

## Quantitation of Adenovirus Particles by TaqMan Analysis

### 1. Purpose

This protocol describes a method for the quantitation of adenovirus viral particles in wild type or recombinant adenoviral stocks by the real-time PCR reaction (TaqMan). The method does not require a DNA extraction step, therefore circumventing issues related to incomplete viral DNA recovery.

### 2. Scope

This assay is applicable to the quantitation of wild-type and E1-deleted adenovirus type 2 and 5 preparations.

### 3. Background

The starting material is purified adenovirus at high titers ( $1-10 \times 10^{12}$  vp/ml). The vector stock is lysed by proteinase K treatment to expose the viral DNA. After proteinase K treatment, serial dilutions of the sample lysates are tested by a real time PCR assay targeting sequences in the adenoviral E4 region of the adenovirus genome. Total adenovirus genomes are quantitated by comparison to a standard curve of adenovirus type 2 genomic DNA.

At the dilutions tested ( $1:10^6$  and above) no interference due to RNA, proteins or formulation buffer components is observed. The TaqMan assay is quantitative over a range of 5 to 50,000 adenovirus E4 copies per reaction. The 96-well format of the microtiter plate and the 2 hours running time of the assay facilitates titer determination of 8 samples per run within 8 hours.

### 4. Reagents

- 4.1 Proteinase K 20mg/ml (Gibco BRL). Catalog # 25530-015. Store at 4°C
- 4.2 Water for irrigation (WFI, Baxter). Catalog # 2F7114
- 4.3 Tris base (Sigma). Catalog # T6687, or equivalent
- 4.4 EDTA (Invitrogen). Catalog # 25300-054, or equivalent
- 4.5 Sodium dodecyl sulfate (SDS), 10% (Sigma). Catalog # L-4522, or equivalent
- 4.6 2x Proteinase K buffer : 20mM Tris pH 8.0, 20mM EDTA, 0.1% SDS, Store at RT for up to one year
- 4.7 Primers (HPLC purified):

AdE4 forward 5'-CAC-CAC-CTC-CCG-GTA-CCA-TA-3' (20-mer)

AdE4 reverse 5'-CCG-CAC-CTG-GTT-TTG-CTT-3' (18-mer)

The AdE4 forward primer is reconstituted in WFI at a 300µM storage stock and is stored at or below -20°C. A 3µM working stock is made by a 100-fold dilution with WFI and stored at or below -20°C. The AdE4 reverse primer is reconstituted in WFI at a 500µM storage stock and is stored at or below -20°C. A 10µM working stock is made by a 50-fold dilution with WFI and is stored at or below -20°C. Both primer stocks are stable for one year when stored at or below -20°C.

- 4.8 Probe

AdE4 probe:

5'-6FAM-AAC-CTG-CCC-GCC-GGC-TAT-ACA-CTG-TAMRA (24-mer)

The probe has been made by Applied Biosystems and is kept at a 100µM storage stock at or below -20°C; the working stock is 10µM after 10-fold dilution with WFI. Probe stock is stable for one year when stored at or below -20°C.

- 4.9 2x PCR master mix, (Applied Biosystems). Catalog #, 4304437
- 4.10 25mM dNTP mix, (Applied Biosystems). Catalog #, N808-0270
- 4.11 DNA hydration solution (Gentra). Catalog # D-50K4
- 4.12 Yeast tRNA 25 mg/ml. (Gibco BRL). Catalog # 15401-011

## 5. Equipment and Supplies

- 5.1 ABI PRISM 7700 Sequence Detection System (SDS) unit (Applied Biosystems)
- 5.2 Heat block for temperatures up to 90°C
- 5.3 MicroAmp Optical 96-well Reaction Plate. Applied Biosystems, Catalog # N801-0560
- 5.4 MicroAmp Optical Caps, 8 Caps/Strip. Applied Biosystems, Catalog # N801-0933
- 5.5 MicroAmp Cap Installer. Applied Biosystems
- 5.6 Micro-pipettes, varying sizes, calibrated
- 5.7 Aerosol resistant pipet tips, suitable for the Micro-pipettes
- 5.8 Micro-centrifuge, Centrifuge 5415 (Eppendorf)
- 5.9 Low-speed centrifuge with adapters for 96-well plate
- 5.10 1.5ml micro-centrifuge tubes
- 5.11 50ml centrifuge tubes

## 6. Positive Control Standards

Adenovirus type 2 DNA (Gibco BRL, Catalog # 15270-010, or equivalent), diluted in WFI to deliver 10,000 copies/µl, 1,000 copies/µl, 100 copies/µl, 10 copies/µl, 1 copy/µl. Positive control standards are stable for one year after manufacturing when stored at or below -20°C.

## 7. Procedure

Perform each step of the procedure in the designated area of the PCR core facility. The assay will be performed two times.

### 7.1 Proteinase K treatment

Adenovirus test article (5µl) is mixed with 50µl 2x Proteinase K buffer and 40µl WFI in a 1.5ml micro-centrifuge tube. Proteinase K (5µl) is added and mixed by pipetting up down for several times. Note that this procedure results in a 20-fold dilution of the adenovirus test article. The test article is incubated at 56°C for 45 minutes, followed by a heat inactivation step at 90°C for 10 minutes. Allow the temperature of the test article to adjust to RT, pulse spin and prepare 10-fold dilutions using DNA hydration solution/t RNA (25µg/ml) as a diluent. Due to the expected high number of adenoviral copies in the test article, it is necessary to dilute the adenovirus test article to final dilutions of 1: 2 x 10<sup>6</sup>, 1: 2 x 10<sup>7</sup>, and 1: 2 x 10<sup>8</sup> to fall within the linear range of the TaqMan assay.

### 7.2 PCR Master Mix Set-up

Determine the total number of reactions for the plate to include:

- a minimum of 3 test sample dilutions
- a positive control dilution series (10,000 copies/µl, 1,000 copies/µl, 100 copies/µl, 10 copies/µl, 1 copy/µl)

- WFI as a negative control
- diluent as a negative control

All test sample dilutions, positive controls and negative controls are tested in triplicate. Determine the master mix volume needed (45µl/reaction) and increase the volume by 20% to allow for pipetting losses. Thaw all the reagents at room temperature in the designated master mix area. Add the master mix reagents in the order listed into a 1.5ml micro-centrifuge tube or a 50ml centrifuge tube.

<b>Reagent</b>	<b>Per reaction</b>
2X PCR Master Mix	25µl
3µM forward primer (AdE4 forward)	5µl
10µM reverse primer (AdE4 reverse)	5µl
10µM FAM probe (AdE4 probe)	0.5µl
2.5mM dNTPs	6µl
WFI	3.5µl
DNA template	5µl
<b>Final volume</b>	<b>50µl</b>

Mix well by swirling or, if the container is a 1.5ml tube, gently vortex the mix. Return remaining reagents to their storage conditions.

### 7.3 PCR Template Set-up and Cycling Conditions

Designate wells for each type of sample (unknown, standard or negative control) on a new SDS run set-up template. This template should match the PCR plate setup under 7.4.

The 7700 unit's default cycling conditions are used:

50°C for 2 min hold  
 90°C for 10 min hold  
 95°C for 20 sec, 62°C for 1 min 30 seconds (40 cycles)

### 7.4 PCR Plate Set-up

Determine where each sample will be placed in the reaction plate. Assemble the test samples, positive control standards, negative controls, a 96-well optical reaction plate and optical caps. Aliquot 5µl of the WFI into the designated wells for a negative control. Add 45µl of the master mix and cap. Aliquot 55µl of diluent into the designated wells for a negative control. Add 45µl of the master mix and cap. Aliquot 5µl of the positive control standards into the designated wells. Add 45µl of the master mix to each of these wells and cap. Aliquot 5µl of the test samples to their designated wells. Add 45µl of master mix to each of these wells and cap. Pulse spin the plate in the low-speed centrifuge up to 500rpm, to help remove any air bubbles. Place the plate in the 7700 unit's heat block and start the run.

## 7.5 TaqMan Data Analysis

Analysis is accomplished by using the SDS software's default settings (baseline 3-15, threshold set to 10x baseline's standard deviations). If the initial threshold is below 0.01, an adjustment is allowed to bring the threshold up to 0.01 and to re-calculate.

## 7.6 Criteria for a valid assay

The assay is considered valid if following criteria are met:

- The slope of the standard curve has a value greater than  $-4.00$ .
- The correlation coefficient (R fit) has a value greater than 0.970.
- All negative controls (WFI, diluent) have a  $C_T=40$ .
- For the positive control standard at 50 to 50,000 copies/reaction, at least 2 of the 3 replicates of each standard concentration have a  $C_T<40$ , with authentic amplification plots.

## 7.7 Data interpretation

For each test article dilution, the TaqMan report will provide a mean value from averaging the results of the triplicates. The mean titer per each dilution set is then determined as follows:

Mean titer per dilution set (particles/ml) = mean value from TaqMan report x final dilution factor ( $2 \times 10^6$  to  $2 \times 10^8$ ).

Usually, three mean titers (one per each dilution tested) are obtained. The final titer is calculated as the mean of means as follows:

$$\text{Final titer (p/ml)} = \text{mean of mean titers per dilution set}$$

The titer of adenovirus test article is reported as "particles/ml" (p/ml).

Note: Test article dilutions that produce TaqMan results that fall outside the positive control standard range are not used for calculation of final titer.

If more than one independent assay is performed on the same test article, the final titer of the test article will be calculated by averaging the titers obtained in each valid assay.