Bid Submission Form
Participation in Assignment of Infectious Titer
RFP 9.0

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Contact Information – RFP 9.0

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***If laboratories are submitting a proposal as a group, a main contact should be provided along with contact information for each participating laboratory (attach additional copies of this form).**

Please indicate if your institution is also submitting proposals for the other activities:

- √ Determination of Particle Concentration
- √ Short-term/Field Stability Studies
- ☐ Long-term Stability Study
- √ Other Characterization
- ☐ Donation of Supplies/Other Services for Characterization Phase

Capability Statement UT College of Pharmacy

Part of Dr. Croyle's research program at The University of Texas at Austin focuses on issues associated with large-scale production, characterization, formulation, delivery and long-term stability of adenoviral vectors for gene therapy under GLP conditions. Laboratory staff has experience with the procedure described in RFP 9.0.

Personnel Qualifications, Experience and Training

A) Personnel Involved in Performing the Procedure

Xuan Cheng M.D. Suzhou Medical College, People's Republic of China
M.S. Molecular Pharmacology, University of Maryland, Baltimore, MD USA

Xuan has 2 years experience in the characterization and production of adenoviral vectors.

Shellie Callahan BA Biology Point Loma Nazarene University, San Diego, CA

Shellie has 2 years experience in the characterization and production of adenoviral vectors.

B) Personnel Involved in Reviewing the Data

Maria Croyle, R.Ph., Ph.D. Ph.D. Pharmaceutics, University of Michigan

Maria has over 10 years experience working with adenoviral vectors for gene therapy. Her graduate work involved the development of adenoviral vectors for oral gene delivery. As a post-doctoral fellow at the University of Pennsylvania's Institute for Human Gene Therapy, she was responsible for initiation and design of stability studies for viral vectors used in clinical trials at the Institute and developed and produced lyophilized clinical grade viral vectors for shipment to external clinical sites.

Equipment

Biological Safety Cabinets
Laboratory is equipped with 2 NuAire Class II Type A/B3 cabinets
Cabinets are inspected yearly by a contract company through UT.

Incubators
Laboratory is equipped with 4 IncuSafe CO_2 incubators (Sanyo Scientific)
Temperature and CO_2 content are assessed manually and recorded weekly.

Waterbath Isotemp 105 (Fisher Scientific) Temperature assessed daily and recorded weekly.

Microscope Laboratory is equipped with a MicrosOptics 1500 IV 900 series inverted microscope with 10x, 20x and 40 x objectives and a 10x eyepiece.

Centrifuge Jouan CR3i refrigerated centrifuge Max speed: 14,000 rpm..

Pipettes Adjustable microliter pipettes (Ranin, Eppendorf) 0.5-10, 1-20, 20-200 and 200-1000 µl
Adjustable microliter pipette (Biohit) 20-200 µl
All are calibrated annually by a contract company through UT.

Timeline

Performance of the procedure outlined in RFP 9.0, data analysis, review and reporting will take place 6 weeks after receipt of material.

Adenovirus 5 WT Reference Material Standard Operating Procedure for Determination of Infectious Titer in 293 Cells in a 96-Well Format

Infectious Titer of Purified Adenovirus Preparations

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.

Participants will perform this assay two times, starting with different dilutions of the Ad5 WT Reference Material. The two assays may be performed on the same day as long as the starting dilution is made separately for each assay.

Scope:

The method described uses the cytopathic effect (CPE) that adenoviruses have on cells as the readout to detect infection in HEK 293 cells 7-10 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.

Background:

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. Several 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.

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.

Reference:

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

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

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.

S. Andreadis, T. Lavery, H.E. Davis, J.M. Le Doux, M.L. Yarmush, and J.R. Morgan, "Toward a 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.

Materials:

NOTE: Use aseptic technique for all steps involving handling of Material nos. 1 and 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.

1. HEK 293 cells, provided by the Working Group
    a. 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 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 Testing Cell Bank that was derived from the CGMP Cell Bank used for production of the reference material.
    b. Freezing medium:
    90% Fetal bovine serum and 10% DMSO
    c. Instructions for vial thaw, propagation and expansion, and cell counting are included in the Methods Section. For the assay, use cells that are 60-85% confluent prior to seeding the 96-well plate.
2. Ad5 WT Reference Material, prepared as:
    a. 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.)
        i. 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.
3. Tubes, centrifuge, 15 mL, sterile
4. Tubes, centrifuge, 50 mL, sterile
5. Tubes, centrifuge, 250 mL, sterile (Fisher, cat. no. 05-538-53)
6. Tube, centrifuge, 500 mL, sterile (Fisher, cat. no. 07-200-621)
7. Pipettes to deliver 1, 5, 10, and 25 mL volumes, sterile
8. Aspiration device for aseptically removing medium from cells
9. Tissue culture flasks, 25 cm^2, sterile
10. Tissue culture flasks, 75 cm^2, sterile
11. Tissue culture flasks, T-225 cm^2, sterile
12. 70% isopropanol
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)
14. 293 Cell Culture Media: Dulbecco's modified Eagle's medium (DMEM, High glucose) containing 4500 mg/L D-glucose, supplemented with 10% defined, bovine calf serum, 2 mM L-glutamine, and 1 mM sodium pyruvate. Sources for media components are:
    a. DMEM, High glucose with L-glutamine and without sodium pyruvate, Invitrogen cat. no. 11995, or Irvine Scientific cat. no. 9031 or equivalent,

b. Sodium pyruvate, BioWhittaker cat. no. 13-115E, or JRH Biosciences cat. no. 59202-77P, or equivalent
c. Bovine calf serum, defined (Hyclone Laboratories cat. no. A-2151-L, or equivalent)

15. "Adenovirus Dilution Media": Dulbecco's modified Eagle's Medium (DMEM High glucose) containing 4500 mg/L D-glucose, supplemented with 2 mM L-glutamine and 1 mM sodium pyruvate [without serum]
16. Trypsin, 0.05%(w/v), 0.53 mM EDTA (Invitrogen cat. no. 25300-054, or equivalent), or Trypsin-EDTA, without calcium and magnesium chloride (Invitrogen cat. no. 25200-049, or equivalent)
17. 0.4% (w/v) Trypan Blue Solution (Sigma cat. No. T-8154, or equivalent)
18. Microcentrifuge tubes or small capped tubes, non-sterile, for cell counting procedure
19. Sterile sample tubes with caps (minimum volume 2 mL) for preparing dilutions of the Ad5 WT Reference Material
20. Sterile microliter pipette tips, with aerosol filters for 1-200 µL and 200-1000 µL volumes
21. 96-well tissue culture plates, flat bottom, sterile (CoStar cat. no. 3598, or equivalent)
22. Media reservoirs, sterile
23. Chlorine Bleach, 5% (v/v), prepared as:
    a. 5 mL Chlorine Bleach (standard household chlorine bleach)
    b. Q.S. to 100 mL with ultra-pure water (Milli-Q or distilled water)
24. Biohazard bags and containers for liquid waste, solid waste, and pipette tips
25. Kimwipes or tissues

Equipment:

1. Biosafety cabinet (laminar flow hood), suitable for BL-2 containment, Class II, Type A/B3
2. Waterbath set to 37°C
3. Centrifuge, low speed, and rotor
4. Incubator, humidified air, 7 ± 1% CO_2, 37°C ± 1°C
5. Pipette aid
6. Inverted light Microscope fitted with a 10X brightfield objective and 10X eyepieces
7. Hemacytometer (Fisher, cat. no. 0267110 or equivalent)
8. Hemacytometer cover slip (Fisher cat. no. 02-671-35 or equivalent)
9. Cell counter, manual (Fisher cat. no. 02-670-11, or equivalent)
10. Adjustable microliter pipettes: 1-20 µL, 20-200 µL, 200-1000 µL
11. Adjustable microliter multi-channel pipette (8 channels): 20-200 µL

Methods:

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.

1. *Initial Vial Thaw:*
   a. Warm 293 Cell Culture Media 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.
   b. 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.
   c. 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.
   d. Decant the supernatant from the pelleted cells and discard.
   e. 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.
   f. Incubate the cells in a CO_2 incubator at 37°C. Cells should become 80% confluent in 24 to 48 hours.
   g. 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 x 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 will grow very quickly. This is normal. If the 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.

2. *Feeding cells:*
   C) 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.
   D) 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.

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E) 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

d. Label the flasks with "re-fed" and the date, and return the flasks to the incubator.
e. Feed 293 cells every other day.

3. *Trypsinization of HEK 293 cells for passaging or counting*:
   4. 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.
   5. 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. 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

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

G) 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

7. Gently rock the flask and decant the solution from the flask.
8. 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.**
9. Examine the cells under the microscope. When they detach from the plastic substratum, they will be rounded up.
10. Gently tap the flask on the side to loosen the cells.
11. 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.

4. *Passaging Cells*
   a. Calculate the concentration of viable cells per section 5 and document all calculations and dilutions required for seeding new flasks.
   b. 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.

c. Decant the supernatant and resuspend the cells in an appropriate volume of fresh medium. Add the following volumes of 293 Cell Culture Media to flasks:

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

d. Prepare dilutions, if necessary, and seed flasks.
e. Label each flask with the following information:
   (1) Cell Line Name
   (2) Date
   (3) Passage Number (add "1" to the previous passage number)
   (4) Number of cells seeded per flask
f. Place the flasks back in a humidified 37°C incubator.

5. *Counting Cells:*
   a. 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.
   b. 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).
   c. *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.*
   d. 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.
   e. 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.
   f. 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.
   g. Record the number of cells counted in each square.
   h. Calculate the mean cell count, the standard deviation and % CV. If the % CV is greater than 20%, repeat steps 5.a. through h.

i. 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 = (cells / mL) x (the original volume from which the cell sample was removed)

j. 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.

Infectious Titer Assay Method:

NOTE: The assay will be performed two times. These can be scheduled so that both assays are initiated on the same day or are set up on different days. Dilutions and assay plates should be labeled to distinguish the assay set (A or B). See step 2.

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".

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

   a. Calculate the total number of cells needed for the assay using a concentration of 1×10^5 cells per mL and 100 µL per well (10,000 cells per well). Eleven mL of cell suspension at 1×10^5 cells per mL will be required per plate, for a total of 1.1×10^6 cells per plate. The total number of plates required is four if both assay sets are being set up on the same day (two plates per assay). Typically 293 cells can be provided in T-225 cm^2 flasks containing $2 – 4 \times 10^7$ cells.
   b. Prepare the appropriate volume of cell suspension, diluting with 293 Cell Culture Media.
   c. Using a multi-channel pipette and a media reservoir, pipette 100 µL of the cell suspension into each well of each 96-well tissue culture plate. Cover.
   d. Place the plate(s) in the 37°C, 7% CO_2 incubator for 18 to 22 hours until ready to apply the assay samples.

2. Sample preparation:

   a. 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 1:2 Ad5 starting dilutions be made to begin each dilution series. Make one series at a time.* Begin by marking sample tubes as follows:
      i. "1:2 Ad5 A" or "1:2 Ad5 B"
      ii. "1:50 Ad5 A" or "1:50 Ad5 B"
      iii. "1:250 Ad5 A" or "1:250 Ad5 B"
      iv. "1:2500 Ad5 A" or "1:2500 Ad5 B"
      v. "1:2.50 x 10^5 Ad5 A" or "1:2.50 x 10^5 Ad5 B"
      vi. "1:2.50 x 10^6 Ad5 A" or "1:2.50 x 10^6 Ad5 B"
      vii. "1:1.25 x 10^7 Ad5 A" or "1:1.25 x 10^7 Ad5 B"
      viii. "1:6.25 x 10^7 Ad5 A" or "1:6.25 x 10^7 Ad5 B"
      ix. "1:3.125 x 10^8 Ad5 A" or "1:3.125 x 10^8 Ad5 B"
      x. "1:6.25 x 10^8 Ad5 A" or "1:6.25 x 10^8 Ad5 B"
      xi. "1:1.25 x 10^9 Ad5 A" or "1:1.25 x 10^9 Ad5 B"
      xii. "1:2.5 x 10^9 Ad5 A" or "1:2.5 x 10^9 Ad5 B"

xiii. "1:5.0 x 10^9 Ad5 A" or "1:5.0 x 10^9 Ad5 B"
xiv. "1:1.0 x 10^{10} Ad5 A" or "1:1.0 x 10^{10} Ad5 B"
xv. "1:2.0 x 10^{10} Ad5 A" or "1:2.0 x 10^{10} Ad5 B"
xvi. "1:4.0 x 10^{10} Ad5 A" or "1:4.0 x 10^{10} Ad5 B"
xvii. "1:8.0 x 10^{10} Ad5 A" or "1:8.0 x 10^{10} Ad5 B"
xviii. "1:1.6 x 10^{11} Ad5 A" or "1:1.6 x 10^{11} Ad5 B"
xix. "1:3.2 x 10^{11} Ad5 A" or "1:3.2 x 10^{11} Ad5 B"
xx. "1:6.4 x 10^{11} Ad5 A" or "1:6.4 x 10^{11} Ad5 B"
xxi. "1:1.28 x 10^{12} Ad5 A" or "1:1.28 x 10^{12} Ad5 B"

b. Thoroughly triturate (pipetting the volume of the tube up and down several times using a 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.
c. Add 200 µL Adenovirus Dilution Media into each "1:2 Ad5"tube and thoroughly mix the sample by trituration. Triturate at least 10 times.
d. Prepare a series of dilutions for each assay (A and B) in individual sterile tubes. Use the Adenovirus Dilution Media to make the dilutions. 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	*Volume of Previous Dilution (µL)*	*Volume of Adenovirus Dilution Media (µL)*	*Total Volume*
"1:50 Ad5"	100 µL	2400 µL	2500 µL
"1:250 Ad5"	200 µL	800 µL	1000 µL
"1:2500 Ad5"	100 µL	900 µL	1000 µL
"1:2.50 x 10^5 Ad5"	100 µL	900 µL	1000 µL
"1:2.50 x 10^6 Ad5"	100 µL	900 µL	1000 µL
"1:1.25 x 10^7 Ad5"	460 µL	1840 µL	2300 µL
"1:6.25 x 10^7 Ad5"	460 µL	1840 µL	2300 µL
"1:3.1250 x 10^8 Ad5"	460 µL	1840 µL	2300 µL
"1:6.25 x 10^8 Ad5"	1800 µL	1800 µL	3600 µL
"1:1.25 x 10^9 Ad5"	1800 µL	1800 µL	3600 µL
"1:2.5 x 10^9 Ad5"	1800 µL	1800 µL	3600 µL
"1:5.0 x 10^9 Ad5"	1800 µL	1800 µL	3600 µL
"1:1.0 x 10^{10} Ad5"	1800 µL	1800 µL	3600 µL

"1:2.0 x 10^{10} Ad5"	1800 µL	1800 µL	3600 µL
"1:4.0 x 10^{10} Ad5"	1800 µL	1800 µL	3600 µL
"1:8.0 x 10^{10} Ad5"	1800 µL	1800 µL	3600 µL
"1:1.6 x 10^{11} Ad5"	1800 µL	1800 µL	3600 µL
"1:3.2 x 10^{11} Ad5"	1800 µL	1800 µL	3600 µL
"1:6.4 x 10^{11} Ad5"	1800 µL	1800 µL	3600 µL
"1:1.28 x 10^{12} Ad5"	1800 µL	1800 µL	3600 µL

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

3. Inoculation with Adenovirus Samples:
   a. After incubating the cells for 18 to 22 hours, check their condition. They will be approximately 50% confluent in the wells, although this will be difficult to assess visually.
   b. Make additions to the 96-well plates, one 96-well plate at a time. Make sure the plate is marked "Assay A1", "Assay A2", "Assay B1", etc.
   c. Remove the 293 Cell Culture Media from each well in the plate. Change pipette tips.
   d. Place 200 µL per well of 293 Cell Culture Media into each of the eight wells in the first column of cells. This is the No Virus Control. There will be a column 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, allowing at least 30 minutes between the end of infecting cells in one plate before beginning the infection of the next 96-well plate.**

   e. Note the time of infection. Infect the cells in the second column 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 8 wells in the second column. Begin with the Ad5 WT sample marked "1:1.28 x 10^{12} Ad5". Change pipette tips.
   f. Infect the cells in the next column of the 96-well plate with 200 µL per well in one column using the Ad5 WT sample marked "1:6.4 x 10^{11} Ad5". Change pipette tips.
   g. Continue infecting columns of cells with 200 µL per well (8 wells per dilution) using the following samples in order: "1:3.2 x 10^{11} Ad5", "1:1.6 x 10^{11} Ad5", "1:8.0 x 10^{10} Ad5", "1:4.0 x 10^{10} Ad5", "1:2.0 x 10^{10} Ad5", "1:1.0 x 10^{10} Ad5", "1:5.0 x 10^{9} Ad5", "1:2.5 x 10^{9} Ad5", and "1:1.25 x 10^{9} Ad5".
   h. Cover the first plate and incubate for 60 minutes from the beginning of the infection at 37°C in a humidified air incubator at 7% CO_2. The incubation time is a critical factor and should be adhered to closely.

i. After waiting 30 minutes, begin adding the No Virus Control column to the next plate of the assay as in step d. above.

j. Then, noting the time of infection, infect the cells in the next column 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 8 wells in the second column. Begin with the Ad5 WT sample marked "1:6.25 x 10^8 Ad5". Change pipette tips.

k. Continue infecting columns of cells with 200 µL per well (8 wells per dilution) using the following samples in order: "1:3.1250 x 10^8 Ad5", "1:6.25 x 10^7 Ad5", and "1:1.25 x 10^7 Ad5". Change pipette tips between each dilution addition.

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

m. Cover the second plate and incubate for <u>60 minutes</u> from the time of infection at 37°C in a humidified air incubator at 7% CO_2. <u>The incubation time is a critical factor and should be adhered to closely.</u>

n. 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	1:1.28 x 10^{12} Ad5	1:6.4 x 10^{11} Ad5	1:3.2 x 10^{11} Ad5	1:1.6 x 10^{11} Ad5	1:8.0 x 10^{10} Ad5	1:4.0 x 10^{10} Ad5	1:2.0 x 10^{10} Ad5	1:1.0 x 10^{10} Ad5	1:5.0 x 10^9 Ad5	1:2.5 x 10^9 Ad5	1:1.25x 10^9 Ad5
B	No Virus Control	1:1.28 x 10^{12} Ad5	1:6.4 x 10^{11} Ad5	1:3.2 x 10^{11} Ad5	1:1.6 x 10^{11} Ad5	1:8.0 x 10^{10} Ad5	1:4.0 x 10^{10} Ad5	1:2.0 x 10^{10} Ad5	1:1.0 x 10^{10} Ad5	1:5.0 x 10^9 Ad5	1:2.5 x 10^9 Ad5	1:1.25x 10^9 Ad5
C	No Virus Control	1:1.28 x 10^{12} Ad5	1:6.4 x 10^{11} Ad5	1:3.2 x 10^{11} Ad5	1:1.6 x 10^{11} Ad5	1:8.0 x 10^{10} Ad5	1:4.0 x 10^{10} Ad5	1:2.0 x 10^{10} Ad5	1:1.0 x 10^{10} Ad5	1:5.0 x 10^9 Ad5	1:2.5 x 10^9 Ad5	1:1.25x 10^9 Ad5
D	No Virus Control	1:1.28 x 10^{12} Ad5	1:6.4 x 10^{11} Ad5	1:3.2 x 10^{11} Ad5	1:1.6 x 10^{11} Ad5	1:8.0 x 10^{10} Ad5	1:4.0 x 10^{10} Ad5	1:2.0 x 10^{10} Ad5	1:1.0 x 10^{10} Ad5	1:5.0 x 10^9 Ad5	1:2.5 x 10^9 Ad5	1:1.25x 10^9 Ad5
E	No Virus Control	1:1.28 x 10^{12} Ad5	1:6.4 x 10^{11} Ad5	1:3.2 x 10^{11} Ad5	1:1.6 x 10^{11} Ad5	1:8.0 x 10^{10} Ad5	1:4.0 x 10^{10} Ad5	1:2.0 x 10^{10} Ad5	1:1.0 x 10^{10} Ad5	1:5.0 x 10^9 Ad5	1:2.5 x 10^9 Ad5	1:1.25x 10^9 Ad5
F	No Virus Control	1:1.28 x 10^{12} Ad5	1:6.4 x 10^{11} Ad5	1:3.2 x 10^{11} Ad5	1:1.6 x 10^{11} Ad5	1:8.0 x 10^{10} Ad5	1:4.0 x 10^{10} Ad5	1:2.0 x 10^{10} Ad5	1:1.0 x 10^{10} Ad5	1:5.0 x 10^9 Ad5	1:2.5 x 10^9 Ad5	1:1.25x 10^9 Ad5
G	No Virus Control	1:1.28 x 10^{12} Ad5	1:6.4 x 10^{11} Ad5	1:3.2 x 10^{11} Ad5	1:1.6 x 10^{11} Ad5	1:8.0 x 10^{10} Ad5	1:4.0 x 10^{10} Ad5	1:2.0 x 10^{10} Ad5	1:1.0 x 10^{10} Ad5	1:5.0 x 10^9 Ad5	1:2.5 x 10^9 Ad5	1:1.25x 10^9 Ad5
H	No Virus Control	1:1.28 x 10^{12} Ad5	1:6.4 x 10^{11} Ad5	1:3.2 x 10^{11} Ad5	1:1.6 x 10^{11} Ad5	1:8.0 x 10^{10} Ad5	1:4.0 x 10^{10} Ad5	1:2.0 x 10^{10} Ad5	1:1.0 x 10^{10} Ad5	1:5.0 x 10^9 Ad5	1:2.5 x 10^9 Ad5	1:1.25x 10^9 Ad5

Plate 2

	1	2	3	4	5	6	7	8	9	10	11	12
A	No Virus Control	1:6.25 x 10^8 Ad5	1:3.125 x 10^8 Ad5	1:6.25 x 10^7 Ad5	1:1.25 x 10^7 Ad5	No Virus Control	No Virus Control	No Virus Control	No Virus Control	No Virus Control	No Virus Control	No Virus Control
B	No Virus Control	1:6.25 x 10^8 Ad5	1:3.125 x 10^8 Ad5	1:6.25 x 10^7 Ad5	1:1.25 x 10^7 Ad5	No Virus Control	No Virus Control	No Virus Control	No Virus Control	No Virus Control	No Virus Control	No Virus Control
C	No Virus Control	1:6.25 x 10^8 Ad5	1:3.125 x 10^8 Ad5	1:6.25 x 10^7 Ad5	1:1.25 x 10^7 Ad5	No Virus Control	No Virus Control	No Virus Control	No Virus Control	No Virus Control	No Virus Control	No Virus Control
D	No Virus Control	1:6.25 x 10^8 Ad5	1:3.125 x 10^8 Ad5	1:6.25 x 10^7 Ad5	1:1.25 x 10^7 Ad5	No Virus Control	No Virus Control	No Virus Control	No Virus Control	No Virus Control	No Virus Control	No Virus Control
E	No Virus Control	1:6.25 x 10^8 Ad5	1:3.125 x 10^8 Ad5	1:6.25 x 10^7 Ad5	1:1.25 x 10^7 Ad5	No Virus Control	No Virus Control	No Virus Control	No Virus Control	No Virus Control	No Virus Control	No Virus Control
F	No Virus Control	1:6.25 x 10^8 Ad5	1:3.125 x 10^8 Ad5	1:6.25 x 10^7 Ad5	1:1.25 x 10^7 Ad5	No Virus Control	No Virus Control	No Virus Control	No Virus Control	No Virus Control	No Virus Control	No Virus Control
G	No Virus Control	1:6.25 x 10^8 Ad5	1:3.125 x 10^8 Ad5	1:6.25 x 10^7 Ad5	1:1.25 x 10^7 Ad5	No Virus Control	No Virus Control	No Virus Control	No Virus Control	No Virus Control	No Virus Control	No Virus Control
H	No Virus Control	1:1.25 x 10^7 Ad5	1:6.25 x 10^7 Ad5	1:3.125 x 10^8 Ad5	1:1.5625 x 10^9 Ad5	1:7.8125 x 10^9 Ad5	1:3.90625 x 10^{10} Ad5	1:1.953125 x 10^{11} Ad5	1:9.765625 x 10^{11} Ad5	1:4.88281 x 10^{12} Ad5	1:2.44141 x 10^{33} Ad5	1:1.22070 x 10^{14} Ad5

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

p. Add 200 µL per well of 293 Cell Culture Media into all wells, changing pipette tips between each well. Cover the plate and return to the .37°C humidified air incubator at 7% CO_2.

q. Repeat for each individual plate at the 60 minute mark.

r. Incubate the 96-well plates for approximately 7 to 10 days in a humidified air 7% CO_2 incubator at 37°C checking them daily (including weekends) for signs of CPE.

4. Sample Analysis:
   a. Examine the 96-well plates daily (including weekends) for signs of CPE in the wells using a light microscope. Compare to the No Virus Control column. If in doubt about CPE, have a supervisor confirm the observations. If a plate becomes contaminated, the assay must be repeated.
   b. CPE should be evident in at least half the dilutions after 7 to 10 days.
   c. Record the results on a daily basis, scoring wells "+" or "-" for CPE using the CPE scoring form. Return the plates to the incubator as soon as daily results are recorded.
   d. Once CPE is evident and the assay data has been recorded and accepted, the plates should be discarded as biohazardous waste according to the procedures of your institution.

Determining the Result:

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 the day where [a] there was at least one sample dilution where CPE was evident in all 8 wells at the end of the assay, [b] at least one sample dilution where CPE was evident in at least 2 but no more than 6 wells at the end of the assay, and [c] at least one sample dilution where no CPE was evident in any of the 8 wells at the end of the assay are the data that are relevant for the results worksheet.
2. Fill out the results worksheet provided by the Ad5 WT Reference Material Working Group using data from the day the CPE in the wells met the acceptability criteria.
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, n is the total number of wells per dilution (i.e., 8), 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 (50%), 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). From the equation, one can see that p_w must be less than 8 ($n = 8$) and greater than zero. Optimally the number of positive wells should be between 20 and 80% of the total wells in a dilution (between 2 and 6 positive wells).

4. Record all calculations.
5. Report results:
   a. Acceptability of the assay
      i. No bacterial or other contamination occurred during the assay.
      ii. The No Virus Control wells did not show any signs of contamination or CPE.
      iii. There was at least one sample dilution where no CPE was evident in any of the 8 wells at the end of the assay.
      iv. There was at least one sample dilution where CPE was evident in all 8 wells at the end of the assay.
      v. There was at least one sample dilution where CPE was evident in at least 2 but no more than 6 wells at the end of the assay.
   b. 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).
   c. Report the infectious titer for each Assay (A and B) in infectious units/mL to the nearest hundredth.
   d. Report the average infectious titer for the Ad5 Reference Material in infectious units/mL to the nearest hundredth.