Determination of infectious titer of E1-deleted adenovirus by FACS analysis for hexon protein

1) Purpose

Determination of infectious titer of wildtype or E1-deleted adenovirus type 5 by intracellular staining of the hexon protein in infected 293 HEK cells.

2) Scope

This report describes a rapid method to determine infectious titer of crude and purified preparations of wildtype or E1-deleted adenovirus irrespective of the transgene. Adenovirus preparations with an expected titer of $3x10^5$ infectious units/ml or above can routinely be quantified.

3) Background

The assay involves inoculating detector cells with the adenovirus test article and measuring the accumulation of the adenoviral hexon structural protein within the infected cells. HEK 293 cells are used as detector cells. Serial dilutions of the test virus are prepared and five dilutions that bracket the expected titer are tested in triplicates. Cells are harvested 24 hours after inoculation and stained with a FITC-conjugated anti hexon antibody. Stained cells are detected and quantified by flow cytometry. A positive control adenovirus is included to ensure the proper performance of the assay. To limit the probability of multiple hits per cell, and thus underestimation of infectious titer, only dilutions that yield an infected population lower than 30% are used for titer calculation. Titer is then calculated on the basis of single hit analysis and standardized as described by Nyberg-Hoffman et al. (Nature Medicine 3, 808, 1997). The titer is expressed as infectious units/ml (IU/ml). In this format, the assay is efficient and capable of providing high throughput and rapid turnaround time.

4) Materials, Reagents and Equipment

HEK 293 cells and all materials used for cell culture of HEK 293 cells are as described in ARMWG RFP 9.0.

4.1 Twelve (12) well cell culture plate, sterile, polystyrene (Corning Costar, cat # 3513, or equivalent)
4.2 IntraPrep Permeabilization reagents (Immunotech, cat # 2389)
4.3 Reagent 1: Fixation reagent (5.5% Formaldehyde in PBS)
4.4 Reagent 2: Permeabilization solution (0.2% Triton X-100 in PBS)
4.5 Intracellular staining (ICS) wash solution (PBS, 4% FBS)
4.6 Anti-hexon Adeno mAb-FITC conjugated (Chemicon International, cat # 5016)
4.7 Cell Genesys Adenovirus Reference Control, lot # 677-107.1a
A working stock of the standard virus is prepared by a 10-fold serial dilution to a final dilution of 1:1,000 in 293 Cell Culture Media and is stored at –80°C
4.8 FACS tubes (Becton and Dickinson, cat # 35-2235)
4.9 16 % paraformaldehyde solution, Electron Microscopy Science, cat # 15710
4.10 Biosafety cabinet (laminar flow hood), in BL-2 laboratory

4.11 Isotemp Waterbath at 37°C +/- 2°C (Fisher Scientific, or equivalent)
4.12 Spinchrome R Centrifuge, low speed and rotor (Beckman, or equivalent)
4.13 Adjustable micro-pipettes (P20, P200, P1000)
4.14 Hematocytometer and cover slip (Fisher, cat # 0267110, or equivalent)
4.15 Inverted microscope, 10x bright field objective
4.16 Incubator, humidified air, 7 ± 1% CO_2, 37°C ± 2°C
4.17 FACScan with Autoloader upgrade (Becton and Dickenson)

5 Methods

5.1 Seeding of cells

The assay will be performed two times. This can be scheduled so that both assays are initiated on the same day or are set up on different days. Dilutions and culture plates should be labeled to distinguish between both assays (A or B). HEK 293 cells will be thawed and cultured according to ARMWG-RFP9.0. For determination of the infectious titer of adenovirus by FACS analysis for hexon protein, cells will be seeded in 12 well culture plates as follows:

5.1.1 Determine a viable cell count and adjust cell density to $5x10^5$ cells/ml.
5.1.2 Calculate the total volume of cell suspension to be seeded. One ml of cell suspension ($5x10^5$ cells/ml) will be required for each well to be seeded. A minimum of 27 wells are required per assay (negative control, positive control at three different dilutions, test article at five dilutions, all samples in triplicate). Prepare cell suspension for 3-4 extra wells to account for pipetting losses.
5.1.3 Seed 1ml of cell suspension to each well of the 12 well cell culture plates.
5.1.4 Place the plates in the 37°C, 7% CO_2 incubator for 18 to 22 hours until cells are approximately 50% confluent.

5.2 Test article preparation

Prepare a series of dilutions of the Ad5 WT Reference Material in Adenovirus Dilution Media for each assay as described in ARMWG-RFP9.0, page 13, 2a through 2e. To prepare the dilutions for the hexon assay, start with the 1:250 Ad5 (A, B) tube and follow this scheme:

To create sample dilution	Volume of previous dilution (µl)	Volume of Adenovirus Dilution Media (µl)	Total volume (µl)
1:250 (A or B)			1000
1:2500 (A or B)	500	4500	5000
1:5000 (A or B)	2000	2000	4000
$1:1x10^4$ (A or B)	2000	2000	4000
$1: 2x10^4$ (A or B)	2000	2000	4000
$1: 4x10^4$ (A or B)	2000	2000	4000
$1: 8x10^4$ (A or B)	2000	2000	4000

Change pipette tips between each dilution step in the series. The Ad5 WT Reference Material will be tested in the hexon assay at following dilutions: 1:5000, $1:1x10^4$, $1:2x10^4$, $1:4x10^4$, $1:8x10^4$ based on an expected physical titer of the Ad5 WT Reference Material of $2\text{-}5x10^{11}$ p/ml.

5.2.1 Dilute Cell Genesys Reference Adenovirus Control in Adenovirus Dilution Media in subsequent 10 or less fold dilution steps to a final dilution of 1:5000, $1:10^4$ and $1:2x10^4$. Change pipette tips between each dilution step in the series. A volume of 500µl/well of each test dilution per triplicate well is required.

5.2.2 Add 500µl Adenovirus Dilution Media to the wells designated as negative control. Negative control is set up in triplicate.

5.2.3 Add 500µl diluted Cell Genesys Adenovirus Reference Material to the wells designated as positive control. Each positive control dilution is set up in triplicate.

5.2.4 Add 500µl diluted test adenovirus article at 1:5000, $1:1x10^4$, $1:2x10^4$, $1:4x10^4$, $1:8x10^4$ per well. Each adenovirus test article dilution is set up in triplicate.

5.2.5 Incubate the cell culture plates for 60 +/- 2 minutes from the beginning of the infection at 37°C in a humidified incubator.

5.2.6 After 60 +/- 2 minutes infection time, remove media and samples from all wells. Change pipette tips between each well.

5.2.7 Add 2ml of 293 Cell Culture Media into all wells. Change pipette tips between all wells.

5.2.8 Incubate cells at 37°C in a humidified incubator for 24 hours.

5.3 Intracellular Staining

5.3.1 Collect supernatant from each well into prelabeled FACS tube. Check the FACS tubes for the absence of cracks. Change pipette tips between each well. Avoid allowing the cell monolayer to dry-out by only working three wells at a time.

5.3.2 Wash with 1ml of PBS per well. Collect PBS wash into same tube. Change pipette tip for each well. Avoid allowing the cell monolayer to dry-out by only working three wells at a time.

5.3.3 Add 100µl trypsin-EDTA per well. Place cell culture plate in a humidified 37°C incubator for 3 minutes until cells are detached. Gently tap to loosen cells from plate.

5.3.4 Add 900µl ICS wash solution per well.
5.3.5 Collect cell suspension in the corresponding FACS tube (total volume: 3 ml). Change micropipette tip for each well.
5.3.6 Pellet cells by centrifuging at 700-1000 rpm for 5 minutes.
5.3.7 Aspirate off supernatant.
5.3.8 Fix cells by adding 100µl of IntraPrep reagent 1 (5.5% paraformaldehyde) to each tube.
5.3.9 Mix well. Incubate for 15 minutes at room temperature.
5.3.10 Add 4ml ICS wash solution per tube.
5.3.11 Pellet cells by centrifuging at 700-1000 rpm for 5 minutes.
5.3.12 Aspirate off supernatant.
5.3.13 Permeabilize cells by adding 100µl of IntraPrep reagent 2 (0.2% Triton X-100 in PBS) to each tube.
5.3.14 Mix well. Incubate for 15 minutes at room temperature.
5.3.15 Add 1 drop (= approximately 35µl) of anti-hexon adeno mAb-FITC conjugated per tube.
5.3.16 Gently vortex each tube. Incubate for 15-30 minutes at room temperature. Keep the tubes protected from light.
5.3.17 Add 4ml ICS wash solution.
5.3.18 Pellet cells by centrifuging at 700-1000 rpm for 5 minutes.
5.3.19 Aspirate supernatant.
5.3.20 Fix cells in 1ml PBS/0.5% paraformaldehyde.
5.3.21 Process samples through flow cytometer to acquire the data.

6. Flow cytometry Set-up

The flow cytometer parameters are set up so that the negative control sample appears in the same location in every run. Using the CellQuest application, set-up is done by opening an acquisition window. The negative control sample is placed in the cytometer and settings are adjusted as follows: A region is drawn on the forward scatter (FSC) versus sideward scatter (SSC) dot plot between 50 and 100 for both parameters. Two distinct cell population may occur in the FSC versus SSC dot blot. The cell population showing lower FSC and lower SSC represent the dead population and will not be acquired. The cell population with higher FSC and SSC (approximately 90%) represents the viable single cells to be acquired. The FSC and SSC voltages and/or Amps are adjusted such that the viable cell population resides within the drawn region. A single parameter histogram displaying the FL1 parameter is also opened. FL1 parameter is displayed in a logarithmic scale. The FL1 voltage is adjusted so that the histogram appears between $1e^0$ and $1e^2$. Once the settings have been adjusted for the negative control, all test and control samples are acquired without changing the settings. A total of $1x10^4$ events are acquired per FACS tube.

7. Generation of analytical plot

The negative control FSC versus SSC dot plot is opened. A gate is drawn selecting the viable cell population (approximately 90%). A single parameter histogram is generated displaying FL1 fluorescent intensity for the negative control. A marker is set to quantitate positive staining cells by placing it just to the right of the negative population

(approximately from $1x10^2$ to $1x10^4$). Approximately 0.1 to 0.5% of the negative cell population should reside within the marker range. This value is used as a background. This histogram is then used to evaluate all the samples tested.

8. Data analysis

% infected population = % within the marker - % background

Data of all dilutions that result in a % infected population between 3% and 30% will be used for the calculation of titer (see 10, Data Interpretation).

9. Criteria for a valid assay

The assay is considered valid if the following criteria are met:

- no bacterial or other contamination occurred during the assay.
- the titer of the positive control is within 0.5 log of the established titer.
- there are 3 to 30 % positive cells in at least one dilution of the test article.

10. Data interpretation

Each replicate of sample that yields an infected population between 3-30% is considered acceptable and used to calculate an infectious titer. Dilutions that yield an infected population greater than 30% are not used since they will underestimate the titer. Due to the limiting dilution conditions the probability of multiple hits per observation are very low. The infectious titer of each acceptable replicate is calculated by normalizing the data by the diffusion equation for single hit analysis:

$IU/ml = h * D / [\varphi * C_t * I * \sqrt{t}]$

h = total hits (infectious events) = fraction of infected population (between 0.03 and 0.3) * total number of acquired cells ($1x10^4$)
D= dilution factor
φ = average cell area for 293 = $6.3x10^{-6}$ cm^2
C_t = total number of acquired cells ($1x10^4$)
I = diffusion coefficient of adenovirus =$2.38x10^{-4}$ cm per particles per $sec^{1/2}$
t = 3600 seconds, time of infection

The infectious titer of the test article is obtained as follows:

A titer is calculated for each replicate of each dilution.
Titers for the 3 replicates of the same dilution are averaged to obtain the mean titer for that dilution.
Mean titers for each dilution are then averaged to obtain the assay titer.
The assay titers for two independent assays will be averaged to obtain the final test article titer.