SRM INSTITUTE OF SCIENCE AND TECHNOLOGY

FACULTY OF ENGINEERING AND TECHNOLOGY

SCHOOL OF BIOENGINEERING

DEPARTMENT OF BIOTECHNOLOGY

18BTC203J- ANIMAL BIOTECHNOLOGY LABORATORY MANUAL

SEMESTER VI

EXP. NO.: 1
DATE:

PREPARATION OF CULTURE MEDIA FOR ANIMAL CELL CULTURE

AIM:

To prepare MEM medium for the given animal cell culture.

PRINCIPLE:

(Highlight the importance of media in cell culture, importance of various components of media, importance of serum, importance of CO_2 , importance of pH, types of media for cell culture)

The components of MEM and DMEM should be tabulated on the left side of the note book (using pencil).

REQUIREMENTS:

- MEM (1 vial)
- Sodium bicarbonate
- Streptomycin (10mg/mL)
- Membrane filter (0.22µ)
- Falcon tube/ centrifuge tube
- Sterile double distilled water 500 mL
- Sterile 500 mL measuring cylinder
- Sterile filter sterilization set-up
- Sterile filtration flask
- Sterile medium storage bottle

PROCEDURE:

- The laminar air flow chamber was sterilized by wiping with 70% ethanol followed by UV irradiation for 10 min.
- The commercially available powdered media (1 vial) weighing 9.6 g was suspended in 800 ml of sterile water with a gentle stirring.
- Once the powder was completely dissolved, 2.2 g of sodium bicarbonate was added and mixed until dissolved.
- 1 ml of streptomycin solution was added and mixed gently.
- The contents were made up to 1000 mL with sterile water.
- The pH was adjusted to 7.2 using 1N HCl or 1N NaOH.
- The medium was sterilized by filtering through a sterile membrane filter with a porosity of 0.22 micron, using negative pressure.
- The sterilized medium was transferred to a storage bottle, and stored in a refrigerator till use (An aliquot of media was kept in CO_2 incubator for 24h to check the sterility).

NOTE: 10% of fetal calf serum can be added before the addition of antibiotic solution for preparing culture medium with serum which can be further used for cell culture.

RESULT:

DISCUSSION:

List the applications of various medium used in cell culture along with specific cell lines.

EXP. NO.: 2
DATE:

ISOLATION AND CULTURE OF SPLENOCYTES

AIM:

To isolate splenocytes from goat spleen and to culture them *invitro*.

PRINCIPLE:

(write on the importance of primary culture, role of splenocytes)

REQUIREMENTS:

- Goat Spleen
- Sterile PBS
- Petri Dish
- Scissors
- Forceps
- Syringe and Needle
- Centrifuge Tubes
- MEM with 10 % FCS
- Tissue Culture Flask (T-25)
- Centrifuge
- CO_2 incubator
- Phase contrast microscope

PROCEDURE:

1. The Laminar Air Flow Chamber was sterilized by wiping with 70% ethanol followed by UV irradiation for 15 min.
2. The spleen was placed on a Petri dish and was thoroughly washed with sterile PBS.
3. After washing with PBS, the used PBS was decanted and then fresh PBS was added to the Petri dish.
4. PBS was taken on a syringe and the needle was pierced into the tissue and the PBS was released with pressure to mash the spleen and release the splenocytes into the Petri dish. This step was repeated several times to maximize the recovery of splenocytes.
5. The cell suspension was transferred to a falcon tube and centrifuged at 1500 rpm for 10 minutes.
6. The supernatant was aspirated and the pellet was resuspended in PBS. It was centrifuged at 1500 rpm for 5 minutes. The supernatant was discarded, and the pellet was re-suspended in 1 ml of Medium-199.
7. A 1:200 dilution of the cell suspension was made (5 µl of cell suspension + 995 µl of PBS). 10 µl of the diluted cell suspension was taken and visualized light microscope.
8. 200 µl of the cell suspension was transferred to the tissue culture flask containing 5 ml of media with 10% FCS under aseptic conditions.
9. The flask was incubated at 37°C for 24 h in a CO_2 incubator.

10. The increase in cell population was monitored using a phase contrast microscope after 24h.

RESULT:

DISCUSSION:

Applications of splenocyte culture. Cite few examples of the cell lines of spleen and their applications.

EXP. NO.: 3
DATE:

CELL COUNTING AND VIABILITY

AIM:

To isolate and count the viable hepatocytes from goat liver.

PRINCIPLE:

What are primary culture? Why is it important? Why goat liver? Use of haemocytometer, Why tryphan blue?

REQUIREMENTS:

- Liver
- Sterile PBS
- MEM with 10% FCS
- Tryphan blue 0.4%
- Petriplates
- Haemocytometer
- Microscope

PROCEDURE:

- The Laminar Air Flow Chamber was sterilized by wiping with 70% ethanol followed by UV irradiation for 15 min.
- The liver was placed on a Petri dish and was thoroughly washed with sterile PBS.
- After washing with PBS, the used PBS was decanted and then fresh PBS was added to the Petri dish.
- PBS was taken on a syringe and the needle was pierced into the tissue and the PBS was released with pressure to mash the liver and release the hepatocytes into the Petri dish. This step was repeated several times to maximize the recovery of hepatocytes.
- The cell suspension was transferred to a falcon tube and centrifuged at 1500 rpm for 10 minutes.
- The supernatant was aspirated and the pellet was resuspended in PBS. It was centrifuged at 1500 rpm for 5 minutes. The supernatant was discarded, and the pellet was re-suspended in 1 ml of MEM.
- Equal volume of trypan blue (0.4%) solution was mixed with cell suspension (10µl each).
- Haemocytometer was placed on the flat surface of the work bench and the coverslip was placed on the counting chamber.
- 10 µl of cell suspension was loaded to a haemocytometer through diffusion by capillary action. It was made sure that there was no air bubbles and over-filling beyond the ruled area.
- The counting chamber was kept idle on the bench for 20 -30 seconds to allow the cells to settle.
- The counting chamber was placed on the stage of the microscope between the clip to hold slide so that the counting chamber can be moved.
- The number of unstained cells, seen on the square of any of the 4 corners which were divided into 16 smaller squares, was counted.

- The concentration of cells in the original suspension in cells/ml was calculated based the formula

  No. of Cells/ml =2 X n X D.F X 10^4

  2 = to extrapolate for the 20µl of cell suspension

  n= total number of cells counted in 1 square corner

  D.F= dilution factor

  10^4= to extrapolate the total number of cells present in 1 mL of the cell suspension

RESULT:

DISCUSSION:

What are the uses of hepatocyte culture? What are the various cell lines from liver? What type of studies can be performed using these cells?

EXP. NO.: 4
DATE:

PRIMARY CELL CULTURE USING CHICK EMBRYO

AIM:

To isolate and culture the chick embryo fibroblast under aseptic condition.

PRINCIPLE:

What are primary cultures? What is fibroblast? Why is it important? Why 8-days old egg is used? Action of trypsin.

REQUIREMENTS:

- Eight-day-old embryonated eggs
- Sterile PBS
- MEM supplemented with 10 % FCS
- Trypsin
- Culture flask- T-25
- Scissors
- Forceps
- Petriplates
- Beaker covered with cheese cloth

PROTOCOL:

1. An eight-day-old embryonated egg was surface cleaned with 70% ethanol.
2. The egg was cut open at top using a pair of scissors, and then with the help of forceps the embryo was removed and placed in the petriplate containing PBS.
3. The embryo was washed with the PBS and then transferred to another petriplate containing PBS.
4. The head, appendages and visceral organs were removed from the embryo.
5. Once it was cleaned, the remaining of the embryos were transferred to another petriplate containing PBS and minced thoroughly with a pair of bent scissors.
6. The suspension of minced tissue was poured into the beaker containing equal amount of trypsin solution (0.25% trypsin in PBS).
7. It was stirred at 37ºC for 30 min using a magnetic stirrer.
8. The cell suspension was filtered through sterile cheese cloth and the filtrate was collected in a beaker.
9. The filtrate was centrifuged at 1200 rpm for 10 min.

10. The supernatant was discarded and the pellet was re-suspended in 5 ml of PBS and centrifuged at 1200 rpm for 10 min. The resultant supernatant was discarded and the pellet was suspended in 1 ml of medium.
11. After performing cell counting, the cells were seeded in a $25 cm^2$ culture flask with the cell density of 2×10^5.
12. The seeded flasks were incubated at 37ºC in a CO_2 incubator for 24 h.

RESULT:

DISCUSSION:

What are the uses of fibroblast culture? What are the various cell lines of fibroblast? What type of studies can be performed using the cells?

EXP. NO.: 5
DATE:

CELL PASSAGING

AIM:

To passage / subculture the cells in order to maintain them viable for extended period of time.

PRINCIPLE:

Importance of cell passaging, confluency, trypsinization, adherence to culture vessel, split ratio

REQUIREMENTS:

- Cell line
- Trypsin-EDTA
- MEM with 10 % FCS
- Tissue culture flask
- Sterile pipettes
- Centrifuge tubes
- CO_2 incubator
- Centrifuge
- Phase contrast microscope

PROCEDURE:

- Flask with cell confluence of 70- 80% was taken for passaging.
- Spent culture media was aspirated from the cell culture vessel.
- Cells were rinsed with 3-4 drops of Trypsin-EDTA solution by gently rocking the flask back and forth and discarded.
- Then 1 ml (100 µL for 24 well plate) of Trypsin-EDTA was added to the flask and it was gently rotated so that the entire monolayer was covered with trypsin-EDTA solution.
- The flask was incubated for 3-5 min until the cells get detached. After incubation, the cells were examined under microscope. Fully trypsinized cells appeared rounded up and no longer attached to the surface of the flask. If the cells were not fully detached, the flask was placed back into the incubator for few more minutes (The cells start to sheet and the media becomes cloudy upon trypsinization)
- Once the cells got detached, 3 ml (200 µL for 24 well plate) of MEM with serum was added to the flask to inactivate trypsin.
- The cells were dispensed with care by suspending the cells in the medium three to four times with the tip of the pipette on the bottom corner of the flask to disaggregate the clumps or sheets of cells.
- Then the harvested cells were collected and pipetted into a centrifuge tube. The cells were centrifuged for 3 min at 1200 rpm.
- Once the centrifugation is over, the supernatant was discarded. The pellet was resuspended with 1ml of fresh medium and the cells were split into new flasks containing fresh media (5mL)according to the split ratio.

- The culture flask should be labelled with the cell type, passage number and date.
- The cells were then maintained in a CO_2 incubator.
- Periodic eyeballing of the cells under microscope is mandatory to check for the shape, growth of the cells and to ensure no contamination.

RESULT:

DISCUSSION:

Uses of passaging, importance of passage number.

EXP. NO.: 6
DATE:

CRYOPRESERVATION OF CELLS

AIM:

To preserve the given cell culture for a long period using cryopreservation.

PRINCIPLE:

What is cryopreservation? Cryoprotectants & its role, about the slow programming of the temperature for preservation.

REQUIREMENTS:

- Cryomedium (5% DMSO, 95% Fetal calf Serum)
- Cryovials/ampoules
- 0.2 μ filter
- Cell culture (Primary cells / cell line)
- Falcon tubes
- MEM
- Sterile pipettes
- Tryphan blue
- Haemocytometer
- Centrifuge
- Liquid nitrogen container

PROCEDURE:

1. Cryo- medium was sterilised using 0.2 μ filter and stored in cryovials.

   For Primary culture:

   - The Laminar Air Flow Chamber was sterilized by wiping with 70% ethanol followed by UV irradiation for 15 min.
   - The spleen/liver was placed on a Petri dish and was thoroughly washed with sterile PBS.
   - After washing with PBS, the used PBS was decanted and then fresh PBS was added to the Petri dish.
   - PBS was taken on a syringe and the needle was pierced into the tissue and the PBS was released with pressure to mash the tissue and release the Splenocytes/hepatocytes into the Petri dish. This step was repeated several times to maximize the recovery of primary cells.
   - The cell suspension was transferred to a falcon tube and centrifuged at 1500 rpm for 10 minutes.

- The supernatant was aspirated and the pellet was resuspended in PBS. It was centrifuged at 1500 rpm for 5 minutes. The supernatant was discarded, and the pellet was re-suspended in 1 ml of MEM.
- $3X10^5$ cells (with above 90% viability) were taken and resuspended in 1.5 ml of cryomedium, and it was then transferred into cryo- vial.

For cell lines:

- The cells were examined microscopically for any microbial contamination. Only the contamination- free flasks were chosen for preservation.
- An aliquot of cells were taken for viable cell counting using tryphan blue on a haemocytometer.
- The culture flasks with more than 90% live cells were taken for cryopreservation.
- The adherent cells were trypsinized (for detaching the cells from the flask) and transferred to the centrifuge tubes.
- The cells were centrifuged at 1500 rpm for 5 min. Using a pipette, the supernatant was removed without disturbing the cell pellet.
- The cell pellet (approx. $3X10^5$ cells) was re-suspended in 1.5 ml of cryomedium, and it was then transfered into cryo- vial.

2. The ampoules containing the cells (suspension/adherent) were placed in 4^0C for 2-4 hrs for freezing.
3. Frozen ampoules were then gradually brought to low temperatures by incubating then in -20^0C for 4 hrs and then at -80^0C for overnight.
4. The vials were then transferred to the cryo-box and placed inside the canister and stored in liquid nitrogen can (-196^0C) for long term storage.

RESULT:

DISCUSSION:

Applications of cryopreservation. Duration of storage for primary cells & cell lines.

EXP. NO.: 7
DATE:

REVIVAL OF CRYOPRESERVED CELLS

AIM:

To revive the cryopreserved cells that were stored in liquid nitrogen.

Principle:

Importance of cell revival, good revival rate, factors affecting cell revival

REQUIREMENTS:

- Cryopreserved cells (Primary cells/ Cell line)
- MEM with 10 % FCS
- Tissue Culture Flask
- Sterile centrifuge tubes
- Sterile pipettes
- Centrifuge
- CO_2 incubator
- Phase contrast microscope

PROCEDURE:

- The cryopreserved cell stock was thawed gradually by transferring from liquid nitrogen to -80°C, -20°C, 0 ^{o}C and then to 4°C.
- After complete thawing, the contents of the cryovial were transferred to the 25cm^2 flask containing 10 ml of medium and checked under microscope.
- The flask was then incubated in the CO_2 incubator for overnight.
- On the next day, the cell suspension was centrifuged at 1500 rpm for 5 min. After discarding the supernatant, the pellet was resuspended in 5ml of fresh media. This step was performed in order to remove DMSO (the growth inhibitor) from the media
- The cell revival was periodically checked using phase contrast microscope.
- In case of adherent cells, the medium was directly discarded (to remove the non-adherent cells & DMSO) and the cells were supplemented with medium containing serum and viewed under the microscope to check for its revival rate.

RESULT:

DISCUSSION:

Applications of cell revival

EXP. NO. : 08
DATE:

NUCLEAR AND MITOCHONDRIAL STAINING OF CELLS

AIM:

To stain the nucleus and mitochondria of adherent cells and view it under fluorescence microscopy.

PRINCIPLE:

Importance of nuclear and mitochondrial staining, Role of Rhodamine 123, Propidium iodide, role of formaldehyde and Methanol. Principle of fluorescence microscope.

REQUIREMENTS:

- CHO Cells/L6 myoblast
- DMEM
- Petri Plates
- Cover Slips
- Slides
- Propidium iodide (0.5mg/ml) (stock)
- Rhodamine 123 (1 mg/ml) (stock)
- Sterile PBS (1X)
- Methanol
- Forceps

PROCEDURE:

- The cells were seeded on the cover slip under aseptic condition and incubated at 5% CO_2 overnight for proliferation.
- The media was decanted using a pipette without disturbing the cover slip.
- The coverslip was then fixed with formaldehyde for 5 minutes.
- The formaldehyde was removed and 1 ml of PI (10 µL from stock was diluted with 990 µL of PBS) and 1 ml of Rh 123 (10 µL from stock was diluted with 990 µL of sterile water) was applied over the cover slip and incubated for 20 minutes in dark.
- The dye was discarded and the coverslip was washed with 1X PBS.
- Then the cover slip was carefully transferred to a clean glass slide and observed under fluorescence microscope using 535 nm/ 617 nm (for PI) and 500nm/536nm (for Rh123).

RESULT:

DISCUSSION:

Applications of nuclear and mitochondrial staining

EXP:NO:9

DATE:

CELL VIABILITY ASSAY USING MTT

AIM:

To determine the viability of the cells using MTT assay.

INTRODUCTION:

Cell proliferation and death are essential processes for self-reproduction including tissue generation and regeneration. Non-physiological alternations in levels of these factors lead to anomalous cytogenetic behavior of cells which in turn leads to cell transformation, uncontrolled cell growth-the initial event for cancer development. Pharmaceutical research is hence largely focused on effect of drugs, cytotoxic agents and biologically active compounds which affect cytogenetics.

Tetrazolium salts have been used to develop a quantitative colorimetric assay to estimate mammalian cell survival and proliferation. The MTT assay detects living, but not dead cells and the signal generated is dependent on the metabolic state of the cells. This method can therefore be used to measure cytotoxicity, proliferation or activation.

PRINCIPLE:

The MTT cell Assay kit is designed for determination of cell viability based on reduction of the yellow colored water soluble Tetrazolium dye 3-[4, 5-diamethylthiazol-2yl]-2, 5-diphenyl tetrazolium bromide (MTT) to formazan crystals.

Mitochondrial lactate dehydrogenase produced by live cells reduces MTT to insoluble formazan crystals, which upon dissolution in an appropriate solvent exhibits purple color, the intensity of which is proportional to the number of viable cells and can be measured spectrophotometrically at 570nm.

APPLICATIONS:

Cell proliferation: Quantification of changes in proliferation activity of cells caused by trophic factors, cytokines and growth promoters.

Cell viability: Evaluation of the effect of drug on cell viability.

Cytotoxicity: Evaluation of effects of inhibitors or inducers of apoptosis, cytotoxic reagents, carcinogens and toxins.

Drug discovery: High-throughput screening of various anti-cancer drugs.

REQUIREMENTS:

- L6 cells/HT-29 cells cultured in 96 well plate
- Sterile tips
- Micropipettes
- MTT

- 1X PBS
- DMSO for solubilisation
- Triton-X
- Quercetin

PROCEDURE:

PREPARATION OF MTT REAGENT:

6mg of MTT was dissolved in 1 ml of 1X PBS and completely dissolved. The reagent appeared bright yellow in color.

Procedure for determining cell proliferation:

1. 75,000 cells/well were seeded in a 96 well plate containing 200µl of DMEM with serum.
2. The plate was incubated at 37^{0}C in 5% CO_2 incubator for 24 hours.
3. After the incubation period, 4 different concentrations (0.1 µM, 1µM, 10µM and 100µM) of anti-cancer agent (Quercetin) was added and incubated.
4. The untreated wells served as control, triton-X (0.01%) treated wells served as positive control and the medium containing wells without the cells served as the blank.
5. After 24 hours, the plate was removed from the incubator and the media was aspirated and 100µl of PBS was added to all the wells.
6. After 10 min, 20µl of MTT reagent was added to all the wells in dark.
7. The plate was wrapped with aluminium foil to avoid exposure of light and then incubated for a period of 3 hours.
8. The contents of the plate were discarded without disturbing the cell monolayer and 100µl of solubilisation buffer (DMSO) was added and incubated for another 1hour in dark.
9. The absorbance was read on ELISA reader/ spectrophotometer at 570nm.
10. The effect of Quercetin on cell proliferation can be determined by plotting a graph for the different concentrations of the drug Vs OD in L6 (normal cells) & HT-29 (cancer cells).
11. The cytotoxic effect of the drug on cell lines can be calculated using the given formula:

    % Cytotoxicity = [(OD of test/OD of control) X 100] -100

RESULT:

INFERENCE:

The effect of the drug on normal cell’s viability & cancer cell’s viability & its importance.

EXP. NO.: 10
DATE:

DETERMINATION OF GLUCOSE UPTAKE BY THE CELLS

AIM:

To determine the uptake of 2-deoxy-D-glucose by the given cell line using GOD-POD method.

PRINCIPLE:

What is diabetes, its types, importance of glucose uptake assay, importance of L6 myotubes, Why 2-deoxy-D-glucose for the assay?

For spectrophotometric method: role of glucose oxidase (GOD) and peroxidase (POD), Its reaction.

REQUIREMENTS:

- L6 myotubes (L6 myoblasts were differentiated to myotubes)
- 2-deoxy-D-glucose
- 24 well plate
- DMEM with 10% FCS
- DMEM with 2% FCS
- KRPH buffer ((118mM NaCl, 5mM KCl, 1.3mM $CaCl_2$, 1.2mM $MgSO_4$,1.2mM $KH2PO_4$ and 30mM HEPES – pH 7.4)
- Insulin (100nM)
- Test drug
- 0.1% SDS
- GOD-POD reagent :
    - Phosphate buffer :100 mmol/L
    - Glucose oxidase : >8U/ml
    - Peroxidase : >0.5U/L
    - 4- amino antipyrine: 0.28mmol/L
- Standard (glucose : 100 mg/dL = 5.55 mmol/L))
- Spectrophotometer

PROCEDURE:

1. The L6 cells were seeded in a 24 well plate at a seeding density of $0.75X10^6$ cells per well and incubated overnight.
2. After the cells have attained 70 % confluency, the cells were induced for differentiation by the addition of DMEM containing 2% FCS.
3. The cells were differentiated for 2 days.
4. The L6 myotubes were serum starved for 1 h.

5. The cell line was incubated with the test (drug) for 30 min.
6. The wells treated with Insulin (100nM) for 20 minutes prior to deoxy glucose uptake assay served as positive control.
7. The untreated wells served as control.
8. Following incubation, the medium was aspirated and 500µl of KRPH buffer was added to each well.
9. 5µl of 10mM 2-deoxy-D-glucose was added to all the wells and incubated at 37°C for 30 min.
10. The uptake process was terminated by aspiration of KRPH solution containing 2-deoxy-D-glucose.
11. The cells were lysed with 100 µl of 0.1% SDS.
12. The lysates were subjected to determination of glucose using GOD-POD.

Measurement of glucose uptake using GOD-POD method

1. In 2ml eppendorfs , 0.1ml of distilled water (blank) was added.
2. 0.1ml of lysates was transferred to the eppendorf.
3. 90 µl of distilled water was added to 10 µl of the standard (glucose) and labeled as standard.
4. 1ml of GOD-POD reagent was added to all the tubes.
5. The contents of each tube were mixed properly.
6. The eppendorf tubes were incubated at 37°C for 10 min.
7. The absorbance was read at 505nm and the values were subtracted using blank.
8. The graph was plotted with % 2-deoxy-D-glucose uptake with respect to control.

Calculation:

$$\text{Concentration of unknown sample} = \frac{(\text{Concentration of standard}) \times \text{absorbance of unknown sample}}{\text{absorbance of standard}}$$

$$\text{\% Glucose uptake with respect to control} = \frac{(\text{concentration of test} - \text{concentration of control})}{\text{concentration of control}} \times 100$$

RESULT:

INFERENCE:

Infer your results