بسم الله الرحمن الرحيم

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Tissue Culture Lab& Techniques

S	Date	Topic
Lab(1)		Tissue Culture Lab and Equipment (Laminar flow hood-Liquid nitrogen container-Autoclave-Centrifuge-Water bath-Ice maker- Inverted microscope-pipettes-flasks-How to use the pipettes (blue and yellow tips))
Lab(2)		Preparation of media (use of magnetic stirrer and pH meter) Sterilization (Filtration & Autoclaving)
Lab(3)		Thawing and growing Primary culture : Liver (adherent) and spleen (suspension) cells Growing cells on cover slips
Lab(4)		Cell count (Haemocytometer) Primary culture: monocyte cell (Method of Extraction &Culture)
Lab(5)		Freezing Fixing and staining cells grown on cover slips
Lab(6)		Photographing cells with digital camera and printing
Lab(7)		Presentation
Lab(8)		Exam

LAB (1)

Tissue Culture Lab and Equipment

Essential Equipment :

- ***Laminar Flow Hood (Safety Cabinet) :***
  Gives best sterile protection to the culture.
- ***CO_2 Incubator :***
  A device with uniform temperature can be maintained, used in growing cultures.
- ***Autoclave :***
  Sterilization under high temperature and pressure.
  Solid materials sterilize in 121°C/15 min and liquid in 121°C/20 min
- ***Inverted microscope :***
  Quantitative analysis of cultured cells for seeing the sample in three dimensional.
- ***Magnetic Stirrer :***
  Rapid stirring action for dissolving chemicals is available with any stirrer for disaggregating or suspension culture.
- ***pH meter :***
  Adjusting the pH.
- ***Liquid Nitrogen Container :***
  To save the sample as it is, for long time in -198°C
- ***Sensitive Balance :***
  To weight the substances.
- ***Vacuum Air Pump :***
  A sterilizing by filtration (fluid pass throw filter membrane to remove all type of microorganism in the culture medium.
  Centrifuge :
  There are two sizes
  Microfuge which used with Eppendrof tubes.
  Macrofuge which used with 3,5,10,15,50ml tubes.
- ***Refrigerator.***
- ***Freezer(-20º,-80º)C.***

- ***Plastic Well Plate**: To* **seeds the cells.**
- ***Cry vial Tubes**: To* **freezes the cell in liquid nitrogen container.**
- ***Hot Plate.***
- ***Homogenizer.***
- ***Water Bath.***
- ***Milex filter.***
- ***Stir Cup filter.***
- ***Shaker.***
- ***Ice maker.***
- ***Pipettes :***

  There are different types:
  Multi pipette – single pipette – simple pipette – automatic pipette – micro pipette – disposable (Pester) pipette.
  Tips:
  Single pipette:
  Crystal tips: 0.5 -10 µl
  Yellow tips: 10-200 µl
  Blue tips: 200-1000 µl
  White tips: 1- 5 ml
- ***Flask :***

  To seed the cells.
  There are two sizes: 75 cm^3 & 25 cm^3
  When we put the flasks inside the incubator we must loosen the caps to allow sufficient gas exchange.

How to use pipettes :

Single pipette –automatic pipette – disposable Pester pipette.

Preparation of solutions with different concentrations

Solutions & dilutions :

$C_1V_1 = C_2V_2$

C_1= starting concentration. V_1= starting volume.

C_2= final concentration. V_2= final volume.

$\frac{\text{Want}}{\text{Have}} \times \text{Final volume}$

w/w (weight / weight) solutions :

1% solution is 1g solute in 100g final weight of the solutions.

1ml H_2O weight 1g

Mix weight of solvent with volume of H_2O

- **10% NaCl = 10g NaCl in 90ml H_2O**

w/v (weight/ volume) solutions :

1% w in v means:

1g solute in a final volume of 100 ml.

Examples :

***Prepare 50 ml of 2% NaCl.**

2 g ⟶ 100 ml H2O

? ⟶ 50 ml

$$\frac{50 \times 2}{100} = 1 \text{ g}$$

***Prepare 25 ml of 2% NaCl .**

2 g ⟶ 100 ml H2O

? ⟶ 25 ml

$$\frac{25 \times 2}{100} = 0.5 \text{ g}$$

***Prepare 200 ml of 0.85% NaCl.**

0.85 g ⟶ 100ml H2O

? ⟶ 200ml

$$\frac{0.85 \times 200}{100} = 17 \text{ g}$$

***Prepare 50 ml of 0.3% NaCl.**

0.3g → 100 ml H2O

? → 50 ml

$$\frac{0.3 \times 50}{100} = 0.15 \text{ g}$$

v/v (volume/ volume) solutions :

Examples:

***Prepare 100 ml of 10% acetic acid.**

10ml acetic acid + 90ml H_2O

***Prepare 20ml of 0.2% acetic acid.**

0.2ml → 100ml H_2O

? → 20ml

$$\frac{0.2 \times 20}{100} = 0.04 \text{ ml}$$

***Prepare 50ml of 0.5% acetic acid.**

0.5ml → 100ml H_2O

? → 50ml

$$\frac{0.5 \times 50}{100} = 0.25 \text{ ml}$$

*** Prepare 70% alcohol.**

70ml alcohol + 30ml H_2O

*** Prepare 400ml (1x) from (10x) stock PBS.**

$$\frac{1}{10} \times 400 = 40 \text{ ml}$$

400 -40 = 360 ml H_2O

Molarity :

To prepare a solution in Moles/Liter = M

The molecular weight (mw) must be known.

E.g. mv of NaOH =40

40g in 1L (1000ml) =1M

Mw × concentration required = amount to weight/liter

40 × 0.5 = 20g in 1L

Volume required = 500ml

Amount/liter × volume required = amount to weight

Examples:

***prepare 100ml of 0.5 M NaCL. (Mw= 58.88)**

0.5 x 58.88 = 29.44

58.88 In L in 1M

29.44 In L in 0.5

? _________ 100ml

$$\frac{100 \times 29.44}{1000} = 2.944\ g$$

***Prepare 200ml of 70mM sucrose. (Mw = 342.3)**

$$70mM = \frac{70}{1000} = 0.07\ M$$

$$200ml = \frac{200}{1000} = 0.2\ L$$

342.3 x 0.07 = 23.961

342.3 In 1L in 1M

23.961 In 1L = 0.07 M

? __________ 0.2

23.961 x 0.2 = 4.7922

***Prepare 100ml of 50mM sucrose. (Mw = 342.3)**

$$50mM = \frac{50}{1000} = 0.05\ M$$

$$100ml = \frac{100}{1000} = 0.1\ L$$

342.3 x 0.05 = 17.115

342.3 In 1L in 1M

17.115 In 1L = 0.05 M

? _________ 0.1

17.115 x 0.1 = 1.7115

Dilutions:

Prepare 400 ml (1 x) solutions from (10x) stock PBS.

$C_1V_1 = C_2V_2$

Examples:

- **How much ml of 12 M HCl is required to prepare 250 ml of 2 M HCl solution.**

$$\frac{2\ M}{12\ M} \times 250 = 41.6\ ml$$

250 – 41.6 = 208.4 H_2O

- **What volume of 10 M acetic acid is required to prepare 1L of 0.50 M acetic acid?**

  $$\frac{0.050}{10} \times 1000 = 50 \text{ ml}$$

  1000 -50 = 950 ml H_2O

- **Dilute 500 ml 325 Mm sucrose to final concentration of 200 mM .**

  $$325 \text{ mM} = \frac{325}{1000} = 0.\ 325 \text{ M}$$

  500 ml = 500 = 0.5 L

  $C_1V_1 = C_2V_2$

  $0.325 \text{ x } 0.5 = 0.2 \text{ x } V_2$

  $$\frac{0.325 \text{ x } 0.5}{0.2} = 0.812 \text{ L}$$

LAB (2)

Preparation of media&Stains

1) Preparation of RPMI media (1L):

1- Add 700ml distilled water to the ready measured powder media (15.8g or as it required in the container).
2- Add 20ml 10% sodium bicarbonate ($NaHCO_3$).
Prepare 100ml of 10% $NaHCO_3$ (10g $NaHCO_3$ +90ml dD.W)
Filter ($NaHCO_3$) (with milex filter 0.45 µm membrane)
3- Add 1% (10ml) L-Glutamine (200mM)
4- Add 1% (10ml) antibiotic (penicillin10.000IU/streptomycin10.000µg\ml).
5- Add 160 ml distilled water.
6- Adjust the pH 7.2 - 7.4.
If pH is high: add HCl (acid).
If pH is low: add NaOH (base).
7- Filtering through Millipore filter with 0, 22µ pore size using vacuum air pump.
8- Add 100ml FCS (Fetal Calf Serum).

Preparation of stains:

A) Giemsa Stain (10%):

1- Add 10ml Giemsa Stain to 90ml distilled water.
(Filter with filter paper every time before using).

B) Trypan Blue (0.4%):

1- Add 0.4g Trypan Blue
2- 100ml Saline (0.9g NaCl in 1L D.W)
3-Filter (0.45 µm membrane).

LAB (3)

Growing Cell Line :

Cell Line	Cell Type	Medium
MCF-12	Adherent	Universal Media
MCF-7	Adherent	RPMI
MDA	Adherent	RPMI
HSFN-1	Adherent	DMEM-F12
THP-1	Suspension	RPMI

Thawing Cry preserved Cells:

1- Wear cry vial gloves and face shield and retrieve the required (check the index) ampoule (cry vial tube) from the liquid nitrogen container.
2- Defrost the vial in a 37°C water bath with constant, moderate agitation.
3- When thawed, swab the vial with 70% alcohol.
4- Working in Sterile area (hood), open the vial and transfer the contents using sterile Pasteur pipette in to 15ml centrifuge tube(V shape).
5- Add 10ml pre-warmed (37°C) culture medium gradually *.
6- Centrifuge the cells 1500 rpm for 5min.
7- Discard the supernatant and gently resuspend the cell pellet in 15ml culture medium.
8- Transfer the cells in a $25cm^3$ culture flask.
9- Observe the cells and place them in the CO_2 incubator.

*this is important with Dimethyl Sulfoxide, where sudden dilution can cause severe osmotic damage and reduce cell survival by half.

Primary Culture :

- Growing Liver Cells (adherent) .
- Growing Spleen Cells (suspension) .

Initiation of a Primary Culture :

1- Dissect the animal.
2- Take the liver and the spleen and wash with sterile normal saline.
3- Homogenize the organs with 10ml media (insert the homogenizer in ice).
4- Transfer the homogenized organs into 15ml centrifuge tubes and Close tightly.
5- Centrifuge 500 rpm for 5min.
6- Transfer the supernatant into a 15ml centrifuge tube.
7- Centrifuge 2500 rpm for 15 min.
8- Discard the supernatant and add 10ml media to the pellet shake well.
9- Take 1ml of the tissue and add to 9ml media.
10-Transfer to a labeled 25cm^3 flask.
11-shake the flask gently and put in CO_2 incubator 37ºC.

Notice :

The liver cells grow as an adherent monolayer.
The spleen cells grow as suspension.

Staining technique:

- **Giemsa Stain.**

1- Dissect a rat (or mice) and cut out a small piece of the liver and spleen.
2- With a forceps take a small piece of both organs.
3- Place a small amount of the specimens (by pressing down) on clean microscope slides.
4- After the specimen in the slide dry fix it in 100%methanol for 1min.
5- Stain with Giemsa stain for 25 min.
6- Wash the slides with running tap water.
7- Dry the slides with filter paper.
8- View with the light microscope.

Sub culturing Adherent Cells:

1- Discard media from the culture flask.
2- Rinse cells with PBS (10ml/75cm^3- 3ml /25cm^3) by gently rocking the flask back and forth, thin remove the PBS and discard.
3- Add trypsin to the flask (3ml/75cm^3-1ml/25cm^3) and rock the flask to ensure that the entire monolayer is covered with trypsin solution.
4- incubate the cells 3-5 min until the cells detach (avoid leaving cells exposed to trypsin longer than necessary and to force cells to detach as this may result in clumping) .
5- Add culture media (pipette the cells up and down) then transfer in a 15ml centrifuge tube.
6- Centrifuge the cells 1500 rpm for 5 min *.
7- Discard the supernatant and resuspend the cell pellet in fresh culture media, pipette the cells up and down.
8- Keep the cells in ice.
9- Count the cells with chamber (hemocytometer) thin dilute to the appropriate concentration for seeding.
10- Add the appropriate volume of cells suspension to a new flask containing media.
11- Place flask in incubator with loosens caps to allow gas exchange.

***(step 6 can be avoided as the serum in the medium will inactivate the remaining trypsin and prevent cell damage).**

Sub culturing Suspension Cells:

1- Transfer the cell suspension in a centrifuge tube.
2- Centrifuge the cells 1500 rpm for 5min.
3- Resuspend the cell pellet in culture medium.
4- Count the cell by hemocytometer and dilute to the appropriate concentration for seeding.
5- Add the appropriate volume of cell suspension to a new flask containing medium.
6- Place flask (standing) in incubator with loosens caps to allow gas exchange.

LAB (4)

Hemocytometer Counting Chamber:

1- Clean the surface of the hemocytometer slide and cover slip with 70% alcohol.
2- Place the cover slip over the hemocytometer counting chamber.
3- Shake the cell suspension well and mix equal volume of cell suspension and Trypan Blue stain (1:1).
4- Transfer 20µl cell suspension to the edge of the chamber and allow it to be drawn into the chamber by capillary action.
5- Place the hemocytometer on the microscope stage.
6- Count cells in the four marginal chambers using 40 X objective.
7- to calculate the number of cells :
   c = n/v
   c = cell concentration (cells/ml).
   n = average number of cells counted.
   v = volume counted = 10^4
   thus : c = n x 10^4
   Concentration of cells/ml = average number of cells x DF* X 10^4
   * DF: dilution factor
8- after calculating the cell concentration for seeding :
   Concentration of cells required x final volume
   Concentration of cell /ml
9- Add the appropriate volume cell concentration required to a new flask containing medium (final volume).
10-place flask in incubator with loosens caps to allow gas exchange.

LAB (5)

Preparation of the freezing media:

RPMI 1640 media 80 ml.
Serum 10 ml.
Dimethyl Sulfoxide (DMSO) 10 ml.

Freezing:

1- Count the cells.
2- Centrifuge the cells 1500 rpm for 5 min.
3- Discard the supernatant and add the required amount of freezing media to the pellet.
*** Amount of freezing media needed to be added to the cells:**

$$\frac{\text{Cell count obtained/ml}}{\text{Cell count required/ml}}$$

(The freezing media requires 10^6/ml cells)

4- Distribute the cells in cry vial labeled tubes.
5- Place the tubes in freezing box (Iso propanol) -1ºC per mints.
6- Place the tubes in the freezer (-80°C) for 3-4 days.
7- Transfer the tubes from the freezer to the liquid nitrogen container.

Growing Cells In 24 Well Plate

1- Subculture the cells.
2- Determine cell concentration.
3- Insert sterilized cover slips (13mm) in a 24 well plate using sterilized forceps and needle.
4- Calculate the seeding density required for each well :
   Cell concentration required X Final volume
   Cell count/ml
5- Place 1ml of the cell concentration required in each well (label the plate)
6- Shake the plate gently, cover and place in CO_2 incubator 37ºC.

Fixing & Staining the Cells with Giemsa - H& E stain

A) Fixing:

1- Remove media from the 24 well plate.
2- Fix the cells on the cover slip with 10% formalin (or methanol) for 10min.
3- Wash with PBS solution.
   (Dissolve one tablet of PBS in 100ml sterile D.W)

B) Staining (Giemsa):

1- Stain the cells with Giemsa for 20 min.
2- Wash in tap water.
3- Remove the cover slips from the plate (wells) .
4- Prepare clean labeled slides.
5- Mount with DPX media.

C) Staining with (H&E):

1- Stain with Haematoxylin for 7-10 min.
2- Wash in tap water and rinse in distilled water.
3- Stain with Eosin for 1min.
4- Wash in tap water and rinse with distilled water.
5- Remove covers lips from wells.
6- Prepare clean labeled slides.
7- Mount with DPX.

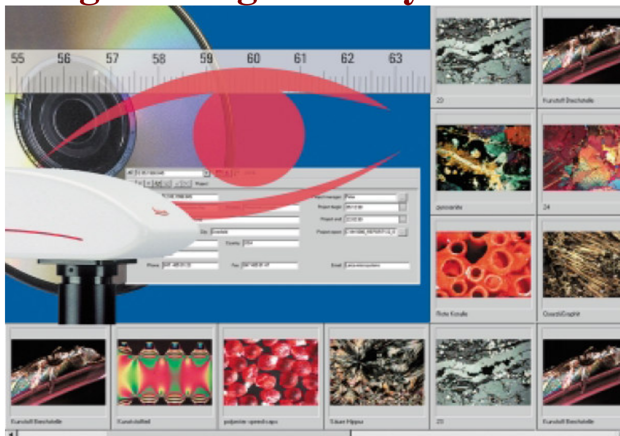
LAB (6)

Photographing cells with digital camera and printing

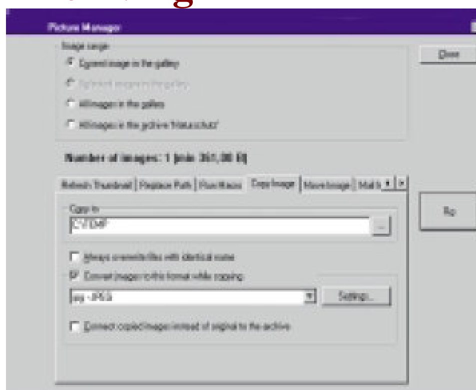
Inverted Microscope

Compound Light Microscope

Image Management System



Archiving



Movie Recorder

Movie Recorder/
Movie Player Module

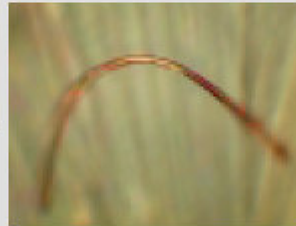


Image 1: Object in focus

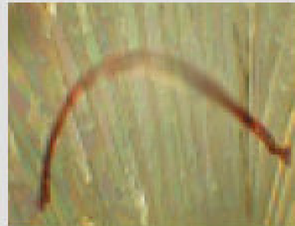


Image 2: Background in focus

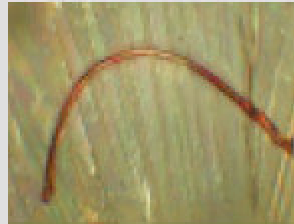


Image 3: Object and Background in focus

Measurement Module