1.0 PURPOSE

To describe a method for determining the infectious titer of adenoviral particles on HEK 293 cells whereby the diffusion of adenovirus particles is taken into account.

2.0 SCOPE

The method described uses the cytopathic effect (CPE) that adenoviruses have on cells as the readout to detect infection in HEK 293 cells 10 days post-infection with an adenovirus sample. The infectious titer of the adenovirus sample is related to the inverse of the sample dilution where virus is detected in the assay wells at a given sample dilution after correcting for the diffusion of the adenovirus particle.

A convenient method for estimating adenoviral infectious titer is an end-point assay set up in a 96-well tissue culture plate. The cells are plated into each well such that they reach approximately 50% confluence after one day of growth. The sample is then diluted so that the final particle concentration falls between 5 and 10^3 particles/mL. Different dilutions are prepared and the cells are infected after first removing the medium from the wells. Each different dilution of virus is placed into at least 8 wells per dilution. The infection time is limited to 60 minutes and then the virus solution is replaced with medium. Cells are incubated for varying times depending on the method of detection. One method of detection is visual inspection for cytopathic effect. This may require one to two weeks of incubation. The dilutions that produce fewer than 100% positive wells are used in the titer calculation per the Spearman-Karber analysis method (which is based on Finney's work). This method converts data such that graphing the data as log dilution verses positive wells approaches a straight line. Spearman-Karber performed an interpolation to a midpoint, providing a log dilution where 50% of the wells would have been positive. Titer is expressed as the negative log of the dilution. The Lynn program transforms this number by taking the reciprocal of the dilution (ten raised to the power of the Spearman-Karber number) and divides by the inoculum volume to get the infectious titer. In the method described the endpoint dilution calculation is made.

Results for quantal assays (plus/minus assays) follow a binomial distribution with parameter p*, where

p* = (number of negative results)/total number of results.

Therefore an alternative method for calculating virus titers is based on Poisson distribution. The concentration (C) of an entity in a test material can be calculated from p* using the equation

$$\mathbf{p^* = e^{-C}}$$

Because adenovirus particles diffuse slowly in solutions, the number of viral particles that may come into contact with the cells during an assay is less than the number present

in the solution. A model derived from Fick's Laws of Diffusion demonstrated that most adenoviral particles are infectious. That work along with the work of others showed the importance of diffusion constraints for virus binding. This limitation can be accounted for by the use of diffusion-normalized analysis. This analysis takes into account the diffusion of the adenovirus particle under the conditions of the assay by deriving normalization equations from Fick's Laws of Diffusion. For a titer plate assay the equation is given by:

$$V = -([\ln(1 - (p_w / n))] * D) / [A_w * C_w * I * \sqrt{t}]$$

where p_w is the number of positive wells per dilution, n is the total number of wells per dilution, D is the dilution factor, A_w is the area of the bottom of the well in cm^2, C_w is the confluence of the well at the time of infection, I is a constant incorporating the diffusion coefficient and is equal to 2.38×10^{-4} cm per particles $sec^{1/2}$, and t is the exposure time in seconds. Time is a critical parameter for this method. From the equation, one can see that p_w must be less than n and greater than zero. Optimally the number of positive wells should be between 20 and 80% of the total wells for a given dilution. This method yields infectious titers that are up to fifty per cent of the particle concentration.

3.0 REFERENCES

3.1 D.J. Finney, "Probit Analysis." (1962) Cambridge University Press, Cambridge, U.K.

3.2 D.E. Lynn, "A BASIC computer program for analyzing endpoint assay," (1992) *Biotechniques 12:* 880-881.

3.3 C. Nyberg-Hoffman, P. Shabram, W. Li, D. Giroux, and E. Aguilar-Cordova, "Sensitivity and reproducibility in adenoviral infectious titer determination," (1997) *Nature Medicine 3:* 808-811.

3.4 S. Andreadis, T. Lavery, H.E. Davis, J.M. Le Doux, M.L. Yarmush, and J.R. Morgan, "Toward a more accurate quantitation of the activity of recombinant retroviruses: alternatives to titer and multiplicity of infection," [corrected and re-published article originally printed in *J. Virol*ogy *74:* 1258-1266] (2000) *J. Virology 74:* 3431-3439.

3.5 ARMWG CPE Scoring Worksheet

3.6 ARMWG Infectious Titer Data Worksheet

4.0 MATERIALS

NOTE: Use aseptic technique for all steps involving handling of material nos. 4.1 and 4.2.

NOTE: Adenovirus 5 WT Reference Material samples and 293 HEK cells should be handled as biohazardous using BL-2 procedures. Wear gloves, safety glasses, and laboratory coat. Properly dispose of waste, including wash solutions from viral culture.

4.1 HEK 293 cells, provided by the Adenovirus Reference Material Working Group

4.1.1 293 cells, a human transformed primary embryonal kidney cell line (see ATCC CRL 1573), provided as a 1.0 mL frozen vial containing approximately 1 x 10^7 cells/mL. An accompanying certificate of analysis/characterization summary will indicate actual cell concentration, passage number, % viability upon thaw, results of sterility testing and testing for the presence of mycoplasma. The cell vials are part of a HEK 293 Testing Cell Bank that was derived from the CGMP Working Cell Bank used for production of the reference material.

4.1.2 Freezing medium:

4.1.2.1 90% Fetal bovine serum and 10% DMSO

4.1.2.2 Sources of FBS include Invitrogen cat. no. 26140-079 (US origin) or 10099-141 (Australian origin).

4.1.3 Instructions for vial thaw, propagation and expansion, and cell counting are included in the Procedure Section. For the assay, use cells that are 60 to 85% confluent prior to seeding the 96-well plate.

4.2 Ad5 WT Reference Material, prepared as:

4.2.1 Thaw frozen sample at room temperature in a biosafety cabinet. It should take less than 15 minutes to equilibrate the sample to room temperature. Mix thoroughly during and/or after the thaw, preferably, by trituration using a sterile pipette. (Triturate at least 10 times.)

4.2.1.1 If the test article is not sampled after 30 minutes it should be stored on wet ice or at 2-8°C until use. Just prior to use, the sample should be warmed to room temperature. Do *not* use a hot water bath to warm the sample.

4.3 Tubes, centrifuge, 15 mL, sterile

4.4 Tubes, centrifuge, 50 mL, sterile

4.5 Tubes, centrifuge, 250 mL, sterile (Fisher, cat. no. 05-538-53 or equivalent)

4.6 Tube, centrifuge, 500 mL, sterile (Fisher, cat. no. 07-200-621 or equivalent)

4.7 Pipettes (serological) to deliver 1, 5, 10, and 25 mL volumes, sterile

4.8 Aspiration device for aseptically removing medium from cells

4.9 Tissue culture flasks, 25 cm^2, sterile

4.10 Tissue culture flasks, 75 cm^2, sterile

4.11 Tissue culture flasks, T-225 cm^2, sterile

4.12 70% isopropanol

4.13 Dulbecco's Phosphate-buffered saline ("D-PBS"), sterile, calcium and magnesium-free [1X] (Invitrogen cat. no. 14190, or Irvine Scientific cat. no. 9240, or equivalent)

4.14 293 Cell Culture Media (one for culture of 293 cells and one for the assay)

4.14.1 <u>293 Cell Culture Media for normal cell culture activities (cell expansion):</u> Dulbecco's modified Eagle's medium (DMEM High glucose) containing 4500 mg/L D-glucose, 2 mM L-glutamine, and 1 mM sodium pyruvate, supplemented with 10% defined, bovine calf serum.

4.14.2 <u>293 Cell Culture Media for assay</u>: Dulbecco's modified Eagle's medium (DMEM Low glucose) containing 1000 mg/L D-glucose, 1 mM sodium pyruvate, and 2 mM-glutamine, supplemented with 10% defined, bovine calf serum.

4.14.3 Sources for media components are:

4.14.3.1 DMEM, High Glucose: DMEM, 4500 mg/L glucose, with L-glutamine and without sodium pyruvate, Invitrogen cat. no. 11995, or Irvine Scientific, cat. no. 9031, or equivalent

4.14.3.2 DMEM, Low Glucose: DMEM, with 1.0 g/L glucose, without L-glutamine, with sodium pyruvate, BioWhittaker cat. no. 12-707F, or Invitrogen cat. no. 10316 or equivalent, *OR* DMEM with 1.0 g/L glucose, with L-glutamine, with sodium pyruvate, Invitrogen cat. no. 11885, or JRH Biosciences cat. no. 56-467-0000 [requires sodium bicarbonate], or equivalent

4.14.3.3 Sodium pyruvate, 100 mM solution, Invitrogen, cat. no. 11360-070, BioWhittaker cat. no. 13-115E, or JRH Biosciences cat. no. 59-20377, or equivalent

4.14.3.4 Glutamine, 200 mM (100X), Invitrogen cat. no. 25030, JRH Biosciences cat. no. 59-202-100M, or equivalent

4.14.3.5 Bovine calf serum, defined, Invitrogen cat. no. 10371-029, or Hyclone Laboratories cat. no. A-2151-L, or equivalent

4.15 "Adenovirus Dilution Media": Dulbecco's modified Eagle's Medium (DMEM Low glucose) containing 1000 mg/L D-glucose, 2 mM L-glutamine, and 1 mM sodium pyruvate [without serum]

4.16 Trypsin, 0.05%(w/v), 0.53 mM EDTA (Invitrogen cat. no. 25300-054, or equivalent), or Trypsin-EDTA (0.25% Trypsin), without calcium and magnesium chloride (Invitrogen cat. no. 25200-049, or equivalent)

4.17 Trypan Blue Solution, 0.4% (w/v) (Sigma cat. No. T-8154, or equivalent)

4.18 Microcentrifuge tubes or small capped tubes, non-sterile, for cell counting procedure

4.19 Sterile sample or conical tubes with caps (2 to 15 mL) for preparing dilutions of the Ad5 WT Reference Material

4.20 Sterile microliter pipette tips, with aerosol filters for 1-200 µL and 200-1000 µL volumes

4.21 96-well tissue culture plates, flat bottom, sterile (CoStar cat. no. 3598, or equivalent)

4.22 Media reservoirs, sterile

4.23 Chlorine Bleach, 5% (v/v), prepared as:

4.23.1 5 mL Chlorine Bleach (standard household chlorine bleach)

4.23.2 Q.S. to 100 mL with ultra-pure water (Milli-Q or distilled water)

4.24 Biohazard bags and containers for liquid waste, solid waste, and pipette tips

4.25 Kimwipes or tissues

5.0 EQUIPMENT

5.1 Biosafety cabinet (laminar flow hood), suitable for BL-2 containment, Class II, Type A/B3

5.2 Waterbath set to 37°C

5.3 Centrifuge, low speed, and rotor

5.4 Incubator, humidified air, 5 to 7% CO_2, 37°C ± 1°C

5.5 Pipette aid

5.6 Inverted light Microscope fitted with a 10X brightfield objective and 10X eyepieces

5.7 Hemacytometer (Fisher, cat. no. 0267110 or equivalent)

5.8 Hemacytometer cover slip (Fisher cat. no. 02-671-35 or equivalent)

5.9 Cell counter, manual (Fisher cat. no. 02-670-11, or equivalent)

5.10 Adjustable microliter pipettes: 1-20 µL, 20-200 µL, 200-1000 µL

5.11 Adjustable microliter multi-channel pipette (8 channels): 20-200 µL

5.12 Biohazard waste container

6.0 PROCEDURE

6.1 293 Cell Culture After Initial Thaw, Expansion, Trypsinization, Passaging, and Cell Counting

NOTE: HEK 293 cells should be handled as biohazardous, using BL-2 procedures. Wear gloves, safety glasses, and laboratory coat. Properly dispose of waste, including solutions.

6.1.1 Initial Vial Thaw:

6.1.1.1 Warm 293 Cell Culture Media (DMEM High glucose, supplemented as described) to 37°C prior to starting. Wipe the media bottle with 70% isopropanol and place in the biological safety cabinet. Pipet 10 mL of 293 Cell Culture Media into a 15 mL sterile centrifuge tube.

6.1.1.2 Quickly thaw a vial of the HEK 293 cells in a 37°C waterbath just until no ice is visible. Immediately wipe the vial with 70% isopropanol and place in the biological safety cabinet.

6.1.1.3 Using a sterile 2 mL pipette, transfer the thawed cells into the 15 mL sterile centrifuge tube containing the 293 Cell Culture Medium. Centrifuge for 5 minutes, approximately 230 *x g* (1000 RPM) at room temperature. Aspirate medium from the cell pellet.

6.1.1.4 Decant the supernatant from the pelleted cells and discard.

6.1.1.5 Resuspend the cell pellet in 10 mL of fresh 293 Cell Culture Medium and place the cell suspension in a 75 cm^2 tissue culture flask, labeled with the cell line name, passage number, culture medium, and date.

6.1.1.6 Incubate the cells in a CO_2 incubator at 37°C. Cells should become 80% confluent in 24 to 48 hours.

6.1.1.7 Expand the HEK 293cell culture by passaging the cells into T-225 cm^2 flasks when the 75 cm^2 flask is approximately 80% confluent. Typically 293 cells grown in T-225 cm^2 flasks contain $2 – 4 \times 10^7$ cells per flask at 80% confluency. See sections 3 and 4.

NOTE: HEK 293 cells grow very slowly when seeded at lower densities. However once cultures become 50% confluent, 293 cells can grow very quickly. This is normal. If the 293 cells are maintained at 50-80% confluency prior to passage,

293 cells remain adherent. Do NOT allow the cells to become fully confluent. Do NOT over-trypsinize the cells during passaging.

6.1.2 Feeding cells:

6.1.2.1 Remove a flask of cells from the incubator and observe the cells under the microscope. Wipe the flask with 70% isopropanol and place the flask in the biological safety cabinet.

6.1.2.2 Decant the spent culture media from the culture vessel into a sterile 250 mL or 500 mL centrifuge tube, without disturbing the cells growing in the vessel.

6.1.2.3 Add the following volumes of 293 Cell Culture Media to the flasks, being careful not to disturb the growing monolayer of cells:

Vessel size:	25 cm^2	75 cm^2	225 cm^2
mL of media added per vessel:	5	10	50

6.1.2.4 Label the flasks with "re-fed" and the date, and return the flasks to the incubator.

6.1.2.5 Feed 293 cells every other day.

6.1.3 Trypsinization of HEK 293 cells for passaging or counting:

6.1.3.1 Remove a flask of cells from the incubator and inspect the cells using an inverted microscope. Check the confluency and the general condition of the cells. Record confluency and any observations.

6.1.3.2 Before beginning, warm 293 Cell Culture Media, D-PBS and the trypsin-EDTA solution to 37˚C in a waterbath. Wipe bottles and tissue culture flasks with 70% isopropanol before placing in the biological safety cabinet.

6.1.3.3 Decant the spent medium from the flask, into a sterile 250 mL or 500 mL centrifuge tube. Add D-PBS without calcium and magnesium salts as follows:

Flask size (cm^2)	25	75	225
Volume (mL)	5 mL	10 mL	10 mL

6.1.3.4 After rocking flask gently, decant the D-PBS wash from the flask.

6.1.3.5 Add the disassociation solution, trypsin-EDTA, using the following scheme:

Flask size (cm^2)	25	75	225
Volume (mL)	2 mL	3 mL	5 mL

6.1.3.6 Gently rock the flask and decant the solution from the flask.

6.1.3.7 Place the flask on the biological safety cabinet workspace for up to 5 minutes. If the cells are especially adherent, add 2 mL fresh dissociation solution, and place the flask into a 37°C incubator for an additional 5 minutes. **Do NOT over-trypsinize the 293 cells.**

6.1.3.8 Examine the cells under the microscope. When they detach from the plastic substratum, they will be rounded up.

6.1.3.9 Gently tap the flask on the side to loosen the cells.

6.1.3.10 Add 10 mL of 293 Cell Culture Media per flask and gently resuspend the cells by triturating with a 25 mL pipette. Remove a small sample for counting cell number.

6.1.4 Passaging Cells

6.1.4.1 Calculate the concentration of viable cells per section 5 and document all calculations and dilutions required for seeding new flasks.

6.1.4.2 Transfer the cell suspension to a sterile 50 mL centrifuge tube and pellet the cells at by centrifuging at ~ 230 x *g* (1000 RPM) for 5 minutes, at room temperature.

6.1.4.3 Decant the supernatant and resuspend the cells in an appropriate volume of fresh medium. Add the following volumes of 293 Cell Culture Media (DMEM High glucose, supplemented as described) to flasks:

Vessel size:	25 cm^2	75 cm^2	225 cm^2
mL of warmed media to add per vessel:	5	10	50

6.1.4.4 Prepare dilutions, if necessary, and seed flasks.

6.1.4.5 Label each flask with the following information:

6.1.4.5.1 Cell Line Name

6.1.4.5.2 Date

6.1.4.5.3 Passage Number (add "1" to the previous passage number)

6.1.4.5.4 Number of cells seeded per flask

6.1.4.6 Place the flasks back in a humidified 37°C incubator.

6.1.5 Counting Cells:

6.1.5.1 Using a sterile 5 mL pipette, vigorously triturate the solution to assure that the cells are suspended. Sterilely remove a small volume using a 1 mL pipette and place it in a non-sterile tube. Remove 100 µL using a micropipette from this small volume and place it in a non-sterile microcentrifuge tube or a small, capped tube.

6.1.5.2 Transfer 60 µL of D-PBS and 40 µL of the 0.04% Trypan blue solution into the tube with the 100 µL of cells. Mix well. Record the dilution factor (2x).

6.1.5.3 Do not let the cells sit in the Trypan blue solution for more than 15 minutes because eventually both viable cells and non-viable will take up the dye.

6.1.5.4 Using a 2-20 µL pipette and a new tip, remove 10 µL of the Trypan blue/cell suspension mixture. With a hemacytometer cover slip in place on the hemacytometer, place the end of the pipette tip on the groove underneath and allow the chamber to fill by capillary action. Do not overfill or underfill the chamber.

6.1.5.5 Place the filled hemacytometer on the microscope stage. Count the viable cells located in the first large corner square, using a cell counter. Include cells on top and left touching the middle line of the perimeter. Do not count cells touching the middle line at the bottom and right sides. Viable cells are distinguished from dead cells by their lack of blue staining (viable cells exclude Trypan blue). If the number is between 50 and 150, continue counting the remaining three large corner squares.

6.1.5.6 If the count is greater than 150 cells, the cell suspension should be diluted so that the cell count will be within the range specified in 5.e. above (50 to 150). If the count is less than 50, the cells should be pelleted by gentle centrifugation (300 *x g*), and resuspended in a smaller volume so that the cell count will be within the range specified. In either case, repeat the count.

6.1.5.7 Record the number of cells counted in each square.

6.1.5.8 Calculate the mean cell count, the standard deviation and % CV. If the % CV is greater than 20%, repeat steps 5.a. through h.

6.1.5.9 Record the cell concentration, the total volume of the cell suspension, and the total number of cells.

Cell concentration:

Cells / mL = (the mean cell count per square) x (the dilution factor) x (10^4)

Total cell number:

Total cells = (number of cells / mL) x (the original volume from which the cell sample was removed)

6.1.5.10 When the procedure is complete, clean the hemacytometer cover slip and hemacytometer with 70% (v/v) isopropanol and a Kimwipe or tissue. Air dry completely before using again.

6.2 Infectious Titer Assay Method:

NOTE: Perform the assay two times. These can be scheduled so that both assays are initiated on the same day or are set up on different days. It is recommended that the duplicate assays be initiated on separate days to reduce variability. Dilutions and assay plates should be labeled to distinguish the assay set (e.g., A or B). CPE will be read and recorded on day 10.

6.2.1 *"Day –1"*: Detach 293 cells from flask with trypsin-EDTA as described above and prepare for plating into 96-well culture plates per steps 3 and 5 under "293 Cell Culture After Initial Thaw, Expansion, Trypsinization, Passaging, and Cell Counting". However, resuspend cells into the DMEM, Low glucose (supplemented as described) media.

NOTE: Passage the 293 cells a minimum of two times after thaw. Do not use 293 cells that became 100% confluent during culture.

6.2.1.1 Calculate the total number of cells needed for the assay using a concentration of 4×10^5 cells per mL and 100 µL per well (40,000 cells per well). Eleven mL of cell suspension at 4×10^5 cells per mL will be required per plate, for a total of 4.4×10^6 cells per plate. The total number of plates required is four if the duplicate assay sets are being set up on the same day (two plates per independent dilution series). Typically 293 cells can be provided in T-225 cm^2 flasks containing 2 to 4×10^7 cells so that a maximum of two (2) T-225 cm^2 flasks should be required for the duplicate assays.

6.2.1.2 Prepare the appropriate volume of cell suspension, diluting with 293 Cell Culture Media (DMEM, Low glucose, supplemented as described).

6.2.1.3 Using a multi-channel pipette and a sterile media reservoir, pipette 100 µL of the cell suspension into each well of each 96-well tissue culture plate. Cover ("Day –1").

6.2.1.4 Place the plate(s) in the 37°C, CO_2 incubator for 18 to 22 hours until ready to apply the assay samples.

6.2.2 "*Day 0":* Sample preparation:

6.2.2.1 Prepare a series of dilutions of the Ad5 WT Reference Material in Adenovirus Dilution Media for each assay set, A and B. If the two assay sets are to be performed on different days, then make only one set per day. If the two assay sets are to be performed on the

same day it is important that **separate** Ad5 starting dilutions be made to begin each dilution series. Make one series at a time. Note that the dilution series starts with a 1:2 dilution, followed by a series of greater dilutions to get closer to the appropriate range for the assay. Finally there are a series of dilutions based on the square root of two, which cover the dilution range over which data will be used for calculating the infectious titer. Do not use serological pipettes to make the dilutions. Use only microliter pipettes. Limiting the analyst to one dilution series per day reduces assay variability.

6.2.2.2 Begin by marking sample tubes as follows:

6.2.2.2.1 "1:2 Ad5 A" or "1:2 Ad5 B"

6.2.2.2.2 "1:50 Ad5 A" or "1:50 Ad5 B"

6.2.2.2.3 "1:250 Ad5 A" or "1:250 Ad5 B"

6.2.2.2.4 "1:2500 Ad5 A" or "1:2500 Ad5 B"

6.2.2.2.5 "$1:2.50 \times 10^5$ Ad5 A" or "$1:2.50 \times 10^5$ Ad5 B"

6.2.2.2.6 "$1:2.50 \times 10^7$ Ad5 A" or "$1:2.50 \times 10^7$ Ad5 B"

6.2.2.2.7 "$1:5.00 \times 10^7$ Ad5 A" or "$1:5.00 \times 10^7$ Ad5 B"

6.2.2.2.8 "$1:1.00 \times 10^8$ Ad5 A" or "$1:1.00 \times 10^8$ Ad5 B"

6.2.2.2.9 "$1:2.00 \times 10^8$ Ad5 A" or "$1:2.00 \times 10^8$ Ad5 B"

6.2.2.2.10 "$1:2.83 \times 10^8$ Ad5 A" or "$1:2.83 \times 10^8$ Ad5 B"

6.2.2.2.11 "$1:4.00 \times 10^8$ Ad5 A" or "$1:4.00 \times 10^8$ Ad5 B"

6.2.2.2.12 "$1:5.66 \times 10^8$ Ad5 A" or "$1:5.66 \times 10^8$ Ad5 B"

6.2.2.2.13 "$1:8.00 \times 10^8$ Ad5 A" or "$1:8.00 \times 10^8$ Ad5 B"

6.2.2.2.14 "$1:1.13 \times 10^9$ Ad5 A" or "$1:1.13 \times 10^9$ Ad5 B"

6.2.2.2.15 "$1:1.60 \times 10^9$ Ad5 A" or "$1:1.60 \times 10^9$ Ad5 B"

6.2.2.2.16 "$1:2.26 \times 10^9$ Ad5 A" or "$1:2.26 \times 10^9$ Ad5 B"

6.2.2.2.17 "$1:3.20 \times 10^9$ Ad5 A" or "$1:3.20 \times 10^9$ Ad5 B"

6.2.2.2.18 "1:4.53 x 10^9 Ad5 A" or "1:4.53 x 10^9 Ad5 B"

6.2.2.2.19 "1:6.40 x 10^9 Ad5 A" or "1:6.40 x 10^9 Ad5 B"

6.2.2.2.20 "1:1.28 x 10^{10} Ad5 A" or "1:1.28 x 10^{10} Ad5 B"

6.2.2.3 Thoroughly triturate (pipetting the volume of the tube up and down at least 10 times using a 0.5 or 1 mL pipette) the thawed Ad5 WT Reference Material prior to pipetting (see Materials section). Add 200 µL thawed Ad5 WT Reference Material into each of the "1:2 Ad5 A" and the "1:2 Ad5 B" tubes.

NOTE: It is especially critical to place the adenovirus sample solution into the tube and then to add the Adenovirus Dilution Media to the adenovirus, rather than the other way around. Use only microliter pipettes, do NOT use serological pipettes.

6.2.2.4 Add 200 µL Adenovirus Dilution Media into each "1:2 Ad5"tube and thoroughly mix the sample by trituration. Triturate at least 10 times.

6.2.2.5 Prepare a series of dilutions for each assay (A and B) in individual sterile tubes. Use the Adenovirus Dilution Media and microliter pipettes to make the dilutions. Always place the adenovirus solution to be diluted into the tube first, adding the Adenovirus Dilution Media second. Mix each dilution gently but thoroughly by triturating 10 times prior to making the next greater dilution. Change pipette tips between dilutions. Prepare a series of dilutions using the "1:2 Ad5" dilution to create the "1:50 Ad5" sample dilution per the following scheme:

To Create Sample Dilution	*Previous Dilution Name*	*Volume of Previous Dilution (µL)*	*Volume of Adenovirus Dilution Media (µL)*	*Total Volume*
"1:2 Ad5"	Undiluted Ad5 WT Reference Material	200 µL	200 µL	400 µL
"1:50 Ad5"	"1:2 Ad5"	100 µL	2400 µL	2500 µL
"1:250 Ad5"	"1:50 Ad5"	200 µL	800 µL	1000 µL
"1:2500 Ad5"	"1:250 Ad5"	100 µL	900 µL	1000 µL
"1:2.5 x 10^5 Ad5"	"1:2500 Ad5"	100 µL	9900 µL	10000 µL
"1:2.5 x 10^7 Ad5"	"1:2.5 x 10^5 Ad5"	120 µL	11880 µL	12000 µL
"1:5.00 x 10^7 Ad5"	"1:2.5 x 10^7 Ad5"	6000 µL	6000 µL	12000 µL
"1:1.00 x 10^8 Ad5"	"1:5.00 x 10^7 Ad5"	6000 µL	6000 µL	12000 µL
"1:2.00 x 10^8 Ad5"	"1:1.00 x 10^8 Ad5"	6000 µL	6000 µL	12000 µL
"1:2.83 x 10^8 Ad5"	"1:2.00 x 10^8 Ad5"	8485 µL	3515 µL	12000 µL
"1:4.00 x 10^8 Ad5"	"1:2.83 x 10^8 Ad5"	8485 µL	3515 µL	12000 µL
"1:5.66 x 10^8 Ad5"	"1:4.00 x 10^8 Ad5"	8485 µL	3515 µL	12000 µL
"1:8.00 x 10^8 Ad5"	"1:5.66 x 10^8 Ad5"	8485 µL	3515 µL	12000 µL
"1:1.13 x 10^9 Ad5"	"1:8.00 x 10^8 Ad5"	8485 µL	3515 µL	12000 µL
"1:1.60 x 10^9 Ad5"	"1:1.13 x 10^9 Ad5"	8485 µL	3515 µL	12000 µL
"1:2.26 x 10^9 Ad5"	"1:1.60 x 10^9 Ad5"	8485 µL	3515 µL	12000 µL
"1:3.20 x 10^9 Ad5"	"1:2.26 x 10^9 Ad5"	8485 µL	3515 µL	12000 µL
"1:4.53 x 10^9 Ad5"	"1:3.20 x 10^9 Ad5"	8485 µL	3515 µL	12000 µL
"1:6.40 x 10^9 Ad5"	"1:4.53 x 10^9 Ad5"	8485 µL	3515 µL	12000 µL
"1:1.28 x 10^{10} Ad5"	"1:6.40 x 10^9 Ad5"	6000 µL	6000 µL	12000 µL

6.2.2.6 Be careful not to confuse the A and B dilution series.

6.2.3 Inoculation with Adenovirus Samples:

6.2.3.1 After incubating the cells for 18 to 22 hours, check their condition. They should be approximately 80% confluent in the wells.

6.2.3.2 Make additions to the 96-well plates, one 96-well plate at a time. Make sure the plates are distinctively marked ("Assay A1", "Assay A2", "Assay B1", etc.).

6.2.3.3 Carefully remove the 293 Cell Culture Media from each well in the first plate. Change pipette tips.

6.2.3.4 Place 200 µL per well of 293 Cell Culture Media into each of the twelve (12) wells in row A. This is the No Virus Control. There will be a row of No Virus Control on every assay plate.

NOTE: Infection time is a critical parameter in the assay. Total infection time is 60 minutes. Time the start of infection and infect only one plate at a time.

6.2.3.5 Infect the cells in row B of the 96-well plate with inoculum consisting of 200 µL of the diluted Ad5 WT Reference Material. Place 200 µL into each of the 12 wells in row B. Begin with the Ad5 WT sample marked "1:1.28 x 10^{10} Ad5". Change pipette tips.

6.2.3.6 Infect the cells in the next row, row C, of the 96-well plate with 200 µL per well using the Ad5 WT sample marked "1:6.40 x 10^{9} Ad5". Change pipette tips.

6.2.3.7 Continue infecting rows D through H with 200 µL per well (12 wells per dilution) using the following samples in order: "1:4.53 x 10^{9} Ad5", "1:3.20 x 10^{9} Ad5", "1:2.26 x 10^{9} Ad5", "1:1.60 x 10^{9} Ad5", and "1:1.13 x 10^{9} Ad5".

6.2.3.8 Cover the first plate and incubate for 60 minutes from the beginning of the infection at 37°C in a humidified air CO_2 incubator. Note the time of infection. The incubation time is a critical factor and should be adhered to closely.

NOTE: Liquid removal after infection must be done VERY CAREFULLY in order not to disturb the 293 cells in the wells. Detachment of 293 cells is exaggerated in the 96-well plate format.

6.2.3.9 After 60 minutes, remove the media and samples from all wells. Change pipette tips between each row (or column or well).

6.2.3.10 Carefully add 200 µL per well of 293 Cell Culture Media into all wells of the assay plate, changing pipette tips between columns or rows or wells. Re-cover plate number 1 and return it to the 37°C humidified air CO_2 incubator.

6.2.3.11 Begin infection of second plate of the assay. Begin adding the No Virus Control to row A of the second plate of the assay as in steps 6.2.3.3 and 6.2.3.4 above.

6.2.3.12 Then infect the cells in row B of the second 96-well plate with inoculum consisting of 200 µL of the diluted Ad5 WT Reference Material. Place 200 µL into each of the 12 wells in row B. Begin with the Ad5 WT sample marked "1:8.00 x 10^8 Ad5". Change pipette tips.

6.2.3.13 Continue infecting rows C through H of cells with 200 µL per well (12 wells per dilution) using the following samples in order: "1:5.66 x 10^8 Ad5", "1:4.00 x 10^8 Ad5", "1:2.83 x 10^8 Ad5", "2.00 x 10^8 Ad5", "1.00 x 10^8 Ad5", and "5.00 x 10^7 Ad5". Change pipette tips between each dilution addition.

6.2.3.14 Add 200 µL per well of 293 Cell Culture Media into each of the remaining wells in the second plate.

6.2.3.15 Cover the second plate and incubate for <u>60 minutes</u> from the time of infection at 37°C in a humidified air CO_2 incubator. Note the time of infection.

6.2.3.16 The plate formats for the assay are:

Plate 1

	1	2	3	4	5	6	7	8	9	10	11	12
A	No Virus Control	No Virus Control	No Virus Control	No Virus Control	No Virus Control	No Virus Control	No Virus Control	No Virus Control	No Virus Control	No Virus Control	No Virus Control	No Virus Control
B	1:1.28 x 10^{10} Ad5	1:1.28 x 10^{10} Ad5	1:1.28 x 10^{10} Ad5	1:1.28 x 10^{10} Ad5	1:1.28 x 10^{10} Ad5	1:1.28 x 10^{10} Ad5	1:1.28 x 10^{10} Ad5	1:1.28 x 10^{10} Ad5	1:1.28 x 10^{10} Ad5	1:1.28 x 10^{10} Ad5	1:1.28 x 10^{10} Ad5	1:1.28 x 10^{10} Ad5
C	1:6.40 x 10^9 Ad5	1:6.40 x 10^9 Ad5	1:6.40 x 10^9 Ad5	1:6.40 x 10^9 Ad5	1:6.40 x 10^9 Ad5	1:6.40 x 10^9 Ad5	1:6.40 x 10^9 Ad5	1:6.40 x 10^9 Ad5	1:6.40 x 10^9 Ad5	1:6.40 x 10^9 Ad5	1:6.40 x 10^9 Ad5	1:6.40 x 10^9 Ad5
D	1:4.53 x 10^9 Ad5	1:4.53 x 10^9 Ad5	1:4.53 x 10^9 Ad5	1:4.53 x 10^9 Ad5	1:4.53 x 10^9 Ad5	1:4.53 x 10^9 Ad5	1:4.53 x 10^9 Ad5	1:4.53 x 10^9 Ad5	1:4.53 x 10^9 Ad5	1:4.53 x 10^9 Ad5	1:4.53 x 10^9 Ad5	1:4.53 x 10^9 Ad5
E	1:3.20 x 10^9 Ad5	1:3.20 x 10^9 Ad5	1:3.20 x 10^9 Ad5	1:3.20 x 10^9 Ad5	1:3.20 x 10^9 Ad5	1:3.20 x 10^9 Ad5	1:3.20 x 10^9 Ad5	1:3.20 x 10^9 Ad5	1:3.20 x 10^9 Ad5	1:3.20 x 10^9 Ad5	1:3.20 x 10^9 Ad5	1:3.20 x 10^9 Ad5
F	1:2.26 x 10^9 Ad5	1:2.26 x 10^9 Ad5	1:2.26 x 10^9 Ad5	1:2.26 x 10^9 Ad5	1:2.26 x 10^9 Ad5	1:2.26 x 10^9 Ad5	1:2.26 x 10^9 Ad5	1:2.26 x 10^9 Ad5	1:2.26 x 10^9 Ad5	1:2.26 x 10^9 Ad5	1:2.26 x 10^9 Ad5	1:2.26 x 10^9 Ad5
G	1:1.60 x 10^9 Ad5	1:1.60 x 10^9 Ad5	1:1.60 x 10^9 Ad5	1:1.60 x 10^9 Ad5	1:1.60 x 10^9 Ad5	1:1.60 x 10^9 Ad5	1:1.60 x 10^9 Ad5	1:1.60 x 10^9 Ad5	1:1.60 x 10^9 Ad5	1:1.60 x 10^9 Ad5	1:1.60 x 10^9 Ad5	1:1.60 x 10^9 Ad5
H	1:1.13 x 10^9 Ad5	1:1.13 x 10^9 Ad5	1:1.13 x 10^9 Ad5	1:1.13 x 10^9 Ad5	1:1.13 x 10^9 Ad5	1:1.13 x 10^9 Ad5	1:1.13 x 10^9 Ad5	1:1.13 x 10^9 Ad5	1:1.13 x 10^9 Ad5	1:1.13 x 10^9 Ad5	1:1.13 x 10^9 Ad5	1:1.13 x 10^9 Ad5

Plate 2

	1	2	3	4	5	6	7	8	9	10	11	12
A	No Virus Control	No Virus Control	No Virus Control	No Virus Control	No Virus Control	No Virus Control	No Virus Control	No Virus Control	No Virus Control	No Virus Control	No Virus Control	No Virus Control
B	1:8.00 x 10^8 Ad5	1:8.00 x 10^8 Ad5	1:8.00 x 10^8 Ad5	1:8.00 x 10^8 Ad5	1:8.00 x 10^8 Ad5	1:8.00 x 10^8 Ad5	1:8.00 x 10^8 Ad5	1:8.00 x 10^8 Ad5	1:8.00 x 10^8 Ad5	1:8.00 x 10^8 Ad5	1:8.00 x 10^8 Ad5	1:8.00 x 10^8 Ad5
C	1:5.66 x 10^8 Ad5	1:5.66 x 10^8 Ad5	1:5.66 x 10^8 Ad5	1:5.66 x 10^8 Ad5	1:5.66 x 10^8 Ad5	1:5.66 x 10^8 Ad5	1:5.66 x 10^8 Ad5	1:5.66 x 10^8 Ad5	1:5.66 x 10^8 Ad5	1:5.66 x 10^8 Ad5	1:5.66 x 10^8 Ad5	1:5.66 x 10^8 Ad5
D	1:4.00 x 10^8 Ad5	1:4.00 x 10^8 Ad5	1:4.00 x 10^8 Ad5	1:4.00 x 10^8 Ad5	1:4.00 x 10^8 Ad5	1:4.00 x 10^8 Ad5	1:4.00 x 10^8 Ad5	1:4.00 x 10^8 Ad5	1:4.00 x 10^8 Ad5	1:4.00 x 10^8 Ad5	1:4.00 x 10^8 Ad5	1:4.00 x 10^8 Ad5
E	1:2.83 x 10^8 Ad5	1:2.83 x 10^8 Ad5	1:2.83 x 10^8 Ad5	1:2.83 x 10^8 Ad5	1:2.83 x 10^8 Ad5	1:2.83 x 10^8 Ad5	1:2.83 x 10^8 Ad5	1:2.83 x 10^8 Ad5	1:2.83 x 10^8 Ad5	1:2.83 x 10^8 Ad5	1:2.83 x 10^8 Ad5	1:2.83 x 10^8 Ad5
F	1:2.00 x 10^8 Ad5	1:2.00 x 10^8 Ad5	1:2.00 x 10^8 Ad5	1:2.00 x 10^8 Ad5	1:2.00 x 10^8 Ad5	1:2.00 x 10^8 Ad5	1:2.00 x 10^8 Ad5	1:2.00 x 10^8 Ad5	1:2.00 x 10^8 Ad5	1:2.00 x 10^8 Ad5	1:2.00 x 10^8 Ad5	1:2.00 x 10^8 Ad5
G	1:1.00 x 10^8 Ad5	1:1.00 x 10^8 Ad5	1:1.00 x 10^8 Ad5	1:1.00 x 10^8 Ad5	1:1.00 x 10^8 Ad5	1:1.00 x 10^8 Ad5	1:1.00 x 10^8 Ad5	1:1.00 x 10^8 Ad5	1:1.00 x 10^8 Ad5	1:1.00 x 10^8 Ad5	1:1.00 x 10^8 Ad5	1:1.00 x 10^8 Ad5
H	1:5.00 x 10^7 Ad5	1:5.00 x 10^7 Ad5	1:5.00 x 10^7 Ad5	1:5.00 x 10^7 Ad5	1:5.00 x 10^7 Ad5	1:5.00 x 10^7 Ad5	1:5.00 x 10^7 Ad5	1:5.00 x 10^7 Ad5	1:5.00 x 10^7 Ad5	1:5.00 x 10^7 Ad5	1:5.00 x 10^7 Ad5	1:5.00 x 10^7 Ad5

NOTE: Liquid removal after infection must be done VERY CAREFULLY in order not to disturb the 293 cells in the wells. Detachment of 293 cells is exaggerated in the 96-well plate format.

6.2.3.17 For each plate, at 60 minutes after the infection time, carefully remove the media and samples from all wells. Change pipette tips between each column (or row or well).

6.2.3.18 Carefully add 200 µL per well of 293 Cell Culture Media into all wells of the assay plate, changing pipette tips between wells. Cover the plates and return to the .37°C humidified air CO_2 incubator.

6.2.3.19 Incubate the 96-well plates for 10 days in a humidified air CO_2 incubator at 37°C. Observations for contamination and general cell health are up to the discretion of the laboratory manager for days 1 through 10.

6.2.4 *"Day 10"*: Sample Analysis/Scoring CPE:

6.2.4.1 Examine each well on day 10 for signs of CPE using a light microscope. Compare to the No Virus Control row. If in doubt about CPE, have a supervisor confirm the observations. If a plate becomes contaminated, the assay must be repeated.

6.2.4.2 CPE should be evident by day 10.

6.2.4.3 Record the results for day 10, scoring wells "+" (CPE positive) or "-" (CPE negative) using the CPE scoring form, FM0200. For each row, calculate the number of positive wells. Record the number for each sample row.

6.2.4.4 Once the assay data has been recorded and accepted, the plates should be discarded as biohazardous waste according to the procedures of your institution.

6.2.5 Determining the Result:

6.2.5.1 Record the raw data on the CPE scoring form provided by the Ad5 WT Reference Material Working Group. Although CPE data may be available from multiple days, only the data from Day 10 will be used to calculate the infectious titer. On Day 10 [a] there should be at least one sample dilution where CPE is evident in all 12 wells, [b] at least one sample dilution where CPE is evident in at

least 3 but no more than 9 wells, and [c] at least one sample dilution where no CPE is evident in any of the 12 wells.

6.2.5.2 Fill out the results worksheet provided by the Ad5 WT Reference Material Working Group using data from the day 10 CPE readings, SW034, ARMWG Infectious Titer Data Worksheet.

6.2.5.3 Viral infectious titer can be determined using the following formula:

Infectious Titer per milliliter (IU/mL):

$$V = -([\ln(1 - (p_w / n))] * D) / [A_w * C_w * I * \sqrt{t}]$$

where p_w is the number of positive wells per dilution and must be less than 12 and greater than zero, n is the total number of wells per dilution (i.e., 12), D is the dilution factor, A_w is the area of the bottom of the well in cm^2 (i.e., 0.32 cm^2), C_w is the confluence of the well at the time of infection (80%), I is a constant incorporating the diffusion coefficient and is equal to 2.38×10^{-4} cm per particles $sec^{1/2}$, and t is the exposure time in seconds (3600 seconds). Optimally the number of positive wells should be between 20 and 80% of the total wells in a dilution (>2 and <10 positive wells).

6.2.5.4 Record all calculations using the ARMWG Infectious Titer Data Worksheet.

6.2.5.5 Report results:

6.2.5.5.1 Acceptability of the assay

6.2.5.5.1.1 No bacterial or other contamination occurred during the assay.

6.2.5.5.1.2 The No Virus Control wells did not show any signs of CPE.

6.2.5.5.1.3 There was at least sample dilution where CPE is evident in all 12 wells.

6.2.5.5.1.4 There was at least one sample dilution where CPE was evident in at least 3 but no more than 9 wells at the end of the assay.

6.2.5.5.1.5 There was at least one sample dilution where no CPE was evident in any of the 12 wells.

6.2.5.5.2 Report the infectious titer for each sample dilution in each Assay (A and B) in infectious units/mL to the nearest hundredth (*e.g.*, a.bc x 10^{d}).

6.2.5.5.3 Report the infectious titer for each Assay (A and B) in infectious units/mL to the nearest hundredth.

6.2.5.5.4 Report the average infectious titer for the Ad5 Reference Material in infectious units/mL to the nearest hundredth.