LABORATORY MANUAL

PRATICAL VETERINARY MICROBIOLOGY

VMC- Unit- 2 & 3

Course title –Veterinary Mycology and Microbial Biotechnology

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FOREWORD

I am glad to see the Laboratory Manual of ***General & Systematic Veterinary Bacteriology, Veterinary Mycology, and Microbial Biotechnology*** *prepared by* ***Prof. G. C. Chaturvedi, Dr. Priyanka Singh and Dr. Neha Singh Assistant Professor, Dept. of Veterinary Microbiology****. It is appreciable to note that the manual covers the practical syllabus of B.V.Sc.& A.H. course as per the standards laid down by Veterinary Council of India.*

Dr. G. C. Chaturvedi, Dr. Priyanka Singh and Dr. Neha Singh have devoted keenly to prepare this manual with their excellent knowledge and expertise in the field of microbiology. They have covered all the aspects like objectives, outline and description, material and methods and observation to be taken care off.

Definitely this manual will be helpful for smooth and effective conduction of practicals and ensure a handbook for students for entire life in the profession.

I congratulate Dr. G. C. Chaturvedi, Dr. Priyanka Singh and Dr. Neha Singh for their strenuous efforts and excellent presentation of this manual.

Dean

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PREFACE

This Laboratory Manual has been prepared for the undergraduate students of B.V.Sc. & A.H. in accordance with the syllabus designed by the Veterinary Council of India. The efforts have been made to make the manuscript worthy, realistic and easily understandable for the students, teachers and Veterinary Practitioners for diagnosis of different microbial diseases of animals. We hope this manual will serve very useful tool to the undergraduate and graduate students of Veterinary Science who are undergoing courses in veterinary microbiology.

It‘s our pleasure to thank Dean Sir, M.J.F College of veterinary and Animal Sciences, Chomu, Jaipur for providing necessary facilities and rendering all helps in preparing this course manual.

Computer operator and typist Mr. Ashutosh Sharma worked hard for very existence of this manual so I acknowledge his efforts.

Course Incharge

Dept. of Veterinary Microbiology

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UNIT-2

Ex -1 MYCOLOGY

Moulds and yeasts can be studied by the same general cultural methods used for bacteria. Nearly all of them grow aerobically on the usual bacteriological culture media at temperatures ranging from 20 to 30°C. Most of them grow more slowly than bacteria, so that media which support bacteria as well as fungi may be overgrown by bacterial contaminates in a mixed inoculum. Where fungi are to be isolated, it is good practice to use a medium that favours their growth, but is not optimal for the growth of bacteria. Acidic (pH 5.6) media that incorporate a relatively high concentration of sugar are tolerated by moulds, but are inhibitory to many bacteria.

One of the best known and oldest media for the growth of fungi was devised by sabouraud and contains maltose and peptone as its principal ingredients. The most common modification used now contains glucose and any one of several specified peptones. This medium is widely used for the isolation of moulds and certain yeasts, and is especially useful for growing pathogenic fungi from infected body fluid and exudates. Its partial selective action is due to the high sugar concentration and low pH. The medium may be made more selective by the addition of chloramphenicol (antibacterial) Cycloheximide can also be inhibitory for some of the pathogenic fungi. Hence, it is advisable to use media with and without cycloheximide, expecially when attempting to isolate the dimorphic fungi.

Emmons, modification of sabouraud dextrose agar with a pH of 6.9 has been suggested for the dematophytes as although they tolerate pH5.6, a pH nearer neutrality is preferred.

Using an incubation temperature of 37°C is in itself a selective procedure. Many of the potentially pathogenic fungi, such as *Aspergillus spp*, prefer lower incubation temperature, but fungi that invade animal tissue can tolerate 37°C. This incubation temperature will exclude many non pathogenic fungi.

The dermatophytes are slow growing and need a lengthy incubation period so there is the danger of the agar media drying up in conventional plastic Petri dishes. This can be overcome by several methods.

1. By taping the plastic Petri dishes. However, as fungi are strict aerobes the tape should be removed and replaced daily to supply the fungus with the required oxygen.
2. By pouring the medium, in 20-25 ml volumes, into sterile glass Petri dishes. The greater depth of agar helps to retain the moisture. These can also be taped if necessary.
3. The medium can be poured as slopes in 30 ml universal bottles. There are inoculated and incubated with loose caps. The disadvantage is that any fungi growing on the slopes are more difficult to examine or to subculture.

Inoculation of media

Culture media for the isolation of yeast can be streaked with an inoculum from the specimen as for bacterial cultures. When attempting to isolate a mould the surface of the agar should be lightly crosshatched in about five sites with a sterile scalpel, the cuts being at right angles to each other. The specimens such as small bits of tissue, scabs or hairs, can then be gently pushed into the agar at these crosshatched areas. If the specimens are merely pushed into the agar surface there is the danger of the agar splitting during incubation.

Sub culturing of fungal colonies

Yeasts can be sub cultured, and pure cultures obtained. In the same manner as for bacteria wit moulds different techniques are needed.

If the mould colony is spouring: A mould colony usually starts to produce spores from the centre outwards and it is often the spores that give a colony its characteristic colour. An inoculating loop can be made slightly moist and sticky pushing it into a portion of sterile agar, and can then be used to collect spores from the colony. The inoculum is introduced just under the surface of the agar in the centre of the new plate if the fungus is a slow grower or produces small colonies.

If a mould colony is not spouring: The stalling phase and then death of a fungal colony starts with the hyphae in the centre of the colony. It is therefore, important to subculture hyphase from the edge of colony. A small block of agar (about 5sq. mm) is out of the centre of the subculture plate with a sterile scalpel and discarded. Using the same scalpel a similar-sized block of agar is cut including fungal hyphae, from the edge of the colony to be

Sub-cultured. The agar block containing hyphae is transferred to the subculture plate and placed smoothly with the mould side up into the previously cut hole. The cut hyphae will regenerate and grow out form surface of the block.

Ex 1.1 Subculture a Mould

Materials : mould culture, sabouraud dextrose agar. Petri plates inoculation loop.

Composition :

Sabouroud's dextrose agar

Peptone (mycological)	10g
Dextrose	20g
Agar	15g
Dis. Water	1000 ml

Dissolve the ingredients by steaming, adjust pH to 5.4-5.6, sterilize by autoclaving at 115°C for 15 min and dispense (20-25ml) in sterile Petri plates.

Procedure

1. Using a sterile moist loop collect few spores from the colony of mould.
2. Aseptically inoculate the spores in a fresh plate of SDA, just beneath the surface.
3. Incubate at 22-25°C temperature for few days, till fungal growth offence.

Ex 1.2 Stain a Mould by Lactophenol Cotton Blue Method.

Materials : mould culture, alcohol (95%) cotton blue stain, glass slide cover slip teasing needle

Composition :

Lactophenol cotton blue stain

Cotton blue (aniline blue) water soluble	0.05g
Lactic acid	10.00g
Phenol crystals	10.00g
Glycerol	20.00ml
Water	10.00ml

1. Weigh the cotton blue and dissolve in the water by gentle warming.
2. Weigh the phenol in a beaker and add the stain solution. Stir to dissolve and transfer to a clean brown bottle.
3. Add the lactic acid and glycerol, and mix well.

Procedure

1. Place a drop of 95% alcohol on a clean glass slide.
2. Transfer a small amount of fungi growth to the alcohol using a sterile teasing needle.
3. Gently tease the fungus to separate its structure.
4. After evaporation of alcohol, add a drop of cotton blue stain over the fungal elements.
5. Place a cover slip over the stain, avoiding air bubbles apply gentle pressure over the colony fragments to spread out the hyphae and other structures.
6. Remove the excess stain using blotting paper and leave the slide for few minutes to allow the stain to penetrate the fungal elements.
7. Examine under the low-powder objective and then under high power dry objective of the light microscope.

Results : draw the figures of fungal elements

Ex 1.3 Demonstration of Sporulation of Fungi by Slide Culture Technique.

Materials : culture of *Aspergillus* spp. Sabouraud dextrose agar plate, scalped, bent glass rod (U-shape), Petri plate (sterile), glass slide cover slip, fitter paper, distilled water (sterile), lactophenol cotton blue stain, inculcating needle, forceps (sterile) alcohol (95%)

Procedure

1. Place a circular piece of filter paper at the bottom of a Petri plate.
2. Place the bent glass rod on the filter paper and a glass slide with the cover slip on rod.
3. Wrap the assembly in paper and autoclave at 121°C for 15 min.
4. Cut 1 cm^2 agar block from SDA plate using a flamed or sterile scalpel and transfer it aseptically on the glass slide placed on the bent glass rod.
5. Inoculate fungal spores on the four corners of the agar block using sterile inoculating needle.
6. Place the sterile cover slip on the top of the agar block with sterile forceps.
7. Moisten the filter paper with sterile distilled water and incubate at 37°C temperature.
8. Check the slide periodically for growth and sporulation.(the fungus frown on the inoculated sides of the agar block and under the edges of the cover slip)
9. When sporulation has taken place remove the cover slip from agar block and apply a drop of 95% alcohol to the centre of cover slip.
10. Add a drop of LPCB stain on a clean glass slide and place cover slip on it with fungal side down. Avoid formation of air bubbles.
11. Apply gently pressure on the cover slip to spread out the hyphae and other structures. Remove excess stain by blotting paper.
12. Examine slide under microscope and note the characteristic structures.

Results:

Genus: *Aspergillus* (Mould)

The species of the genus *Aspergillus* and penicillium are the common moulds seen on damp bread or almost any other organic matter. They are also considered as common laboratory contaminants. Of the 300 species of aspergilla, *A. fumigates* is highly pathogenic for birds, and occasionally causes invasive disease in human beings. Other potentially pathogenic species are *A. flavus, A.nidulans* and possibly *A. niger* and *A. terreus*. Several manifestations of aspergillosis are seen in domestic fowl, turkeys, and other wild and domestic avian species including penguins in captivity. These include diffuse infection of air sacs, diffuse pneumonic form and nodular form involving the lungs. The disease is called –brooders pneumonia‖ in chicks and poults. The principal gross lesion consists of yellow nodules in lungs and airsacs. In cattle, the infection involves the uterus, the foetal membranes and skin and may occasionally result in abortions. In horses, the infection of *Aspergillus* causes abortions, keratomycosis and occasional pulmonary Aspergillosis mainly in stabled horses. In dogs, cats and sheep, the lungs are often involved. Diagnosis may be made by microscopic examination and by culture. The fungus grows rapidly on culture media like sabouraud's dextrose agar (SDA). Identification of *Aspergillus* is based on growth characteristics and morphology. Aspergilla have septate hyphae. Asexual conidia are arranged in chains and originate from elongated cells called sterigmata borne on the expanded ends (vasicles) of conidiophores as shown in Figure. The typical condial heads are seen only in the lungs and air sacs, where there is access to oxygen.

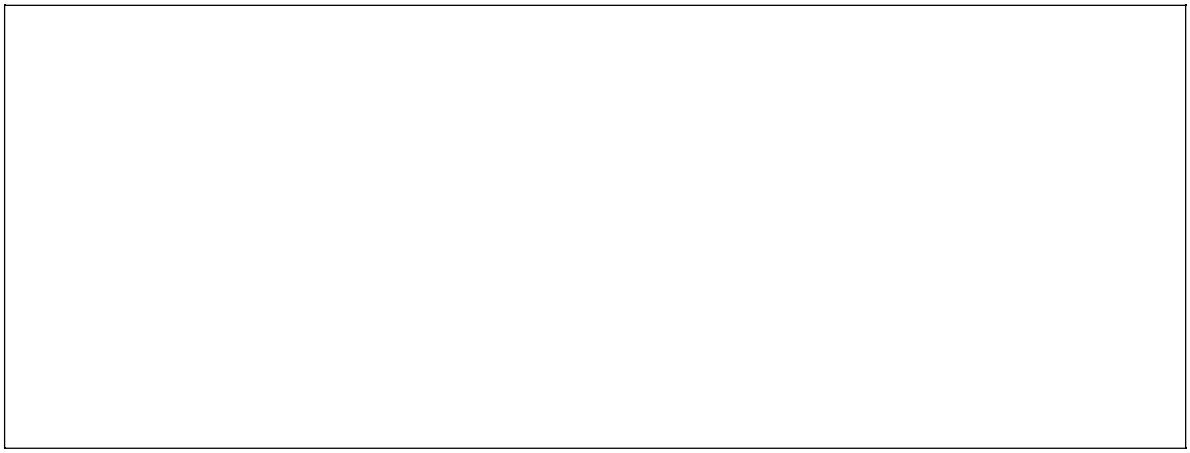


Fig. *Aspergillus* fumigates with (a) conidia (b) sterigmata (c) vesicle (d) conidiophore

Exercise No. – 2

Experiment: To study the morphological and cultural characteristics of *Aspergillus fumigatus*

Culture: Slant of known culture of *Aspergillus fumigatus*

Materials:

1. Bacteriological loop/chiseling needle, test tubes, slides, cover slips, Durham's tube
2. Lacto phenol cotton blue stain, 10% NaOH
3. Media:
   (a) Sabouraud's dextrose agar (SDA)
   (b) Sabouraud's dextrose booth (SDB)
   (c) Corn meal agar (CMA)

(A) Morphological characteristics:

1. Put 2-3 drops of lactophenol cotton blue stain on a grease-free slide.
2. Mix small amount of *aspergillus fumigatus* (mould) culture uniformly with the help of chiseling needle and cover it with the cover slip.
3. Examine the slide (mount) under microscope (40x).
4. Note the morphology and staining characteristics of the mould.

Results/observations

Fig. *Aspergillus fumigatus* showing flask shaped structure and conidiophores

1. Inoculate sabouraud's dextrose agar (SDA) plate by streaking with the help of sterile chiseling needle.
2. Inoculate corn meal agar medium by slide culture technique.
3. Inoculate sabouraud's dextrose broth (SDB)
4. Incubate the inoculated media at room temperature (22-25^{o} C) for 2 to 3 days in an incubator.
5. Examine the colony characteristics on SDA and SDB media.
6. Prepare slide mount of each of the above culture separately and examine under the microscope (40x).
7. Compare morphological characteristics of the organism (mould) grown under different conditions as above.

Results/observations:

S.No.	Media	Observation
1	Growth on sabouraud's dextrose agar (SDA)	
2	Growth on sabouraud's dextrose agar (SDB)	
3	Growth on corn meal agar medium (CMA)	
4	Presence of vesicle formation by *A. fumigatus*	
5	Presence of conidiophore	
6	Presence of conidia	
7	Presence of sterigmata	

Conclusions

Genus: *Candida* (Yeast)

Yeast is unicellular eukaryotic fungus. Of all pathogenic yeasts, members of the genus *Candida*, particularly *C. albicans* are most commonly associated with human and animal disease conditions. Candidiasis is a disease of varied manifestations involving skin, mucous membrane and natural orifices. The disease has been reported in chickens, turkeys, ducks, geese, pigeons, pheasants, quails, dogs, cattle, pigs, colts, lambs and monkeys. *Candida albicans* is an ovoid or spherical budding cell, which produces pseudomycelia both in culture and in tissues as shown in figure. *Candida* species are normal inhabitants of skin and mucosa. Diagnosis of candidiasis can be done by microscopy and cultural methods. Wet films or gram-stained smears from lesions or exudates show budding gram-positive cells. As *Candida* can be seen on normal skin or mucosa as well, its presence in abundance is of concern. Demonstration of mycelia forms indicates colonization and tissue invasion, and is therefore of great significance. Cultures can be obtained readily on *sabouraud's* dextrose agar (SDA) and on an ordinary bacteriological culture media. Colonies of *Candida* are creamy white, smooth and with a yeasty odour. *Candida albicans* can be differentiated from other species *(C.tropicalis, C. pseudotropicalis and C. krusei*) on the basis of growth characteristics and sugar assimilation and fermentation tests *C. albicans* alone forms chlamydospores on corn meal agar at 20^{o} C. all *Candida* strains are sensitive to nystatin but as it is poorly absorbed from gut, it is not useful in systemic disease.

Exercise No. – 3

Experiment: To study the morphological and cultural characteristics of *Candida albicans* and other yeasts.

Culture: Slant of known culture of *Candida albicans*

Materials:

1. Bacteriological loop/chiseling needle, test tubes, slides, cover slips, durhum's tube
2. Lacto phenol cotton blue stain, 10% NaOH
3. Media:
    (a) Sabouraud's dextrose agar (SDA)
    (b) Sabouraud's dextrose booth (SDB)
    (c) Corn meal agar (CMA)

(A) Morphological characteristics:

1. Put 2-3 drops of lactophenol cotton blue stain on a grease-free slide and mix it with a loopful of culture (*C. albicans*).
2. Put a coverslip over it and examine the slide (mount) under microscope (40x).
3. Note the morphology and staining characteristics of the mould.

Results/observations

Fig. *Candida albicans* in a stained smear

1. Inoculate sahouraud‘s dextrose agar (SDA) plate by streaking with the help of sterile chiseling needle.
2. Inoculate corn meal agar medium by slide culture technique.
3. Inoculate sabouraud’s dextrose broth (SDB)
4. Incubate the above inoculated/streaked media at room temperature 22-25^{o} C for 2 to 3 days in an incubator.
5. Examine the colony growth characteristics on SDA and SDB mediums.
6. Prepare slide mount of each of the above culture separately and examine under the microscope (40x).
7. Compare morphological characteristics of the organism (mould) grown under different conditions as above.

Results/observations:

S.No.	Media	Observation
1	Growth on sabouraud’s dextrose agar (SDA)	
2	Growth on sabouraud’s dextrose agar (SDB)	
3	Growth on corn meal agar medium (CMA)	
4	Presence of hyphal elements	
5	Presence of chlamydospores	

Conclusions

UNIT- 3

Ex. No.	Title	Page No.	Date	Signature
1	Extraction and quantification of nucleic acid			
2	Plasmid isolation and profiling			
3	Polymerase Chain Reaction			
4	Electrophoresis			
5	Use of multimedia and audio-visual aids for molecular biology aspects			
6	General Buffers and Reagents			

UNIT-3

PRACTICAL No. 1

Extraction and quantification of nucleic acid

Many different methods and technologies are available for the isolation of genomic DNA. In general, all methods involve disruption and lysis of the starting material followed by the removal of proteins and other contaminants and finally recovery of the DNA. Removal of proteins is typically achieved by digestion with proteinase K, followed by salting-out, organic extraction, or binding of the DNA to a solid-phase support (either anion-exchange or silica technology). DNA is usually recovered by precipitation using ethanol or isopropanol.

In general, the separation of DNA from cells and cellular components can be divided into four stages:

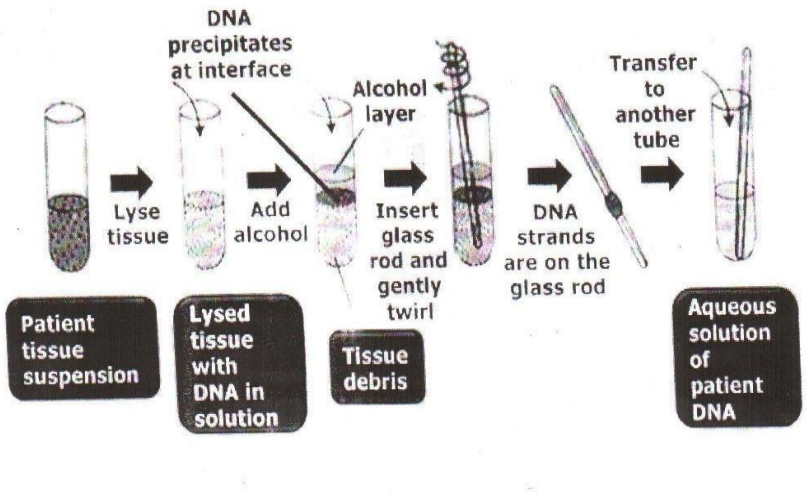
1. Cell disruption
2. Lysis of Cell
3. Removal of Proteins and Contaminants
4. Recovery of DNA

Extraction of DNA from whole blood

1. Collect approximately 20 ml of fresh blood in l ml of 2.7% EDTA solution. Blood should not be collected into heparin, which is the inhibitor of the polymerase chain reaction.
2. Centrifuge at 1500 rpm for 15 mts and discard the supernatant plasma. Carefully transfer the buff coat to a fresh tube and recentrifuge.
3. Resuspend the buffy coat in l5ml of extraction buffer. Incubate the solution for l hour at 37°C
4. Add proteinase K to a final concentration of 100 microgram/ml using a glass rod gently mix the enzyme into the viscous solution.
5. Place the suspension of lysed cells in a water bath for 3 hrs at 50^{o} C. Swirl the viscous solution periodically.
6. Cool the solution to room temperature.
7. Add an equal volume of phenol equilibrated with Tris- HCl (pH 8.0) and gently

mix the two phases by slowly turning the tube for l0mts. If the two phases have not formed an emulsion at this stage, place the tube on a roller apparatus for l hour.

8. Separate the two phases by centrifugation at 5000 rpm for 15 mts at room temperature. It is essential that pH of the phenol be approx 8.0 to prevent DNA from becoming trapped at the interface between the organic and aqueous phase.
9. With a wide pipette transfer the viscous aqueous phase to a clean centrifuge tube, and repeat the extraction once each with phenol, phenol: chloroform: Isoamyl alcohol (24:24:1) and Chloroform: IAA (24:1).
10. Transfer aqueous phase to afresh centrifuge tube and add l/10th volume of 3M Ammonium acetate. Add 2 volumes of ethanol and swirl the tube until the solution is thoroughly mixed. DNA will immediately form a precipitate that can be removed from pipette tip with a glass rod if the DNA precipitate becomes fragmented.
11. Centrifuge at 5000 rpm for 5mts at room temperature
12. Wash DNA precipitates twice with 70% ethanol and store the pellet in an open tube at room temperature until the lust traces of ethanol hut e evaporated.
13. Add l ml of TE (pH 8.0).
14. Allow the DNA to dissolve. This takes l2-24 hrs.
15. Store DNA at 4^{o} C



PRACTICAL No. 2

PLASMID ISOLATION AND PLASMID PROFILING

The word plasmid introduced by Joshua Lederberg in 1952. Plasmids are small extra chromosomal circular molecules from l kb to more than200 kb in size, which exists in multiple copies (up to a few hundred) within the host *E. Coli* cells.

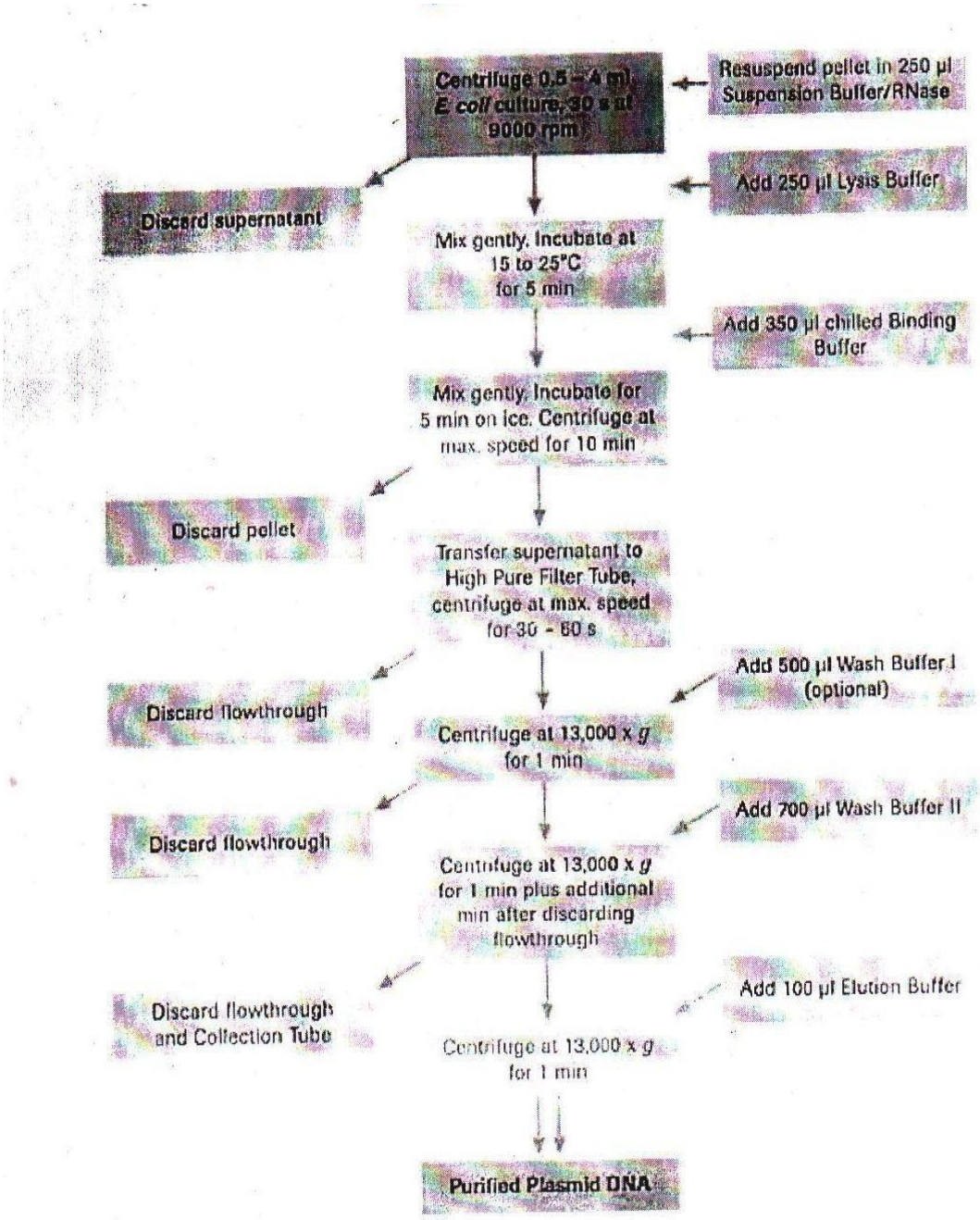
Plasmids are considered –replicons‖, capable of autonomous replication within a suitable host. Plasmids can be found in all three major domains: Archaea, Bacteria, and Eukaryotes. Similar to viruses, plasmids are not considered by some to be a form of –life‖. Unlike viruses, plasmids are –naked‖ DNA and do not encode genes necessary to encase the genetic material for transfer to a new host, though some classes of plasmids encode the sex pilus necessary for their own transfer.

They contain an origin of replication. Which enables them to replicate independently, although it normally relies on polymerases and other components of the host cell machinery. They usually carry a few genes, one of which may confer resistance to antibacterial substances such as Ampicillin or 'Tetracyclins. One of the major applications of plasmids is its use as a cloning vector in recombinant DNA protocols. The most commonly used method for purification of plasmid DNA is called alkaline lysis.

Methodology of Plasmid Isolation

1. Take bacterial culture in a centrifuge tube
2. Centrifuge it at 6000 rpm for l0mts.
3. Discard the supernatant, keep only 50µl and dissolve the pellet.
4. Add TENS 300µl, which causes lysis of bacterial cell wall
5. Vortex to mix the content for 30 sec
6. Add P3 (acidic pH) sodium/ Potassium acetate 150µl.
7. Keep the tubes in ice at room temperature for 10mts.
8. Centrifuge at 10,000 rpm 40C for 10mts
9. Take the supernatant in separate tubes and add isopropanol (0.6 volume)
10. Keep for 30mts at room temperature

11. Centrifuge at 12,000 rpm for 10mts at 40C
12. Discard the supernatant and add 80% ethanol (300µl) in each tube.
13. Centrifuge at 12,000 rpm fro 5mts at 40C
14. Keep the tubes in dry bath. Add TE buffer and RNAse (l5µl). TE buffer gives rigidity to DNA.



PRACTICAL No. 3

POLYMERASE CHAIN REACTION

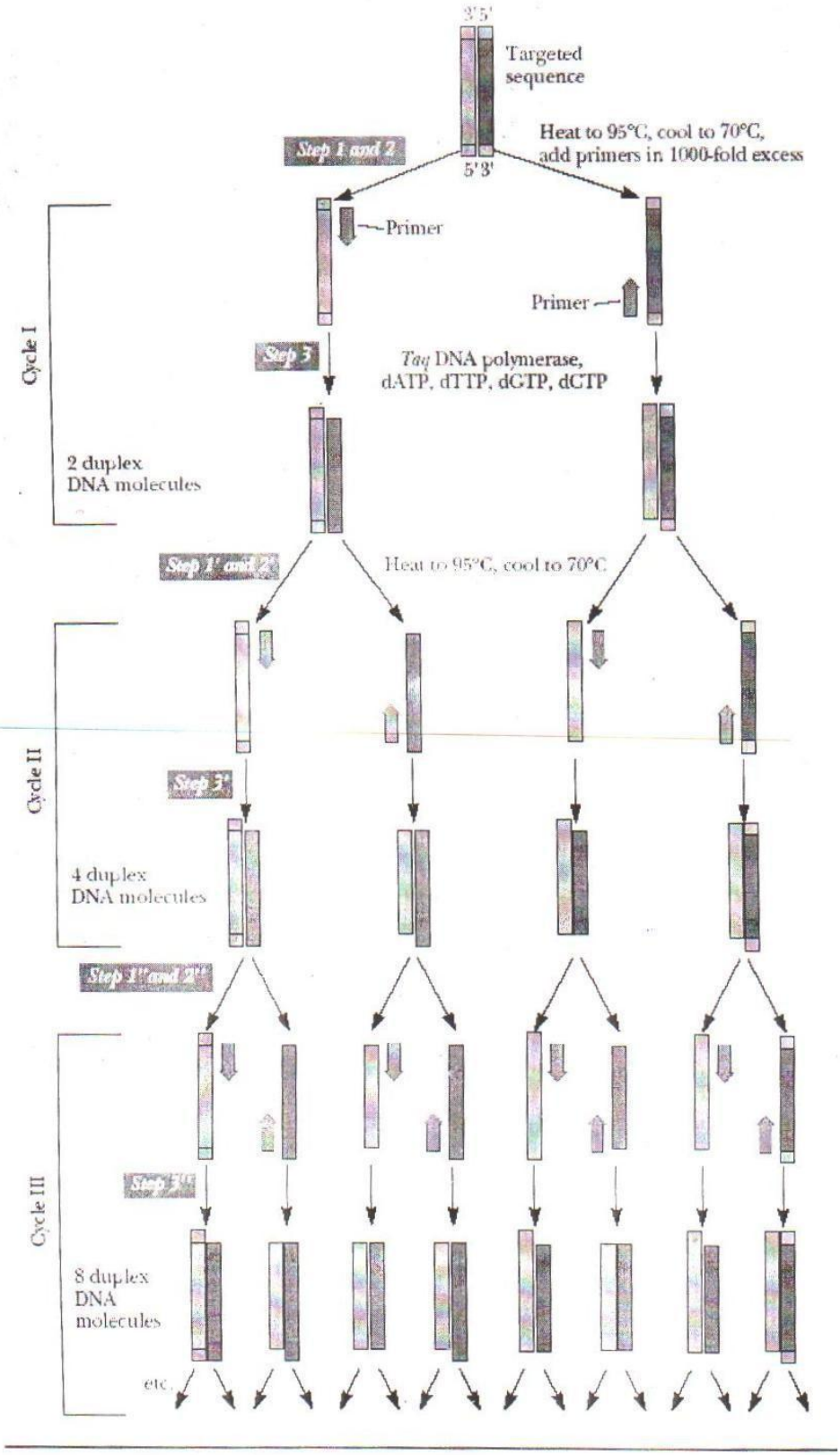
In the 15 years since its introduction the polymerase chain reaction (PCR) has become a basic and initial tool in both research and analytical laboratories. The invention of Polymerase Chain Reaction by KaryMullis, in 1983 followed by its practical application by Saiki et al., 1985 has revolutionized the field of` molecular biology. The technique consists basically of the enzymatic synthesis of a target sequence, using thermo stable DNA polymerase and a succession of cycles that include denaturation of template DNA, hybridization of specific DNA primers to the template and extension of the primers, so that it become possible to generate multiple copies of the target region, enzymatically. This amplification of a small region of DNA sequence into million-fold is accomplished via a succession of incubation steps at different temperatures. Thus PCR provides a method for obtaining large quantities of DNA sequence from small amounts of` DNA. Its simplicity and speed makes it ideally suited for characterization of the etiological agents of the bacterial diseases. PCR has simplified many procedures for gene cloning, analyzing and modifying nucleic acid fragment from complex genome. Desired gene fragment can be generated in a matter of hours. Hence, by using this technique direct detection of` the nucleic acid of` bacterial pathogen, even when present in negligible quantities, become possible. PCR is also useful tor rapid detection bacterial pathogens that are difficult to cultivate e.g. certain Mycobacteria, slow growing Mycoplasma. PCR can also be applied to fixed tissues (frozen or formalin fixed) reducing or eliminating the risk involved in transport and handling of specimens infected with live virulent pathogens. Although PCR, though extremely useful has certain shortcomings such the problems caused by contaminants and inhibitors or lack of suitable sequences for designing specific primers. The development of newer PCR reagents and procedures has, however, increased both its specificity and efficacy). The increasing availability of PCR reagents, increased availability of oligonucleotides and its synthesis at low cost and the tremendous increase in the availability of-nucleotide sequence data have favored the application of PCR diagnosis.

Despite unforeseen obstacles, the prospects for PCR technology application in the clinical laboratories remain excellent, as these techniques become more automated accessible and affordable. The potential application and the results obtained in our

laboratory with some of the bacterial pathogens will be discussed.

The important bacterial diseases affecting livestock and/or poultry include anthrax blackleg, listeriosis, brucellosis, tuberculosis, salmonellosis, enterotoxaemia, John‘s disease and pasteurellosis. These diseases are of serious economic concern in animal farming. So it becomes necessary to use improved diagnostic tools to alert ourselves about the health of the animals at the; earliest stages of infection, so that proper corrective measure can be taken up in the shortest possible time and at the minimum cost. The recent developments in molecular biotechnology have provided DNA based highly sensitive methodologies that early on the detection of DNA of the pathogens in body fluids and tissues.

PCR can be used to determine the sex of embryos. Thus the sex in vitro fertilized cattle embryos could be determined before their implantation in the uterus. Although PCR has some shortcoming, such as the problems caused by contaminant and Inhibitors or the lack of suitable sequences for designing specific primers, the outstanding research effort focused on this technique have minimized the deficiencies and allowed its increased general use as a diagnostic tool.


3'5'
Targeted sequence
Heat to 95°C, cool to 70°C, add primers in 1000-fold excess
Step 1 and 2
5'3'
Primer
Primer
Cycle I
Step 3
Taq DNA polymerase, dATP, dTTP, dGTP, dCTP
2 duplex DNA molecules
Step 1' and 2'
Heat to 95°C, cool to 70°C
Cycle II
Step 3'
4 duplex DNA molecules
Step 1'' and 2''
Cycle III
Step 3''
8 duplex DNA molecules
etc.

POLYMERASE CHAIN REACTION METHODOLOGY

Materials required:

1. Isolated DNA
2. Primers
3. Taq DNA polymerase
4. 10X PCR buffer
5. 10mM $MgCl_2$
6. 50% Glycerol
7. 10mM dNTPs
8. DW

Methodology

1. Using of above isolated DNA. PCR is carried out adding following reagents to a thin walled PCR tube.
   - Isolated DNA 5 µl
   - Primers I 1 µl
   - Primer II 1 µl
   - 10X PCR buffer 5 µl
   - 10mM MgCl2 1:5 µl
   - 50% glycerol 10 µl
   - 10mM dNTPs 1 µl
   - DW to make 48.5 µl
2. Heat in boiling water bath for 5 min and immediately chill on ice.
3. After spinning down the contents add l.5 µl (2-3 units) of Taq DNA polymerase and put for cycling in a thermal cycler using following cycles.
   I. 94^0 C l min
   II. 62^0 C 1 min
   III. 72^0 C 1 mm Repeat steps I through III 35 times.IV 72^0 C 5 mm
4. Analyse l0 µl of PCR product in l% agarose gel running along with the marker.

PRACTICAL No. 4

ELECTROPHORESIS

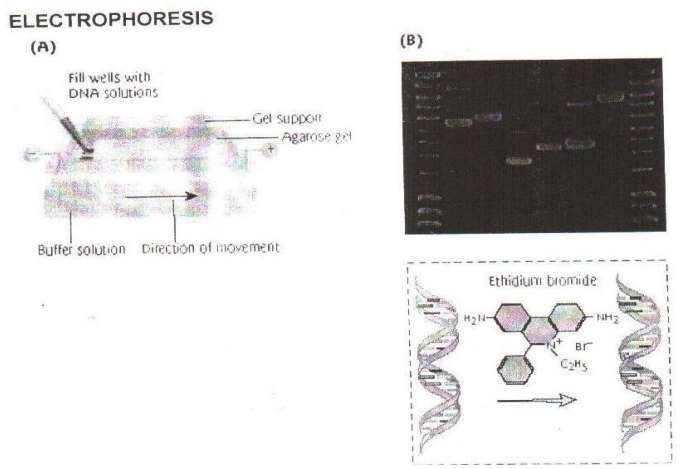
Electrophoresis is the standard method for analyzing, identifying, and purifying fragments of DNA or RNA that differ in size, charge, or conformation. It is one of the most widely used techniques in molecular biology. When charged molecules are placed in an electric field, they migrate toward the positive (anode, red) or negative (cathode, black) pole according to their charge. In contrast to proteins, which can have either a net positive or net negative charge, nucleic acids have a consistent negative change due to their phosphate backbone, and they migrate toward the anode. Proteins and nucleic acids are separated by electrophoresis within a matrix or –gel‖. Most commonly, the gel is cast in the shape of a thin slab, with wells for loading the sample. The gel is immersed within an electrophoresis buffer that provides ions to carry a current and some type of buffer to maintain the pH at a relatively constant value. The gels used for electrophoresis are composed either of agarose or polyacrylamide. agarose gels are used in a horizontal gel apparatus, while polyacrylamide gels are used in a vertical gel apparatus. These two differ in resolving power. agarose gels are used for the analysis and preparation of fragments between --100 and 50.000 bp in size with moderate resolution, and polyacrylamide gels are used for the analysis and preparation of small molecules with single nucleotide resolution. This high resolution is required-for applications such as for DNA sequencing. In contrast to agarose, polyacrylamide gels are also widely used for the electrophoresis of proteins agarose is a polysaccharide extracted from seaweed. Agarose gels are easily prepared by mixing agarose powder with butler solution, boiling in a microwave to melt, and pouring the gel into a mold where the agarose (generally 0.5-2.0%) solidifies into a slab. By varying the concentration of agarose, fragments of DNA from about 100 to 50,000 bp can be separated using standard electrophoretic techniques. A toothed comb forms wells in the agarose. The agarose slab is submerged in a buffer solution and an electric current is passed through the gel, with the negatively charged DNA (due to the phosphate in the sugar—phosphate backbone) moving through the gel from the negatively charged electrode (cathode) towards the positive electrode (anode). Pores between the agarose molecules act like a sieve that separates the molecules by size.

In gel filtration chromatography nucleic acids flow around the spherical agarose beads. In contrast, in an electrophoretic gel, nucleic acids migrate through the pores; thus fragments separate by size with the smallest pieces moving the fastest and farthest through the gel. Because DNA by itself is not visible in the gel, the DNA is stained with a fluorescent dye such as ethidium bromide (EtBr). EtBr intercalates between the bases causing DNA to fluoresce orange when the dye is illuminated by ultraviolet light.

To fractionate large DNA molecules such as YACs (see Section 8.4), agarose gel electrophoresis is carried out with a pulsed electric field. The periodic field causes the DNA molecule to reorient; longer molecules take longer to realign than shorter ones, thus delaying their progress through the gel and allowing them to be resolved. DNA molecules up to 200-400 Mb in size have been separated by various pulsed field-based methods.

Polyacrylamide gel electrophoresis

Polyacrylamide is a cross-linked polymer of acrylamide. The length of the polymer chains is dictated by the concentration of acrylamide used, which is typically between 3.5 and 20%. Polyacrylamide gels are significantly more cumbersome to prepare than agarose gels. Because oxygen inhibits the polymerization process, Polyacrylamide gels have a rather small range of separation, but very high resolving power. In the ease of DNA, polyacrylamide is used for separating fragments of less than about 500 bp. However, under appropriate conditions, fragments of DNA differing in length by a single base pair are easily resolved. Bands in polyacrylamide gels are usually detected by autoradiography, although silver shutting can also be used.



Agarose gel electrophoresis is used to separate DNA (and RNA) molecules according to size.

(a) A pipet is used to load DNA samples on an agarose gel in a horizontal gel apparatus. The negatively charged nucleic acids move toward the positive electrode. Larger molecules move more slowly than smaller molecules so the DNA (or RNA) molecules are separated according to size.

(b) Photograph of an agarose gel stained with ethidium bromide (Et-Br) to make the DNA bands visible. Et-Br molecules intercalate between the bases (see inset) causing the DNA to fluoresce orange when the gel is illuminated with UV light. (Photograph courtesy of Vinny Roggero. College of William and Mary).

Agarose Gel Electrophoresis

Agarose, which is extracted from seaweed, is a linear polymer; Commercially available agarose is not completely pure; it is contaminated with other polysaccharides, salts and proteins. These differences can affect both the migration of the DNA and the ability of the DNA recovered from the gel to serve as a substrate in enzymatic reactions. So, most manufacturers now prepare special grades of agarose that are screened for the presence of inhibitors and nucleases and tor minimal background fluorescence after staining with ethidium bromide.

Materials required:

1. Agarose
2. TBE 0.5X
3. Ethidium bromide
4. 6X loading buffer

Methodology

1. Seal the open ends of the electrophoresis tray with adhesive tape. Place the comb on the tray.
2. Weigh appropriate amount of agarose powder to prepare 0.8% agarose gel. Add appropriate volume of electrophoresis buffer (0.5 X TBE). Heat the slurry until the agarose dissolves.
3. Cool the solution to about 60^{o} C and add 2-3µl of ethidium bromide and pour the gel on sealed tray. Leave it without disturbing until gel solidities.
4. Remove comb and adhesive tape and place the tray on electrophoresis tank. Add enough electrophoresis buffer (0.5 X TBE) to cover the gel to a depth of about l mm.
5. Mix the DNA samples with 6 X loading buffer. Slowly load the sample into the slots of submerged gel.
6. Connect the gel tank with electrical leads so that the DNA migrates from cathode towards anode. Apply a voltage of 5V/cm. Run the gel until the tracking dye (bromophenol blue) reaches to the other end of gel.
7. Turn off the electric current. Take out the gel tray.
8. Observe the gel under UV transilluminator (320 nm)

SDS (sodium dodecyl sulphate)-PAGE

Materials required:

1. Acrylamide — bisacrylamide
2. Stacking buffer (0.5 M pH 6.8)
3. Resolving butter (1.5 M pH 8.8)

4. 10 % SDS
5. 1.5% APS
6. TEMED
7. Tris Glycine electrophoresis buffer
8. 1X SDS gel loading buffer

Methodology:

1. Determine the volume of the gel mould. Prepare the volume of solution containing the desired concentration of acrylamide for resolving gel as given below

   Recipe for Gel Preparation Using SDS

   Resolving

   Stacking 15% 12% 10%

   Acrylamide - Bisacrylamide (30:8) 2.5 15 12.5 10

   Stacking gel buffer (0.5 M pH 6.8) 5.0 -

   Resolving gel buffer (1.5 M pH8.8)- 7.5 7.5 7.5

   10% SDS 0.2 0.3 0.3 0.3

   1.5% 1.0 1.5 1.5 1.5 1.5

   Water 11.3 5.7 8.25 10.7

   TEMED 0.015 0.015 0.015 0.015

2. Mix the components in the order shown. Polymerization will begin as soon as the TEMED has been added. Without delay, swirl the mixture rapidly and proceed to next step.
3. Pour the acrylamide solution into gap between the glass plates.
4. After polymerization is complete, pour off the overlay and wash the top of gel several times with deionized water to remove any unpolymerized acrylamide.

5. Prepare the stacking gel as follows. In a disposable plastic tube, prepare the appropriate volume of solution containing the desired cone. of acryl amide using the values given in table. Mix the components in order shown. Polymerization will begin as soon-as the TEMED has been added. Without delay, swirls the mixture rapidly and proceed. Stacking gel buffer (0.5 M. pH 6.8) — 6.0 gm Tris adjust pH to 6.8 with l M HCI (48 ml) and make volume to 100 ml. Resolving gel buffer (1.5 M pH 8.8) l8.l5 g Tris and 48.0 ml l M HCL and mixed and brought to 100 ml final volume with water to make the pH 8.8.
6. Pour the stacking gel solution directly onto the surface of the polymerized resolving gel. Immediately insert a clear Teflon comb into the stacking gel solution being careful to avoid trapping air bubble.
7. While the stacking gel is polymerizing, prepare the samples by heating them to 100°C for 3 minutes in l X SDS gel loading buffer to denature the protein.

   1 X SDS gel loading buffer:

   50 mM Tris Cl (pH 6.8)

   100 mM dithiothreiotol

   2% SDS

   0.1% bromophenol blue

   10% glycerol
8. After polymerization is complete, remove the Teflon comb carefully mount the gel in the electrophoresis apparatus. Add Tris glycine electrophoresis buffer to the top and bottom reservoirs.

   Tris Glycine electrophoresis buffers 25 mM glycine (pH 8.3):

   0. 1 % SDS

   30.3 gm Tris

   144 gm glycine

   10 gm SDS

   1 liter distilled water

9. Take up to 20 µl of each of unindueed, induced and purified protein samples and mix with 2X sample butter, boil and load in a predetermined order in the wells.
10. Attach the electrophoresis apparatus to an electric power supply.

Staining SDS-PAGE with Coomassie Brilliant Blue

Polypeptide is separated by SDS-PAGE can be simultaneously fixed with methanol, glacial acetic acid and stained with Coomassie brilliant blue.

Materials required:

1. Coomassie brilliant blue
2. Staining solution
3. Destaining solution

Methodology:

1. Dissolve 0.25 g of commassie Brilliant Blue R250 in 90 ml of methanol: H_2 O (1:1 v/v) and 10 ml of glacial acetic acid. Filter the solution through a Whatrnan No.1 filter.
2. Immerse the gel in 5 volumes of staining solution. Keep at room temperature for 4 hours.
3. Remove the stain.
4. Destain the gel in destain solution lot 12 hours (methanol: Aceticacid: Water = 30:10:60).
5. After destaining, gels may be stored indefinitely in water.

PRACTICAL No. 5

USE OF MULTIMEDIA AND AUDIO-VISUAL AIDS FOR MOLECULAR BIOLOGY ASPECTS

The main objective of this study is to outline specific manners by which learning and teaching processes can be enhanced in the university campuses for the sake of superior learning capabilities and experiences in the fields of molecular biology. Multimedia and audio visual aid may help to improve the teaching of molecular biological courses, including the disciplinary, the problem-oriented and combined approaches. In the disciplinary approach, the previous courses are taught in the classical manner as a coherent subject, covering basic concept behind DNA& RNA structure and function, techniques concern to molecular biology, pathology and immunology, as well as clinical manifestations, diagnosis, therapy, control and prevention of parasitic diseases. Problem-oriented teaching approaches the subject, starting from basic principles of DNA replication, hybridization and basic technical approaches like chromatography and electrophoresis. It also tackles training of skills for problem solving and self-learning. Combined approaches include elements of the disciplinary approach and those of other methods. A list of the developed courses and the way of presentation has been discussed. The course syllabus put in student guide to be given to the student in the beginning of the semester. Improving the practical courses has been done by recording them in video. The strategy in the lectures has been changed with the students to minimize the distance with them. Moreover, three workshops have been hold during improving the courses to enhance the efficacy of the technician in the using the computer and modern equipments. Based on the observations and experiences regarding teaching molecular biology. There are few websites which may help student to get much information about the molecular biology.

There are few traditional audio and visual aids which may be useful in molecular biology study:

CD

COMPUTER

PROJECTOR

CHART

POSTER

LEAFLET

TELIVISION

FLANNEL GRAPH

But now days use of internet make possible easy to learn and understand the molecular biology. Followings are the few resources given which helps to obtain the information regarding molecular biology.

Web Sites with Molecular Biology Links

Frontiers in Bioscience: Guides, Tutorials, Manuals, and Protocols

This site has provides access to Frontiers in Bioscience, a journal focused on the area of molecular biology. If you need to obtain a pdf copy of an article, please speak with Chemistry/Physics Library Staff.

Harvard University Department of Molecular and Cellular Biology: List of Links

This is a list of molecular and cellular biology links provided by the Department of Molecular and Cellular Biology at Harvard University.

Selected worldwide web sites

The following is a list of web sites that readers may find useful. The sites have been visited at various times by one or more of the authors. Most are located in the United States, but many provide extensive links to international sites and to databases (eg, for protein and nucleic acid sequences) and online journals. RKM would be grateful if readers who find other useful sits would notify him of their URLs by e-mail (rmurray6745@rogers.com) so that they may be considered for inclusion in future editions of this text.

Readers should note that URLs may change or cease to exist.

Access to the biomedical literature

High wire press: http:/highwire.stanford.edu/

Extensive lists of various classes of journals – biology, medicine, etc – and offers also the most extensive list of journals with free online access.

National library of medicine: http://www.nlm.nih.gov; (Free access to medicine via PubMed.)

General resource sites

The biology project (from the University of Arizona): http://www.biology.arizona.edu/default.html (contains excellent biochemical coverage of enzymes, membranes, etc.)

Harvard University Department of Molecular & Cellular Biology links: http://mcb.harvard.edu/BioLinks.html (Contains many useful links.)

Sites on specific topics

American heart association: http://www.americanheart.org/ (useful information on nutrition, on the role of various bimolecular – eg, cholesterol, lipoproteins-in heart disease, and on the major cardiovascular diseases).

Cancer genome anatomy project (CGAP): http://www.cgap.nci.nih.gov.

(An interdisciplinary program to generate the information and technical tools to decipher the molecular anatomy of the cancer cell.)

Carbohydrate Chemistry and Glycobiology: A web tour: http://sciencemag.org/feature/data/carbohydrates.shl

(Contains links to organic chemistry, carbohydrate chemistry and glycobiology.)

European Bioinformatics Institute: http://ebi.ac.uk/index.html

(Maintains the EMBL nucleotide and SWISS-PROT databases as well as other databases.)

Gene Cards: http://www.genecards.org/

(A database of human genes, their products, and their involvements in disease; from the Weizmann Institute of Science.)

GeneTests: http://www.geneclinics.org/

(A medical generics information resource with comprehensive articles on many genetic diseases.)

Genes and Disease: http://www.ncbi.nlm.nih.gov/disease/

(Coverage of the genetic bases of many different diseases.)

Howard Hughes Medical Institute: http://www.hhmi.org/

(An excellent sire for following current biomedical research. Contains a comprehensive Research News Archive.)

Human Gene Mutation Database: http://www.hgmd.cf.ac.uk/hgmd0.html

(An extensive tabulation of mutations in human genes from the Institute of Medical Generics in Cardiff Wales.)

Human Genome Project Information: http://www.doegenomes.org

(From the U.S. Department of Energy; also contains general information on genomics and on microbial genomes.)

Karolinska Institute: Diseases and Disorders: http://www.mic.ki.se/Diseases/Cl8.html

(Contains extensive links pertaining in nutritional and metabolic diseases.)

Lipids Online: http://lipidsonline.org/

(A resource from Baylor College of, Medicine for health care practitioners with an interest in atherosclerosis, dyslipidemias, and lipid management.)

MITOMAP: http://www.mitomap.org/

(A human mitochondrial genome database.)

National Center for Biotechnology Information: http://ncbi.nlm.nih.gov/

Information on molecular biology and how molecular processes affect human health and disease.)

National Human Genome Research Institute: http://www.genome.gov/

(Extensive information about the Human Genome Project and subsequent work.)

National Institutes of Health: http://www.nih.gov/

(Includes links to the separate Institutes and Centers that constitute NIH, covering a wide range of biomedical research.)

Office of rare disease: http://rarediseases.info.nih.gov

(Access to information on more than 7,000 rare diseases, including current research.)

OMIM (Online Mendelian Inheritance in Man): http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=OMIM

(A fantastically comprehensive resource on human genetic disease, initiated by Dr Victor A. McKusick, considered by many to be the father of modern human genetics.)

Protein Data Bank: http://www.rcsb.org.pdb/

(A worldwide repository for the processing and distribution of three-dimensional biologic macromolecular structure data.)

Society for endocrinology: http://www.endocrinology.org.default.htm

(The site aims to advance education and research in endocrinology for the public benefit.

Society for neuroscience: http://web.sfn.org

(Contains useful information on a variety of topics in neuroscience.)

The broad Institute: http://www.broad.mit.edu/

(The broad Institute is a research collaboration of MIT, Harvard and its affiliated hospitals, and the whitehead Institute and was created to bring the power of genomics to medicine.)

The Institute for Genetic research: http://www.tigr.org/

(Contains sequences of various bacterial genomes and other information.)

The protein Kinase resource: http://www.kinasenet.org/

(Information on the protein Kinase family of enzymes.)

The Signaling Gateway: http://www.signalling-gateway.org/

(A comprehensive resource for anyone interested in signal transduction.)

The welcome Trust Sanger Institute: http://www.sanger.ac.uk/

(A genome research center whose purpose is to increase knowledge of genomes, particularly through large-scale sequencing and analysis.)

Biochemical Journals and Reviews

The following is a partial list of biochemistry journals and review series and of some biomedical journals that contain biochemical articles. Biochemistry and biology journals now usually have Web sites, often with useful links, and some journals are fully accessible without charge. The reader can obtain the URLs For the following by using a search engine.

Annual Reviews of Biochemistry, Cell and Developmental Biology, Genetics, Genomics and Human Genetics Archives of Biochemistry and Biophysics (Arch Biochem Biophys)

Biochemical and Biophysical Research Communications (Biochem Biophys Res Commun)

Biochemical journal (Biochem J)

Biochemistry (Biochemistry)

Biochemistry (Moscow) (Biochemistry [Mosc])

Biochimica et Biophysica Acta (Biochim Biophys Acta)

Biochimie (Biochimie)

European Journal of Biochemistry (Eur J Biochem)

Indian Journal of Biochemistry and Biophysics (Indian J Biochem Biophys)

Journal of Biochemistry (Tokyo) (J Biochem [Tokyo])

Journal of Biological Chemistry (J Biol Chem)

Journal of Clinical Investigation (J Clin invest)

Journal of Lipid Research (J Lipid Res)

Nature (Nature)

Nature Genetics (Nat Genet)

Proceedings of the National Academy of Sciences USA (Proc Natl Acad Sci USA)

Science (Science)

Trends in Biochemical Sciences (Trends Biochem Sci)

PRACTICAL No. 6

GENERAL BUFFERS AND REAGENTS

TENS (Tris/EDTA/NaOH/SDS)-20ml

Tris (l M)	200µl
NaOH (10N)	200 µl
EDTA (0.5M)	40 µl
SDS (10%)	l ml

Distil water to make 20 ml.

TSS

LB Medium	50 ml
10% Polyethylene glycol	5.0ml
$MgSO_4$ or $MgCl_2$	20 ml
DMSO	2.5 ml

Adjust pH to 6.5 and store at 4^o C

SOB medium

Tryptone	2 gm
Yeast Extract	0.5 gm

NaCl	0.05 g
Distilled water	90 ml
KCl	2.5mM

Adjust pH to 7.0 with NaOH. Make volume to 100ml and sterilized by add sterile $MgCl_2$ to 10mM

Phospahte Buffered Saline (PBS) pH -7.4

Sodium chloride	8 g
Disodium hydrogen phosphate	1.16 gm
Potassium chloride	0.2 gm
Potassium dihydrogen phosphate	0.24 gm

Add sterile water to make 1000 ml. sterilize with autoclaving for 20mts at 151b/Sq.in. Store at room temperature

Sodium Dodecyl Sulphate (SDS) 10%

SDS	1 gm

Distilled water 10 ml

EDTA 0.5M, pH-8.4

EDTA	18.612 gm
Distilled water	80 ml

Stir vigourously on a magnetic stirrer to mix. Adjust pH to 8.0 and make the volume 100 ml. autoclave and store.

Sodium acetate 3 M

Sodium acetate	24.6 gm

Distilled water	100 ml

Adjust pH to 5.2 with glacial acetic acid or adjust the ph to 7.0 with dilute acetic acid. Sterilize by autoclaving.

Proteinase K (10mg/ml)

Proteinase K	10 mg
Distilled water	1 ml

Store at -20^{o} C

RNAase (10mg/ml)

RNAas	10 mg
Distilled water	1 ml

Heated in boiling water bath for 14 minutes. Stored at -20^{o} C.

Tris EDTA (TE) Buffer (pH-8.0)

Tris HCL-10mm

EDTA-1mM

Isotonic lysis buffer (pH-9.0)

Tris HCL	10mM
NaCl	140mM
MgCl2	0.5mM
CaCl2	1mM
DTT	1mM
EDTA	1mm

Triton x-100	0.5%
Phenyl sulphonyl fluoride	1mM

Tris-Glucose-EDTA

Tris (1M) pH-8.0	5ml
EDTA (0.5M) pH-8.0	2.5ml
Glucose (1M)	33ml
Distilled water made to	100ml

Lysozyme solution (20mg/ml)

Lysozyme	100 mg
Tris-Glucose-EDTA	5 ml

Tris-Borate-EDTA (TBE) buffer (5X stock solution)

Tris base	54 gm
Boric acid	27.5 gm
EDTA (0.5 M) pH8.0	20 ml

Distilled water to make 1000 ml

6X loading dye for agarose gel Electrophoresis

Glycerol	300 µl
10% bromphenol	25 µl
Distilled water	675 µl