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PUERTO RICO NUCLEAR CENTER

MEDICAL SCIENCES AND RADIOBIOLOGY DIVISION

COURSE IN

TISSUE CULTURE AND RADIOISOTOPE TECHNIQUES

AT CELLULAR AND SUBCELLULAR LEVEL

PROGRAM AND LABORATORY EXERCISE MANUAL

Jone 18. daly 3

1970

?OPERATED BY UNIVERSITY OF PUERTO RICO UNDER CONTRACT

No, AT (W8-1I-IEBD FOR U. & ATOMIC ENERGY COMMISSION

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COURSE IN

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Introdvetvien Lo Heme Cette Techniques

(Guaje 15 to 29, 1970)

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_ Drs Je Chirivoga, Director

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2 (une 2 to 25, 1970)

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?Tissue CULTURE Cour

(une 29 to July 2, 1970)

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39:00 am, he Belence of Virology

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10:00 ame

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1:00 pm, Medsuring Virve InltipMeation

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9:00 10 Teoture

10:00 am. Methods for Detecting Wren.

Maltipaicetion ip Tinme

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10:00 a.m. Laboratory eeroise £9

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Tayors of Celie

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10:00 a.m. Inboratory Bueroine 3.19

to Woutralizing 4

2:00 pam, An Cold Cultures

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to Lecture

10:00 a.m. ?Application of tesse Coitnre

in Virus Teolatien avd Voecine

Prouietion

Laboratory:

vation aud interpretstion

of Previous Fxporironts

IypLication of Sieeue Culture Techniques to Virology.

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TABORATORY RERRCTSE #1

PREPARATION OF MATERIAL. STERILIZATION AND STERILITY

?TESTS. STANMG OF CELLS

orsoctive

?ALL components in a cell or orga culture systen met

ve free of contarinant microcrgantons, Routine testing

Procedures should be carried out in order to mule ont the

Presence of bacteria, fing and aycoplasna.

tatertats:

?ho tibes of 1 cette

so thes of tela celts

SMe thes of ne 2 cots

Bie tubes of thyvglicolate median

?Six tubes of Sabourand agar .

fe tubes of PPL0 bot

Suelve tes of PO agar

tenes" stain

Procedure:

Observe under the microscope and describe the different

cell, typos.

2 Tests for otertiity

2:1 Inoculate 0.2 ml of each cell eulture tube into 10 x1 of

?tnyoglicolate medium. Incubate at 37°C. ead aid record

Fesults every 24 hours. If negative, discard after 5 days.

2:2 Streak @ loopfull of each cell culture on Sabourand Agar.

Incubate at room temperature, Read and record results

during © week, at 24 hour intervate,

---Page Break---

ach

Yahoratony Benetse #1

With @ pipette deposit sone drops of each coll culture
?eauta on Pio broth and agar (Bife0). streak the ager
with @ bacteriological loop, invert the plate and
Ancubate et 37° for seven days. Ineubste the broth
?at the same texperature for four days and after this
Period, place cose drops on a PPLO agar plate, spreading
with a bacteriological oop.
Without renoving the cover of the plates, inoculated
?the Ist and lth days, Look for colonies of PPLO under
?the microscope stage focusing through the agar. Use 4
10K objective and 10, 12.5 or 15 x ocular.

Most FFLO colonies ce round, with
?8 dense center end a less dense periphery, giving the
appearance of a fried egg. YFLO colonies have been
Aoolated from tiseve cultures, however, they do not

confora strictly to this appearance on primary solution,

?They may appear to lack a distinct periphery and appear

to be totally eubedded in the agar. ?these coteates are

usually very ssi11 and look "gramlar" or "eathery".

YELO colonies vary from 10 to 500 mlcrons in atoncter

(0.02 to 0.5 mn) and characteristically the center omly,

OF al of the eoleuy, 4s exbedded in the ager, Tndividael

organisns cannot be resolved since they are the size of an

sverage virus particle, Occasionally, at the periphery

Of PELO colonics, "large bodies" characteristic of this

group of orginions are found, After locating the colontes,

---Page Break---

mBReeEe ee eS eS es as a2 eeCUlcer EY OE eeOUDT,llOllU

Inboratory Frereise #1

they are vsuelly marked out on the petri dish with a glass

?parking penest.

Confirastion of PELO colontes depends, in addition to

morphological charactere, on:

1, Insbitity to renove the inbeddrd portion of the
colony from the agar surface by stroking the colony
with @ loop. this denonstrates the fact that the
colony {s eabedied. lncterisl colonies will rub off.

2. The non-reversien to bacteria vhich subsequent passages
of the colontes vill reveal. Reversion to bacterial
form would be typical of L forma.

3. A requireaent for native protein.

4, Reaction with the Denes stain.

The Dlenes stain is prepared by dissolving 2.5 os

methylene blue, 1.25 gus. azur IT, 10,0 ene maltose, end
0.25 gms sodium carbonate in 100 si of distilled water. with
cotton svab moistened in the stain, stroke the area of an
agar plate just saJacent to the suspected colony. ?The stein
W121 diffuse to the colony vnich 4s then examined under the
microscope as described above. The FPLO colonics stend out
distinctly vith densely blue ataining centers and Light blue

peripheries. Becterial colontes are also stained but these

fare decolorized in sbout 30 minutes. ?The FPLO colontes

never decolorize the stain.

Staining of cols ty Glensa method.

Renove the groxth nediun of the tubes provided for sterility testing.

---Page Break---

Laboratory Bxercise #1

2. Wash twice with Hank's solution.

3. Add methyl alcohol covering the surface of the slide. After 5

mimates the cellular sheet wil1 be fixed.

Renove the slechol ané add 1 ml of Giensa? stain (make a fresh

stain by diluting 1 drop into 1 ml of aistitied vater).

5. After staining during 30 minutes, vach with tap water.

6. Remove the slide; let dry; mount folloving the instructions.

---Page Break---

8.

LABORATORY EXERCISE #2

PREPARATION OF TISSUE CULTURE CoNSETTURNTS

Objective:

?To obtain the componente which will provide the cell "in vitro"

with the organic end inorgenie substances necessary for ite nutrition,

and will protect it againet changes in physical state, teaperature,

PH, ete.

Materiels: 7

4 cnick eabryoe

Scissors

syrioge 50 m2.

Centrifuge tubes

Graduated cylinder

Antibiotics (Teniei1iin and streptoayein)

11, HANKS BALANCED SALT SOLUTION (R88)

A. 10 X Bolution

wait A Nalco; 3.5 am.

Discoive in 250 mi, diatilted water. Dispense in a convenient

Lottie (50 ml. screw-cep prescription bottle) and autoclave at

320°C for 15 minutes.

unit #2

wc 60.0 gn.

a 1.0 wn.

e904, 759 2.0 en.

a04.28,0 0.6 ox:

atucove 30.0 ex.

10370, 0.6 em

Pistolve in 800 ml, distitied vater.

---Page Break---

Lanoratory Broreise #2

unit #3

o0 Lb gn.

Dissolve in 100 nl, aistilted water.

unit #

Phenol Red O.M ame

Nix Phenol Red in a smell amount of water until @ paste,dilute

to 150 ml. with distilled water, titrate to pi 7 with 1/20 acti.

Make up to final! voluse of 200 ml. Preserve with 1-2 ml, Chioroform.

MA 100 wi. of untt fli to unit #2 and then add unit #3 to make

1,000 nl. Pour solution into glass stoppered bottle end odd 3-4 ml.

chloroform se @ preservative. ?hie solution may be kept at room

| tenperature for 6 nonths-1 year.

NOT: Mintitee transfor of chiorofors Sn preparation of the workdng

solution. ve certain that bottle caps are loosened during

mtoclaving to ingure thet all chloroform 1s driven off.

Working solution

?the working BSS is prepared by ailuting 10K Stock 1:10 with Asetilled water. Dispense in convenient size rerew cap bottles and outociave at 120°C for 15 minutes, Asoptieally afd 2.5 ml. of sterile codiun bicarbonste solution (Unit #1) to each 100 mh. of BSS. ?The pil my be adjusted with C02. ?The Dalanced salt solution is now ready for use, Do not tighten cape unti2 pi of BSS 48 7.4.

2. NUTRIENT YEDIA

Begle'e minimum e

ential medium containe bigher concentrations of

anino acids then the basal medium first described by Eagle, which

---Page Break---

?he medium iy be prepared with Menks PSS vere.

Te mediua 18 prepared,

At the time of use,

red st 20°C) and NaHCO; are added to the

1X solution,

Solution Per Liter 10x negiun

L-Arginine. Hen

1.05 gn.

A-tystiaine. vo 0.33 em,

Atysine, Her 0.58 gm,

tryptophane 0.20 gm,

. 1-PhenyLatanine 0.32 an.

Lsthreonine © O48 gm,

Asteueine 0.52 gm,

l-Vatine 0.06 ee

I-Tsoleveine 0.52 em,

I-Methionine 0.15 om.

Solution B:

Antyrosine 0.32 gn.

aecyetine 0.24 gn,

?Tere anino acids are Aiseclved in 200 ml, of 0.075 Hea with

gentle heating (60%),

Solution 0:

Meotinanide 200 me.

Pyridoxal, 200

?Thienine 200 ag

Pantothenic Acta 200 ng

Choline 200 ng,

SvInositor 400

Riboflavin 20 me.

---Page Break---

=

Laboratory Exercise £2

Components are aissolved in approxinetely:175 ml. of double

aistinted water ana then brought to e final volume of 200 ml. with

double distilled water. the solution 2 dispensed n 10 xi. ancunts

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

of 10x meatus.

solution Br

200 mi. of Biotin ere Gissolved in 150 ml. of double atstiiied

water. To inerense stability during storage, 1.0 ml. of 1.0 HCL

4s added. the total volume 4s brought to 200 mi. ?with double

aistinted water and the solution dfepensed fn 10 al. ancunts and

stored at -20°C. 10 xl. of Solution D are added to esch Liter of

20K medi.

Seiution

200 mg. folie acta (crystalline) are Glesclved in 200 ad. 1X Hanks!

BES pit 7.8. The eolution is dispensed in 20 ni. snouts and stored

ab -20°Cs 10 ml. of Solution ¥ are added to each Liter of 10X nediue,

Gutanine Solution 36 - (To be added at the time of use) 12 ens. of

AsGlutamine are dissolved in 400 ml. of double distitied weter and

sterilized by i2tration through Seitu-type ped. The solution ie

stored at -20°C and 1.0 ml. is added to each 100 ml. of 1X Bagle's

medium.

Preparetion of the final nixtu

iY BSS

fa, The following are dissolved in solution Bt

Wac 80.0 gx.

Ke 4.0 gee

2.0 gn.

---Page Break---

a2

Laboratory Exercise #2

B. Te following are @issolved in 50 ml. double distitied water and

?2ded to the pool.

apHPOy 12,0 1.52 em.

WLP, 0.60 on.

20 grans of Glucose are dissolved in 50 ml. of double aistiiied

water with 20 ml, of 141 Phenol Red solution and added to the poo.

4. Te voluse of the pool is brought to 600 ml. with double distitiea

water and the following solutions sre added:

Per 1,0 Liter 10x meatun

Solution ¢ 10.0 mL.

Sotution D 10.0 m.

Solution F 1 20.0 mi.

fe, In 8 separate flask containing 160 mi. double distiiied vater 2.0 gas.

anhydrous CeCly are Atssolved and added to the pool elovly vith

vigorous shaking.

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

is brought to approximately 950 ml. with double distilied water.

6. A solution containing 20,000 units of PenicsI2in an 20,000

micrograns of Streptomycin ?per ml. {8 added in @ volume of 5.0 ml.

Por liter and the mixture is held in the refrigerator overnight.

The total volume de brought to exactly 2,000 ml. with double

Aistsiled water end the solution ts sterilized through Soitz

type pad.

A. Por use,

¢ solution is diluted to 1X with sterile double aistiiied

water and 24 of the 3f Glutamine Solution nd 1.25 to 2.59, of @

2.04 watc0, are added,

---Page Break---

Laboratory Exeretee #2

D. The following are dissolved in 50 ml. double distilled water and

aided to the pool.

NepHPO4 1290 1.52 on.

101%), 0.60 ex.

©. 20 grans of Glucose are dissolved in 50 ml. of double aietitiea

water with 20 12. of 1% Menol Red solution and added to the pool.

4. The volume of the pool 4s brought to 600 mi, with double distitiea

water snd the following solutions are added:

Per 1,0 iter 10x medium

Solution ¢ 10.0 al.

setution D 10.0 m.

solution E 1 10.0 mt.

6. Jn a separate flack containing 160 ml. double distilled vater 2.0 gms. anhydrous CeCly ere Aiasolved and added to the pool slowly with vigorous shaking.

£. Me wnino acids of Solution A are added to the pool and the vous is brought to approximately 950 ml. with double distitied water.

&. A colution containing 20,000 unite of Penlei21in and 20,000 mlcrograns of Streptonycin per ml. 4s added in a volune of 5.0 ml. or Liter end the mixture is held in the refrigerator overnight.

1h, the totel volune is brought to exactly 2,000 ml. with double Aistiiied veter end the solution fs sterilized through a Seitz type ped.

4. Por use,the solution is diluted to 1x with sterile double distitied

water and 1¢ of the 3% Glutamine Solution and 1.25 to 2.5% of a

2.84 natCo, are added.

---Page Break---

. * laboratory Exercise #2

For Laboratories occasionally using sunli atounts of Pagle' s rediua, t, 4¢ recomended that the 1X wediun be prepared by attuting 4 10X Stock So2ution of the nino acide and the 20K stock solution

of the Vitenins (stored at -20°C) appropriately An Farle or Hanks?

299 adding Glutaaine, antibiotics and Nel003 as indicated shove.

?Mais prevents deterioration of the vitanine during? long term storage

at ice box temperature.

3. CELL DISPERSTNG AGENTS

A, Srypain Solution 1.0%

2 ge. of powdered trypein is dissolved in 100 ml. of phosphate buffer saline and the solution is passed through ash-free filter paper (Schleicher and Schull #589). ?The solution is then sterilized by filtration through a deite-type pad and stored at -20°C.

1B, Versene Solution (Bthyelenediamine tetraacetic acid)

Mech 8.0 om.

= HPO, 0.2 en.

Ke. 0.2 em

TiaghPO, 0.35 en.

Versene 0.20 gm.

Dissolve in 1,000 ail. of distilled water, Dispense in convenient amounts and sterilize by autoclaving at 120°C for 15 mimites.

4, AWETBIOTICS OLUTION

Fenieii2in (20,000 units per mi.) and Streptoryein (20,000 microgamta per mi.)

1. Ald 10 wd. of Marks" eolution to 1,000,000 unite of Penfci1iin,

2, Add 210 wl. of Honks' solution to 1 viel with a gram of Streptonycin.

---Page Break---

Iaboratory Frereise #2

3, Mix the contents of both vials end ada up to 50 ml. of Hanks! solution.

1, Dispense jo viels and keep at -20°C.

5. CHICK manyo EXIRACT 50%

Bach student will be provided with 5 exbryos 9-10 days of age.

A. Harvest the enbryos end place then in @ sterile Fetri-dish vhere the eyes, beake, lege and wings are removed.

B. The renaining tissvee are washed in a beaker containing Hanks! BSS, ?thon minced with uterine selssors.

©. the minced tiesve te passed through a 50 ml. ejringe into a

graduated cylinder or contrifuge tube.

D, An equal volume of Hanks" BSS is added to the tissue culture and the mixture is etirred and allowed to stand for 30 minutes.

B, ?the mspenston is centrifuged at 1,500 rpa for 20 minutes end the supernetant fluid (constituting the 50% extract) ir removed and stored at -20°C.

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

?at 2,000 rpa for 10 minutes.

---Page Break---

a5.

TABORATORY RXERCTSE #3

MONOLAYER CULTURES OF CHICK EMBRYO CELLS

coast

satptind yates atures tren sick any were aoe

Sm frat to te nied for vine peopegation, evelomant of nor

testes hae Hoorensd the ute of chee eahryo firobst fr

fru fctaten ad engen geodetic.

Material,

i 4 enick enbryos

Petes dimes

Beaker

S| sefssors

Forceps

syringe

Erleaseyer flask

magnetic stirrer

Centrifuge tibes

Procedure

1. Chick exbryos 9 days old are harvested and placed in e sterile

Petri dish vere eyes, beaks, legs and wings are removed end aiscarded.

2: the embryos are transferred to beaker containing Ranke? BSS and

washed in 3 changes of the solution.

3. the extryos are minced Snto pieces approximately 3 mi. sn ataneter,

with uterine seiscore, and the minced tissue ie washed with 3

changes of Hanks? BSS.

4, Me minced tiasuc 4s passed through a 50 ml. syringe (without needle

into a 500 ml. Erlenmeyer Mask, vhore it 1e vashea twice with

90-100 mi. of Hanks" BSS.

---Page Break---

Laboratory Exeretse #3

5. After the fluid from the second washing has been resoved, an sppropoiate volune of .25f solution of trypsin in Hanks" BSS is added, ?The volune of trypsin solution exployed is determined by ?the musmer of embryos being processed (5-20 enbryosy 200 ml., 32-20 enbryoe, 300 na.)

6. A magnetic etirring bar 1s added to the flask which is placed on ?the magnetic stirrer and the euepension agitated for 1 hour et

room temperature,

After one hour of trypsinization the flask 4s slanted to sedinent large tiscue particles end the supernstant fluid (cell euspension) is decanted through a stainless steel wire cloth (72 mesh wire ieneter .0037 inches) into contrisuge tubes,

8. the cell euspension 48 centrifuged horizontally at 600 rpm for 20 minutes and the supernatant fluid containing trypsin is aspirated Smodiately. The celle are washed once by resuspending in Hanke! BOS, centrifuging at GOO xp for 10 minutes and aspirating the

supernatant fluid.

9. he celts are then resuspended in 15 to 30 ml. of Hanks? BSS,

?transferred to 15 el. or 30 ml. graduated, conical centrituye

?tubes and centrifuged horizontally at 600 rps for 10 minutes.

10. The volume of packed celle ie noted and after removal of the

supernates

Muid, the celle are diluted in the following medium:

Povine ecru 2.0 ma.

5 Lectatbunin bydrolyacte

in physiological saline 5.0 ml,

---Page Break---

an.

ae

Laboratory Exercise #3

50% Chick Bibryo Extract 4.0m.

Honks' Bss. 05.5 mi.

2.84 WalGO, 2.5 me

Penicillin-streptosyein solution 1.0 ml.

For tube culture the cells ere diluted 1 to 200 and dispensed in ml, volumes, For plaguing in stoppered bottles, the cells ere algo diluted 1 to 200 end alepensed in @ xi. volumes into three ounces preseription bottles, For plaguing in 60 mm, Petri dishes 5 ml, of 1:200 ailution of the cells ere added and incubation is conducted in 9§ atmosphere.

After Sncubation at -37°C for 1 to 2 days, complete monolayers of celle are formed and the cultures sre ready for inoculation with

viruses or clinical material.

---Page Break---

TADORATORY RKCESE gh

?TROHITLQUES FOR GROWING CELL LINES IN TISSUE CULTURE

crgectt

Tio experinent Ss designed to maintain a coll Line (tteta, ne 2 or 1)

for the duration of the course. ach student will be provided with 1 bottle

of cells.

Materiales

1 bottle with a confluent cel) monolayer

Haenocytoneter

Solvtion trypan tue

14 tudes

Procedure: .

1. Observe the cells under the microscope end describe then.

2. Remove modiun with a pipette. Ws

h 2 times with Hank's B99. Aaa

10 mi of 0.25 per centtrypein solution, alloxing the trypsin to

cover the eels for exactly one minute at room texper-

ature, Renove all of the trypsin and place the tutes in 37°C

Ameubator for 10 minutes. At the end of this tine the col2s

?should be almost completely detached from the vall of the bottle.

420 10 ml, of fresh medium and suspend the cells homogeneously

with a pipette,

3+ Cot the cells in the haenocytoneter

3-1, With @ Pasteur pipette carefully exprese a drop of the cell

suspension made up of 0.5 ml. cells plus 1.0 ml. of txypan

Blue under the haenocytoncter coverglaiss, avolding any

overflow into the moat,

---Page Break---

Iaboratory Ereretse gh

3-2, Detemine the everage munber of vieble cells (dead cells

* tain ?iue) in the 4 large corner squares used for

counting white blood cells, multiply ty 10,000 the

nunber of viable celis to obtain the muber of cells

Ber ml. Adjust to 50,000 cells/ml. usiug Eagle's meatun,

Tranter 50,000 cells into exch of 1 test tubes. stopper

tubes with rubber stoppers. Incubate at 37°C in en

horizontal plane.

4, Keep record and observe cells every any.

---Page Break---

LABORATORY EXERCISE 45

CONSERVATION AND TRANSFOR? OF CELL, CULTURES

Objectives:

To maintain in the laboratory with a minimum of handling vieble

cel ines not in continuous use.

Materinter

cen culture

Haewacytoneter

?Trypan biue solution

Aespoures

A, Storage

?1, Obtain a bottle with a culture of § day old cells from vnieh tho

out-grovth medium has been rexoved

id replaced with 10 wl. of

fresh mediun,consisting of 10 per cent hores serum end 90 per cent

Jactaltuain hydrolysate yeast extract medium. After a2 day

Sncubation period, the medium 4e removed, the cells trypsinized and

counted. f bottle should yield between 10 end 20 x 108 cette,

othervise the cells are not suiteble for storege.

2. the pit of the medium is adjusted to 7.4 by xewno of an 8.0% HalCOs

solution. Add 21.0 mi. of sterile glycerol to the 0 xl, of medina

4m each culture.

3. Transfer the coll cuspension to ampoules (xhich can be Mane-sented)

or to tubes, tightening the scoppere and soning with adhesive tape,

4, Bring to MC during 1 hour and thea place the tubes (ar anpoutes) in

?the Reveo (~70°C) where the temperature will drop 1°¢ per minute,

Once the tenperature inside the empoule has reachéa -20°C, the celis

---Page Break---

Laboratory Brerciee #5

can be stored at -197°C in the Liquid nitrogen refrigerator.

Under these conditions celle can be stored for periods up to 5 years.

5. To revive the frozen celle, the smpoule is removed from the Liquid nitrogen refrigerator ond thaved rapidly in a 37°C water bath, A volume of the cell suspension containing 1.5 to 2.0 x 10° is added to 10 mi. of outgrowth medium and cultures initiated in @ 200 mi. rottle.

1B, Transport

1. srypsine a bottle of Hela cells and @ilute in growth medium to obtain © suspension of no nore than 0.6 x 10° celis/a.

2, Refrigerate at °c for 24 hours,

\3. centrifuge at 200 rpm for 30 minutes and discard the supernatant.

. Ada medium to obtain @ cell suspensfon of 2.4 x 10° per ml.

5. In this state the celle can be shipped in an iced container and pon receipt sedinented by centrifugation at 200 rpm for 30 minutes

and resuspended in fresh growth medium at a concentration of 0.6 x 10°

cells/ml. A satisfactory method to ship cell cultures is obtained

PiaLing the vessel with nutrient medina, preventing the trauma to the

celis by the movenent of the mediua. Upon receipt, the medium should

?be renoved and the cells fed with a new one.

---Page Break---

22.

?LABORATORY EXERCISE #6

TUABELING OP POLYSONES

Objective:

?To etudy the incorporation of RNA precursors into cellular polysones.

Material:

1 Liter RSB butter

0.02 M trie-H CL pit 7.

0.01 MK cL

0.0015 M mg Cly

100 ml 10% euerose w/w dn SB

1100 mi 10% euerose w/w in RSR

\ 20 m2 ReB containing 5 x 10-7 gn/a/ nydrocorticone

500 ma Liquid scintillator .

2 lake bottles with confluent

monolayer of L-celis

?Trypsin for renoval of celle

Procedur

Monday:

1. ake up eotut tone.

2. Add 30 y cH uridine to each Blake bottle.

3: take 6 10-204 mucrose gradients.

4, set up minp and flow celt for wonttoring gradtente

5. Set up paper discs for fractionation.

Tuesday:

1, Add 10 y © cM urtaine to Make bottles and incubate 10 min.

2, trypainize celle,

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Laboratory Exercise #6

3. Centrifuge cells in conteal centrifuge bottle.

4, Take up.in 1,0 ml RSB containing 5 x 10°? gn/m hydrocortisone.

5. Womogenize in glass homogenizer.

6. Ada 0.33 ml 20% sucrose to hosogenate and 8000 RPM for 5 min.

7. Aaa Wi ng BoC to supernatant and place 0.4 nl on each of 3 sucrose eredients.

8. Centrifuge in SW 39 rotor at 35,000 RMI for 30 min.

9. Pump gradients through Mow cell and on to propor aises.

20, Wash @iees 10 minut

in 10f TCA and 5 min. in 95% ethanot.

11, Iny @isce and adé to viele.

22. Count 1 min/viad.

---Page Break---

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1 OF FOLYSOMES

Tis is a laboratory exercise, vhose purpose is to fantltarize the

student with cone techniques widely used in cellular and molecular biclogy.

Jn the cell, proteins are synthestzed on the polyscnes. These consist of individual ritosones held together by the messenger RNA vhich specifics the anino acid composition of the protein. The size of the Polysone 1s proportional to the alze of messenger ang,consequently, of ?the peptide to be synthesized,

Tt would appoer that the ribosomes are stable and can be revsed by ?the cellswhereas most neasenger RRA is unstable. Hence the ribosanes, are ?vert labeled by a long exposure to an RNA,precursor (in this case 13 ?wridine) Whereas the messenger RNA can be labeled by a short exposure to precureor (cl uriaine),

Atter breskage of the cell and removal of miclet, mitochondria, and eel wall by low speed centrifugation, the principal particulate matter eft in the supernatant 1s the polysones. these are best anelyzed by sucrone density gradient centrifugation, In this technique the ample is layered on top of & Linear mecrose gradient in a centrifuge tube. There 18 @ Linear increase of sucrose with depth of the tube in onder to steb{2ize the process of centrifugation.

After centrifugation ine svinging tuoket rotor, it 1e necessary to analyze the contents of the tube. This can be done. in various ways. However, we chal Alsplace the contents of the tube by injecting @ more

dense Liquid into the botton of the tube, Total miclet? acid can be measured

---Page Break---

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by allowing the displaced fluid to flow through a flow cell which 4e monitored at 260 my in a spectropbotoneter. Fractions are collected on paper? discs for analysis of radioactivity.

It is convenient to collect samples on dises since inthis form they can be easily processed to yield defined biocheniead fractions with unffors physical properties for counting (no necessity for quenshing corsection).

Liquid ecintiation counting currently provides the simplest: nethoa for detection of § -enitters and the most frequently used technique for feperation of double Lebel. By an appropriate setting of the dieriminators in to separate channels, both 13 and cl ean be counted accurately end

efficiently.

---Page Break---

26.

?LABORATORY EXERCISE #7

METABOLIC STUDIES USING TSOTOPES TH TISSUE CULTURE

cagects

Uso of Innetnfonine-cMi, in the biosynthesis of phosphatiail? choline.

tncorpuratfen of Cle for tranrdethiations mos

Materiade:

Hunan Liver cel cultures (Chang's cell Line)

Tamethionine-cUn, (Hew Mngland Tuclear Corp., Mecs.), sterile

Gatution (0-1 m2 2.5 ye).

?Teypein solution 0.25% in pit 7.4 phosphate buffer.

n-butencl. .

Teotenio sodium chloride solution (0.94).

Developing solvent for chronatograrhy:

+ ?ehloroform, methanol, water (65:25:

Gass plates with 250 uw Loyer of silicecgel G.

Preparation: Silicergei 6-15 ge," 30 ml distilled water; emlettied

and layered in each plate.

Chromatography chesbers.

Todine vayor chanbers.

15 md centrifuge tubes.

Sterile pipettes (0.5, 5.0 snd 10.0 rl)

Seintilleting Liquid (FPO 0.4f, FOROP 0.00f in toluene).

Radioactivity counting viels.

Pecknan Liquid seintilistor.

stirring rode and Pasteur pipettes

Proceai

Ls Add 0.1 mh (2.5 ye) of Iemethionine-clltig dissolved in 10 wl of

culture nediun to a Chang's cell culture. Tncubate for 2 hours

at 37°C.

---Page Break---

Laboratory Exercise #7

2. Srypsinizo the cel culture with 5.0 ml of trypsin solution.

Resuspend the cells in 5.0 nd of culture mediue,

3. Ceutrituge at 1,000 RAF for 10 minutes.

'k, Resvapend the cell pack in 2 mi. saline with gentle agStation.

Centrifuge ut 1,000 REM for 10 mimites,

Repeat the stove prisedure tice.

6. Resuspend the cele 4n 1,0 ml saline ant transfer to a Totter

Bivejetm houcgentzer vial with « Fastour pipette, Rinse the

centeiiige tube with on additions) 0.5 at aatine portion,

TT. slomagenize 3-5 minutos,

8. Aaa 1.0 nd of Bebutanci. Shake for 2 houre,

9. Centrizuge at 3,000 RPM for 25 mimtes.

20. transfer 20 & of the butanol phase to « counting Vial adding

5.0m of the seintintating Meuta.

UL. Prepare @ st24ca-get plate.

22, Dey tn @ 200% oven for 2 hour.

35. Allow the plate to coo and place an aliquot of ?he ttand

Phase 2 em from the Border, the volume depending on the

Pedloactivity cout, Next to thts sample epot « standard of

208 of Ine -tecithine,

2, Plece 50 a. of the developing solvent in the chanier and then the

stienuge2 pate,

15. AUlow to develop untii the solvent level has reached the top

border.

---Page Break---

Laboratory Beeresce #7

1G. Mow to dry at room tenperature, Develop the sample components

in un?fodine vapor chaser,

1. Once the spots are identified the gel is collected from each one

fend Placed in individuel viele, Add 5 nl ecintiiating quid

fand count in the Bechnen Liquid scintiletor using the carbon 1

tracer.

28. Discus

of the results obteined.

---Page Break---

retov vot a) Us of Santee the wee of Suctpes in the saty

ot cetutr fnctiens ia based on the property of these elemnte to reve

as molecular markers, whose fate 1s followed through the metsbolie pathways

sn do tore connie are wibizel, the propery of areentiatieg &

state or raiontiveSsoape fron the ordinary eleente, due toner

ascteent nist vast or by partic ot radetionenasn, sakes 18

saga vo tntarmtine or fant eomouitof te neaelinn 1 Setatd.

fale, ny tnt oa property {8 te pootig to mor te Snterenngewuee

of \ marked compound within the cell cr other level of higher organizatior.,

ars ma vith tris of Instremte of vying ttotecian arty 0

guid scintittetion rystem hes a great sdveotege over others in the oat

radioactive enicsione (# radiations of 13, oc),

D) Tranmethylation mechantsn to fora phorphatidilcholine - his transne-

thylation process hes been studied at different levels of organization.

of low ener

Jn intect aninats, in homogenates, in cell fractions, in the presence

of isolated enzyme aystens, etc.

4m intact oninels (Du Vigneoul et. al.), the first studies made

on rats fod on a diet defticient in methionine and eystine but in the

presence of choline end honocystine, led te a denoustration of the

existence of u metabolic process thet forms choline frén methionine.

---Page Break---

fn evident proot of this operational necheatua was gotten "in vivo!

using rediouctive end stable Ssotopes. the use of CDs-nethionine,

tht, methionine and CDs-chotine choved Ubet Were yas ay "ia toto!

Incorporating nechanin of the methyl groups tran the esthlonine into

the structure choline and viceversa and thet the radtoactivity rocafned

{tn the phomphotypide of the liver.

otter studios in liver homogenates and éeti fractions ted to the

enanstration of the sane tyye of molecules fnvelved in this process

(aromer, J. and Greonborg, D.). In thie vay the redtoactivity frou

CMhig-nothionine war incorporated in the shospholypide of these frestione and

Ubty-methionine in the presence of phosphatiailethenolanine, ATP, and ig °*

cofactors formed phosphatidiicholine, phosphatsé!lnononcthylethanolansine.

Previously, work dealing vith §-Adenosiinethionine (Cantont et el.) elioved

methionine, APP, and Mg °* to be replaced by said compound in cell fractions

?These vorks established the participation of at least two enzymes

?that catalyze the formation of phosphaties2 choline by means of transnethyletion

?using methionine and a phosphatidic derivative. One that eatalyzes the

a Me PF

(s-@enosii methionine synthetase) end enother that catalyzes the evecessive

formtion of S-adenosil methionine using methionine, ATP,

?trenenethylation steps (§-Adenosii nethtonine: phoephstiéilethanolamine-

ethyl. transferase).

There 4s no information available on the study of this netabolfe process

im oninal or human coli cultures, fut the studies that have been made and

briefly published are valid for this Level of organization, the cell level.

---Page Break---

CH, -0-0c-k'

© bH-e-0e-a"

e ¢

BN = Clee CI 0-P 0-8 Hy

ey ou Phrafatects whsvecle

aus Crene

whe CH ~CH~9 ~ pH ?O- CH,

Cle on

pie

BOW-ON CN. OP ~0-0 Hy

cu, by

Gus

i rt 1 ~O-

4 HCW, - Cl oP O-eu,

ey ow

---Page Break---

ae

TARORATORY EXERCISE 48

Memions OF UETECTTHG OR MEASURING VIRUS MULTIPLICATION IN ISSU CULTURE

CHTOPATHIC EFFECT OF VIRUS MILITPLICATION

Qojectiv

4p determine the virus @i1ution that gives rise to cytopcthic

changes in 50 per cent of the inoculated eel cultures.

Material

Poliovirus suspension

mbes

Pipettes

Proceaur

<1, Observe the Heta cell cultures prepared during the third day of

antoratory work.

2. Pipette off the grovth media aid replace St with .9 ml, cach of

Ragie's nedtun containing 2% serun.

3. Prepare tenfold attutions of polfovirus type 1 as follows:

a. Set up @ row of & Wassernon'e tubes munber

2 through ana

Aiepenee 1.8 ml, of media into each of then.

?b. Take 0.2 ml. of the poliovirus suspension end edd to the first,

?tube in the row. Mix thoroughly with a sterile pipette,

c, Take 0.2 ml. from the dilution in the tube #l and pass At to tube

#2 wixing the contents.

@. Repeat the operation with the resainder tubes,

4, mocutete .1 of esch dilution of the virus and deliver to each of

4 tubes of lieta cells. A seperate pipette should be used for each

Aiutions hovever in"the interest of laboratory glarsuare economy

use one 0.2 mi. pipette for adding the virus dilution to the Hela

---Page Break---

Laboratory Exercise #8

cette beginning with the highest Afiution and working back to the

dover.

5. Set up to 2 Fela tubes for control without inoculation.

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

T. Calculate the TEIgy by the Reed-ttiench method.

In the following table an exanple ir given of data derived from an

ideal experizent for illustrating the procedure of secumlation:

we

7 ?ACCUMULATED VALUES

a a cs

dilution ' Ratlo CPE ?Wo. CFE _' CPE "No. CPs" Ratio "Per Cent

yiot + Wife tw "0 'a2 ' 6 ' 32/2 + 100

wes Wr w+ 6 § 8 + ot BB + 20

ws a gt a kh at st oo

ao ahh. * 3B fat kt apt 20

wF + of + o * rr ee °

Accumated velues for the totel muaber of tybes that shoved a CPB

or were intact are obtained Ly adding in the direction indiested by the

?srrows, the accumilated CTE ratio reprosente the acoumiated maber of

tubes with eytopathte changes over the secumated total mber inoculated.

Jn this example the cytopathic change in the 10°3 asiution, 18

higher than 50%5 4m tho next lower @itution, 1074, it is considerably

Jower. the necessary proportionate distance of the 50 per cent CPE end

---Page Break---

-33+

Taboratory Brereise £8

8 obtained as follows:

oint Lies between these tvo dilutions end

Per cent CPE at dilution next above 50f) ~ Sof = Proportionate

Per cont CPE at dilution next above 50%) - (per cent CPR _adatance

?at ablution

next below)

or 80 - 50: 30 x o,5

B20 bo

Since logarithmically the distence between two ailutions is @

funtion of the incremental steps used in preparing the series, st is

necetsary to correct the proportionate aistance by the dilution factor.

In the case of seriat ten-fold dilution the factor 4s 1 (10g 10 © 2) and

Yuin is disregarded. In our exemple we have:

Negative logarithm of CTD, and point titer = negative logarithm of the

D,

30 @ilution above the 50 per cent CPE

\ plus the proportionate aictence

\ Fhotor

that de:

Negative logeritha of the aliution above 50% -3

Proportionate distance (0.5 X dftution factor (log 10): 05

TCTs 35

= 10-35

Log TOM yy = 10

---Page Break---

he

LABORATORY EXERCISE 49

PLAQUE METHOD FOR CONFLUBHT LAYERS OF CELLS

hsective:

Jo produce eircunscribed infected areas by vaccinia virus in chick

esbryo fibroblasts which do not take the neutral red vital stata

smd appear as clear unstained areas against a background of viable

stained cells,

vatertares

Caick exbryo fibroblasts

Veceinta virus suspension

Water bath

?| Neutral rea

Petri atshes :

Proseaur

1. Set up three Wasserman'e tubes runbered 1 through 3 and edd 1.8

?nl, of Fagle's media to all tubes. From the pool of vaccinia virus

supplied sda .2 ml. to tute #1

16 mix thoroughly with @ sterile

Pipette, Withdraw 0.2 ml. and edd to tube #2. Repeat the previous

step with new pipettes so that you will have virus aitutions 1072,

10-2, ana 1073,

3. Remove the out-growth medium from the Petri dishes previously

Prepared with chick exbryo fibroblasts and vush once with sxline.

Inoculate a plate with 0.5 ml. of each virue dilution.

3. Incubate at 37°C for 2 houre for virve adeorption with occastonal

rocking to distribute the virus particle:

4. After the adsorption period, remove the fluid ?and overlay the

fibroblast sheets with 5 mL. of the following agar medium:

Agar 1.0 gm.

---Page Break---

1

Yeast Bxtract (Difco) oa em

actalbunin hydrotysate oh am.

Horse seri 34.0 mi.

Take? Bee 85.0

Fenici22in 50 u/s. end 50 Streptomycin to complete medium.

5. Allow the agar to solidify and tum the plate upside down and

Ancubate at 37°C for 3h days with the cell monolayer dow.

6. Add 3 ml. of a 1/1000 1+ 7 neutral-red solution; incubate at room

?temperature for 2-h houre and overnight at °C,

7. The dilutions used should produce distinct end separated plagues.

Observe against a vbite background. Hy counting the munber of

plaques at the Ailution sere they appear distinct, and by

multiplying by the cocr-cpondent ?idution fector, the number of

plaque forming units (PF) por ml. of the virus suspension can

be ealeuisted.

---Page Break---

TABORATORY EXERCISE 410

UBUYRALT2ING ANDTBODIRS ASSAYS IN CELL CULTURES

Sajectiver

to eteviate the cepeeity of a serum to neutralize the eytopathse

effect of apoliovirus in a Hela cells systens.

Materials:

Serum

Follovirus euspension

Subes with HeLa ces

Water bath at 56°C.

Procedure:

1. the serum specimen 1s inactivated at 56°C for 30 minutes to

Gestroy heat non-specific vires inhibitory substances.

2. Set up a row of tubes to make serum @ilutions of 1:4, 1:16,

h, 11256,

1024 prepared in either balanced salt solutions

or the mintenance medin to b2 used in the cell cultures,

3, TeLiovinus 48 aiiuted to contain 100 Fy 4n & volume of

0.1 ni, (an determined by a previous titration of the virus).

?The virel dilutions are made in the sase medium employed for

the preperation of the serum Slutions.

4, Nqual volumes of the serum dilutions (0.5 ml.) and of the ailuted

test virus (0.5 ml.) are mixed, ?the volune of serum virus mixture

prepared is dependent upen the musber of coll cultures to be

inoculated with the mixture. For virus control the test virus

@llution fe mixed? with en equal volume of diluent (or known.

noms serum) ond incubated under the cue conditions as the serum

---Page Break---

?a aboratory Breretse #20

virus mixture. For serum control, the 1/4 dilution is mixed

with aiavent, Tt 1e necessary to perform a concurrent titration

of the virus to establish that a test dose acwally contetne amproxinately 100 TTB.

?he conditions recomended for ineubstion of eerun virus mixtures

vary widely for certain agentss 1t hae been demonstrated that some prelitdnary ineubetion dose increase the neutralizing capacity cf somim. the inportant consideration 49 to avotd incubation conditions under viteb the vires may be labelled for a eanple for long periodsor 37°C. For most neutralization ?tests; incubation of the serum virue mixtures 49 conducted for 30 minutes to one hour at room temperature or at HC.

[After incubstion period, serum virus mixtures, virus controls ?nd serun controls, are inoculated ia wlunes of .2 ml. into monglayer tube cultures: At least two cultures ere exployed for each serun mixture «

the Snocudated cultures are incubated at 37°C end exeninet microscopically for abidity of the serum to ?uhibSt CPE of the virus.

?The cytopathic effect of the inoculated tubes Le recorded

find the neutralizing end point ie expressed av that elution

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

---Page Break---

ER EEE EE SE'SSE_? eo eel! lL

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Iaboratory Exeroise #10

of the virus, as {lusteated in the following examie:

sem DIRION * CRE? CRE "Ho cHPE + ope ino con | MORTALITY

ae ?sotto 1 1 MOS patio ter cont

uh Foe to 2 te 6 toe °

me ige fe la le la im los

a:6h tye tas a ta te tap B

wee tue fa fa ba fa tap lg

10h app eo tk te Am + 100

SSPE gee oy os

logarithm 50 per cent stralizing end point = -1.8 + (0.5 y (0. 6)

«28+ 60.)

= 2a

antilogaritim -2.1 = 120

---Page Break---