⟨162⟩ DIPHTHERIA ANTITOXIN POTENCY TESTING FOR HUMAN IMMUNE GLOBULINS

An in vitro method is provided that is suitable for determining the potency of diphtheria antitoxin (antibodies against the diphtheria toxin) in preparations of plasma-derived human immune globulins. Diphtheria toxin is produced by *Corynebacterium diphtheriae* and has the ability to produce a cytopathogenic effect on susceptible epithelial cell lines. The test is based on the ability of diphtheria antitoxin to neutralize the diphtheria toxin, decreasing its cytotoxic effect. Specifically, the test determines the potency of the diphtheria antitoxin based on its ability to inhibit the cytotoxic effect of diphtheria toxin on cultured Vero cells (African green monkey kidney epithelial cells) relative to a reference standard. The mitochondrial dehydrogenases of live Vero cells can reduce the dye 3-4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide (MTT) to a blue/black product that is then measured by absorbance at 540 nm. If no or little diphtheria antitoxin is present, then diphtheria toxin induces cell death and the inability of cells to reduce MTT, resulting in the presence of white or colorless wells. Acceptance criteria are defined by the appropriate regulatory agencies.

ASSAY

- **PROCEDURE**

DMEM-5 solution: Dulbecco's Modified Eagle's Medium[1], supplemented to contain 5% fetal bovine serum (FBS), 2 mM L-glutamine, 50 µg/mL of gentamicin, and 2.5 µg/mL of fungizone. [NOTE—Alternatively, a combination of 0.1 mg/mL of kanamycin sulfate, 0.1 units/mL of penicillin, and 0.1 mg/mL of streptomycin can be used in place of gentamicin.]

DMEM-2 solution: Dulbecco's Modified Eagle's Medium[1], supplemented to contain 2% fetal bovine serum (FBS), 2 mM L-glutamine, 50 µg/mL of gentamicin, and 2.5 µg/mL of fungizone. [NOTE—Alternatively, a combination of 0.1 mg/mL of kanamycin sulfate, 0.1 units/mL of penicillin, and 0.1 mg/mL of streptomycin can be used in place of gentamicin.]

MTT solution: 5 mg/mL of MTT in phosphate-buffered saline.[2] Prepare just before use and minimize exposure to room light.

Extraction solution: 0.4 N hydrochloric acid in isopropanol

Toxin test solution: Obtain a liquid preparation of toxin from a culture of *C. diphtheriae*.[3] It may have been rendered sterile by filtration, and sterility may be maintained by the inclusion of a suitable antimicrobial preservative. Store the filtrate in the dark at 2°–8° for several weeks until the activity is considered to be constant, as determined by testing. Dilute the toxin in *DMEM-5 solution* to obtain a suitable *Toxin test solution* that provides a working equivalence with the *Standard solution*. [NOTE—An appropriate *Toxin test solution* may be empirically determined as follows. Titrate the toxin against a fixed concentration of antitoxin (e.g., 0.125 units/mL) using volumes and media as defined below. Determine the lowest concentration of toxin that causes Vero cell toxicity in the presence of the antitoxin concentration chosen. This can be defined as LCD/20 (where "LCD" stands for limit of cytotoxic dose, and "/20" refers to 1/20 units/mL = 0.05 units/mL). This is a minimum concentration that should be used in the *Assay*.]

Trypsin–EDTA solution: Dissolve 0.4 g of trypsin, 0.2 g of ethylenediaminetetraacetic acid (EDTA), and 0.85 g of sodium chloride in 100 mL of sterile water. Use cell culture grade and sterile reagents to prepare. Alternatively, a premade sterile preparation may be used.

Cell culture preparation: Prepare Vero cells[4] by culturing in 75-cm^2 tissue culture flasks in *DMEM-5 solution* at 36 ± 1°, 5% carbon dioxide (CO_2), and a humidified environment. [NOTE—Alternative flask sizes may be used by adjusting the volumes used below.] After 2–3 days of growth, the medium may be replaced by *DMEM-2 solution*, with growth allowed to continue. When the cells have reached a confluent monolayer, discard the culture media. Wash the cell layer by pipetting 5 mL of *Trypsin–EDTA solution* into the flask and gently rocking the flask back and forth for approximately 30 s. Remove and discard the *Trypsin–EDTA solution*. Add an additional 5 mL of *Trypsin–EDTA solution*, and gently rock back and forth for 1 min. Remove and discard all but 1 mL. Incubate at 36 ± 1°, 5% carbon dioxide (CO_2), in a humidified environment for approximately 10 min or until the cell sheet begins to slough from the flask, and tap the flask to release the cells. Add 10 mL of *DMEM-5 solution* to the trypsinized cells, count the cells, and adjust the cell suspension to 1×10^5 cells/mL in the same medium.

Standard solutions: In sterile tubes, prepare four concentrations of the standard by diluting U.S. Standard Diphtheria Antitoxin[5] with *DMEM-2 solution* to 1 unit/mL, followed by serial dilutions in the same media that result in the additional concentrations of 0.5, 0.25, and 0.1 units/mL.

Sample solutions: In sterile tubes, dilute each human immune globulin test sample to 1.25% protein with *DMEM-2 solution*. Using the diluted sample, further dilute the samples in *DMEM-2 solution* to achieve an expected diphtheria antitoxin concentration that is within the range of the *Standard solutions* (i.e., 1 to 0.1 units/mL).

Analysis

Samples: *Toxin test solution*, *Standard solutions*, and *Sample solutions*

Test each *Standard solution* or *Sample solution* in duplicate. Label 96 well culture plates by marking off the 8-well columns in groups of two; all four *Standard solutions* and one *Sample solution* can be tested per plate. As described below, columns 11 and 12 can be used for the untreated cell control and the toxin control, respectively. [NOTE—Alternative plate layouts maintaining the dilutions can be used.] Add 112.5 µL of *DMEM-2 solution* to each well in row A, columns 1–10 only (these are standard- or sample-nonspecific toxicity controls). Add 75 µL of *DMEM-2 solution* to each well in rows B–H, columns 1–10 only.

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[1] 11885-092 Life Technologies, or equivalent.
[2] 14190-250 Life Technologies, or equivalent.
[3] Diphtheria toxin code 12/282 from NIBSC or suitable alternative.
[4] American Type Culture Collection CCL-81.
[5] Use appropriate U.S. Standard Diphtheria Antitoxin as designated by the Center for Biologics Evaluation and Research, Food and Drug Administration.

Add 37.5 µL of a *Standard solution* dilution or a *Sample solution* to wells A1–A2, and add 75 µL of the same solution to wells B1–B2. Add 37.5 µL of a second *Standard solution* dilution or *Sample solution* to wells A3–A4, and add 75 µL of the same solution to wells B3–B4. Continue to make similar additions of *Standard solution* dilutions or *Sample solutions* to each group of two columns in columns 1–10. Make two-fold serial dilutions by transferring 75 µL from each well in row B to each well in row C of the same column. Mix and then continue transferring 75 µL in the same manner down each column. Discard the last 75 µL after mixing row H. Add 75 µL of the *Toxin test solution* to rows B–H in columns 1–10. Add 150 µL of *DMEM-2 solution* to all wells in column 11 (cell control), and add 75 µL of *DMEM-2 solution* plus 75 µL of *Toxin test solution* to all wells in column 12 (toxin control).

Cover the plates, and incubate them for 1 h at 36 ± 1°, at 5% carbon dioxide (CO_2), and in a humidified environment. Add 150 µL of cell suspension to all wells, cover the plates, and incubate them at 36 ± 1°, at 5% carbon dioxide (CO_2), and in a humidified environment. Incubate for 4–5 days, checking periodically for microbial contamination by microscopic examination. Once toxicity control wells in row A containing the lowest concentration of a *Standard solution* are nearly confluent, add 15 µL of *MTT solution* to each well. Cover the plates, and incubate them in the carbon dioxide (CO_2) incubator at 37° for 3 h. Discard the medium, and add 150 µL of *Extraction solution* to each well. Cover the plates, and place aluminum foil over them to minimize light exposure. Shake gently to solubilize the blue formazan formed by viable cells. Using a suitable plate reader, read the absorbance of each well at a wavelength of 540 nm.

Calculation: The cutoff dilution is the highest dilution in which there are still viable cells in the well but beyond which there are no viable cells. Cutoff dilution is defined by a corresponding absorbance (Abs). [NOTE—The average absorbance value can be calculated for the cell control wells in column 11 and divided by 2 to obtain a 50% control Abs value. This value can be used to determine cutoff dilutions. The cutoff dilution for each *Standard solution* and *Sample solution* can therefore be defined as the highest dilution where the Abs value is greater than the 50% control Abs value.] Determine the cutoff dilutions for each *Standard solution* and *Sample solution*. Plot the cutoff dilutions obtained for all *Standard solutions* (expressed as the reciprocal of the cutoff dilution) versus U.S. Standard Diphtheria Antitoxin units. Calculate a linear regression line from the data. Calculate the geometric mean for the four cutoff dilutions associated with each *Sample solution*. Compare the mean cutoff dilution to the linear regression of the *Standard solution* data to determine a titer, in units/mL, for each *Sample solution*. To obtain the final potency value for the *Sample solution*, multiply the titer by any dilution that was made to the *Sample solution* prior to the *Assay*.

System suitability: The correlation coefficient of the standard curve must be >0.995. The slope of the standard curve must be between 26 and 38. The test is valid if the cells in wells of row A, columns 1–10, appear normal and similar to the cell control wells in column 11. In addition, the cutoff dilution values for *Standard solution* and *Sample solution* replicates should be within one serial dilution of each other, and the cutoff dilution must be within the range of the *Sample solution* serial dilutions.