Laboratory Manual

VETERINARY MICROBIOLOGY

VMC Unit-III

Department of Veterinary Microbiology
College of Veterinary & Animal Sciences, Udgir
MAHARASHTRA ANIMAL & FISHERY SCIENCES UNIVERSITY, NAGPUR

LABORATORY MANUAL
FOR
VETERINARY MICROBIOLOGY

VMC

(New Syllabus As Per MSVE 2016)

Unit – III

Veterinary Microbiology

Compiled
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CERTIFICATE

Certified that this is a bonafide record of practical work done in the laboratory for the course of **VETERINARY MICROBIOLOGY (VMC) Unit III** during the year.

Name of the student:_____

Registration No.: _____

Exam seat No.:_____

CourseTeacher

ANNUAL EXAMINATION

Evaluated the practical record submitted for the Annual Practical Examination held on _____.

Course Teacher **Sectional Head**

Examiner

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Practical 1

EXTRACTION AND QUANTITATION OF NUCLEIC ACID

EXTRACTION OF DNA

The genomic DNA isolation needs to separate total DNA from RNA, protein, lipid, etc. Initially the cell membranes must be disrupted in order to release the DNA in the extraction buffer. SDS (sodium dodecyl sulphate) is used to disrupt the cell membrane. Once cell is disrupted, the endogenous nucleases tend to cause extensive hydrolysis. Nucleases apparently present on human fingertips are notorious for causing spurious degradation of nucleic acids during purification. DNA can be protected from endogenous nucleases by chelating Mg^{2++} ions using EDTA. Mg^{2++} ion is considered as a necessary cofactor for action of most of the nucleases. Nucleoprotein interactions are disrupted with SDS, phenol or proteinase K. Proteinase enzyme is used to degrade the proteins in the disrupted cell soup. Phenol and chloroform are used to denature and separate proteins from DNA. Chloroform is also a protein denaturant, which stabilizes the rather unstable boundary between an aqueous phase and pure phenol layer. The denatured proteins form a layer at the interface between the aqueous and the organic phases which are removed by centrifugation. DNA released from disrupted cells is precipitated by cold absolute ethanol or isopropanol. The organism should be harvested in late log to early stationary phase for maximum yield.

Materials Required:

Luria Bertani Broth, *E. coli* culture, Autoclaved Distilled Water, Eppendorf tubes 2 ml

Reagents:

TE buffer (pH 8.0), 10% SDS, Proteinase K, Phenol-chloroform mixture, 5M Sodium Acetate (pH 5.2), Isopropanol, 70% ethanol.

1. **Proteinase K:** Dissolve 10 mg of Proteinase K in 1 ml autoclaved distilled water.
2. **Phenol – Chloroform Mixture:** The pH is very important. For RNA purification, the pH is kept around pH 4, which retains RNA in the aqueous phase preferentially. For DNA purification, the pH is usually 7 to 8, at which point all nucleic acids are found in the aqueous phase. Mix equal volume of phenol with chloroform. Keep the mixture on ice & add 20 ml TE buffer, extract by shaking for 15 minutes. Remove the dust on the surface layer using a pipette. Repeat 4-5 times. Add 30-40 ml of TE buffer, store on ice.
3. **5M Sodium Acetate:** Dissolve 41 g of sodium acetate in 100 ml distilled water and adjust pH with dilute acetic acid (pH 5.2).
4. **Isopropanol**
5. **70% Ethanol**

Procedure:

1. 2 ml overnight culture is taken and the cells should be harvested by centrifugation for 10 minutes
2. 875 µl of TE buffer should be added to the cell pellet and the cells are resuspended in the buffer by gentle mixing.
3. 100 µl of 10% SDS and 5 µl of Proteinase K should be added to the cells.
4. The above mixture is mixed well and incubated at 37º C for an hour in an incubator.
5. 1 ml of phenol-chloroform mixture is added to the contents, mixed well by inverting and incubated at room temperature for 5 minutes.
6. The contents should be centrifuged at 10,000 rpm for 10 minutes at 4º C.
7. The highly viscous jelly like supernatant should be collected using cut tips and is transferred to a fresh tube.
8. The process is repeated once again with phenol-chloroform mixture and the supernatant is collected in a fresh tube.
9. 100 µl of 5M sodium acetate is added to the contents and is mixed gently.
10. 2 ml of isopropanol is added and mixed gently by inversion till white strands of DNA precipitates out.
11. The contents are centrifuged at 5,000 rpm for 10 minutes.
12. The supernatant is removed and 1ml 70% ethanol is added.
13. The above contents are centrifuged at 5,000 rpm for 10 minutes.
14. After air drying for 5 minutes 200 µl of TE buffer or distilled water is added.
15. 10 µl of DNA sample is taken and is diluted to 1 or 2 ml with distilled water.
16. The concentration of DNA is determined using a spectrophotometer at 260/280 nm.
17. The remaining samples should be stored for further experiments.

Precautions:

- Cut tips should be used so that the DNA is not subjected to mechanical disruption.
- Depending on the source of DNA the incubation period of Proteinase K should extended.
- The phenol chloroform extraction should be repeated depending on the source of DNA to obtain pure DNA.
- DNase free plastic wares and reagents should be used.

Removal of proteins from nucleic acids can be achieved by extraction with phenol: chloroform solutions. For crude mixtures of nucleic acids, digestion with a broad range proteolytic enzyme prior to the phenol: chloroform extraction may be beneficial. Although proteins are efficiently denatured by phenol, RNase activity is not completely inhibited. Therefore, a small amount of isoamyl alcohol is added to further ensure the deactivation of RNase activity. For DNA extraction, the pH of the phenol phase can be adjusted to 8.0 by equilibration with tris buffer.

Protocol for removal of proteins form nucleic acids follows:

1. Mix equal volume of phenol: chloroform: isoamyl alcohol solution with the nucleic acid solution in polypropylene tube with a cap. Mix briefly until an emulsion form.

2. Centrifuge at 12,000 g for 3-5 minutes at room temperature. The aqueous phase (upper) and organic phase (lower) should be well separated. The interface, which typically appears as an opaque disc, contains the denatured proteins.

3. Transfer the aqueous phase to a new tube. Discard the interface and organic phase.

4. Repeat steps 1-3 until no protein is visible at the interface.

5. (Optional) Mix equal volume of chloroform with the aqueous phase. Mix briefly and centrifuge at 12,000 g for 3-5 minutes. This step will remove any residual phenol.

6. Pipette upper phase into a new tube and precipitate the nucleic acid as desired.

Quantitative Analysis of DNA

Aim: To determine the amount, concentration and purity of the given DNA sample. Principle: This experiment is purely an application of the Beer Lamberts' Law which states that the concentration of the sample is directly proportional to the absorbance of light done by the sample. It is given by following expression:

The device UV spectrophotometer works on this principle and used to find the concentration of the sample. Concentration and quality of a sample of DNA is measured with a UV spectrophotometer. A standard graph can be drawn using different concentrations of DNA and OD (optical density) values. The diagram above shows that a beam of monochromatic radiation (Io) is directed to a sample solution. Absorption takes place by the sample and the beam of radiation is leaving out (I).

Materials Required: DNA sample, TE buffer UV spectrophotometer

Procedure:

1. Take the DNA sample (10 ul) in TE buffer.

2. Now dilute the above sample by the factor of 100 i. e, by taking 10µl of the sample in 990µl of TE buffer.

3. After doing this take the optical density value at A260 & A280 and calculate the amount of DNA recovered.

4. Use the following formula to determine the concentration of DNA:

Total DNA (ug) = (A260) (50 ug/ml/A260) (100) (0.1 ml)

where 100 is the dilution factor and 0.1 ml is the total volume of the DNA; Quality: DNA quality measurement is based on the fact that OD at 260 nm is twice that at 280 nm if the solution contains pure DNA. If there is a contaminant, there is some additional OD, which decreases the OD ratio between 260 and 280 nm. Clean DNA has a OD260/OD280 between 1.8 and 2.0

EXTRACTION OF RNA

Extraction of rotavirus dsRNA from faecal sample

All the plastic ware and glassware used for the extraction of dsRNA of rotavirus should be treated with 0.1% Diethylpyrocarbonate (DEPC) and properly autoclaved in order to make it free of RNAse activity. One control sample should be used for the standardization of extraction of dsRNA and RNA-PAGE. A 10% faecal suspension was made in lysis buffer (appendix) and centrifuged at 12,000 g for 20 min to remove coarse particles and cellular debris. The clarified supernatant should be stored at -20ºC till further used or immediately used for extraction of dsRNA of rotavirus.

Extraction of rotavirus dsRNA by phenol chloroform method

1. An aliquot of 1 ml clarified faecal supernatant should be treated with 0.1 volumes of 10% of sodium dodecyl sulphate (SDS) and incubated for 1 h at 56ºC in water bath.
2. An equal volume of phenol: choloroform: isoamyl alcohol (25:24:1) should be added to this clarified faecal supernatant. It should be vortexed and centrifuged at 12,000 g for 15 min at 4ºC.
3. The upper aqueous layer should be carefully transferred to another tube (DEPC treated) without disturbing the interphase.
4. It should be mixed with equal volume of chloroform: isoamyl alcohol (24:1), vortexed and then the mixture should be centrifuged again at 12,000 g for 10 min. and upper clear aqueous phase should be transferred to fresh microcentrifuge tube (DEPC treated).
5. To this aqueous phase,0.1 volume of 3 M sodium acetate and equal volume of isopropanol should be added and mixed gently.
6. The RNA should be precipitated by keeping it at -20ºC for overnight.
7. The RNA should be pelleted by centrifugation at 10,000 g for 15 min.
8. The pellet obtained should be washed with 1 ml of 70% chilled ethanol to remove excess salts by centrifuging at 10,000 g for 5 min.
9. The pellet should be airdried and suspended in nuclease free water and stored at -20ºC.

TRI reagent

1. Rotaviral dsRNA should be extracted from 10% clarified faecal suspension. Briefly, about 0.75 ml of TRI REAGENT LS(R) should be mixed with 0.25 ml of sample (10% faecal extract) and homogenized well.
2. Homogenate should be stored for 5 min at room temperature to permit the complete dissociation of nucleoprotein complexes.
3. It should be followed by addition of 0.2 ml chloroform to the homogenate and shaken vigorously for 15 sec.
4. The resulting mixture should be stored at room temperature for 15 min and immediately centrifuged at 12,000 g for 15 min at4ºC.
5. The colorless upper aqueous phase should be transferred to fresh centrifuge tube, to which 0.5ml of isopropanol should be added for RNA precipitation.
6. The mixture should be stored at room temperature for 10 min and centrifuged at 12,000 g for 10 min at 4ºC.
7. The supernatant obtained should be discarded and the RNA pellet should be washed with 1 ml ethanol (75%) by vortexing, followed by centrifugation at 7500 g for 5 min at 4ºC.
8. The ethanol wash should be removed and the RNA pellet should be air dried for 3-5 min.
9. The RNA pellet should be dissolved in 20 µl nuclease free water(NFW) and stored at -20ºC till further use.

Reagents for RNA extraction

3M Sodium acetate

Sodium acetate . 3 H2O 40.81g Dissolved in 80ml distilled water and the pH was adjusted to 5.2 with glacial acetic acid. Final volume adjusted to 100ml. It was then filtered through Whatman no.1 filter paper and stored at room temperature.

2M Sodium acetate, pH 4.6

Sodium acetate. 3 H2O 27.21gm Dissolved in 80ml distilled water and the pH was adjusted to 4.6 with glacial acetic acid. Final volume adjusted to 100ml. It was then filtered through Whatman no.1 filter paper and stored at room temperature.

SDS (10%) SDS (Sigma) 10gm Dissolved in 80ml of distilled water at 68^0C and final volume was adjusted to 100 ml. filtered through Whatman no.1 filter paper and stored at room temperature.

Quantitative Analysis of RNA

Aim: To determine the amount and concentration of RNA sample isolated from bacterial cells.

Principle: This experiment is purely an application of the Beer Lamberts' Law which states that the concentration of the sample is directly proportional to the absorbance of light done by the sample. It is given by following expression:

$$A = \varepsilon * c * l$$

The device UV spectrophotometer works on this principle and used to find the concentration of the sample.

Materials required: RNA sample TE buffer, UV spectrophotometer

Procedure:

1. Remove a 10 µl aliquot of total RNA and dilute with 990 µl of TE buffer (10 mM Tris, 1 mM EDTA, pH 7.0).
2. Read at A260 and A280 blanked against TE buffer and calculate the amount of RNA obtained. The RNA obtained may be determined by the formula:

Total RNA (ug) = (A 260) (40 ug) (100) (0.05 ml)

- A260 is the absorbance of the solution at 260 nm
- 1 OD of RNA equals to 40 ug/ml/A 260
- 100 is the dilution factor, and 0.05 ml is the total volume.
- Concentration = Total ug/50 ul = ug/µl or mg/ml.

Exercise

1. Write the 260/280 ratio of pure DNA and RNA.
2. What does it indicates if 260/280 ratio of nucleic acid is more?

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Practical 2

PLASMID PROFILING
Bacterial Plasmid DNA Extraction Using Alkaline Lysis Method

Materials and reagents

1. Luria-Bertani broth (LB) medium: Bacto-tryptone, yeast extract
2. RNAase
3. Isopropanol
4. Ethanol

Equipment

1. Table-top centrifuge

Procedure

1. Grow bacterial (*E. coli*) culture in LB medium with appropriate antibiotics at 37 °C overnight (O/N) with shaking. For > 10 copies plasmid, 3 ml cell culture is usually enough.
2. Transfer O/N culture to a 1.5 ml Eppendorf tube, and spin down cell culture (twice) at high speed for 1 min at table-top centrifuge.
3. Discard the supernatant. To remove the liquid completely by upside down tube onto a piece of paper towel for a few seconds.
4. Add 100 µl of resuspension solution (P1 buffer) into each tube, and vortex to completely resuspend cell pellet
5. Add 100 µl of lysis solution (P2 buffer) and mix by gently inverting the tube 5-6 times. The solution should quickly turn transparent and become more viscous indicating bacterial lysis has taken place.
6. Add 150 µl of neutralizing solution (P3 buffer) and mix by inverting the tubes several times. At this point bacterial chromosomal DNA is usually seen as a white precipitate.
7. Centrifuge the tubes at high speed for 10 min.
8. Carefully transfer the supernatant (try to not disturb the white precipitate) to a new labelled 1.5 ml Eppendorf tube with a 1 ml pipette.
9. Add 2.5-3 volume of 200-proof cold ethanol (stores at -20 °C) to each tube and mix by inverting the tubes for a few times.
   Note: In order to increase DNA yield, you can keep the tubes at -20 °C for 30 min or longer before centrifuge (next step).
10. Spin down plasmid DNA precipitate (transparency pellet) at high speed at 4 °C for 10 min.
11. Discard the supernatant and remove the remaining liquid as much as possible by leaving the tube upside-down on a piece of paper towel, then keep the tubes in a tube holder and air dry for 10-20 min. To dry faster, keep tubes at 37 °C heat blocker. DNA precipitate turns to white when dry.
12. Resuspend the DNA pellet with 50 µl TE. To completely dissolve the pellet by pipetting solution several times.

Note: lots of RNA is present in the DNA sample. So, for subsequent reaction, for example, to digest plasmid DNA, add 1-5 µl (1 mg/ml) RNAase to the digestion solution to completely remove RNA. Or, add RNAase directly to resuspension solution with a final concentration of 1 mg/ml.

Reagents & Media:

1. LB medium: 1% Tryptone, 0.5% yeast extract, 200 mM NaCl.
2. Resuspension solution (P1 buffer): 50 mM glucose, 10 mM EDTA, 25 mM Tris, pH 8.0. Store at 4 °C.
3. Lysis solution (P2 buffer): 0.2 N NaOH, 1% SDS. Store at room temperature.
4. Neutralizing solution (P3 buffer): 3 M KOAc, pH 6.0. For 100 ml solution, 60 ml 5 M potassium acetate (49.07 g potassium acetate in 100 ml H_2O), 11.5 ml glacial acetate and 28.5 ml H_2O. Store at room temperature.
5. TE: 1 mM EDTA, 10 mM Tris-HCl, pH 8.0.

Exercise

Q1. How genomic DNA is removed during Plasmid DNA extraction procedures?

References

1. Birnboim H.C., Doly J. (1979). A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Res* 7(6): 1513-23.
2. Birnboim H.C. (1983). A rapid alkaline extraction method for the isolation of plasmid DNA. *Methods in Enzymology* 100: 243-55.

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Practical 3

AGAROSE GEL ELECTROPHORESIS FOR DIAGNOSIS OF NUCLEIC ACID OF MICROBES

Agarose gels are used to analyze DNA molecules. These gels are simple to construct, because they rely only on the gelling properties of agarose. Molecules are separated by size and visualized with fluorescent intercalating dyes.

Agarose gel electrophoresis is a method used to separate DNA fragments. DNA molecules will be separated according to their sizes and charges. The location of DNA fragments within the gel can be determined directly by staining with appropriate dyes, and can be detected with naked eyes.

Aim: To separate and visualise DNA bands by Agarose gel electrophoresis.

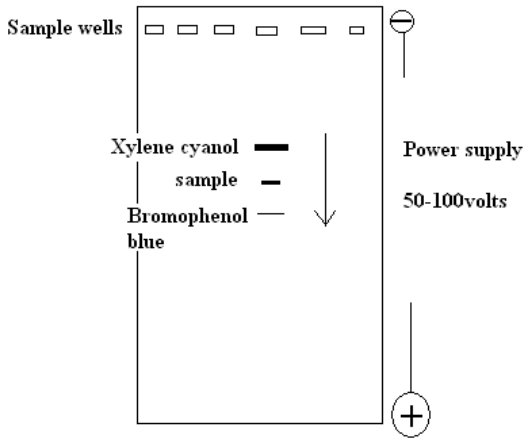
Principle: The agarose gel contains molecule sized pores, acting like molecular sieves. The pores in the gel control the speed that molecules can move. DNA molecules possess a negative charge in their backbone structure due to the - presence of PO_4 groups thus this principle is exploited for its separation.

Smaller molecules move through the pores more easily than larger ones. Conditions of charge, size, and shape interact with one another depending on the structure and composition of the molecules, buffer conditions, gel thickness, and voltage. Agarose gels are made with between 0.7% (provides good resolution of large 5–10 kb DNA fragments) and 2% (good resolution for small 0.2–1 kb fragments).

The gel setup provides wells for loading DNA in to it. The loaded DNA molecules move towards the positively charged electrode (anode) and get separated along the length of the gel. Ethidium bromide (EtBr), a chromogen is added to the gel to visualize the separated DNA under UV trans- illumination. EtBr intercalates between the bases and glows when UV radiation is passed through the gel.

Purpose of gel loading buffer

The loading buffer gives colour and density to the sample to make it easy to load into the wells. Also, the dyes are negatively charged in neutral buffers and thus move in the same direction as the DNA during electrophoresis. This allows you to monitor the progress of the gel. The gel loading dye possesses bromophenol blue and xylene cyanol. Density is provided by glycerol or sucrose**.**



Xylene xyanol gives a greenish blue colour while bromophenol blue provides bluish colored zone. The successful DNA run is determined by the presence of both the colored dye in thegel.

Materials required

Electrophoresis buffer: 1x TAE buffer, Agarose ultra pure (DNA graded), electrophoresis tank, gel tray, sample comb and power supply,Plastic or insulation tape, Ethidium bromide: 10 mg /ml stock solution, 5x Gel loading dye, DNA marker solution, DNA sample and gloves.

Procedure

Making a 1% Agarose Gel

1. Weigh 0.5 g agarose and dissolve it in 50 mL of 1x TAE Buffer. (Note: Use 250 ml conical flask for preparing 50 ml solution to avoid overflow of gel solution while heating and to avoid its loss.)
2. Heat the solution over a hot plate to boiling constituency marked with a clear solution
3. Leave the solution to cool and add 2µl of EtBr solution mix it well by gentle swirling.
4. Pour it in the gel tray-comb set up. Also, be sure the gel plates have been taped securely and contain the well combs prior to pouring
5. Allow the solution to cool and harden to form gel.

Loading of Samples

1. Carefully transfer the gel to the electrophoresis tank filled with 1x TAE buffer.
2. Prepare your samples [8 ul of DNA sample (0. 1 ug to 1 ug) & 2 ul of 5x gel loading dye]
3. Remove the comb and load the samples into the well.
4. Connect appropriate electrodes to the power pack and run it at 50- 100volts for 20min.
5. Monitor the progress of the gel with reference to tracking dye (Bromophenol blue).
6. Stop the run when the marker has run 3/4th of the gel.
7. Examining the gel
8. Place the gel on the UV-transilluminator and check for orange colored bands in the gel.

Precautions

1. Wear gloves during the addition of EtBr and while handling the casted gel (EtBr is a potent carcinogen).
2.Handling the gel should be careful as the gel may break due to improper handling.
3. While performing the UV-trans illumination for visualising the bands, avoid direct contact and exposure to eyes.

Exercise

1. Write the composition of Bromophenol Blue – Gel Loading Buffer, TBE Buffer.
2. Write the wavelength of UV light used to visualize DNA during electrophoresis.

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Practical 4

SDS PAGE ELECTROPHORESIS FOR DIAGNOSIS OF PROTEINS OF MICROBES

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) is used to separate proteins with relative molecular mass no smaller than 10 KD. Very small proteins (< 10 KD) are difficult to resolve due to low ability of binding to SDS, which can be solved by gradient gels or using different electrophoresis conditions, like Tricine-SDS-page.

Materials and reagents: Pre-stain Protein MW marker , TEMED, Ammonium persulfate, SDS, 30% Acrylamide stock (37.5:1 acrylamide:bisacrylamide), Bromophenol Blue, b-mercaptoethanol, Tris Base, Glycine, EDTA, Glycerol.

Equipment

Protein mini gel cassettes (Bio-Rad), Heating block module, Power supply

Procedure

1. **Making SDS-PAGE gel**

a. Clean and completely dry glass plates, combs, spacers, and assemble gel cassette by following manufactory instructions.

b. Prepare 10% lower gel (separating gel) by adding the following solutions (wear gloves when prepare gel solution) (total volume= 5 ml)
   2 ml dH_2O
   1.67 ml 30% acrylamide/Bis
   1.25 ml 1.5 M Tris, pH 8.8
   25 µl 20% SDS
   25 µl 10% ammonium persulfate (Make it fresh and store at 4 °C up to a month)
   2.5 µl TEMED (add it right before pour the gel)
   Note: change ration of dH_2O to 30% acrylamide/Bis to get different percentage of separating gel.

c. To avoid polymerization, after adding TEMED, mix well and quickly transfer the gel solution by using 1 ml pipette to the casting chamber between the glass plates and fill up to about 0.7 cm below the bottom of comb when the comb is in place.

d. Add a small layer of isopropanol to top of the gel prior to polymerization to straighten the level of the gel

e. Once the gel has polymerized, start to prepare stacking gel (5%) by adding the following solutions (total volume = 3 ml)
   2.088 ml dH_2O
   0.506 ml 30% acrylamide/Bis
   0.375 ml 1 M Tris, pH 6.8
   15 µl 20% (w/v) SDS
   15 µl 10% ammonium persulfate
   1.5 µl TEMED (add it right before pour the gel)

f. Remove the isopropanol layer by using filter paper. Rinse the top layer of the gel with dH_2O and dry off as much of the water as possible by using filter paper.
g. Add TEMED and mix the stacking gel solution well. Quickly transfer the gel solution by using 1 ml pipette till the space is full then insert the appropriate comb.
h. Allow the top portion to solidify and then carefully remove the comb.
Note: The gels can be stored with the combs in place tightly wrapped in plastic wrap and put them in a second container with wet tissue towel (keep the gels moist) at 4 °C for 1 to 2 weeks.

2. Sample Preparation

a. Prepare same amount of protein samples according to BCA assay result, see BCA (bicinchoninic acid) protein assay.
b. Add the same volume of 2x protein sample buffer to each protein sample, mix and boil the samples at 95 °C heating block module for 10 min.
c. Spin the samples at the maximal speed for 1 min (samples from some tissue/cell sources may need longer spin) in table-top centrifuge and leave the samples at room temperature until ready to load onto the gel.
Note: can store extract protein samples (containing sample buffer) at -20 °C and reheat at 95 °C for 5 min when use next time.

3. Electrophoresis

a. Remove the gel cassette from the casting stand and place it in the electrode assembly with the short plate on the inside. Press down on the electrode assembly while clamping the frame to secure the electrode assembly and put the clamping frame into the electrophoresis tank.
b. Pour some 1x electrophoresis running buffer into the opening of the casting frame between the gel cassettes. Add enough buffer to fill the wells of the gel. Fill the region outside of the frame with 1x running buffer.
c. Slowly load the same amount of protein samples into each well as well as load 10 µl of protein MW marker.
d. Connect the electrophoresis tank to the power supply.

4. **Protein detection**
If protein of interest is about 0.2 µg or more in the sample, typically use Coomassie blue staining (see Coomassie blue staining). Otherwise, use silver staining (sliver staining), which is more sensitive and can detect as little as 5 ng protein.

Reagents

1. 10x Running Buffer:
30.3 g Tris-base
144.0 g glycine
10.0 g SDS
Completely dissolve in about 800 ml dH_2O and then more dH_2O up to 1 liter.

2. 2x SDS protein sample buffer:
   1.25 ml 1 M Tris-HCl pH 6.8
   4.0 ml 10% (w/v) SDS
   2.0 ml glycerol
   0.5 ml 0.5 M EDTA
   4 mg Bromophenol Blue
   0.2 ml b-mercaptoethanol (14.3 M)
   Bring the volume to 10 ml with dH_2O.

Exercise

1. Write the role of Ammonium persulfate.
2. Write the applications of SDS PAGE.

Reference

1. Laemmli U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227(5259): 680-5.

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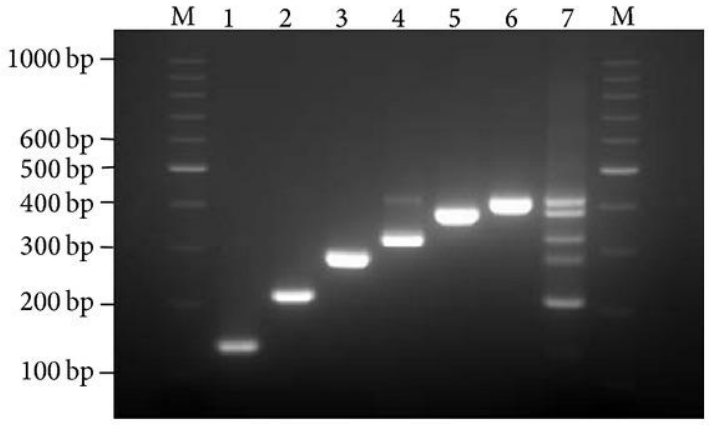
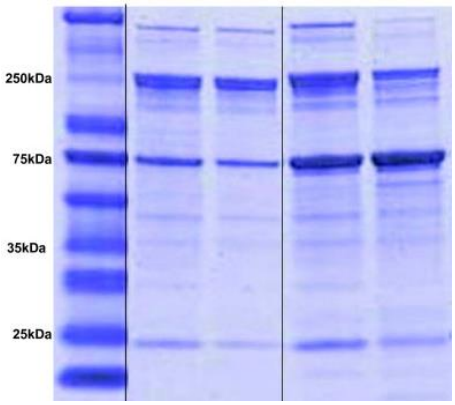
PLATE 1



Gel Electrophoresis-PCR Product visualization



SDS PAGE-Commassie Blue stain

SDS PAGE- Silver stain

Thermocycler

Gel Documentation Unit

Practical 5

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

Genbank: GenBank ®sequence database is an open access, annotated collection of all publicly available nucleotide sequences and their protein translations. This database is produced and maintained by the National Center for Biotechnology Information (NCBI) as part of the International Nucleotide Sequence Database Collaboration (INSDC). The National Center for Biotechnology Information is a part of the National Institutes of Health in the United States. https://www.ncbi.nlm.nih.gov/

International Nucleotide Sequence Database Collaboration, which comprises the DNA Data Bank of Japan (DDBJ), the European Nucleotide Archive (ENA), and GenBank at NCBI. These three organizations exchange data on a daily basis.

GenBank and its collaborators receive sequences produced in laboratories throughout the world from more than 100,000 distinct organisms. The database started in 1982 by Walter Goad and Los Alamos National Laboratory.

Protein Data Bank: RCSB PDB, a global resource for the advancement of research and education in biology and medicine. Along with our Worldwide PDB collaborators, RCSB PDB curates, annotates, and makes publicly available the PDB data deposited by scientists around the globe. The RCSB PDB then provides a window to these data through a rich online resource with powerful searching, reporting, and visualization tools for researchers. https://www.rcsb.org/

BLAST: The Basic Local Alignment Search Tool (BLAST) finds regions of local similarity between sequences. The program compares nucleotide or protein sequences to sequence databases and calculates the statistical significance of matches. BLAST can be used to infer functional and evolutionary relationships between sequences as well as help identify members of gene families. https://blast.ncbi.nlm.nih.gov/Blast.cgi

MEGA: Molecular Evolutionary Genetics Analysis - MEGA is an integrated tool for conducting sequence alignment, inferring phylogenetic trees, estimating divergence times, mining online databases, estimating rates of molecular evolution, inferring ancestral sequences, and testing evolutionary hypotheses. MEGA is used by biologists in a large number of laboratories for reconstructing the evolutionary histories of species and inferring the extent and nature of the selective forces shaping the evolution of genes and species. https://www.megasoftware.net/

Primer Blast: Primer designing tool. https://www.ncbi.nlm.nih.gov/tools/primer-blast/

Metagenomics: Metagenomics is the culture-independent genomic analysis of a community of microorganisms. It provides a community-wide assessment of metabolic function and bypasses

the need for the isolation and laboratory cultivation of individual species. The analysis of metagenomic data provides a way to identify new organisms and isolate complete genomes from unculturable species that are present within an environmental sample. https://www.ebi.ac.uk/metagenomics/

Exercise

1. Explore the above links and mine for information.

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APPENDIX

REAGENTS USED FOR RNA-PAGE

Acrylamide/Bisacrylamide mix (30%)

Acrylamide 29.2gm
N',N'-bismethylene acrylamide 0.8gm
DW to make 100ml. Filterd and stored at 40C in dark amber coloured bottle.

1.5M TrisHCl, pH 8.8

Tris base 18.15gm
DW 60ml
Adjust pH 8.8 with concentrated HCl
DW to make 100ml
Filtered through whatman no.1 filter paper. It was stored at 40^{O}C.

0.5M Tris HCL, pH 6.8

Tris base 6.0gm
DW 60ml
Adjust to pH 6.8 with concentrated HCl
DW to make 100ml. Filtered through Whatman no.1 filter paper. Stored at 40C.

Ammonium persulfate (10%)

Ammonium per sulphate 100mg, DW 1ml

2X RNA-PAGE sample buffer

0.5M TrisHCl pH 6.8 5.0ml
Glycerol 3.0ml
10% SDS 200µl
0.05% Bromophenol blue 400µl, DW 11.4ml

Tris-glycine running buffer (1X)

Tris base 3.0gm
Glycine 14.4gm, DW to make 1000ml

REAGENTS FOR SILVER STAINING

Fixative solution : 0.5% glacial acetic acid, 10% ethanol
Staining solution : 0.185% silver nitrate in GDW
Developer : 3gm of NaOH Pellets in 100ml GDW and 0.75 ml of formaldehyde
Stopper : 5% glacial acetic acid
Storing solution : 10% ethanol

SILVER STAINING

1. The gel was taken out from plates and marked at the corner. Subsequently, the gel was fixed in the fixative solution (appendix) for 30 min at room temperature with intermittent gentle shaking.
2. The fixative was removed and the gel was stained with silver nitrate solution (appendix) for 30minutes with gentle shaking.
3. The silver nitrate solution was drained off and the gel was quickly rinsed with distilled water twice to remove excess silver nitrate to minimize back ground staining.
4. Subsequently, the developer (appendix) was added in darkened room to develop the stained RNA bands by gentle shaking for 10 min.
5. The reaction was stopped by adding stop solution (5% glacial acetic acid).
6. The gel was kept in the solution for 15 min. The stained gel was observed for (11) RNA segments (of rotaviral dsRNA) and photographed if found positive and stored in 10% ethanol.

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Veterinary Microbiology

Practical Manual

Unit III (As per MSVE 2016)

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COVASU

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