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CHAPTER 11

VIRUS PLAQUE CONFIRMATION PROCEDURE

1. INTRODUCTION

This chapter describes a procedure that may be used for confirming virus plaques in cell cultures adhering to glass or plastic surfaces or in cells suspended in overlay agar. Although the procedures and reagents are for use with the Buffalo green monkey kidney (BGM) cell line, they may also be used for cells other than BGM. Where large numbers of plaques are observed and confirmation of each plaque is not practical, select no less than ten plaques per sample. Select only plaques that are well separated.

The virus plaque confirmation procedure may also be used to obtain sufficient virus quantities from plaque isolates with which to identify the isolated viruses in Chapter 12. Use aseptic techniques and sterile materials and apparatus only. Sterilize all contaminated materials before discarding them (see Chapters 2 and 3).

2. RECOVERY OF VIRUS FROM PLAQUE

2.1 Apparatus, Materials and Reagents

2.1.1 Pasteur pipettes, disposable, cotton plugged--229 mm (9 inches) tube length. Flame pipette gently about 2 cm from end of tip until tip bends to approximate angle of 45 degrees.

Place pipettes into a 4-liter beaker, cover beaker with aluminum foil, and dry heat sterilize for not less than 1 hour at 170 degrees C.

2.1.2 Rubber bulb--1 mL capacity.

2.1.3 Cell culture in tubes. See Chapter 9 (January, 1987 revision), Section 7.2 for preparation of cell culture tubes.

2.1.4 Tissue culture roller apparatus--1/5 rpm speed (product no. TC-1, New Brunswick Scientific, or equivalent).

2.1.5 Culture tube drum for use with roller apparatus (product no. ATC-TT16, New Brunswick Scientific, or equivalent).

2.1.6 ELAH--Earle's base with 0.5 percent lactalbumin hydrolysate and without NaHCO3 (Hazleton Kansas City Biological, product no. DM-303, or equivalent) supplemented with antibiotics (dihydrostreptomycin sulfate, penicillin G, tetracycline and amphotericin B; Sigma Chemical Co., or equivalent). ELAH--Earle's base solution supplemented with antibiotics and serum (from Section 2.1.8) is employed as a maintenance medium in the cell culture tubes used for virus plaque confirmation testing (See Step (a) in Section 2.2.2). ELAH--Earle's base solution supplemented with antibiotics, but without serum is used as a storage medium, if plaque sample material must be stored before confirmation procedure is completed (See Step (c) in Section 2.2.2). Whenever possible, plaque sample material should be inoculated onto a cell culture immediately, because storage of such sample material even at -70 degrees C may result in some reduction in confirmation counts. Only small volumes of the antibiotic supplemented ELAH --Earle's base solution are required. Employ stock antibiotic and ELAH -- Earle's base solutions prepared for use in Chapter 10 (December, 1987 revision). If unavailable, see Chapter 10, Section 2.1.3 for procedure for preparation of stock antibiotic solutions and Section 2.1.4 for preparation of ELAH--Earle's base solution and for supplementation of ELAH--Earle's base solution with antibiotics. Remaining reagents may be stored for subsequent use. Store antibiotic stock solutions at -20 degrees C and ELAH--Earle's base solution at 4 degrees C for periods no greater than 4 and 2 months, respectively. Reagents should be held in tightly stoppered or capped containers.

2.1.7 Vial, screw-capped (with rubber insert)--3.7 mL (1 dram) capacity. Place 1 mL of antibiotic supplemented ELAH--Earle's base solution (storage medium) from Section 2.1.6 in the screw-capped vial.

2.1.8 Fetal calf serum, filter-sterilized, heat inactivated at

56 degrees C for 30 min, certified free of viruses, bacteriophage and mycoplasma (GIBCO Laboratories, or equivalent).

2.2 Procedure

2.2.1 Procedure for obtaining viruses from plaque.

Decision to test plaque material for viruses immediately or to store material at -70 degrees C for later testing must be made before proceeding further.

(a) Place rubber bulb onto upper end of pasteur pipette.

(b) Remove screw-cap or stopper from plaque bottle (if plaque is in petri dish, raise cover from dish sufficiently to allow entry into dish).

(c) Squeeze rubber bulb on upper end of pasteur pipette to expel air.

(d) Penetrate agar directly over edge of plaque with tip of pasteur pipette.

(e) Gently force tip of pipette through agar to surface of vessel, and scrape cells from edge of plaque. If cells are present as a monolayer on the surface of the vessel, surface must be repeatedly scratched and gentle suction applied to insure that virus-cell-agar plug enters pipette. If cells are suspended in the agar, scraping of vessel surface with pipette is unnecessary.

(f) Aspirate plug from plaque into pipette.

(g) Remove pipette from plaque bottle (or petri dish).

(h) Replace and tighten down screw-cap or stopper on plaque bottle (if plaque is in petri dish, replace cover on dish). If sample is to be tested in cell culture immediately, proceed to Section 2.2.2, Step (b.3). If sample must be stored, proceed to Section 2.2.2, Step (c.2).

2.2.2 Procedure for inoculating viruses obtained from plaques onto cell cultures.

(a) Cell culture processing. If at all feasible, use a laminar flow hood while processing cell cultures. Otherwise, use an area restricted solely to cell culture manipulations. Viruses or other microorganisms must not be transported, handled, or stored in cell culture transfer facilities.

(a.1) Add fetal calf serum to the antibiotic supplemented ELAH --Earle's base solution (see Section 2.1.6) for a final concentration of 5 percent on day samples will be tested.

(a.2) Pour spent medium from cell culture tubes and discard the medium. To prevent splatter, a gauze-covered beaker may be used to collect spent medium.

(a.3) Replace discarded medium with a 2-mL volume of the 5 percent serum-antibiotic supplemented ELAH--Earle's base solution (maintenance medium) from Step (a.1). To reduce shock to cells, warm the maintenance medium to 36.5 plus or minus 1 degree C before placing on cell monolayer. To prevent disturbing cells with the force of the liquid against the cell monolayer, add the maintenance medium to the side of cell culture test tube opposite the cell monolayer.

(b) Procedure for samples tested immediately.

(b.1) Prepare cell culture tube in accordance with instructions given in Steps (a.1) through (a.3).

(b.2) Remove cap from cell culture tube.

(b.3) Place tip of pasteur pipette containing virus-cell-agar plug from Section 2.2.1, Step (h) into the maintenance medium in cell culture tube. Tilt cell culture tube as necessary to facilitate procedure and avoid scratching cell sheet with pipette.

(b.4) Force virus-cell-agar plug from pasteur pipette into the maintenance medium by gently squeezing rubber bulb. Squeeze bulb repeatedly to wash contents of pipette into the maintenance medium.

(b.5) Withdraw pipette from cell culture tube, replace and tighten down screw-cap on tube, and discard pipette.

(b.6) Place cell culture tube in drum used with tissue culture roller apparatus.

(b.7) Place in drum three additional culture tubes which have not been inoculated with agar sample. These tubes will serve as negative controls.

(b.8) Incubate cell culture at 36.5 plus or minus 1 degree C, while rotating tube at a speed of 1/5 rpm.

(b.9) Examine cells daily microscopically for 1 week, starting with day 3, for evidence of cytopathic effects (CPE). CPE may

be cell disintegration or changes in cell morphology. Rounding-up of infected cells is a typical effect seen with enterovirus infections. Incubation of BGM cells in roller apparatus for periods greater than 1 week is not recommended as cells under these conditions tend to die-off if held longer. Recovered virus (enteroviruses) preparations may be stored in a -70 degrees C freezer. Procedures are given in Chapter 12 for identification of confirmed viruses. If identification of confirmed viruses is to be directly undertaken, see Chapter 12 for sample requirement. Store remaining sample portion at -70 degrees C until no longer needed.

(c) Procedure for samples to be stored at -70 degrees C before testing.

(c.1) Remove cap from vial containing storage medium.

(c.2) Place tip of pasteur pipette containing virus-cell-agar plug from Section 2.2.1 Step (h) into vial.

(c.3) Force virus-cell-agar plug from pasteur pipette into storage medium by gently squeezing rubber bulb. Squeeze bulb repeatedly to wash contents of pipette into storage medium.

(c.4) Withdraw pipette from vial, replace and tighten down screw-cap onto vial, and discard pipette.

(c.5) Store vial at -70 degrees C.

(c.6) Place at -70 degrees C three additional vials containing storage medium that have not been inoculated with agar sample. These vials will be used to prepare the negative controls. When confirmation is to be completed proceed to Step (c.7).

(c.7) Prepare cell culture tube in accordance with instructions given in Steps (a.1) through (a.3).

(c.8) Thaw sample quickly in warm water (30-37 degrees C).

(c.9) Remove cap from cell culture tube.

(c.10) Remove cap from storage vial containing thawed sample.

(c.11) With a 1-mL pipette, transfer entire contents of vial containing sample into cell culture tube. Tilt cell culture tube as necessary to facilitate procedure and avoid scratching cell sheet with pipette. Place tip of pipette into the maintenance medium in cell culture tube. Squeeze bulb repeatedly to wash into the maintenance medium any of the

remaining test sample.

(c.12) Withdraw pipette from cell culture tube, replace and tighten down screw-cap on tube, discard pipette and sample vial.

(c.13) Place cell culture tube in drum used with tissue culture roller apparatus.

(c.14) With a 1-mL pipette, transfer entire contents of vials serving as negative controls into cell culture tubes. Transfer contents of vial in accordance with instructions given in Step (c.11).

(c.15) Place the three cell culture tubes in drum used with tissue culture roller apparatus.

(c.16) Incubate cell culture at 36.5 plus or minus 1 degree C, while rotating tube at a speed of 1/5 rpm.

(c.17) Examine cells daily microscopically for 1 week, starting with day 3, for evidence of cytopathic effects (CPE). CPE may be cell disintegration or changes in cell morphology. Rounding-up of infected cells is a typical effect seen with enterovirus infections. Incubation of BGM cells in roller apparatus for periods greater than 1 week is not recommended as cells under these conditions tend to die-off if held longer. Recovered virus (enteroviruses) preparations may be stored in a -70 degrees C freezer. Procedures are given in Chapter 12 for identification of confirmed viruses. If identification of confirmed viruses is to be directly undertaken, see Chapter 12 for sample requirement. Store remaining sample portion at -70 degrees C until no longer needed.

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