SEMESTER V

17BTU514A ANIMAL BIOTECHNOLOGY PRACTICAL 4H - 2C

Total hours/week: L: 0 T: 0 P: 4 Marks: Internal: 40 External: 60 Total: 100

Practical

1. Sterilization techniques: Glass ware sterilization, Media sterilization, Laboratory sterilization.
2. Sources of contamination and decontamination measures.
3. Preparation of Hanks Balanced salt solution
4. Preparation of Minimal Essential Growth medium
5. Isolation of lymphocytes for culturing
6. DNA isolation from animal tissue
7. Quantification of isolated DNA.
8. Resolving DNA on agarose gel.

References

1. Glick, B.R., & Pasternak, J.J. (2009). Molecular biotechnology- Principles and applications of recombinant DNA (4th ed.). Washington, USA: ASM press.

2. Griffiths, A.J.F., Miller, J.H., Suzuki, D.T., Lewontin, R.C., & Gelbart, W.M. (2009). An introduction to genetic analysis (9th ed.). NY:USA, Freeman & Co.

3. Watson, J.D., Myers, R.M., Caudy, A., & Witkowski, J.K. (2007). Recombinant DNA genes and genomes- A short course (3rd ed.). NY:USA, Freeman & Co.

4. Butler, M. (2004). Animal cell culture and technology: The basics (2nd ed.). Bios scientific publishers.

EX. NO-1.1 STERILIZATION TECHNIQUES: THEORY AND PRACTICAL

A) GLASSWARE STERILIZATION

AIM: -

To prepare and sterilize the given glassware.

MATERIAL REQUIRED:-

1. Disinfectant-hypochlorite, 300 ppm available chlorine.
2. Detergent-7x, Decon
3. Soaking baths
4. Bottle brushes
5. Stainless steel baskets
6. Aluminum foil
7. Sterility indicators
8. Sterilizing oven (upto 160°C)

PROTOCOL:-

1. **Collection and washing of glassware**

i. Immediately after use, collect glassware into detergent containing disinfectant. It is very important that glassware does not dry before soaking.

ii. Soak overnight in detergent.

iii. Rinsing: - a. Brushing, b. Machine

Rinse 3-4 time using deionized water and then sensing is done without detergent with deionized or RO water.

iv. After rinsing thoroughly invert bottles etc. in stainless steel wire basket and dry up side down. After that cap bottles with aluminum foil and cool.

2. Sterilization of Glassware:-

i. Attach Small Square of sterile indicating tape or other indicators labeled to glassware and tape.

ii. Place glassware in oven with fan circulated air and temperature set to 160°C.

iii. Ensure that the packing is not very tight. Leave room for circulation of hot air.

iv. Close the oven check the temperature to 160° and seal the oven and leave it for one hour. Time should be recorded.

v. After one hour, switch off the oven and allow it to cool with the door closed.

vi. Use glassware within 24-48 hours.

OBSERVATION/ RESULT:

The given chemicals and glasswares were sterilized

EX. NO-1.2 STERILIZATION TECHNIQUES: THEORY AND PRACTICAL

B) MEDIA STERILIZATION

AIM:-

To perform media sterilization

THEORY:-

Most of the commonly used media are available for commercially but for special and sterilize the media. Some may be auto cleaned while labile solutions like media, trypsin and serum must be filtered through 0.2 porosity membrane filter.

There may be some microbial contamination in the media. The characteristic features of microbial contamination are:-

1. Sudden change in PH, usually decrease with bacterial growth and slightly increase with fungal growth.
2. Sometimes cloudiness appeal in the medium with a slight flame as scum surface or spot on the growth surface that dissipates when flask is moved.
3. Under a low power microscope (around 10X) space between cells will appear granular and may shimmer with bacterial contamination.
4. Yeast appear as separate round or void practical that may or may not be in budding rate.
5. Fungus produce thin filamentous mycelium and sometimes dense lamps of spores are formed.

PROCEDURE:-

i) For heat stable solutions:-

For heat stable solution (i.e. the substance those constituents does not breakdown into simples substances) components are sterilized by autoclaving them at 121°C for 15 mins.

ii) For heat liable solutions:-

For heat liable solutions, filtrations are the only method. Heat liable solutions are those containing disaccharide or polysaccharide which on their autoclaving changes into monosaccharide. Thus, the method used for their sterilization is filtration.

Two types of filters used are:-

1. Absolute
2. Depth

PRECAUTIONS:-

1. Do not autoclave heat liable solutions
2. Heat stable solution should be autoclaved for 15-20 mints for proper sterilization.
3. Microbial infection should be checked carefully, it is sometimes confused with precipitates of co media that are mainly proteins.

OBSERVATION/ RESULT:

The given media were sterilized

EX. NO-1.3 STERILIZATION TECHNIQUES: THEORY AND PRACTICAL

C) LABORATORY STERILIZATION

AIM:-

To perform laboratory sterilization.

REQUIREMENT:-

Chemicals used for disinfection are:-

1. Acids & esters: - Benzoic acid, sulphur dioxide, sulphoides and metabisulphides.
2. Alcohols: - Ethanol, isopropanol, benzyl alcohol, chloroform, chloroputanol, phenyl alcohol, phenyl alcohol.
3. Fumigation: - Formaldehyde [H (CHO)], hydrogen cyanide (HCN), U.V. rays.

THEORY: -

Sterilization of laboratory is very important as contamination may come from laboratory environment. For this purpose, sterilization of the laboratory is done by different methods.

1. Cleaning with disinfectants:-

A wide range of disinfectants are present in the market. These have the potential to kill the different types of microbial forms. Thus autoclaving is not possible to avoid contamination of cultures. Culture rooms are cleaned with the help of disinfectant.

2. Eradication with U.V. lights:-

U.V. light is also supported to kill all microbial forms. These culture rooms are lightened with U.V. light before & after doing the practical work.

PRECAUTIONS:-

i. Remove the shoes after entering the incubation room.

ii. Sterilization of apparatus should be done carefully before the inoculation.

iii. Personal cleanliness is very essential in the laboratory. Avoid contamination by mouth near the laminar air flow.

iv. Hands should be properly sterilized by 70% alcohol or ethanol.

OBSERVATION/ RESULT:

Laboratory were sterilized using disinfectant

EX. NO-2

SOURCES OF CONTAMINATION AND DECONTAMINATION MEASURES

AIM:-

To study the sources of contamination and decontamination measures in ATC lab.

REQUIREMENTS:-

Tissue culture media in addition to provide cells is also ideal substrate for the growth of micro-organisms. So, it is necessary to sterile the media, culture vessels, tools and instruments and surface disinfect the explant as well.

It is important to avoid cross contamination, (when contamination or bacteria is carried from one object/flask/ petriplete to another).

The characteristic features of microbial contamination are:-

i. A certain change in pH usually occur with bacterial growth. There is slight change in pH during fungal growth. There is either no or very little narration in pH during the growth of yeast.

ii. Cloudiness in the medium is sometimes with the slight film or scum on the surface or the spots on the growth surface that dissipates when flask is moved.

iii. Under low power microscope, spaces between cells will appear granular and may shimmer with bacterial contamination yeast appears as separate, round or avoid particles that may or may not be in the budding state. Fungus produces thin filamentous mycelia and sometimes denser clumps of spores.

iv. Under high power microscope it may be possible to solve individual bacteria depending upon its shape (round and cocci).

With the slide preparation the morphology of bacteria can be resolved at 1000 x. Microbial infection sometimes may be consumed with the precipitates of media that are mainly:-

EQUIPMENT & MEDIA	SOURCES OF CONTAMINATION	DECONTAMINATION MEASURES
i. Manipulation pipetting and dispersing.	• Non-sterilized surface and equipment. • Spillage of necks and outside of bottles and on the working surface. • Pouching or holding pipette too low.	• Clean working area of lab and also items present. • Swab properly with 70% ethanol • Dispensing or transferring should be done by pipettes. • Filter or autoclave before use • Test the filter before use.
ii. Solutions:- • Non sterile reagents and media • Dirty storage conditions • Inadequate sterilization.	• Poor commercial supply. • Dust and spores from storage	• Dry heat autoclave, sterilization. • Don‟t store the unsealed materials for more than 24 hrs.
iii. Glassware and screw caps.	• Ineffective sterilization • Contact with non-sterile surface.	• Sterilization by dry heat before use. • Rasterize instruments (70% ethanol)
iv. Tools, instruments and pipettes.	• Invasion insects or dust.	• Use screw caps in preference to stoppers.
v. Culture/flask and media bottles. vi. Facilities, rooms and air.	• Dust and aerosol	• Wipe flasks and bottles with 70% ethanol. • Filtered or clean air is used.
vii. Work surface	• Dust and spillage.	Wipe the floor and work surface regularly.
viii. While operating, hairs, hands, cloths, breathe.	• Dust room skin, hair, and clothing dropped or blown into culture.	Wipe the floor and work surface regularly.

ix. Hoods		• Swab the work surface with 70% ethanol. • Wash hands thoroughly. • Less talking, wear a mask, tie hails.
x. Tissue sampling	Infected at the source during dissection.	• Check the filters. • Wash hands thoroughly, wear a mask, tie hails. • Check the filters.
xi. Co_2 incubators	Growth of molds and bacteria in humid atmosphere also on the wall.	• Check the tissue. • Do not bring the tissue directly to lab and disinfect the tissue surface properly. • Clean out weekly with detergent and 70% ethanol.

OBSERVATION/ RESULT:

EX. NO-3 PREPARATION OF HANKS BALANCED SALT SOLUTION

AIM:-

Isolation of rat macrophages from peritoneum for culturing.

RESQIREMENTS:-

10ml syringe, ice cold PBS & 2% BSA, centrifuge, rat, scissors, vials leishman‟s stain, microscope etc.

Composition of PBS & BSA

1. PBS (Phosphate buffer saline)
2. $Na_2HPO_4.2H_2O$-----1.56 gm (Basic)
3. $NaH_2PO_4.2H_2O$----1.78gm (Acidic)
4. Set the pH at 7.4 (100 ml)
5. Add NaCl (for 100ml – 0.9gm)
6. 100 ml = 0.9x100/105=0.945gm
7. 10ml of PBS + 0.1gm of BSA

PROCEDURE:-

i. The rat was killed with chloroform. The skin was drenched in 70% alcohol to sterilize.

ii. Infinite small incision was made in abdominal cavity skin and skin was sterilized.

iii. 10ml of solution of PBS & BSA (Bovine) serum albuminwas taken in syringe & carefully injected by lifting peritoneum cavity.

iv. The peritoneum was adjusted slowly to suspend the fluid uniformly after 5 mins withdraw as much fluid from body and pour it into vials.

v. Now centrifuge the vial containing fluid for 10 minutes at 200 rpm.

vi. Discard the pellet and suspend it in Leishman’s stain for 1-2 minutes.

PRECAUTION:-

i. The experiment should be performed under aseptic conditions.

ii. Rat should be properly washed with 70% alcohol to avoid any infection.

iii. Ice cold solution has to be injected.

iv. The vessel of the peritoneum should not get damaged while fluid is being injected in or in other words, blood contamination must not occur

COMPOSITION:-

Liquid					
	HBSS 1X	**HBSS 10X WITHOUT Ca & Mg**	**HBSS 1X WITHOUT phenol Red**	**HBSS 1X WITHOUT Ca & Mg**	**HBSS 10X WITHOUT Ca & Mg & phenol Red**
Sodium Chloride	**8000**	**80,000**	**8000**	**8000**	**8000**
Potassium Chloride	400	4000	400	400	40
Potassium phosphate, monobasic KH_2 PO4	60	600	60	60	60
Glucose	1000	10,000	1000	1000	1000
Phenol red Na salt	10	100	-	10	-
Sodium phosphate, dibasic NaH2PO4, anhydrous	48	479	48	48	47.86
Magnesium sulfate ahhydrous, MgSO4	98	-	98	140	-
Calcium chloride anhydrous	140	-	140	-	-
Sodium bicarbonate	350	-	350	350	350

OBSERVATION/ RESULT:

EX. NO-5 ISOLATION OF LYMPHOCYTES FOR CULTURING

AIM:-

To isolates the lymphocytes for culturing.

REQUIREMENTS:-

Sterile phosphate buffer saline ficoll, histopaque of density 7.1 mg/l, centrifuge tube, sterile pipette tips.

THEORY:-

The most commonly used method for the separation of lymphocytes is the sedimentation through high density medium of a monocular cells can be obtained by centrifuging whole blood or the ficoll plate and the commercial preparation are from ficoll.

PROCEDURE:-

1. Collect peripheral blood in the vial containing anti-coagulant.
2. Dilute blood 1:2 with phosphate buffer saline (PBS) layer not more than 3 times the volume of the distilled water into the layer of ficoll.
3. Always hold a tube at an angle while carrying out the layering so that the ficoll and blood do not get mixed.
4. Centrifuge at room temperature at 400 rpm for 30 mins.
5. Take care that the centrifuge doesn't accelerate so rapidly.

Mononuclear lymphocytes and monocytes are recovered at ficoll plasma interphase when they from a white band. The erythrocytes sediment through ficoll plasma and from a pellet.

PRECAUTION:-

i. Layering should be done carefully.
ii. Carefully dilute the blood.
iii. Take care that centrifuge should not decelerate**.**
iv. Observe the white layer carefully.

OBSERVATION/ RESULT:

EX. NO-6 DNA ISOLATION FROM ANIMAL TISSUE

AIM:-

To isolates the DNA from animal tissue.

REQUIREMENTS:-

Animal tissue, centrifuge, eppendrof tubes, EDTA, proteinase K, ammonium acetate.

THEORY:-

Animal cell cultures and most animal tissues can be efficiently lysed using lysis buffer and protease or proteinase K. Fresh or frozen samples should be cut into small pieces to aid lysis. Mechanical disruption using a homogenizer or mortar and pestle prior to lysis can reduce lysis time. Skeletal muscle, heart, and skin tissue have an abundance of contractile proteins, connective tissue, and collagen, and care should be taken to ensure complete digestion with protease or proteinase K.

For fixed tissues, the fixative should be removed prior to lysis. Formalin can be removed by washing the tissue in phosphate-buffered saline (PBS). Paraffin should be similarly removed from paraffin-embedded tissues by extraction with xylene followed by washing with ethanol.

PROCEDURE:-

1. Obtain animal tissue 10-20mg from the sample. Transfer to an eppendrof 1.5ml tube labeled with an identification number.
2. Add liquid nitrogen and grind the tissue for 1 minutye with a pestle.
3. Add 300 µl Extraction (NaCl 10mM; Tris HCl ; 10mM; pH 7.5; EDTA, 10mM; pH 8.0 and 100 µl SDS 5%.
4. Add 15µl Proteinase K
5. Grind the tissue with a pestle
6. Incubate the tube in a water bath or heat block at 60°C for at least 30' minutes.
7. Prepare a new set of eppendrof tubes labeled with identification codes for the next step
8. Centrifuge at 13,000 rpm for 15minutes.
9. Transfer 150 µl supernatant to a new tube.

10. Add 150 µl ammonium acetate 5M and 300 µl isopropanol.
11. Mix inverting the eppendrof tubes (5X)
12. Leave at room temperature for 10 minutes.
13. Centrifuge at 13,000 rpm for 5minutes.
14. Eliminate the supernatant carefully. Make sure that the pellet remains at the bottom of the tube.
15. Wash pellet by adding 500 µl ethanol 70%.
16. Centrifuge at 13,000 rpm for 5minutes.
17. Eliminate the supernatant. dry the pellet for 10-15 minutes at 60°C to evaporate remaining ethanol .
18. Resuspend in 50µl TE + RNAse.

OBSERVATION/ RESULT:

EX. NO-7 QUANTIFICATION OF ISOLATED DNA

AIM:-

To quantify DNA from animal tissue.

THEORY:-

Reliable measurement of DNA concentration is important for many applications in molecular biology. Spectrophotometry and fluorometry are commonly used to measure both genomic and plasmid DNA concentration. Spectrophotometry can be used to measure microgram quantities of pure DNA samples (i.e., DNA that is not contaminated by proteins, phenol, agarose, or RNA). Fluorometry is more sensitive, allowing measurement of nanogram quantities of DNA, and furthermore, the use of Hoechst 33258 dye allows specific analysis of DNA.

SPECTROPHOTOMETRY

DNA concentration can be determined by measuring the absorbance at 260 nm (A260) in a spectrophotometer using a quartz cuvette. For greatest accuracy, readings should be between 0.1 and 1.0. An absorbance of 1 unit at 260 nm corresponds to 50 µg genomic DNA per ml (A260 =1 for 50 µg/ml; based on a standard 1 cm path length. This relation is valid only for measurements made at neutral pH, therefore, samples should be diluted in a low-salt buffer with neutral pH (e.g., Tris·Cl, pH 7.0). An example of the calculation involved in nucleic acid quantification when using a spectrophotometer

When working with small amounts of DNA, such as purified PCR products or DNA fragments extracted from a garose gels, quantification via agarose gel analysis may be more effective.

Effects of solvents on spectrophotometric readings

Absorption of nucleic acids depends on the solvent used to dissolve the nucleic acid. A_{260} values are reproducible when using low-salt buffer, but not when using water. This is most likely due to differences in the pH of the water caused by the solvation of CO_2 from air. A_{260}/A_{280} ratios measured in water also give rise to a high variability between readings (see figure Effect of solvent on A_{260}/A_{280} ratio) and the ratios obtained are typically <1.8, resulting in reduced

sensitivity to protein contamination.. In contrast, A_{260}/A_{280} ratios measured in a low-salt buffer with slightly alkaline pH are generally reproducible.

OBSERVATION/ RESULT:

EX. NO-8 RESOLVING DNA ON AGAROSE GEL

AIM:-

To resolve the obtained DNA from animal tissue.

THEORY:-

Pouring an agarose gel

Agarose concentration

The concentration of agarose used for the gel depends primarily on the size of the DNA fragments to be analyzed. Low agarose concentrations are used to separate large DNA fragments, while high agarose concentrations allow resolution of small DNA fragments. Most gels are run using standard agarose, although some special types of agarose are available for particular applications. For example, low-melt agarose allows in situ enzymatic reactions and can therefore be used for preparative gels. Genomic DNA can be isolated directly from cells immobilized in low-melt agarose gels.

Concentration of agarose used for separating fragments of different sizes

Agarose concentration (% w/v)	DNA fragment range (kb)
0.3*	5–60
0.5	1–30
0.7	0.8–12
1.0	0.5–10
1.2	0.4–7
1.5	0.2–3
2.0*	0.1–2

Electrophoresis buffers

The most commonly used buffers for agarose gel electrophoresis are TBE (Tris·borate–EDTA) and TAE (Tris·acetate–EDTA). Although more frequently used, TAE has a lower buffering capacity than TBE and is more easily exhausted during extended electrophoresis. TBE

gives better resolution and sharper bands, and is particularly recommended for analyzing fragments <1 kb.

The drawback of TBE is that the borate ions in the buffer form complexes with the cis-diol groups of sugar monomers and polymers, making it difficult to extract DNA fragments from TBE gels using traditional methods.

TAE

1x working solution composition	50x stock solution components per liter	Amount per liter
40 mM Tris·acetate	Tris base	242 g
1 mM EDTA	Glacial acetic acid	57.1 ml
–	0.5 M EDTA, pH 8.0	100 ml

TBE

0.5x working solution composition	5x stock solution components per liter	Amount per liter
40 mM Tris·borate	Tris base	54 g
1 mM EDTA	Boric acid	27.5 ml
–	0.5 M EDTA, pH 8.0	20 ml

Gel loading buffer

* 15% Ficoll (Type 400) or 30% glycerol can be used instead of sucrose.

6x working solution composition	5x stock solution components per 10 ml	Amount per 10 ml
0.25% bromophenol blue	Bromophenol blue	25 mg
0.25% xylene cyanol FF	Xylene cyanol FF	25 mg
40% (w/v) sucrose*	Sucrose	5 ml

Pouring the gel

1. Prepare enough 1x electrophoresis buffer both to pour the gel and fill the electrophoresis tank.
2. Add an appropriate amount of agarose (depending on the concentration required) to an appropriate volume of electrophoresis buffer (depending on the type of electrophoresis apparatus being used) in a flask or bottle.

   Tip: The vessel should not be more than half full. Cover the vessel to minimize evaporation.

   Tip: Always use the same batch of buffer to prepare the agarose as to run the gel since small differences in ionic strength can affect migration of DNA.
3. Heat the slurry in a microwave or boiling water bath, swirling the vessel occasionally, until the agarose is dissolved.

   Tip: Ensure that the lid of the flask is loose to avoid build-up of pressure. Be careful not to let the agarose solution boil over as it becomes super-heated.

   Tip: If the volume of liquid reduces considerably during heating due to evaporation, make up to the original volume with distilled water. This will ensure that the agarose concentration is correct and that the gel and the electrophoresis buffer have the same buffer composition.
4. Cool the agarose to 55–60°C.

5. Pour the agarose solution onto the gel tray to a thickness of 3–5 mm. Insert the comb either before or immediately after pouring the gel. Leave the gel to set (30–40 min).

   Tip: Ensure that there is enough space between the bottom of the comb and the glass plate (0.5–1.0 mm) to allow proper formation of the wells and avoid sample leakage.

   Tip: Make sure that there are no air bubbles in the gel or trapped between the wells.

6. Carefully remove the comb and adhesive tape, if used, from the gel. Fill the tank containing the gel with electrophoresis buffer.

   Tip: Add enough buffer to cover the gel with a depth of approximately 1 mm liquid above the surface of the gel. If too much buffer is used the electric current will flow through the buffer instead of the gel.

Running an agarose gel

Agarose gel electrophoresis allows analysis of DNA fragments between 0.1 and 25 kb (e.g., genomic DNA digested with a frequently cutting restriction endonuclease), while pulse-field gel electrophoresis enables analysis of DNA fragments up to 10,000 kb (e.g., undigested genomic DNA or genomic DNA digested with rare cutting restriction endonucleases). The amount of genomic DNA loaded onto a gel depends on the application, but in general, loading of too much DNA should be avoided as this will result in smearing of the DNA bands on the gel.

Gel loading buffer (see table Gel loading buffer) must be added to the samples before loading and serves three main purposes:

- To increase the density of the samples to ensure that they sink into the wells on loading.
- To add color to the samples through use of dyes such as bromophenol blue or xylene cyanol, facilitating loading.
- To allow tracking of the electrophoresis due to co-migration of the dyes with DNA fragments of a specific size.

Preparation of samples

- Add 1 volume of gel loading buffer to 6 volumes DNA sample and mix.
- Samples should always be mixed with gel loading buffer prior to loading on a gel.
- Ensure that no ethanol is present in the samples, as this will cause samples to float out of the wells on loading.

Agarose gel electrophoresis

1. Apply samples in gel loading buffer to the wells of the gel.
2. Prior to sample loading, remove air bubbles from the wells by rinsing them with electrophoresis buffer.
3. Turn on the power supply and run the gel at 1–10 V/cm until the dyes have migrated an appropriate distance. This will depend on the size of DNA being analyzed, the concentration of agarose in the gel, and the separation required.

Visual analysis of the gel

Staining

To allow visualization of the DNA samples, agarose gels are stained with an appropriate dye. The most commonly used dye is the intercalating fluorescent dye ethidium bromide, which can be added either before or after the electrophoresis.

Visualization

Ethidium bromide–DNA complexes display increased fluorescence compared to the dye in solution. This means that illumination of a stained gel under UV light (254–366 nm) allows bands of DNA to be visualized against a background of unbound dye. The gel image can be recorded by taking a Polaroid photograph or using a gel documentation system.

OBSERVATION/ RESULT: