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Chapter 8
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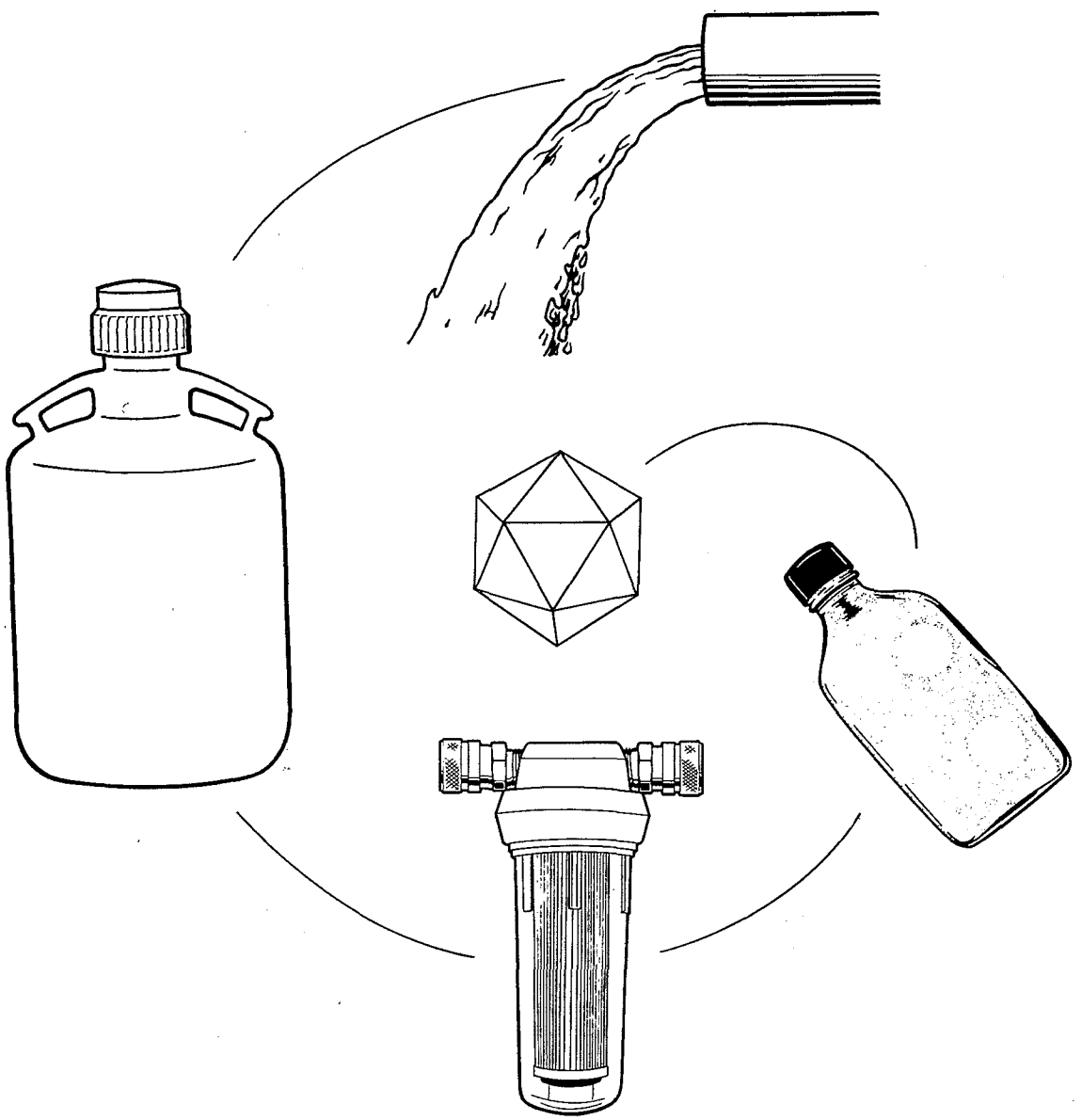

Chapter 8
Method for Reduction of Cytotoxicity of Sample Concentrates

This method may be used for the reduction of cytotoxicity in the recovery of viruses from surface waters, waste waters, drinking waters, ground waters, sludges, soils and dredge spoils found toxic to mammalian cells used for the assay of enteroviruses. The method may also be used for deeply colored sample concentrates that, if left on the cell monolayer, would result in inaccurate plaque counts. The procedure may result in less than usual recovery of viruses. A titer reduction of about 30% may be anticipated; thus, the procedure is to be applied only if there is a likelihood that inocula will be toxic to cell cultures, or may be so darkly colored as to obscure plaques.

Use aseptic techniques and sterile materials and apparatus only. Sterilize all contaminated materials before discarding them (See Chapters 2 and 3).

1. Virus Recovery from Samples

1.1 Process samples containing large amounts of solids, such as sludge, soil and dredge spoil using the procedures described in Chapter 7.

1.2 Process water and wastewater samples by following the VIRADEL procedure described in Chapters 5 and 6.

2. Storage of Sample Concentrates

2.1 Maintain samples at 4°C if samples can be processed within eight hours.

2.2 Store samples immediately at -70°C if processing cannot be undertaken within eight hours.

3. Predetermine Cytotoxicity of Sample Concentrate

Pretest all samples which may be cytotoxic.

3.1 Following the instructions in Sections 5.3.1 thru 5.3.13, inoculate one cell culture monolayer with each of the sample concentrates requiring testing.

In the absence of experience in identifying sample cytotoxicity, it is advisable also to process an uninoculated cell culture which will serve as a control in later comparisons to determine any reduction in sensitivity or survival of the mammalian cells due to the toxicity of the samples.

3.2 Incubate cell cultures at room temperature (22-25°C) for 80 min.

3.3 Add overlay medium to bottle(s) in accordance with the instructions given in Sections 6.1 and 6.2 of this chapter.

3.4 Invert cell culture bottles and incubate for three days in the dark at 36.5° ± 1°C.

3.5 Examine monolayer for cytotoxicity.

Determine cytotoxicity by macroscopic examination of the appearance of the cell culture monolayer. Cytotoxicity should be suspected when the agar color is more subdued, generally yellow to yellow-brown. This change in color results in a mottled or blotchy appearance instead of the evenly diffused color observed in "healthy" cell monolayers. Also viral plaques may be difficult to distinguish from the surrounding monolayer.

4. Processing of Deeply Colored Sample Concentrates

4.1 Identify color interference on the basis of past experience or from results obtained in the pretesting of sample concentrates (Section 4.2).

4.2 Predetermine color interference from sample concentrates.

In the absence of experience, it is advisable to predetermine whether the presence of a particularly prominent color in a virus assay sample will interfere with subsequent plaque counting. To pretest the sample concentrate, add a portion of concentrate equal to that of the inoculum to a single cell culture bottle. It is also advisable to process an uninoculated cell culture that will serve as a control in later comparisons with the test samples. Immediately add overlay medium to the bottle(s) in accordance with the instructions given in Sections 6.1 and 6.2 of this chapter. Invert the cell culture bottles and incubate for 30 min in the dark at 36.5° ± 1°C. Examine the bottles for clarity of the agar overlay medium.

4.3 Process colored sample concentrate that may interfere with virus assay by following the procedure used for reduction in sample toxicity (Section 5).

5. Reduction of Toxicity of Sample Concentrate

5.1 Apparatus and Materials

5.1.1 Cornwall syringe or equivalent with cannula or syringe needle.

A pipetting device will expedite virus assay when large numbers of cell cultures must be treated.

5.1.2 Magnetic stirrer and stir bars.

5.1.3 Cell culture bottles.

See Chapter 9 for the preparation of cell culture bottles. Chapter 9 outlines procedures using the Buffalo Green Monkey (BGM) kidney cells. These cells are very sensitive to many enteroviruses; however, cells other than BGM may also be used.

5.1.4 Waterbath set at 36.5 ± 1°C.

5.2 Media and Reagents

5.2.1 Sodium chloride (NaCl).

5.2.2 GG-free newborn calf serum (Grand Island Biological Co., or equivalent).

5.2.3 ELAH—Earle's base, with 0.5% lactalbumin hydrolysate and without $NaHCO_3$ (Kansas City Biological, cat. no. DM-303, or equivalent).

5.2.4 Washing solution.

(a) To a flask containing an appropriate volume of deionized distilled water, add sufficient NaCl to result in a final concentration of 0.85%.

The volume of NaCl solution required will depend on the number of bottles to be processed and the cell surface area of the bottles used for plaque assay. For determining volume, see Secton 5.3.14.

(b) Mix contents of flask on magnetic stirrer at speed sufficient to dissolve salt.

(c) Autoclave the salt solution at 121°C for 15 min.

(d) Cool salt solution to room temperature.

(e) Add 2% (volume/volume) GG-free newborn calf serum to salt solution.

(f) Mix on a magnetic stirrer at a speed sufficient to uniformly suspend the serum.

(g) Store the washing solution at 4°C.

Although the washing solution may be stored at 4°C for an extended time period, it is advisable to prepare solutions on a weekly basis, thereby lessening the possibility of microbial contamination.

5.3 Procedure

5.3.1 Decant growth medium from cell culture bottles previously prepared in accordance with instructions given in Chapter 9.

The medium is changed from one to four hours before cultures are to be inoculated and carefully decanted so as not to disturb the cell monolayer.

5.3.2 Discard medium.

5.3.3 Replace discarded medium with an equal volume of maintenance medium.

For BGM cells use ELAH—Earle's base solution without serum as maintenance medium.

To reduce shock to cells, warm maintenance medium to 36.5 ± 1°C before placing on cell monolayer.

To prevent disturbing cells with the force of the liquid against the cell monolayer, add maintenance medium to the side of cell culture bottle opposite the cell monolayer.

5.3.4 Return cell culture bottles to 36.5 ± 1°C incubator and hold at that temperature until the bottles are to be inoculated.

5.3.5 Identify culture bottles by coding them with an indelible marker.

5.3.6 Decant maintenance medium from cell culture bottles.

Do not disturb the cell monolayer.

5.3.7 Discard medium.

5.3.8 Inoculate onto each cell monolayer a volume of test sample concentrate appropriate for the cell surface area of the cell culture bottles used.

Inoculum volume should be no greater than 1 mL for each 40 cm^2 of surface area.

5.3.9 Rock inoculated cell culture bottles gently to achieve uniform distribution of inoculum on surface of cell monolayers.

5.3.10 Place cell culture bottles on a leveled stationary surface at room temperature (22-25°C) so that the inoculum will be distributed evenly over the cell monolayer.

5.3.11 Incubate inoculated cell cultures for 80 min to permit viruses to adsorb onto and infect cells.

5.3.12 Decant inoculum from each cell culture bottle.

5.3.13 Discard inoculum.

5.3.14 Into each cell culture bottle, add the volume of washing solution appropriate for the cell surface area of the bottles used.

Add 0.25 mL of washing solution for each cm^2 of cell surface area. Use washing solution as prepared in Section 5.2.4.

To reduce thermal shock to cells, warm washing solution to 36.5 ± 1°C before placing on cell monolayer.

To prevent disturbing cells with the force of the liquid against the cell monolayer, add washing solution to the side of the cell culture bottle opposite the cell monolayer. Also, avoid touching either the cannula or syringe needle of the pipette or the pipetting device to the inside rim of the cell culture bottles to avert the possibility of transporting contaminants to the remaining culture bottles.

5.3.15 Rock the washing solution gently across the cell monolayer a minimum of two times.

It may be necessary to gently rock washing solution across the monolayer more than twice if sample is deeply colored or oily and difficult to remove from the cell monolayer surface.

5.3.16 Decant spent washing solution in a manner that will not disturb the cell monolayer.

5.3.17 Discard washing solution.

5.3.18 Proceed immediately to Section 6.

6. Plaque Procedure for Titrating Viruses

6.1 Preparation

6.1.1 Apparatus and Materials

(a) Waterbath set at 36° ± 1°C.

Used for maintaining the temperature of the overlay medium. See Section 6.1.2 (a) to (h).

(b) Waterbath set at 50° ± 1°C.

Used for maintaining the agar temperature. See Section 6.1.2 (i).

(c) Incubator capable of maintaining the temperature of cell cultures at 36.5° ± 1°C.

6.1.2. Media and Reagents

(a) 2X Medium 199 with Earle's salts (Grand Island Biological Co., cat. no. 400-1100, or equivalent).

(b) HEPES (Sigma Chemical Co., cat. no. H-3375, or equivalent).

(c) GG-free newborn calf serum (Grand Island Biological Co., cat. no. 210-6400, or equivalent).

(d) Magnesium chloride ($MgCl_2$).

(e) Sodium bicarbonate ($NaHCO_3$).

(f) Neutral red, biological stain (Fisher Scientific Co., cat. no. N-129, or equivalent).

(g) Bacto skim milk (Difco Laboratories, cat. no. 0032-01, or equivalent).

(h) Antibiotics—penicillin G, dihydrostreptomycin sulfate, amphotericin B and tetracycline hydrochloride (Sigma Chemical Co., or equivalent).

(i) GIBCO bacteriological agar (Grand Island Biological Co., cat. no. M00010B, or equivalent).

6.2 Procedure

6.2.1 Mix equal portions of overlay medium and agar.

For BGM cells use the following or equivalent ingredients for the final agar overlay medium: 39.5% of a 2X Medium 199 with Earle's salts, 2% of a 1M solution of HEPES, 2% of GG-free newborn calf serum, 3% of a 7.5% solution of $NaHCO_3$, 1% of a 1% solution of $MgCl_2$, 1.5% of a 0.1% solution of neutral red, 1% of a 1% solution of Bacto skim milk and 50% of a 3% suspension of GIBCO bacteriological agar. For each 1 mL of overlay medium prepared, supplement with 100 units of penicillin G, 100 µg of dihydrostreptomycin sulfate, 1 µg of amphotericin B and 0.0125 mg of tetracycline hydrochloride.

6.2.2 To each cell culture bottle, add the volume of warm (42-46°C) agar overlay medium appropriate for the cell surface area of the bottles used.

For each cm^2 of cell surface area, add 0.5 mL of agar overlay medium.

To prevent disturbing cells with the force of the liquid against the cell monolayer, add agar overlay medium to the side of the cell culture bottle opposite the cell monolayer.

6.2.3 Place cell culture bottle, monolayer side down, on a leveled stationary surface at room temperature (22-25°C) so that the agar will distribute evenly before it solidifies.

Agar is fully solidified within 30 to 60 min.

6.2.4 Cover cell culture bottles with a sheet of aluminum foil, a tightly woven cloth, or some other suitable cover to reduce light intensity and thus, prevent damage to the cell monolayer.

6.2.5 Invert cell culture bottles and incubate in the dark at 36.5° ± 1°C.

6.3 Counting Viral Plaques

6.3.1 Count, mark and record plaques in cell culture bottles on days two, three, four, six, eight and twelve after overlaying.

Counting procedure described is for enteroviruses. Depending on the virus density and virus types present in the inoculated sample, rescheduling of virus counts at plus or minus one day may be deemed preferable.

Virus titers are calculated from total count.

6.3.2 Examine cell culture bottles on day sixteen.

If no new plaques appear after 16 days, discard cell culture bottles; otherwise continue to count, mark and record plaques every two days until no new plaques appear between counts.

6.3.3 Calculate virus titer in plaque forming units (PFU) for each virus-containing sample concentrate.

To determine the number of PFU per mL in water, sewage sludge, soil, or dredge spoil sample concentrate, multiply the number of PFU by the reciprocal of the inoculum volume. If the inoculum volume was diluted, also multiply the number of PFU by the reciprocal of the dilution made.

6.3.4 Calculate virus content of original sample.

To obtain virus content of the original sample in terms of PFU per mL, multiply the product from Section 6.3.3 by the concentration factor which is calculated by dividing the volume of the original sample by the volume of the sample concentrate. For soil, digested dewatered sludge and dredge spoil samples, correct for water content and report in PFU per gram of dry weight.

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