PRNC091

PRNC - 91

PUERTO RICO NUCLEAR CENTER

COURSE IN

TISSUE CULTURE AND RADIOISOTOPIC TECHNIQUES AT

CELLULAR AND SUBCELLULAR LEVEL

LABORATORY EXERCISES

J orinario ay unavensity OF PUERTO RICO UNDER CONTRACT

HO. AT (40-11-1859 FOR U. 5. ATOMIC ENERGY COMMISSION

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TISSUE CULTURE AND RADIOISOTOPIC TECHNIQUES AT

CELLULAR AND SUBCELLULAR LEVEL

PUERTO RICO NUCLEAR CENTER

U.S. ATOMIC ENERGY COMMISSION

Division of Medical Sciences and Radiobiology

and

Department of Microbiology, School of Medicine*

UNIVERSITY OF PUERTO RICO

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LABORATORY EXERCISE #1

PREPARATION OF MATERIAL. STERILIZATION AND STERILITY

TESTS,

Objective

All components in a cell or organ culture syste must

be free of contaminant mlcroorzantsns. Routine testing

Procedures should be carried out in order to rule out the

presence of bacteria, fungi and mycoplasma.

Two tubes of chick enbryo Fibroblasts

?wo tubes of HeLa cells

two

tubes of 1-38

Stx tubes of thyoglicolate medium

Stx tubes of Sabouraud agar

Six eubes of PPLO broth

?Twelve tubes of PFLO agar

pienes' stain

Procedur

1, Observe under the microscope and make description of the different

cell types.

2. Tests for stertiity

2:1 Incubate 0.1 ml of each cell culture tube into 10 al. of

ehyogltcolate medium, Incubate at 37°C. Read and record

results. Tf negative, discard after 5 days.

2:2. Streak a loopfull of each cell culture on Sabouraud Agar.

Incubate at rocn temperature, Read and record results

during a week.

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a3

With a pipette deposit some drops of each cell culture

flutd on PPLO broth and agar (Difco). Streak the agar

vith a bacteriological loop, invert the pla

and

Ancutate at 37° for seven days. Tacubate the broth

at the sae tenperature for four days and after this

period, place sone drops on a PPLO agar plate spreading

vith @ bactertologteat Loop.

ithout renoving the cover of the plates, inoculated

the Int and Gth daya, lock for colonies of +

PRLO under the microscope stage focusing throurh the

agar. Use a 10K objective and 10, 12.5 oF 15 X ocular.

Most FPLO colonies appear as round colonies with

4 dense center and a less dense perifery, giving the appearance of a fried egg. PPLO colontes have been

tsolated fron tissue cultures, hovever, that do not

conform strictly to thts appearance on primary Asolation. They may appear to lack 2 dlstinet periphery ?and appear to be totally imbedded tn the agar. These colonies are usually very small and look "granular" oF "feathery". PPLO colontes vary fron 10 to 500 microns in dimeter (0.01 t0 0.5 mn) and characteristically

the center only or all of the colony ts enbedded in the ?agar. Individual organisms cannot be resolved since they are the size of an average virus particle.

oe

onally, at the periphery of PPLO colonies,

"large bodies characteristic of thts group of

forganions are found.? After locating the colonies,

---Page Break---

they are voually marked out on the petrt dish with « glass

raving ponett.

Confirmation of PPLO colontes depends, in addition to

morphological characters, on:

1, InabtLtey to renove the inbedded portt on of the

colony fron the agar surface by stroking the colony with

?4 loop. This denonstrates the fact that the colony

ts embedded. Bacterial colontes will rub off.

2. The non-reversion to bacteria which subsequent passages

of the colontes will reveal. Reversion to bacterial

form would be typical of L forms.

3. A vequirenent for native protein.

4. Reaction with the Dienes stain.

?The Dienes stain ts prepared by dissolving 2.5 ene

vwethylene blue, 1.25 gus. azur TT, 10.0 gas maltose, and 0.25 gr.

sodiun carbonate in 100 ml of distitied water. vith a cotton

?suab moistened in the stain, stroke the area of an agar plate

in vill dt ef

Just adjacent to the suspected colony. The o

the colony which {a then examined under the microscope as

described above. The PPLO colontes stand our distinctly

with densely blue statning centers and Light blue peripheries.

Bacterial colonies are also stained but those are decotortzed

n about 30 minutes. The PPLO colonies never decolorize the

stain.

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LABORATORY. BXERCTSE 2

PREPARATION OF TISSUE CULTURE CONSTITUENTS

Objective:

To obtain the coupogents which will provide the cell

in vitro"

with the organte and inorgente substances necessary for its nutrition,

and respiration and vill protect it against changes in physical stats

temperature, pit, ete.

Materials

4 chtek enbryos

setesors

Syringe 50 nl.

Conteituge tubes

Graduated cylinder

Anttbtottes (PentetIlin and Sereptonyeta)

1, HAWKS BALANCED SALT SOLITEOH (85S)

A. 10 X Solueton

ote #1 nanco, 3.5 em

Dissolve in 250 nl. distilled water. Dispense in a conventent

bottle (50 ml. scrav-cap prescription bottle) and autoclave

at 120°C for 15 minut

vate #2

mac 80.0 gm.

xet 4.0 mm

Me50, .78,0 2.0 wm.

NegHPO, .21,0 0.6 gm.

Giucose 10.0 mm.

1,70, 0.6 mm

Dissolve in 800 wl. distilled vater.

---Page Break---

nore:

2

unte #3

act, 1b ome

2

Dissolve in 100 ml, distilled water.

vate #6

Phenol Red 0.4 pm

Mix Phenol Red {n @ small anount of water until a paste

Atiuce to 150 al, with dtecilled vater, titrate co ph 7

with 1/20 NaOH, Make up to final volume of 200 mt.

Preserve with 1-2 ml. chloroform.

?Add 100 a. of unke #4 co unit #2 and then add unte #3

t0 make 1,000 mi. Pour solution into glass atoppered bottle

?and add 3-4 ml, chloroform as a preservative. This solutton

nay be kept at room tenperature for 6 months-1. year.

: Mintalze transfer of chloroform tn preparation of the working

solution, Be cartain that bottle caps are loosened during

autoclaving to {nsure that all chloroform ts driven off.

Horketng,

?The working BSS ts prepared by diluting 10X Stock 1:10 wieh

elution

dtsetiied water. DLepense tn a conventent size screw cap

bottles and autoclave at 120°C for 15 simutes. Aseptically add

2.5 al. of sterile sodium bicarbonate solution (Untt #1) to each

100 at. of 855. the pH may be adjusted with co,- The balanced

salt solution 4s nov ready for use. Do not tighten caps until

pit of BSS Lo 7.4

NUTRIENT MEDIA

Eagle's minimum essential medium contains Mgher concentrations

of amino acide than the basal medium first described by Eagle, which

---Page Break---

permits cultures to be kept for longer pertods of time without

feeding. ?The medium may be prepared with Hanks B55 base. The

medium Ls prepared, concentrated 10% and stored tn the refrigerator.

At the tine of use, plutanine and antibfotics:(etored at -20°C) and

Yanco, sre added to the 1X solution,

Solution a: Per Liter 10x medtun

AeArginine, Ht 1.05 ene

Aetiysetdine. Het 0.31 mm.

Vetysine, Het 0.58 me

Atryptophane 0.10 on.

1-Phenylalanine 0.32 we

1-Threontne 0.08 mm.

AsLeucine 0.52 gm

levatine 0.665 ems

I-taoleveine 0.52 me

L-Methionine 0.15 am

Solution

A-tyroaine 0.32

Lecystine 0.26 wm

?These anino acids are dissolved in 200 nl. of 0.075 Hcl with

gentle heating (60°C).

Solutton ¢:

Meotinantde

Pyrtdoxal 200 mg.

?Thtanine 200 we.

Pantothente Acid 200 mae

Choline 200 mg.

A-tnosttot 400 me.

Riboflavin 20 wg.

---Page Break---

4

Components are dissolved in approximately 175 al. of double

distilled water and then brought to a final volume of 200 al. with

double distilled water. The solution

dispensed im 10 wl, amounts

and stored at -20°C, 10 ml. of Solution C are added to each Liter

of 10% mediee.

Solution

200 ml. of Biotin are dissolved tn 150 ml. of double disttiled

water. To increase stability during storage, 1.0 al. of 1.0 mHcl

49 added. The total volume te brought to 200 ml. with double

Atottlled vater and the solution dispensed in 10 ml. amounts

and stored at -20°C. 10 al, of Solution D are addi

£0 each

Liter of 10x mediun,

Solvtion

200 ng. folic acid (crystalline) are dissolved in 200 ml. 1X Hanks!

BSS pit 7.8. The solution ts dispensed in 10 ml. amounts and stored

at -20°C; 10 al. of Solution & are added to each iter of 10x sodium.

Glutamine Solution 37 - (To be added at the tine of use) 12 gas. of

I-Glutamine are dissolved tn 400 ml. of double distilled water and

sterilized by fAleration through @ Se{ts-type pad. The solution

Ae stored at -20°C and 1,0 nl. ts added to each 100 mi, of 1x Eagle!

medium.

Preparation of the final mixture of 10x Eagle's medium in

Hanke" BSS

4, The following are étssolved in solution 8:

Nac 80.0 gm.

ket 4.0 mm.

Hg50, 71,0 2.0m.

---Page Break---

s

b. The following are dissolved in 50 ml. double dtsttlied water

and added to the pool.

Ma fPO, 1280 1.52 em.

1,70, 0.60 mm.

ce. 10 grams of Glucose are dissolved in 50 ml. of dosble distilled

water with 20 ml. of 12 Phenol Red solution and added to the

oot.

4. the volune of the pool 12 brought to 600 ml. with double

Aietilled vater and the following solutions are at

Per 1.0 liter 10x medium

solution ¢ 10.0 ml.

Solution D 10.0 al.

Solution E 10.0 et.

fe. In a separate flask contstning 160 ml. double distilled water

2.0 gas. anhydrous CaCl ere dissolved and added to the pool

slovly with vigorous shaking.

£, The amino acids of Solution A are added to the pool and the

volume is brought to approxinately 950 sl. with double distilled

water.

A solution contatning 20,000 units of PentctIiin and 20,000

micrograns of Streptomycin per ml. 1s added tn « volune of 5.0 ml.

per Liter and the mixture 12 held in the refrigerator overnight.

hh. The total voline 42 brought to exactly 1,000 ml. vith double

dLettlled water and the solution fe sterilized through a setts

type pad.

1. For use the solution te diluted to 1X with stertle double

---Page Break---

distilled water and 17, of the 3% Glutanine Solution and 1.25 £0

2.50% of 4 2.8% MARCO, are added.

For laboratories occassionally uetng small ancunte of Eagle's

medium, it 1s recomended that the 1X medivm be prepared by

Atluting 2 10x Stock Solution of the amino acids and the

10X stock soltuton of the Vitamins (stored at -20°C) appropriately

tn Barle or Hanke" BSS a

adding Glutamine, antibiotics and

Manco,

e)

vitamins during long term storage at ice box tenpersture,

Andicated above, This prevents detertoration of the

3. CELL DISPERSING AGENTS

Ay Trypsin Solutton 1.0%

1 gn. of powdered trypsin 1s dissolved in 100 al. of phosphate

buffer saline and the solution 42 p:

ed through ash-free filter

paper (Schleicher and Schull #569). The solutton te then

sterilized by f{1tratton through a Setts-type pad and stored

at 20%.

B. Versone Solution (Ethyelenedtanine tetraacetic actd)

act 8.0 m.

Po, 0.2 mm.

1,70, w=

kel 0.2m.

a, 80, AAS gm

Versene 0.20 gm.

Dissolve in 1,000 al. of distilled water. Dispense in

conventent anounts and sterilize by autoclaving at 120°C for

15 minutes.

---Page Break---

4. aertatorics soUUTTON

Penicillin (20,000 units per ml.) and Streptomycin (20,000 mterogeemas

per at.)

1. Add 10 a1. of Hanks? solution £0 1,000,000 units of Pentctiiin,

2, add 10 at. of Hanks? solution to 1 vial with a gram of Streptomycin.

3. Wix the contents of both vials and add up to 50 al. of Hanks" sotutton.

4, Dispense Ao vials and keep at -20°C.

5. CHICK BABRYO EXTRACT 50%

Each student will be provided with 5 enbryos 9-10 days of age.

?A, Harvest the enbryos and place then in a sterile Petri-dtsh where

tho eyes, beaks, Legs and wings are renoved.

1B, The renaining tissues are washed in a beaker contatntng Hanks" BSS,

then minced with uterine setasors.

C. The theaue minced 1s passed through a 50 al, syringe into a

graduated cylinder or centrifuge tube.

D, fin equal volume of Hanks' BSS te added to the tissue culture

and the mixture {s stirred and allowed to stand for 30 minutes

B, The suspension 1s centrifuged at 1,500 rpm for 20 minutes and

the supernatant fluid (constituting the 50% extract) ts

removed and stored at -20°

F. After thawing for use, the extract 19 clarified by centrifugation

at 2,000 rpm for 10 minutes.

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LABORATORY EXERCISE # 3

TECHNIQUES FOR GROWING CELL LINES IN TISSUE CULTURE

Ob jectsve:

This experinent is designed to maintain a cell line (HeLa) for the

duration of the cours;

Bach student will be provided with 1 bortle

of Mela cells.

Mate

1 noceie

Racnocytoneter

Solution trypan blue

16 tubes

Procedure:

1, Ghserve the cells under the microscope and describe them.

2. Remove medium with a pipette. Add 10 ml of 0.25 per cent

?Trypsin solution; allowing the trypsin to renain on celle

for exactly one minute at 37°C. Resove all of the trypsin

and place the tubes tn a 37%C incubator for 10 minutes. At

the end of this tine the cells should be alnost completely

detached from the wall of the bottle, Add 10 ml. of fresh

medism and aspirate the cell suspension very well with a pipette,

3. Count the celts in the haenocytoneter

3-1, with a Pasteur ptpette carefully express a drop of cell

suspension made up of 0.5 al, cells plus 1.0 ml. of Trypan

blue under the haenocytoneter coverslass, avolding any

overfloy into the moat,

3-2. determine the average number of viable cells (dead cetts

stato blue) in the 4 large corner squares used for

counting white blood cells, Multiply by 10,000 the

mumber of viable cells to obtain the number of cells

---Page Break---

Per ml. Adjust to 50,000 celts/at. using Eagle's medium.

3-3, Transfer 50,000 cells into each of 14 test tubes, stopper

tubes with rubber stoppers. Incubate at 37°C in an horizontal

plane.

4. Keep record and observe cells every day.

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LABORATORY EXERCISE #4

MONOLAYER CULTURES OF CHICK EMBRYO CELLS

Objocetve

Cell cultures of the Maintland type fron chick exbryo were among the first to be used for viral propagation. Developnent of new techniques has increased the use of chick embryo Fibroblast for virus {solation and antigen production.

Material

4 chtek enbryos

Petrt dishes

Beaker

Setssors

Forceps

syringe

Erlemeyer flask

Megnetic stirrer

centrifuge tvbes

Procedure:

1, chick embryo 9 days old are harvested and placed tn a sterile Petri dish where eyes, beaks, lege and wings are removed and discarded,

2, The exbryos are transferred to a besker containing Hanks! BSS ?and washed in 3 changes of the solution.

3. The enbryos are minced into pieces approxinately 3m. tn dtaneter, with uterine setssors, and the ttssue minced Ls washed with 3 changes of Hanks? ass.

4, The minced tissue 4s passed through a 50 nl. syringe (without

---Page Break---

%.

10.

2

needle) nto a 500 al. Erlemeyer flaw where it ts washed twice

with 50-10001. of Hanks? BSS.

After the fluld fron the second washing has been renoved, an appropriate volume of .25% solution of trypsin im Hanks' BSS 1s added. The volune of trypsin solution employed 1s determined by the nunber of eabryos

being processed (5-10 embryos, 200 ml., 11-20 enbryos, 300 al.).

«A magnetic stirring bar ie added to the flask which ts placed

fon the magnetic stirrer and the suspension agitated for 1 hour at oon temperature.

Atter one hour of trypatnization the flask {s slanted to sediment large tissue particles and the supernatant flutd (cell suspension) Ss decanted through a statoless steel wire cloth (72 mesh utre Alaneter .0037 {nches) into centrifuge tubes.

?The cell suspension is centrifuged horizontally at 600 rpm for

10 minutes and the supernatant flutd containing trypsin ts asptrated Amediately. The celle are washed once by resuspending in Hanks"

BSS, centrifuging at 600 rpm for 10 minut

and aspirating the

supernatant Fluid.

?The celle are then resu

jended in 15 £0 30 mi. of Hanks? BSS,

transferred to 15 ml. graduated, conical centrifuge tubes and

centrifuged horizontally at 600 rpm for 10 minutes.

?The volume of packed calle 4 acted and after removal of the

supernatant fluid, the cells are dfluted tn the following,

Moen:

Bovine serum 2.01.

51 Lactalbunin hydrolysate tn

physiological saline 5.0 m1.

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50% Chick mbryo Extract 4.0 wl.

Hanks! ss 85.5 at.

2.81 MaHco, 2.5m.

Pontettlin-steeptonycin solution 1.0 ml.

UL, For tube culture the cells are diluted 1 t0 200 and dlapensed
4 al, volunes. For plaguing in stoppered bottles, the cella
are also @iluted 1 to 200 and dispensed in 8 wl, volumes {nto
three ounces prescription bottles. For plaguing in 50 ml.
Petri dishes 8 ml. of 1:200 diluation of the cells are added
and incubation {s conducted in 5% CO, atmosphere.

12, After incubation 36°-37°C for 1 to 2 days, complete monolayers of
cells are formed and the cultures are ready for tnoculation with

viruses or clinteal material.

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LABORATORY EXERCISE # 5

VIRUS TITRATION: CPE

PURPOSE:

?To determine the virus dilution that gives rise to cytopathic

changes in 50 per cent of the inoculated cell cultures.

Maver tat:

Poliovirus suspension

Tebes

Pipettes

Procedure:

1, Observe the HeLe cell cultur

prepared during the third day of

laboratory work.

2, Pipette off the growth media and replace it with .9 al. each of

Ragle's nediim contataing 2% serun.

3. Prepare tenfold dilutions of poltovirus type 1 as follows:

1a. Set up « row of & Wusserman's tubes numbered 1 through 4 and

Atopense tnto aaah of thes 1.8 ml, of media.

b. Take 0.2 nl, of the poltovirus euspension and add to the first

tube in the row, Mix thoroughly with a sterile pipette

cs Take 0.2 ml, fron the dilution in the tube #1 and pass 1 to tube

(2 wixing the contents.

4. Repeat the operation with the renainder tub

4s noculate .1 of each dilution of the virus and deliver to each of

4 tubes of HeLa colle. A soparate pipette should be used for

Atlution; hovever in the interest of laboratory glassware econony

use one 0.2 al. pipette for adding the virus dilution to the Hebe

celle beginatag vith the highest dtlution and working back to the

lower.

---Page Break---

5+ Set up to 2 Hela tubes for control without inoculation.

6. Bring to the incubator at 37°C and read and record the

results everyday.

7. Calculation of the TCID,, by the Reed-Huench method.

In the folloving table an example 1 given of data derived fron

fan Adeal experiment for illustrating the procedure of

accunulat ton:

ACCUMULATED VALORS

Per cent

CPE : No CRE: Ratto

Wes & + 0 es 0 sie: 100

10 a 4+ 0 + &: 0 : 8/8 = 100

wo + 74 Boa Ss 1: ws 80

10 vs 1 3 1 a Sys 20

ws | oe oo a) 8 08

Accumulated valu

for the total number of tubes that shoved

OPE or were intact are obtained by adding in the directions indteated by the

orrows. The accumulated CPE ratio represents the accumulated number of

tubes with eytopathic changes over the

ceumulated total musher inoculated.

In this example the cytopathic changes in the 10°? ?dilution, is

higher than 50%; that in the next lower dilution, 0, 1s considerably

lower, The necessary proportionate distance of the 50 per cont CPE end

potnt Mes betwoon these tuo dlutfons and te cbtatned as follows:

Ger cone GFE HE alivtion-aest shove SOD > tee cone cre * Rroporttonace

?at dilution next S#9tance

below)

or 80 = 50

w= BOS

50

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Since logarittmtently the distance betwoen two dilutions ts 2

funetion of the incremental steps weed in preparing the sertes, tt 49

Necessary to correct the proportionate distance by the dilution factor.

Im the case of serial ten-fold dilution the factor 1s 1 (log 10 = 1)

and 80 18 disregarded, In our example ve have:

Negative logartthn of TCID,, end point titer = negative logaritim of the

bd atlution above the 50 per cent

GPE plus the proportionate distance

that te:

Negative logarithm of the dilution above 50%

Proportionate distance (0.5 X dilution factor (log 10

eID,

50

Log Tero, = 10°F

50

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LABORATORY EXERCISE #6

?VIRUS "PLAQUE"ASSAY TECHNIQUE

Objective:

To produce circumscribed infected areas by vaccinta virus in chick

?enbryo fibroblasts which do not take the neutral red vital stain

land appear as clear unstained areas against a background of viable

stained cell:

Matertals

Chick embryo fibroblasts

vaccinia virus suspension

ater bath

Neutral red

Petrt dishes

Procedore

1, Set up three Wssernan's cubes nusbered 1 through 3 and add 1.8

lal. of Eagle's media to all tubes, Fron the pool of vaccinia virus

supplied add .2 ml, to cube #1 and mix thoroughly with a sterile

pipette, Withdraw 0.2 ml. and add to tube #2, Repeat the antertor

step with new pipettes so that you will have virus ét1uetons 1077,

10, ane 10".

3, Remove the out-growth mediun from the Petri dishes previously

Prepared with chick eabryo fibroblasts and wash once with saline,

Inoevlate a plate with 0.5 al. of each virus dtlutton.

3. Incubate at 37°C for 2 hours for virus adsorption with occasional

rocking to distribute the virus particles.

4, After the a¢sorption pertod, renove the flutd and overlaid the

fibroblasts sheets with 6 al, of the following agar medium:

Agar 1,0 gm.

---Page Break---

5.

the sane conditions ae the scrum virus mixtures. For serum

control, the dilution 1/4 ts afxed uith dtiuent. Te ts

necessary to perforn a councurrent titration of the virus

to establish that a test dose actually contain approxinately

too texD,,.

?The conditions recamended for incubation of serum virus

mixtures vary widely for certain agents and has been demonstrated

?that sone prelisinary tnevbation does increase the neutralizing capacity of serum. The taportant consideration ts to avotd incubation conditions under which the virus may be labelled

for a sanple for ong period of 37°C. For sost neutral ization teats incubation of the serun virus mixtures 1s conducted for

30 minutes to one hour at roon tenperature or at 4°.

After incubation period, serum virus mixtures, virus controls and serum controls, are tnoculated in volunes of .2 al. into nonolayer tube cultures. At least two cultures are enployed

for each serum mixtor

?The inoculated cultures are incubated et 37°C and examined nteroscoptcally for ability of the serum to inhibit CPE of the virus.

?The cytopathic effect of the Inoculated tubes ts recorded and the neutralizing end point 4s expressed as that dtlution

of serum which protects 50% of the tubes against the test dose

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of the virus, as {1lustrated in the following example:

SERUM DILUTION : CRE: eRe: NOCPE: CRE + NO CPE: _MomaLTTY

Ratio Ration Fer cont

a

Pr a

hee a ae de a ke

Te 7 rr

rnom 2 2: 0: 6: 0 rae 100

?

50% = CPE at dtlution next below

ea ee

Logarithm 50 per cont neutralizing end point = -1.8 + (0.5 x (0.6)

= 1.8 + (0.3)

eta

antilogarittie -2.1 = 120

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?LABORATORY EXERCISE # 7

NEUIRALIZING ANTIBODIES ASSAYS Tt CELL CULTURES

Sbtecttve:

To calculate the capacity of a serun to neutralize the

cytopathic effect of a poliovirus in a Hela colle systen:

rae

Serum

Poliovirus suspension

?Tubes with HeLe celts

eter bath at 56°C.

Procedur.

1, The serum epectnen {2 inactivated at 56°C for 30 minutes to destroy heat non-apectfte virus inhibitory substances.

2. Sot up a rou of tubes to make serum dilutions of 1:4, 1:16, 1:64, 1:256, 1:1024 prepared in etther balanced salt soluttons oF the watntensnce medin £0 be used in the cell cultures,

3. Poliovirus ts diluted co contata 100 TerD,, in a volume of O.1 al. (as determined by a previous titration of the virus). The viral dtluetons are made in the sme medium eoployed for the preparation of the serun dilutions.

4, Equal volumes of the serum dilutions (0.5 ml.) and of the attuted cost views (0.5 ml.) are mixed. The volune of serum virus mixture prepared ts dependent upon the number of cell cultures to be inoculated with tho mixture. For virus contol the teat virus dilution 4 atxed with an equal volume

of dtluent (or know normal serun) and incubated under

---Page Break---

5. To revive the frozen cell lines, the tube 1s renoved fran

the deop-freczer and thawed rapidly in 9 37°C water bath. A

volune of the cell suspension containing 1.5 to 2.0 x ro is added to

10 mi. of out grovth mediun and cultures initiated tn 2 200 al.

boretes

B. Transport

1, Trypsinize a bottle of Hels colls and dtlute in grovth medten

to obtain a suspension of no more than 0.6 x 10° cells/ml.

2. Refrigerate ot 6%C for 24 hours,

3. centrifuge

st 200 rpn for 30 minutes and discard the supernatant.

4, Add medium to obtain a coll suspension of 2.6 x 10° per al.

5. In this state the cells can be shipped in an iced container

?and upon receipt sedimented by centrifugation at 200 rpm

for 30 mintes and resuspend in fresh grovth medium at a

concentration of 0.6 x 10° celle/al. A satisfactory method

to ship cell cultures {s obtained filling the vessel with

nutrient medium preventing to trauma to the cells by the

Bovenent of the medius. Upon receipt the medium should be

renoved and the cells £

with new one.

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LABORATORY EXERCTSE #8

?CONSERVATEON AND TRANSPORT OF CELL CULTURES

Dectives:

?to maintain in the Inboratery with 2 niniaun of handling viable

cell Lines not in continous use.

Maters

Cott culture

Racnacytoncter

?Trypan blue solution

?smpoutes

As ftorage

1, obtain a bottle with a culture of 5 days old cells fron which the

out-sroveh medium has been renoved and replaced with 10 ol.

of fresh medium consisting of 10 per cent hor

serum and 90

per cent lactalbunin hydrolysate yeast extract mediun.

After further 2 days incubation period, the medium ts renoved,

the cells trypsinized and counted. A bottle should yield

between 10 and 20 x 10° celle, otherwise the cells are not

sultable for storage.

2. The pil of the medium 1s adjusted to 7.4 by means of an

8.8% NaHCO, solution. Add 2.0 al. of sterile glycerol to the

10 wl. of medium tn each culture.

3, Transfer the cell suspension to anpovles (which can be flane-sealed)

oF to tubes, thghtentng the stoppers and sealing with adhestve tape,

4, Bring at 4°C during 1 hour and then another hour at -20°C, After

this period place the tubes (or anpoules) at 70°C, Under these

conditions, the celle can be stored for perfods up to 18 sonths.

---Page Break---

5. No revive the froren cell lines, the tube 4 resoved fren

the doep-feozer and thaved raphdly in a 37°C water bath. A

volume of the cell suspension containing 1.5 to 2.0 x 10 is added to

10 m1, of out growth median and cultures tatttated tn a 200 at.

bottle.

3. Transport

1, Teyprtnize 2 bottle of Hels celts and dtlute in growth nedten

0 obtain a suspension of no aore than 0.6 x 10° celle/al.

Refrigerate at 4°C for 26 hours.

3+ Centrifuge at 200 rpm for 30 minutes and discard the supernatant.

4, Add medium to obtain a cell suspension of 2.4 x 10° per ml.

s.

Im thte state the cells can be shtpped in an teed container

and upon receipt sedimented by centrifugation at 200 rpm

for 30 mintes and resuspend in fresh growth mediin at

?concentration of 0.6 x 10° cells/ml. A satisfactory method

to shtp cell cultur

is obtatned ftIling the

el with

nutrient medium preventing to trauma to the cells by the

ovenent of the mediums. Upon receipt the medium should be

Fenoved and the cells fed with new one,

---Page Break---

---Page Break---

LABORATORY EXERCISE #9

PLATING EPPICEENCY AND ISOLATION OF CLONES

Qbjeceive:

?This experiment is designed to determine the plating effietency of D2

cell using the formule

Per cent plating effictency = Colontes formed x 100

Cells inoculated

land to obtain clones fron single DC2 cells.

Mevorisls:

De 2 cell monolayer

Hacoocytoneter

?tubes

Patet dishes

Procedure:

1, Trypsinize monolayer cultures of DC? celle

2. Count in haenocytoneter using trypan blue to obtain viable count.

43. Dilute the cell suspension in nutrient media to obtain « concentration

of 50,000 cetle/al.

3:1 Make tenfold dtiuttons (0.1 al. suspension plus 0.9 =i.

Ragle's medium) so that the suspension will contain 5,000

?and 500 celle per-el.

3:2. Make a dilution 1/2 of the 500 cells suspension, in order to

have 250 celle per ml.

?transfer .1 ml of the cell suspension (25 celle) to each of 2 Petri

Atshes; 0.2 al. (50 celle) to 2 Patri dishes and 0.5 ml. (125 cells)

toa third group. Complete the volune up to 4 al. with fresh Eagle's SZ.

---Page Break---

5. Incubate the plates at 37°C in an atmosphere containing CO,

6. After 8 or 9 days cultures say be exanined mtcroscoptealty.

to observe discrete colonies:

7, Staining of one of the series.

7:1 Wash once with saline

7:2. Fix with methyl alcohol during 5 stnutes.

7:3. stain with Gtensa for 10 minutes.

7:h Rinse and observe under the microscope. Usiny the above

formula determine the plating effictency.

8, In order to isolate a single colony fron one of the series cultured

with different anounts of cells:

8:1 Select the colony to be tsolated and mark the area with @ vax

penetl.

8:2 Renove all media from the culture dish.

8:3. Place over the colony to be {solated a sterile bended Pasteur

pipette vatch contains sone trypein.

8:4 Gently add nutrient medium and @eapend the colony with «

P

wor pipette

8:5 Transfer the celle to a nev culture flask or dish.

8:6 Add enough nutrient medium and incubate at 37°.

---Page Break---

---Page Break---

LABORATORY EXERCTSE # 10

ORGAN COLTURE

Objective:

?The cultivation of fragments of organs in # tissue culture eystes

Watch will peratt to study celts tn a group of associated tissues.

?Taio technique provides a good syaten for research on the actt on

of hormones, vitenin, caretaogens, ote.

Material

Petrt dtehes (dteposable)

Paso

Chick exbryoe

Setasors

Forceps

Seal

1, Prepare culture dishes by placing @ watch g

8 inside a petet

dioh and a ftlter paper ring. These dishes aust be sterilized

at 170°C for 2 hours,

2. Add approxinately 9 drops of plasma to the watch glass, followed

by 3 drope of EE 50 and quickly, but gently, mix before medion

clots.

3, Remove eabryo fram its shell and transfer toa sterile Petri dish.

Dissect out tisoue to be cultivated and rinsein sterile BSS.

4, Cut explants to approximately 2 mm? and rinse 3 tim

ta B68,

5. Depostt with a ptpette 4 fragments onto clot. Renove the

excess of B55 carried over, using # fine pipette.

6, Incubate at 37°C, Observe datly, recording cheractertetice of

growth,

---Page Break---

1

vei

fe Extract (Difco) 0.1 gas

Lactalbunin hydrolysate 0.6 pa.

Horse serum 14.0 a1.

Hanks" 85s 85.0

Pentettltn 50 uel. and 50 Streptenyein to complete medium.

Allow the agar to soltdtfy and turn the plate upside dow and

Ancubate at 37°C for 3n4 days with the cell monolayer dom.

Add 3 ol. of a 1/1000 1 +7 neutral-red solutions incubate at room

temperature for 2-4 hours and overnight at 4°C.

?The {lutions used should produce distinct and separated plaques.

Observe against a vhite background. By counting the mnber of

Plaques at the dtlution vhere they appear distinct and by aulttplying

by the correspondent dtlution factor, the mber of plaque forming

units (FFU) per ml. of the virus suspension can be calculated.

---Page Break---

---Page Break---

LABORATORY FXERCESE #11

macroscorte TecITOUES

Ob ieceive:

This exercise 13 desipned to denonstrate recent mlcroscopte

methods that are helpful to conduct further studies on the cell.

Materials:

Procedur

Gtomsa stain

Anti E. coll conjugated globutin

+ Staining with Fluorescent Antibody.

1, Make @ smear on aide fron a tube with HeLa cel!s previously

tnoculated with E. colt.

2. Air dry. Fix with acetone.

3. Cover the slide wteh an anti

colt fluoreseetn conjugated

globulin

4. Bring the slide co a Petri dish with « moistened cotton plece.

5. After 30 minutes contact, renove the FA r

went by dipping the

slide into a vi

'8e1 of saline golution.

6. Place the slide tn a Jar of buffered saline (pH 7.5) for 5 to

10 minutes.

Ronove the slides, allow to drain and gently blot off exce:

saline with absorbent paper.

8. Place a drop of mounting fluid on the snear and cover with «

coversitp.

9. Pxamtne snears under a steroscope fitted with a cardiotd type

darkfietd condenser and {1¥uninated by an intense light source.

Use fluorescence-free imersion of.

---Page Break---

3. Demonstration of phase microscopy.

©. Glensa stainiy of DC-? colts in Leighton tubes.

---Page Break---

---Page Break---

LABORATORY EXERCTSES 412, 13, 14

(CHROMOSOHE, PREPARATION

Objective:

To cerry out the emmeration of chronosemes ina human diploid

cell for application co genetic studies and cell characterization.

Mavertal,

Human diptotd cell (W1-32) tn Leighton tub

Centrifuge tubes

colchicine (

0,000 tn Bss)

Hypotonte solution

Fixative (1 part acetic acid + 3 parts absolute ethanol)

Acetic orcein stain (2 pp. Orcein dissolved tn 100 mi

botling acette acid)

Procedure:

1, Add colehteine (or Colcentd) to the tubes during the eatly log

phase of grovth, Colehteine thould be used tn a final

concentration of 0.4 us per mi. colcentd in a concentration

of 0.05 we.

2. Bring ** tneubator at 37°C for 16 to 18 hours, in order to

harvest coll at the metaphase period,

3. After Ancubation, take off the mediun and add $ ml. of

hypotonte solution (sodium eftrate 0.52).

4, Place the tubes under a lanp (60 vatt bulb) for 30 minutes,

Discard the hypotonte solution and add $ al. of freshly

Prepared Carboy's fixative.

6. After 30 minutes of contact vith the fixative renove the

coverslip froa the tube, Let it dry overntsht.

7, Stain with oreein during 15 minutes

8, Rinse with distilled water.

---Page Break---

9. Dehydrate through 2 rapid changes each of 95% ethanol,

absolute ethanol and xylol,

10. Mount {n Permount .

---Page Break---

---Page Break---

MICROAUTORADTOGRAPHY

Ob jectty

?Taie experinent is desioned to demonstrate the incorporation

of thymidine Iabetled with tritium {nto the DNA of the cellular

nucleus.

Materials:

?Boulston(Kodak HTB)

Water bath

Contin jars

Slide storage boxes

Developer (Kodak D-19)

Aetd Fixer (Kodak)

Gtensa Stain

Procedur

Change the mediun from MK cells cultivated during 2-3

im Letphton tubs

2. Add new medtus vith eLertated thymidine in» concentration of

1.0 mefot.

3. Allow the ehyntdtne to be in contact «ith the cells 39 minutes

and 1 hour at 37%,

4, After adequate tine vash with Hanks! nes.

5. with a fine forcep remove the coverslip fron the tube,

6. Pix with methyl alcohol for 10 minutes,

7, Mount the coverslip on a nicroscope slide with the cells

factor up. Use Euparol as the mounting medium.

8. Place the slide at 45-50°C for 26 hours or at room

Cenperature for 48 hours in order to dry the Euparol.

---Page Break---

%.

10.

12, Stain for 5 minutes sith Clans:

Be

Expose the slid

11:1 Place slides tn developer for 2 stnute

Cover with photographic envision.

9:1 Place bottle of envision in a vater bath

at 43°C for 15 minutes. Rotate the bottle gently.

9:27 Pour into Couplin Jar.

9:3 Place the slides im an enpty copii Jar that t# tn the

water bath,

9:4 once warmed up pas

the slides to the Jar with the

9:5 Afeer 15 seconds renove the slides and drain the emulsion

fon a paper towel.

9:6 Allow the slides to dry plactne thon before «

fan for 15 minutes or 1-2 hours at rocm temperature.

9:7. Store tn black slide boxes and seal with dark tape in

order to prevent the entrance of Ltght.

at roc temperature for 10 to 30 days.

Develop the slides.

11:2 Minse tn tap water.

1:3 Place in actd fixer 2-5 minutes.

11:6 Rinse in water for 20 minutes.

ANS Rinse in distilled water, Air dry.

Observe with tmnersion ofl.

---Page Break---

---Page Break---

LABORATORY EXERCISES #16, 17, 18

TRTRODUCTION TO RADIOACTIVE ISOTOPE TECHNIQUES FOR STUDY GP BIOLOGICAL SYNTHESIS.

Orotie Acid and Thymidine Incorporation into the nucleic acid of Hela

celle

Introduction:

Te would be extremely difficult to eateuiate the value of the

adtotsotope as a tool in biological research. Certainly,auch of

the progress achieved in this field in recent yeare would have been

impossible prior to the development of the atomic pile, Two baste

facts account for the unusual utility of these radioactive clenents.

?We can tell where they

(location), and how such

Beesent (quantity),

?even after they have becone intermixed with large numbers of stable

atoms of the sane element:

Objective

1, Introduction to apparatus and techniques for use of teotopes

18 tracers tn biological synthetic reactions.

2, Application of ¥:

Hochenteal techniques to the study of

muclete aetds,

Mavoriats

Orotte acid tabeled with ch

Thymtdine Labeled uteh

Ue My celta

Reagents for nuclete acta extraction Schneider's method

Reagents for DUM determination by Cerlotti's method

Reavents for RNA determinations by Orctnol method

Ribonuclease and Deoxyribonuclease

---Page Break---

Exper inet

Procedures:

1h penera! the procedure fe to incubate the LIC MK, cells in @

mediea containing orotic acid labeled cl4 and thymidine H Labeled for

17 hours. The cells are vashed with unlabeled orotic acid and thyntdine..

?The cells are tryprintzed and honogenized, The Nuclete acid are extracted

?and the radioactivity 4s determined,

The procedure will be applied to study orotic actd incorporation

into RNA and Ehymtdine incorporation ino the DNA fraction of the Het

cells.

1, Three 4& hours old bottles will be piven to each student,

The student will add Crotic act 0.1 ue per mt, and thymtdine

O.1jre per ml. and incubate arain for 17 hours.

After 17 hours incubation, renove the media and vash the

nonolayers 2 times with

fine A, Sint. each tine,

3. Wash the monolayers 2 tines nore with saline A containing

unlabeled orotic actd and thymidine (0.1 mp per m1.).

4, Teypeintze the cella, remove the cells fran the botete

and wash 2 tines with saline A in the contrifure (Ten

toutes at 1,000 rpa).

5. Resuspend cells in 5 m1 of phosphate buffer, pil 7.4 and

henopentze for 2 minutes in the Cant-nixer honorentzer.

6. Tal

am aliquots of 0.5 mt for dry wotsht determination,

7, Take an aliquot of 4 m1 and do @ total muclete acid extraction

by the Schneider's Brief method. See appendix A.

Take a 1 el aliquot of the fins? total auclete acid extract

4

and apply to a piece of Filter paper, Do counts for c

and ¥W?, the machine will do these counts stoultancously.

---Page Break---

9. Take an aliquot of 2 nl. of the final total nuctete actd

extra ct and do a DMA determination by Ceriottt's method.

See appendix 8.

10, Take an aliquot of 0,5 nt. of the final total nuctete

actd extract and do a RNA determination by the Oretno?

method. See appendix C.

M1, Take an aliquot of 2 mi of the total muclete acid extract

and Incubate with DNase for 30 minutes at 37C, (100,.p of

DNase per mt.).

22, Add enoush cotd absolute ethanol to make £62 alcohol and

incubate at -10C for 30 minutes

13, Resuapend the precipitate in phosphate buffer, pit 7.4,

16. Do counts for cl (rnay,

15, Repeat step #11 but incubate the aliquot with Ribonuclease

for 30 minutes (100 .p per al).

16, Repeat steps #12 and 13.

17, Do mints for (DNA).

18, Turn tn Report.

---Page Break---

APPENDIE &

Reagent:

SCHNEIDER'S BRIEF METHOD OP MUCLERC ACIDS

EXTRACTION

10% Tea

5% Tea

952 Ethyl alcohol

Procedur

i

(Im tee bath). To the honozenate aliquot? add 4 m) of tee-cold 10% TCA, mix well with stirring rod and centrifure in the cold 4 about 2,500 r.p.m. for 7-10 minutes. Discard the supernatant.

To the residue add 1 nl of ice-cold distilled water, resuspend

WIth a rod, add 4 m1 of 957 ethanol and mix well with a rod. Contrifure at room temperature for 15 minutes. Discard supernatant.

(At room tenperature). To the residue add 5 al of 95% ethanot ?and resuspend with the rod, Centrifure as before and dtecard the supernatant, (The procedure can be interrupted here).

Repeat this step for liver homorenate.

+ (At room temperature), To the residue add 5 nt. of 5% TCA.

Re

juspend with rod. Place the tubes in water bath at

90°C for 15 minutes, sttrrins occasionally. Let then coo!

to room tenperature. Centrifuge for 30 ainutes. Save

Supernatant n 10 ml flasks.

To the residue add 5 nl of 5% TCA, resuspend with the rod,

contrifage as before. Pool the supernatant with that fran

(4), and wake up to 10 wl, final nuclete acta

extract.

---Page Break---

* Homopenates:

Gells = 4 at of a 3 Bettle suspension homogenize in 5 ml.

Liver = 2 m1 of a 20% howopenate

Spleen - 2 m1 of a 107

Kidney - 2 ml. of @ 10n

Lang = @ ml of a 10%

Thysus - 2 mt of a10n

Lyaphnodes = 0.5 81 of a 107"

Bone Marrow - 0.5 al.of a 10%"

Take an aliquot for dry weight determination at the sane tine. The dry

weights are determined by drying the sample in tared bottles at 105%C-

MOC tn an oven, to constant weiphe (24-48 hours).

---Page Break---

APPENDIX 8

DNA Determination by Cerfotti's Method

J. Biol. Chem., 198:297-303 (1952),

1, 0,047 indole (CP) solution in distiiled water. Tt was dissotved tn

warm water. The solution 4s then cooled under ruaning water and

Atluted to volume. Store in refrigerator.

Procedur

1

2

3

2 ml (or an aliquot) of DNA solution or extract in a test tube.

2 Bl of Indole solution

2 mi of HCI concentration

contantnat ion.

+ Place then in a botling water bath for 10 minutes.

+ Cool tn running water.

+ The solution is extracted 3 times with 4 m1 chloroform each

ime and centrifuged £0 pive a complete clear vater phase.

+ Read the water leyer against a blank in the Spectrophotoneter

at 490 24.

---Page Break---

APPENDIX ¢

RNA DETERITIMTION BY THE cRCTHOL METHOD

Reference: Btochen. et Biophys. Acta 1, #3 (1947),

1. 150 mn oreinot

2, 7,5 m1 CuCl, in HCI concentration (0,04)

(0.0538 sm CuCl, tn HCI concentration)

3. 67.5 ml of concentrated HCI

4, 60.0 m1 of diseitied water

Procedure:

Dissolve the 150 mp of orcinol in the 47.5 nl of HCL concentration.

4207.5 nl of the CuCl solution. Mix vel). To the solution add «0 nt

of disetited water.

To 0.5 ml of extract RNA standard solution add 4.5m! of the Reagent.

Mix well. Place the tube tna botling water bath for 40 minutes. Cool

then in an ice-cold water bath, Read the absortance against a blank

in the Spectropheneter at 660 mis.

---Page Break---