PRNC - 91 PUERTO RICO NUCLEAR CENTER COURSE IN TISSUE CULTURE AND RADIOISOTOPIC TECHNIQUES AT CELLULAR AND SUBCELLULAR LEVEL LABORATORY EXERCISES J orinario ay Universidad OF PUERTO RICO UNDER CONTRACT HO. AT (40-11-1859 FOR U.S. ATOMIC ENERGY COMMISSION ---Page Break--- COURSE IN 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 October 17 to November 10, 1966 ** With the partial support of NIH Grant #ST1-AI-171-06 ---Page Break--- LABORATORY EXERCISE #1 PREPARATION OF MATERIAL, STERILIZATION AND STERILITY TESTS, Objective All components in a cell or organ culture system must be free of contaminant microorganisms. Routine testing procedures should be carried out in order to rule out the presence of bacteria, fungi, and mycoplasma. Two tubes of chick embryo fibroblasts, two tubes of HeLa cells, two tubes of 1-38 Stx, two tubes of thioglycolate medium, Stx tubes of Sabouraud agar, six tubes of PPLO broth, twelve tubes of PPLO agar, and Pienes' stain. Procedure: 1. Observe under the microscope and make a description of the different cell types. 2. Tests for sterility: 2:1 Incubate 0.1 ml of each cell culture tube into 10 ml of thioglycolate medium. Incubate at 37°C. Read and record results. If negative, discard after 5 days. 2:2. Streak a loopful of each cell culture on Sabouraud Agar. Incubate at room temperature. Read and record results during a week. ---Page Break--- 3. With a pipette, deposit some drops of each cell culture fluid on PPLO broth and agar (Difco). Streak the agar with a bacteriological loop, invert the plate and incubate at 37° for seven days. Incubate the broth at the same temperature for four days, and after this period, place some drops on a PPLO agar plate spreading with a bacteriological loop. Without removing the cover of the plates, inoculate the 1st and 6th days, look for colonies of PPLO under the

microscope stage focusing through the agar. Use a 10K objective and 10, 12.5 or 15 X ocular. Most FPLO colonies appear as round colonies with a dense center and a less dense periphery, giving the appearance of a fried egg. PPLO colonies have been isolated from tissue cultures; however, they do not conform strictly to this appearance on primary isolation. They may appear to lack distinct periphery and appear to be totally embedded in the agar. These colonies are usually very small and look "granular" or "feathery." PPLO colonies vary from 10 to 500 microns in diameter (0.01 to 0.5 mm) and characteristically, the center only or all of the colony is embedded in the agar. Individual organisms cannot be resolved since they are the size of an average virus particle. Occasionally, at the periphery of PPLO colonies, "large bodies characteristic of this group of organisms are found.” After locating the colonies, ---Page Break--- they are usually marked out on the petri dish with a glass marking pen. Confirmation of PPLO colonies depends, in addition to morphological characters, on: 1. Inability to remove the embedded portion of the colony from the agar surface by stroking the colony with a loop. This demonstrates the fact that the colony is embedded. Bacterial colonies will rub off. 2. The non-reversion to bacteria which subsequent passages of the colonies will reveal. Reversion to bacterial form would be typical of L forms. 3. A requirement for native protein. 4. Reaction with the Dienes stain. The Dienes stain is prepared by dissolving 2.5 g of methylene blue, 1.25 g of azur TT, 10.0 g of maltose, and 0.25 g of sodium carbonate in 100 ml of distilled water. With a cotton swab moistened in the stain, stroke the area of an agar plate in a well-defined area just adjacent to the suspected colony. The area of the colony is then examined under the microscope as described above. The PPLO colonies stand out distinctly with densely blue staining centers and light blue peripheries. Bacterial colonies are also stained but those are

decotortzed in about 30 minutes. The PPLO colonies never decolorize the stain. ---Page Break---
---Page Break--- LABORATORY. EXERCISE 2 PREPARATION OF TISSUE CULTURE CONSTITUENTS Objective: To obtain the components which will provide the cell "in vitro" with the organic and inorganic substances necessary for its nutrition, respiration, and will protect it against changes in physical states: temperature, pH, etc. Materials: 4 chicken embryos, scissors, syringe 50 ml, conical tubes, graduated cylinder, antibiotics (Penicillin and Streptomycin) 1. HAWKS BALANCED SALT SOLUTION (BSS) A. 10 X Solution: Note #1: 3.5 gm. Dissolve in 250 ml. distilled water. Dispense in a convenient bottle (50 ml. screw-cap prescription bottle) and autoclave at 120°C for 15 minutes. Note #2: Make 80.0 gm. NaCl, 2.0 gm. KCl, 0.6 gm. $NaHPO_4$, 0.6 gm. Glucose in 800 ml. distilled water. ---Page Break--- Note #3: Add 1 gm. of $NaHCO_3$ Dissolve in 100 ml. distilled water. Note #6: Phenol Red 0.4 gm. Mix Phenol Red in a small amount of water until a paste forms, dilute to 150 ml. with distilled water, titrate to pH 7 with 1/20 NaOH. Make up to a final volume of 200 ml. Preserve with 1-2 ml. chloroform. Add 100 ml. of Note #4 to Note #2 and then add Note #3 to make 1,000 ml. Pour solution into glass-stoppered bottle and add 3-4 ml. chloroform as a preservative. This solution may be kept at room temperature for 6 months to 1 year. Minimize transfer of chloroform in preparation of the working solution. Be certain that bottle caps are loosened during autoclaving to ensure that all chloroform is driven off. Working: The working BSS is prepared by diluting 10X Stock 1:10 with distilled water. Dispense in convenient size screw-cap bottles and autoclave at 120°C for 15 minutes. Aseptically add 2.5 ml. of sterile sodium bicarbonate solution (Note #1) to each 100 ml. of BSS; the pH may be adjusted with CO_2. The balanced salt solution is now ready for use. Do not tighten caps until pH of BSS is 7.4. NUTRIENT MEDIA

Eagle's minimum essential medium contains higher concentrations of amino acids than the basal medium first described by Eagle, which permits cultures to be kept for longer periods of time without feeding. The medium may be prepared with Hanks B55 base. The medium is prepared, concentrated 10%, and stored in the refrigerator. At the time of use, putrescine and antibiotics (stored at -20°C) and Yanco are added to the 1X solution.

Solution A: Per Liter 10x medium
L-Arginine, 1.05 mM
L-Aspartic acid, 0.31 mM
L-Valine, 0.58 mM
L-Tryptophan, 0.10 mM
L-Phenylalanine, 0.32 mM
L-Threonine, 0.08 mM
L-Leucine, 0.52 mM
L-Isoleucine, 0.52 mM
L-Methionine, 0.15 mM

Solution A: Tyrosine, 0.32 mM
L-Cystine, 0.26 mM

These amino acids are dissolved in 200 ml of 0.075 HCl with gentle heating (60°C).

Solution C:
Pyridoxal 200 mg
Thiamine 200 mg
Pantothenic Acid 200 mg

Choline 200 mg
Adenosine 400 mg
Riboflavin 20 mg

Components are dissolved in approximately 175 ml of double distilled water and then brought to a final volume of 200 ml with double distilled water. The solution is dispensed in 10 ml amounts and stored at -20°C. 10 ml of Solution C are added to each liter of 10% medium.

Solution D:
200 mg of Biotin are dissolved in 150 ml of double distilled water. To increase stability during storage, 1.0 ml of 1.0 M HCl is added. The total volume is brought to 200 ml with double distilled water and the solution is dispensed in 10 ml amounts and stored at -20°C. 10 ml of Solution D are added to each liter of 10x medium.

Solution E:
200 mg of folic acid (crystalline) are dissolved in 200 ml of 1X Hanks' BSS pH 7.8. The solution is dispensed in 10 ml amounts and stored at -20°C; 10 ml of Solution E are added to each liter of 10x sodium glutamine solution.

37 - (To be added at the time of use)
12 g of L-Glutamine are dissolved in 400 ml of double distilled water and sterilized by filtration through a sterile pad. The solution is stored at -20°C and 10 ml is

added to each 100 ml of 1x Eagle's medium. Preparation of the final mixture of 10x Eagle's medium in Hank's BSS: The following are dissolved in solution: NaCl 80.0 gm, KCl 4.0 mm, MgSO4 7.0 2.0 mm. ---Page Break--- The following are dissolved in 50 ml double distilled water and added to the pool: NaHCO3 1280 1.52 gm, CaCl2 0.60 mm. 10 grams of Glucose are dissolved in 50 ml of double distilled water with 20 ml of 12 Phenol Red solution and added to the pool. The volume of the pool is brought to 600 ml with double distilled water and the following solutions are added per 1.0 liter of 10x medium solution: A 10.0 ml, Solution D 10.0 ml, Solution E 10.0 ml. In a separate flask containing 160 ml double distilled water, 2.0 gm anhydrous CaCl2 are dissolved and added to the pool slowly with vigorous shaking. The amino acids of Solution A are added to the pool and the volume is brought to approximately 950 ml with double distilled water. A solution containing 20,000 units of Penicillin and 20,000 micrograms of Streptomycin per ml is added in a volume of 5.0 ml per liter and the mixture is held in the refrigerator overnight. The total volume is brought to exactly 1,000 ml with double distilled water and the solution is sterilized through a sterile type pad. For use, the solution is diluted to 1X with sterile double ---Page Break--- distilled water and 1.7 ml of the 3% Glutamine Solution and 1.25 ml of 2.50% of 4 2.8% MARCO are added. For laboratories occasionally using small amounts of Eagle's medium, it is recommended that the 1X medium be prepared by diluting 2 10x Stock Solution of the amino acids and the 10X stock solution of the Vitamins (stored at -20°C) appropriately in Hank's BSS, adding Glutamine, antibiotics, and MARCO, and vitamins during long-term storage at ice box temperature indicated above. This prevents deterioration of the 3. CELL DISPERSING AGENTS. A. Trypsin Solution 1.0%: 1 gm of powdered trypsin is dissolved in 100 ml of phosphate buffer saline and the solution is p.

Filtered through ash-free filter paper (Schleicher and Schull #569). The solution is then sterilized by filtration through a Setts-type pad and stored at 20°C. B. Versene Solution (Ethylenediaminetetraacetic acid) at 8.0 mM, 0.2 mL, 1.70 w= kel 0.2 mL, AAS gm Versene 0.20

gm. Dissolve in 1,000 mL of distilled water. Dispense in convenient amounts and sterilize by autoclaving at 120°C for 15 minutes. ---Page Break--- 4. Antibiotics Solution Penicillin (20,000 units per mL) and Streptomycin (20,000 micrograms per mL). 1. Add 10 mL of Hanks' solution to 1,000,000 units of Penicillin. 2. Add 10 mL of Hanks' solution to 1 vial with a gram of Streptomycin. 3. Mix the contents of both vials and add up to 50 mL of Hanks' solution. 4. Dispense into vials and keep at -20°C. 5. CHICK EMBRYO EXTRACT 50% Each student will be provided with 5 embryos 9-10 days of age. A. Harvest the embryos and place them in a sterile Petri dish where the eyes, beaks, legs, and wings are removed. B. The remaining tissues are washed in a beaker containing Hanks' BSS, then minced with uterine scissors. C. The minced tissue is passed through a 50 mL syringe into a graduated cylinder or centrifuge tube. D. An equal volume of Hanks' BSS is added to the tissue culture and the mixture is stirred and allowed to stand for 30 minutes. E. The suspension is centrifuged at 1,500 rpm for 20 minutes and the supernatant fluid (constituting the 50% extract) is removed and stored at -20°F. After thawing for use, the extract is clarified by centrifugation at 2,000 rpm for 10 minutes. ---Page Break--- ---Page Break--- LABORATORY EXERCISE #3 TECHNIQUES FOR GROWING CELL LINES IN TISSUE CULTURE Objective: This experiment is designed to maintain a cell line (HeLa) for the duration of the course. Each student will be provided with 1 bottle of HeLa cells. Materials: 1. Nocodazole 2. Trypan blue 16 tubes Procedure: 1. Observe the cells under the microscope and describe them. 2. Remove medium with a pipette. Add 10 mL of 0.25 percent Trypsin.

solution; allowing the trypsin to remain on cells for exactly one minute at 37°C. Remove all of the trypsin and place the tubes in a 37°C incubator for 10 minutes. At the end of this time, the cells should be almost completely detached from the wall of the bottle. Add 10 ml. of fresh medium and aspirate the cell suspension very well with a pipette. 3. Count the cells in the hemocytometer. 3-1, with a Pasteur pipette, carefully express a drop of cell suspension made up of 0.5 ml cells plus 1.0 ml of Trypan blue under the hemocytometer coverslip, avoiding any overflow into the moat. 3-2. Determine the average number of viable cells (dead cells stain blue) in the 4 large corner squares used for counting white blood cells. Multiply by 10,000 the number of viable cells to obtain the number of cells ---Page Break --- Per ml. Adjust to 50,000 cells/ml 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 a horizontal plane. 4. Keep record and observe cells every day. ---Page Break --- ---Page Break --- LABORATORY EXERCISE #4 MONOLAYER CULTURES OF CHICK EMBRYO CELLS Objective Cell cultures of the Maitland type from chick embryo were among the first to be used for viral propagation. Development of new techniques has increased the use of chick embryo fibroblasts for virus isolation and antigen production. Material 4 chick embryos Petri dishes Beaker Scissors Forceps syringe Erlenmeyer flask Magnetic stirrer centrifuge tubes Procedure: 1. Chick embryos 9 days old are harvested and placed in a sterile Petri dish where eyes, beaks, legs and wings are removed and discarded. 2. The embryos are transferred to a beaker containing Hanks' BSS and washed in 3 changes of the solution. 3. The embryos are minced into pieces approximately 3 mm in diameter, with uterine scissors, and the tissue minced is washed with 3 changes of Hanks' BSS. 4. The minced tissue is passed through a 50 ml syringe (without ---Page Break --- %. 10. 2 needle) into a 500 ml.

Erlenmeyer flask where it is washed twice with 50-100 ml. of Hanks' BSS. After the fluid from the second washing has been removed, an appropriate volume of 0.25% solution of trypsin in Hanks' BSS is added. The volume of trypsin solution employed is determined by the number of embryos being processed (5-10 embryos, 200 ml., 11-20 embryos, 300 ml.). A magnetic stirring bar is added to the flask which is placed on the magnetic stirrer and the suspension agitated for 1 hour at room

temperature. After one hour of trypsinization, the flask is slanted to sediment large tissue particles and the supernatant fluid (cell suspension) is decanted through a stainless steel wire cloth (72 mesh, diameter 0.0037 inches) into centrifuge tubes. The cell suspension is centrifuged horizontally at 600 rpm for 10 minutes and the supernatant fluid containing trypsin is aspirated immediately. The cells are washed once by resuspending in Hanks' BSS, centrifuging at 600 rpm for 10 minutes and aspirating the supernatant fluid. The cells are then resuspended in 15 to 30 ml. of Hanks' BSS, transferred to 15 ml. graduated, conical centrifuge tubes and centrifuged horizontally at 600 rpm for 10 minutes. The volume of packed cells is recorded and after removal of the supernatant fluid, the cells are diluted in the following: Bovine serum 2.0 ml., Lactalbumin hydrolysate in physiological saline 5.0 ml., 50% Chick embryo Extract 4.0 ml., Hanks' BSS 85.5 ml., 2.81 mM Malco, 2.5 mM Penicillin-streptomycin solution 1.0 ml. For tube culture the cells are diluted 1 to 200 and dispensed in 4 ml. volumes. For plating in stoppered bottles, the cells are also diluted 1 to 200 and dispensed in 8 ml. volumes into three ounces prescription bottles. For plating in 50 ml. Petri dishes, 8 ml. of 1:200 dilution of the cells are added and incubation is conducted in 5% CO2 atmosphere. After incubation at 36°-37°C for 1 to 2 days, complete monolayers of cells are formed and the cultures are ready for inoculation with viruses or clinical material.

---Page Break--- ---Page Break--- LABORATORY EXERCISE # 5 VIRUS TITRATION: CPE PURPOSE: 'To determine the virus dilution that gives rise to cytopathic changes in 50 percent of the inoculated cell cultures. Material: Poliovirus suspension, Tebes Pipettes. Procedure: 1. Observe the HeLa cell culture prepared during the third day of laboratory work. 2. Pipette off the growth media and replace it with 0.9 ml each of Eagle's medium containing 2% serum. 3. Prepare tenfold dilutions of poliovirus type 1 as follows: 1a. Set up a row of 5 Wasserman's tubes numbered 1 through 4 and add 1.8 ml of media to each of these. b. Take 0.2 ml of the poliovirus suspension and add it to the first tube in the row. Mix thoroughly with a sterile pipette. c. Take 0.2 ml from the dilution in tube #1 and transfer it to tube #2, mixing the contents. 4. Repeat the operation with the remainder of the tubes to inoculate 0.1 ml of each dilution of the virus and deliver to each of 4 tubes of HeLa cells. A separate pipette should be used for dilution; however, in the interest of laboratory glassware economy, use one 0.2 ml pipette for adding the virus dilution to the HeLa cells, beginning with the highest dilution and working back to the lower. ---Page Break--- 5. Set up 2 HeLa tubes for control without inoculation. 6. Bring to the incubator at 37°C and read and record the results every day. 7. Calculation of the TCID50 by the Reed-Huench method. In the following table, an example is given of data derived from an ideal experiment for illustrating the procedure of accumulation: ACCUMULATED VALUES Per cent CPE: No CPE: Ratio 0 + 0 = 0: 100 10 4+ 0 + 0: 8/8 = 100 0 + 74 80 10 1 3 1 20 0 0 0: 8 0 8 Accumulated values for the total number of tubes that showed CPE or were intact are obtained by adding in the directions indicated by the arrows. The accumulated CPE ratio represents the accumulated number of tubes with cytopathic changes over the accumulated total number inoculated. In this example, the cytopathic changes in

the 10° dilution is higher than 50%; that in the next lower dilution, 0, is considerably lower. The necessary proportionate distance of the 50 percent CPE endpoint must be obtained as follows: Given concentration GFE HE dilution - as shown SOD > the concentration ratio * Proportionate distance (at dilution next distance below) or 80 = 50 w = 50. Since logarithmically the distance between two dilutions is a function of the incremental steps used in preparing the series, it is necessary to correct the proportionate distance by the dilution factor. In the case of serial ten-fold dilution, the factor is 1 (log 10 = 1) and 80 is disregarded. In our example, we have: Negative logarithm of TCID50 endpoint titer = negative logarithm of the dilution above the 50 percent CPE plus the proportionate distance that is: Negative logarithm of the dilution above 50% Proportionate

distance (0.5 X dilution factor (log 10)). Log Tero = 10°F 50.

LABORATORY EXERCISE #6 VIRUS "PLAQUE" ASSAY TECHNIQUE Objective: To produce circumscribed infected areas by vaccinia virus in chick embryo fibroblasts which do not take the neutral red vital stain and appear as clear unstained areas against a background of viable stained cells. Materials: Chick embryo fibroblasts, vaccinia virus suspension, water bath, neutral red, Petri dishes. Procedure: 1. Set up three Wasserman's cubes numbered 1 through 3 and add 1.8 ml of Eagle's media to all tubes. From the pool of vaccinia virus supplied, add 0.2 ml to cube #1 and mix thoroughly with a sterile pipette. Withdraw 0.2 ml and add to tube #2. Repeat the anterior step with new pipettes so that you will have virus dilutions 10^7, 10^6, and 10^5. 3. Remove the out-growth medium from the Petri dishes previously prepared with chick embryo fibroblasts and wash once with saline. Inoculate a plate with 0.5 ml of each virus dilution. 4. Incubate at 37°C for 2 hours for virus adsorption with occasional rocking to distribute the virus particles. 4. After the

absorption period, remove the fluid and overlay the fibroblast sheets with 6 ml of the following agar medium: Agar 1.0 gm. ---Page Break--- 5. The same conditions as the serum virus mixtures. For serum control, the dilution 1/4 is mixed with diluent. It is necessary to perform a concurrent titration of the virus to establish that a test dose actually contains approximately 100 TCID50. The conditions recommended for incubation of serum virus mixtures vary widely for certain agents and it has been demonstrated that some preliminary incubation does increase the neutralizing capacity of serum. The important consideration is to avoid incubation conditions under which the virus may be labeled for a sample for a long period at 37°C. For most neutralization tests, incubation of the serum virus mixtures is conducted for 30 minutes to one hour at room temperature or at 4°C. After the incubation period, serum virus mixtures, virus controls, and serum controls are inoculated in volumes of 0.2 ml into non-layer tube cultures. At least two cultures are employed for each serum mixture. The inoculated cultures are incubated at 37°C and examined microscopically for the ability of the serum to inhibit CPE of the virus. The cytopathic effect of the inoculated tubes is recorded and the neutralizing end point is expressed as that dilution of serum which protects 50% of the tubes against the test dose ---Page Break--- of the virus, as illustrated in the following example: SERUM DILUTION: CPE: NO CPE: CPE + NO CPE: 2: 0: 6: 0 Total 100 — 50% = CPE at dilution next below. Logarithm 50 percent neutralizing end point = -1.8 + (0.5 x (0.6) = 1.8 + (0.3) antilogarithm -2.1 = 120 ---Page Break--- ---Page Break--- LABORATORY EXERCISE # 7 NEUTRALIZING ANTIBODIES ASSAY IN CELL CULTURES Objective: To calculate the capacity of a serum to neutralize the cytopathic effect of a poliovirus in a HeLa cell system: Serum Poliovirus suspension Tubes with HeLa cells.

bath at 56°C. Procedure: 1. The serum is inactivated at 56°C for 30 minutes to destroy heat non-infective virus inhibitory substances. 2. Set up a row of tubes to make serum dilutions of 1:4, 1:16, 1:64, 1:256, 1:1024 prepared in either balanced salt solutions of the maintenance medium to be used in the cell cultures. 3. Poliovirus is diluted to contain 100 TCID50 in a volume of 0.1 ml (as determined by a previous titration of the virus). The viral dilutions are made in the same medium employed for the preparation of the serum dilutions. 4. Equal volumes of the serum dilutions (0.5 ml) and of the diluted virus (0.5 ml) are mixed. The volume of serum-virus mixture prepared is dependent upon the number of cell cultures to be inoculated with the mixture. For virus control, the test virus dilution is mixed with an equal volume of diluent (or known normal serum) and incubated under ---Page Break--- 5. To revive the frozen cell lines, the tube is removed from the deep-freezer and thawed rapidly in a 37°C water bath. A volume of the cell suspension containing 1.5 to 2.0 x 10^6 is added to 10 ml of outgrowth medium and cultures initiated in 200 ml bottles. B. Transport:

1. Trypsinize a bottle of HeLa cells and dilute in growth medium to obtain a suspension of no more than 0.6 x 10^6 cells/ml. 2. Refrigerate at 6°C for 24 hours. 3. Centrifuge at 200 rpm for 30 minutes and discard the supernatant. 4. Add medium to obtain a cell suspension of 2.6 x 10^6 per ml. 5. In this state, the cells can be shipped in an iced container and upon receipt sedimented by centrifugation at 200 rpm for 30 minutes and resuspended in fresh growth medium at a concentration of 0.6 x 10^6 cells/ml. A satisfactory method to ship cell cultures is obtained by filling the vessel with nutrient medium preventing trauma to the cells by the movement of the medium. Upon receipt, the medium should be removed and the cells resuspended with new one. ---Page Break--- ---Page Break--- LABORATORY EXERCISE #8 CONSERVATION AND TRANSPORT OF CELL CULTURES Objectives: to

maintain in the laboratory with 2 minimum of handling viable cell lines not in continuous use. Masters Cell culture Racanacytoncter ‘Trypan blue solution’ smpoutes As storage 1, obtain a bottle with a culture of 5 days old cells from which the out-grown medium has been removed and replaced with 10 ml. of fresh medium consisting of 10 percent horse serum and 90 percent lactalbumin hydrolysate yeast extract medium. After a further 2 days incubation period, the medium is removed, the cells trypsinized and counted. A bottle should yield between 10 and 20 x 10^6 cells, otherwise the cells are not suitable for storage. 2. The pH of the medium is adjusted to 7.4 by means of an 8.8% NaHCO3 solution. Add 2.0 ml. of sterile glycerol to the 10 ml. of medium in each culture. 3. Transfer the cell suspension to ampoules (which can be flame-sealed) or to tubes, tightening the stoppers and sealing with adhesive tape. 4. Bring to -80°C during 1 hour and then another hour at -20°C. After this period place the tubes (or ampoules) at -70°C. Under these conditions, the cells can be stored for periods up to 18 months. ---Page Break--- 5. To revive the frozen cell lines, the tube is removed from the deep-freezer and thawed rapidly in a 37°C water bath. A volume of the cell suspension containing 1.5 to 2.0 x 10^6 is added to 10 ml. of outgrowth medium and cultures transferred to a 200 ml. bottle. 6. Transport 1. Trypsinize 2 bottles of cells and dilute in growth medium to obtain a suspension of no more than 0.6 x 10^6 cells/ml. Refrigerate at 4°C for 26 hours. 7. Centrifuge at 200 rpm for 30 minutes and discard the supernatant. 8. Add medium to obtain a cell suspension of 2.4 x 10^6 per ml. 9. In this state the cells can be shipped in an iced container and upon receipt sedimented by centrifugation at 200 rpm for 30 minutes and resuspended in fresh growth medium at a concentration of 0.6 x 10^6 cells/ml. A satisfactory method to ship cell culture is obtained filling the ampoule with nutrient medium preventing trauma to the cells by the movement of the

mediums. Upon receipt the medium should be changed and the cells fed with a new one, ---Page Break--- ---Page Break--- LABORATORY EXERCISE #9 PLATING EFFICIENCY AND ISOLATION OF CLONES Objective: ‘This experiment is designed to determine the plating efficiency of D2 cells using the formula Percent plating efficiency = Colonies formed x 100 Cells inoculated and to obtain clones from single DC2 cells. Materials: D2 cell monolayer Hemocytometer tubes Petri dishes Procedure: 1. Trypsinize monolayer cultures of DC2 cells. 2. Count in hemocytometer using trypan blue to obtain viable count. 3. Dilute the cell suspension in nutrient media to obtain a concentration of 50,000 cells/ml. 3.1 Make tenfold dilutions (0.1 ml suspension plus 0.9 ml Eagle's medium) so that the suspension will contain 5,000 and 500 cells per ml. 3.2. Make a dilution 1/2 of the 500 cells suspension, in order to have 250 cells per ml. Transfer 0.1 ml of the cell suspension (25 cells) to each of 2 Petri dishes; 0.2 ml (50 cells) to 2 Petri dishes and 0.5 ml (125 cells) to a third group. Complete the volume up to 4 ml with fresh Eagle's medium. ---Page Break--- 5. Incubate the plates at 37°C in an atmosphere containing CO■. 6. After 8 or 9 days cultures may be examined microscopically to observe discrete colonies. 7. Staining of one of the series. 7.1 Wash once with

saline. 7.2. Fix with methyl alcohol for 5 minutes. 7.3. Stain with Giemsa for 10 minutes. 7.4. Rinse and observe under the microscope. Using the above formula determine the plating efficiency. 8. In order to isolate a single colony from one of the series cultured with different amounts of cells: 8.1 Select the colony to be isolated and mark the area with a wax pencil. 8.2 Remove all media from the culture dish. 8.3. Place over the colony to be isolated a sterile bent Pasteur pipette which contains some trypsin. 8.4 Gently add nutrient medium and suspend the colony with a pipette. 8.5 Transfer the cells to a new culture flask or dish. 8.6 Add enough nutrient medium and

incubate at 37°. ---Page Break--- ---Page Break--- LABORATORY EXERCISE # 10 ORGAN CULTURE Objective: 'The cultivation of fragments of organs in tissue culture systems will permit to study cells in a group of associated tissues. 'This technique provides a good system for research on the action of hormones, vitamins, carcinogens, etc. Material: Petri dishes (disposable) Paste Chick embryo Scissors Forceps Seal 1. Prepare culture dishes by placing a watch glass inside a Petri dish and a filter paper ring. These dishes must be sterilized at 170°C for 2 hours. 2. Add approximately 9 drops of plasma to the watch glass, followed by 3 drops of EGF 50 and quickly, but gently, mix before adding clots. 3. Remove embryo from its shell and transfer to a sterile Petri dish. Dissect out tissue to be cultivated and rinse in sterile BSS. 4. Cut explants to approximately 2 mm and rinse 3 times in BSS. 5. Deposit with a pipette 4 fragments onto the clot. Remove the excess of BSS carried over, using a fine pipette. 6. Incubate at 37°C. Observe daily, recording characteristics of growth. ---Page Break--- 1 vial of Extract (Difco) 0.1 g Lactalbumin hydrolysate 0.6 g. Horse serum 14.0 ml. Hank's BSS 85.0 ml. Penicillin 50 u/mL and Streptomycin to complete medium. Allow the agar to solidify and turn the plate upside down and incubate at 37°C for 3-4 days with the cell monolayer down. Add 3 ml. of a 1/1000 neutral-red solution. Incubate at room temperature for 2-4 hours and overnight at 4°C. 'The solutions used should produce distinct and separated plaques. Observe against a white background. By counting the number of plaques at the dilution where they appear distinct and by multiplying by the corresponding dilution factor, the number of plaque forming units (PFU) per ml. of the virus suspension can be calculated. ---Page Break--- ---Page Break--- LABORATORY EXERCISE #11 MACROSCOPIC TISSUES Objective: This exercise is designed to demonstrate recent microscopic methods that are helpful to conduct further studies on the cell. Materials:

Procedur Gtomsa stain Anti E. coli conjugated globulin + Staining with Fluorescent Antibody. 1. Make a smear on a slide from a tube with HeLa cells previously inoculated with E. coli. 2. Air dry. Fix with acetone. 3. Cover the slide with an anti E. coli fluorescent conjugated globulin. 4. Bring the slide to a Petri dish with a moistened cotton piece. 5. After 30 minutes contact, remove the FA reagent by dipping the slide into a vial of saline solution. 6. Place the slide in a jar of buffered saline (pH 7.5) for 5 to 10 minutes. Remove the slides, allow to drain, and gently blot off excess saline with absorbent paper. 8. Place a drop of mounting fluid on the smear and cover with a coverslip. 9. Examine smears under a stereoscope fitted with a cardioid type darkfield condenser and illuminated by an intense light source. Use fluorescence-free immersion oil. ---Page Break--- 3. Demonstration of phase microscopy. ©. Giemsa staining of E. coli in Leighton tubes. ---Page Break--- ---Page Break--- LABORATORY EXERCISES 412, 13, 14 (CHROMOSOME PREPARATION Objective: To carry out the enumeration of chromosomes in a human diploid cell for application to genetic studies and cell characterization. Material: Human diploid cell (WI-32) in Leighton tubes, Centrifuge tubes, colchicine (0.0001 mg/ml), Hypotonic solution, Fixative (1 part acetic acid + 3 parts absolute ethanol), Acetic orcein stain (2 pp. Orcein dissolved in 100 ml boiling acetic acid) Procedure: 1. Add colchicine (or Colcemid) to the tubes during the early log phase of growth. Colchicine should be used in a final concentration of 0.4 μg per ml. Colcemid in a

concentration of 0.05 μg/ml. 2. Bring the incubator to 37°C for 16 to 18 hours, in order to harvest cells at the metaphase period. 3. After incubation, take off the medium and add 5 ml of hypotonic solution (sodium citrate 0.56%). 4. Place the tubes under a lamp (60 watt bulb) for 30 minutes. Discard the hypotonic solution and add 5 ml of freshly prepared Carnoy's fixative. 6. After 30 minutes of contact with the fixative.

remove the coverslip from the tube, Let it dry overnight. 7. Stain with orcein during 15 minutes 8. Rinse with distilled water. ---Page Break--- 9. Dehydrate through 2 rapid changes each of 95% ethanol, absolute ethanol and xylene, 10. Mount in Permount. ---Page Break--- ---Page Break--- LABORATORY PRACTICE. A115 MICROAUTORADIOGRAPHY Objective: The experiment is designed to demonstrate the incorporation of thymidine labeled with tritium into the DNA of the cellular nucleus. Materials: Boulston (Kodak HTB) Water bath Container jars Slide storage boxes Developer (Kodak D-19) Acid Fixer (Kodak) Giemsa Stain Procedure Change the medium from MK cells cultivated during 2-3 days in Leighton tubes 2. Add new medium with radiolabeled thymidine in a concentration of 1.0 mCi. 3. Allow the thymidine to be in contact with the cells for 39 minutes and 1 hour at 37°C. 4. After adequate time wash with Hank's buffer. 5. With fine forceps remove the coverslip from the tube, 6. Fix with methyl alcohol for 10 minutes, 7. Mount the coverslip on a microscope slide with the cells facing up. Use Euparol as the mounting medium. 8. Place the slide at 45-50°C for 26 hours or at room temperature for 48 hours in order to dry the Euparol. ---Page Break--- 10. Stain for 5 minutes with Giemsa. 11. Expose the slide. 12. Place slides in developer for 2 minutes. Cover with photographic emulsion. 13. Place bottle of emulsion in a water bath at 43°C for 15 minutes. Rotate the bottle gently. 14. Pour into Couplin Jar. 15. Place the slides in an empty coupling jar that is in the water bath, 16. Once warmed up pass the slides to the jar with the emulsion. 17. After 15 seconds remove the slides and drain the emulsion on a paper towel. 18. Allow the slides to dry placing them before a fan for 15 minutes or 1-2 hours at room temperature. 19. Store in black slide boxes and seal with dark tape in order to prevent the entrance of light. at room temperature for 10 to 30 days. Develop the slides. 20. Rinse in tap water. 21. Place in acid fixer for 2-5 minutes. 22. Rinse in water for 20 seconds.

minutes. ANS Rinse in distilled water, air dry. Observe with immersion oil. ---Page Break--- ---Page Break--- LABORATORY EXERCISES #16, 17, 18 INTRODUCTION TO RADIOACTIVE ISOTOPE TECHNIQUES FOR STUDY OF BIOLOGICAL SYNTHESIS. Orotic Acid and Thymidine Incorporation into the nucleic acid of HeLa cells Introduction: It would be extremely difficult to evaluate the value of the radioisotope as a tool in biological research. Certainly, much of the progress achieved in this field in recent years would have been impossible prior to the development of the atomic pile. Two basic facts account for the unusual utility of these radioactive elements. We can tell where they (location), and how such present (quantity), even after they have become intermixed with large numbers of stable atoms of the same element: Objective 1. Introduction to apparatus and techniques for use of isotopes as tracers in biological synthetic reactions. 2. Application of biochemical techniques to the study of nucleic acids. Reagents for nucleic acid extraction Schneider's method Reagents for DNA determination by Carlotti's method Reagents for RNA determinations by Ortonol method Ribonuclease and Deoxyribonuclease ---Page Break--- Experimental Procedures: In general, the procedure is to incubate the HeLa cells in a media containing orotic acid labeled with C14 and thymidine H3 labeled for 17 hours. The cells are washed with unlabeled orotic acid and thymidine. The cells are trypsinized and homogenized. The nucleic acids are extracted and the radioactivity is determined. The procedure will be applied to study orotic acid incorporation into RNA and thymidine incorporation into the DNA fraction of the HeLa cells. 1. Three 48-hour-old bottles will be given to each student. The student will add orotic

acid 0.1 µM per ml, and thymidine 0.1 µM per ml, and incubate again for 17 hours. After 17 hours incubation, remove the media and wash the monolayers 2 times with fine A, Silt, each time.

3. Wash the monolayers 2 times more with saline A containing unlabeled orotic acid and thymidine (0.1 mp per ml). 4. Typenize the cells, remove the cells from the bottle and wash 2 times with saline A in the centrifuge (Ten minutes at 1,000 rpm). 5. Resuspend cells in 5 ml of phosphate buffer, pH 7.4 and henopentize for 2 minutes in the Cont-mixer homogenizer. 6. Take an aliquot of 0.5 ml for dry weight determination. 7. Take an aliquot of 4 ml and do a total nucleic acid extraction by the Schneider's Brief method. See appendix A. Take a 1 ml aliquot of the final total nucleic acid extract and apply to a piece of filter paper. Do counts for c and YW, the machine will do these counts simultaneously. ---Page Break--- 9. Take an aliquot of 2 ml of the final total nucleic acid extract and do a DNA determination by Ceriotti's method. See appendix B. 10. Take an aliquot of 0.5 ml of the final total nucleic acid extract and do an RNA determination by the Oretno? method. See appendix C. 11. Take an aliquot of 2 ml of the total nucleic acid extract and incubate with DNase for 30 minutes at 37°C (100 µl of DNase per ml). 12. Add enough cold absolute ethanol to make 62% alcohol and incubate at -10°C for 30 minutes. 13. Resuspend the precipitate in phosphate buffer, pH 7.4. 14. Do counts for c (may). 15. Repeat step #11 but incubate the aliquot with Ribonuclease for 30 minutes (100 µl per ml). 16. Repeat steps #12 and 13. 17. Do counts for (DNA). 18. Turn in Report. ---Page Break--- APPENDIX A Reagent: SCHNEIDER'S BRIEF METHOD OF NUCLEIC ACIDS EXTRACTION 10% TCA 5% TCA 95% Ethyl alcohol Procedure (In ice bath). To the homogenate aliquot add 4 ml of ice-cold 10% TCA, mix well with stirring rod and centrifuge in the cold at about 2,500 rpm for 7-10 minutes. Discard the supernatant. To the residue add 1 ml of ice-cold distilled water, resuspend with a rod, add 4 ml of 95% ethanol and mix well with a rod. Centrifuge at room temperature for 15 minutes. Discard supernatant. (At room temperature). To the residue add 5

all of 95% ethanol and resuspend with the rod. Centrifuge as before and discard the supernatant. (The procedure can be interrupted here). Repeat this step for liver homogenate. (At room temperature), to the residue add 5 ml of 5% TCA. Resuspend with the rod. Place the tubes in a water bath at 90°C for 15 minutes, stirring occasionally. Let them cool to room temperature. Centrifuge for 30 minutes. Save supernatant in 10 ml flasks. To the residue add 5 ml of 5% TCA, resuspend with the rod, centrifuge as before. Pool the supernatant with that from (4), and make up to 10 ml, final nucleic acid extract. ---Page Break--- * Homogenates: Gels = 4 ml of a 3% Bovine serum albumin suspension homogenize in 5 ml. Liver = 2 ml of a 20% homogenate. Spleen = 2 ml of a 10% homogenate. Kidney = 2 ml of a 10% homogenate. Lung = 2 ml of a 10% homogenate. Thymus = 2 ml of a 10% homogenate. Lymph nodes = 0.5 ml of a 10% homogenate. Bone Marrow = 0.5 ml of a 10% homogenate. Take an aliquot for dry weight determination at the same time. The dry weights are determined by drying the sample in tared bottles at 105°C in an oven, to constant weight (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 distilled water. It was dissolved in warm water. The solution is then cooled under running water and adjusted to volume. Store in refrigerator. Procedure 1. 2 ml (or an aliquot) of DNA solution or extract in a test tube. 2. 1 ml of Indole solution. 3. 2 ml of HCl concentration. Place them in a boiling water bath for 10 minutes. Cool in running water. The solution is extracted 3 times with 4 ml chloroform each time and centrifuged to give a complete clear water phase. Read the water layer against a blank in the spectrophotometer at 490 nm. ---Page Break--- APPENDIX C RNA DETERMINATION BY THE CRCTHOL METHOD Reference: Blotchen et Biophys. Acta 1, #3 (1947), 1. 150 mg protein. 2. 7.5 ml CuCl2 in HCl concentration (0.04) (0.0538 g CuCl2 in HCl concentration). 3. 67.5 ml of

concentrated HCl. 4. 60.0 ml of distilled water.

Procedure: Dissolve the 150 mg of orcinol in the 47.5 mL of HCl concentration. 4207.5 mL of the CuCl solution. Mix well. To the solution add 10 mL of distilled water. To 0.5 mL of extract RNA standard solution add 4.5 mL of the Reagent. Mix well. Place the tube in a boiling water bath for 40 minutes. Cool then in an ice-cold water bath. Read the absorbance against a blank in the spectrophotometer at 660 nm. ---Page Break---