the control line region indicating that proper volume of specimen has been added and membrane wicking has occurred.

Performance Characteristics

- Relative sensitivity: 97.8%
- Relative specificity: 97.7%
- Accuracy: 97.8%

Storage Conditions

Store the kit between 2-30°C and ensure the kits are not frozen or stored in direct sunlight. The test is valid until the expiration date printed on the foil wrapping.

14. Administering, reading and interpreting a tuberculin skin test.

Tuberculin skin test (TST) is the intradermal injection of a combination of mycobacterial antigens that elicit an immune response (delayed-type hypersensitivity), represented by induration, which can be measured in millimetres.

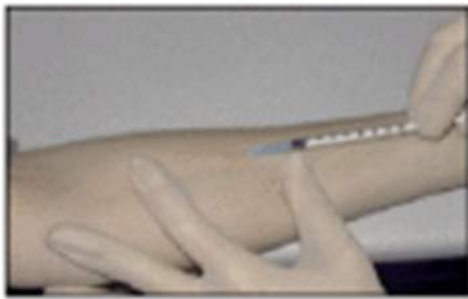
The standard method of identifying people infected with M. *tuberculosis* is the TST using the Mantoux method. Multiple puncture tests should not be used as these tests are unreliable (because the amount of tuberculin injected intradermally cannot be precisely controlled).

This annex describes how to administer, read and interpret a TST using 5 tuberculin units (TU) of tuberculin PPD-S. An alternative to 5 TU of tuberculin PPD-S is 2 TU of tuberculin PPD RT 23.

Administration

1. *Locate and clean injection site 5–10 cm (2–4 inches) below elbow joint*
   - Place forearm palm-up on a firm, well-lit surface.
   - Select an area free of barriers (e.g. scars, sores, veins) to placing and reading.
   - Clean the area with an alcohol swab.
2. *Prepare syringe*
   - Check expiry date on vial and ensure vial contains tuberculin PPD-S (5 TU/0.1 ml).
   - Use a single-dose tuberculin syringe with a short (¼- to ½-inch) 27-gauge needle with a short bevel.
   - Clean the top of the vial with a sterile swab.
   - Fill the syringe with 0.1 ml tuberculin.

3. *Inject tuberculin*
    - Insert the needle slowly, bevel up, at an angle of 5–15°.
    - Needle bevel should be visible just below skin surface.
4. *Check injection site*
    - After injection, a flat intradermal wheal of 8–10 mm diameter should appear. If not, repeat the injection at a site at least 5 cm (2 inches) away from the original site.
5. *Record information*
    - Record all the information required by your institution for documentation (e.g. date and time of test administration, injection site location, lot number of tuberculin).



Administration of the tuberculin skin test using the Mantoux method.

Reading

The results should be read between 48 and 72 hours after administration. A patient who does not return within 72 hours will probably need to be rescheduled for another TST.

1. *Inspect site*
    - Visually inspect injection site under good light, and measure induration (thickening of the skin), not erythema (reddening of the skin).
2. *Palpate induration*
    - Use fingertips to find margins of induration.
3. *Mark induration*
    - Use fingertips as a guide for marking widest edges of induration across the forearm.
4. *Measure diameter of induration using a clear flexible ruler*
    - Place "0" of ruler line on the inside left edge of the induration.
    - Read ruler line on the inside right edge of the induration (use lower measurement if between two gradations on mm scale).
5. *Record diameter of induration*
    - Do not record as "positive" or "negative".

- Only record measurement in millimetres.
- If no induration, record as 0 mm.

Interpretation

Interpretation of TST depends on two factors:

–diameter of the induration;

–person's risk of being infected with TB and of progression to disease if infected.

Induration of diameter ≥5 mm is considered positive in:

–HIV-positive children;

–severely malnourished children (with clinical evidence of marasmus or kwashiorkor).

Induration of diameter ≥10 mm is considered positive in:

–all other children (whether or not they have received BCG vaccination).

Causes of false-negative and false-positive TSTs are listed in Table.

Causes of false-negative and false-positive tuberculin skin tests

Causes of false-negative TST	**Causes of false-positive TST**
Incorrect administration or interpretation of test	Incorrect interpretation of test
HIV infection	BCG vaccination
Improper storage of tuberculin	Infection with non-tuberculous mycobacteria
Viral infections (e.g. measles, varicella)	
Vaccinated with live viral vaccines (within 6 weeks)	
Malnutrition	

Causes of false-negative TST	Causes of false-positive TST
Bacterial infections (e.g. typhoid, leprosy, pertussis)	
Immunosuppressive medications (e.g. corticosteroids)	
Neonatal patient	
Primary immunodeficiencies	
Diseases of lymphoid tissue (e.g. Hodgkin disease, lymphoma, leukaemia, sarcoidosis)	
Low protein states	
Severe TB	