

**Purpose:** To determine the concentration of infectious particles in the AAV8 reference standard material. This process involves serial dilution of the vector in a TCID50 format and endpoint determination through real-time PCR measurement of replicated AAV vector genome DNA.

## **Procedure:**

### 1. Split and Plate Cells

#### 1.1. Materials

##### 1.1.1. Complete Media

##### 1.1.1.1. Complete Media contains:

DMEM (Cellgro Cat # 10-013-CM)

10%FBS (Hyclone Cat #SH30071.02)

1% Penicillin/ Streptomycin solution (Pen/Strep) (Cellgro Cat #03-002-C1)

##### 1.1.2. Phosphate Buffered Saline (PBS) (Cellgro Cat #21-031-CM)

##### 1.1.3. Trypsin (Cellgro Cat #25-053-C1)

##### 1.1.4. 50ml Conical Tubes (2)

##### 1.1.5. tissue culture 96-well Plates (1 plate/ 2 samples)(Falcon Cat #35-3072)

##### 1.1.6. Sterile Solution Basin (Labcor Cat#730.004)

##### 1.1.7. T150 Culture Flasks with vent cap (4) (Corning Cat #430825)

##### 1.1.8. T150 Culture Flasks containing confluent monolayer of RC32 HeLa cells (ATCC # CRL-2972)

##### 1.1.8.1. RC32 cells are transformed HeLa cells expressing AAV2 *rep/cap* genes

#### 1.2. Warm complete media, PBS, and trypsin to 37°C in a water bath for 10 minutes

*Carry out all subsequent steps in the biosafety cabinet.*

#### 1.3. Aspirate media from the confluent monolayer of RC32 cells

#### 1.4. Wash cells with PBS and trypsinize (2mL) to remove cells from culture flask

Add 8ml complete media and transfer the cell suspension to a 50ml conical tube

Repeat for additional flasks transferring cells into the same 50ml conical tube

Mix cell suspension in a 50ml conical tube by vortexing.

#### 1.5. Count living cells using a hemacytometer and trypan blue dye

##### 1.5.1. Calculate cells/ml for the suspension

##### 1.5.2. Calculate average number of cells per flask

#### 1.6. Dilute cell suspension with complete media

##### 1.6.1. The desired concentration of the cell suspension is $(4 \times 10^4)$ cells in 50 $\mu$ l or $(8 \times 10^5)$ cells/ml. Determine the dilution factor by dividing the concentration of cells counted above by $(8 \times 10^5)$

##### 1.6.2. Total volume of diluted cell suspension needed = 5ml X (total number of plates +1)

- 1.6.3. Volume of cell suspension to be used = total volume of diluted cell suspension needed divided by the dilution factor
- 1.6.4. Volume of complete media in diluted cell suspension = Total volume of diluted cell suspension needed) – Volume of cell suspension used in dilution
- 1.6.5. Pipette the appropriate volume of complete media into a 50ml conical tube
- 1.6.6. Vortex the cell suspension and transfer the appropriate volume by pipette into the complete media
  
- 1.7. Plate cells in 96-well plates  
Vortex the diluted cell suspension and pour into a sterile basin.  
Using a 12-channel pipette, pipette 50µl of the cell suspension into each well of a 96-well plate (hold the plate at an angle and dispense the dilution along side of wells to minimize air bubbles in the media). Incubate the plates in a 37°C incubator overnight
  
2. Infection
  - 2.1. Materials
    - 2.1.1. AAV8 reference material (ATCC # VR1816) Aliquots obtained from ATCC should be directly stored at -80°C and thawed only before use for this assay.
    - 2.1.2. Human adenovirus 5 (H5) reference material (ATCC VR-1516; titer 5.8x10<sup>11</sup> p/mL). Upon receipt from ATCC the adenovirus should be thawed on ice, immediately aliquoted (25ul) and snap frozen on dry ice/ ethanol.
    - 2.1.3. Sterile Cryovials (9 per test vector)
    - 2.1.4. Serum-free media
      - 2.1.4.1. Serum-free media contains DMEM , 25mM Hepes (Gibco Cat #15630-080), and 1% Pen/Strep
    - 2.1.5. RC32 cells in 96-well plates from Section 1 above
    - 2.1.6. Wet ice
    - 2.1.7. Multi-well basin (2 per plate of cells to be infected) (USA Scientific Cat #1301-1212)
    - 2.1.8. Complete Media (see 1.1.1.1 above)
    - 2.1.9. 50ml Conical tube (1)
    - 2.1.10. Adhesive air-pore film (1 sheet per plate) (Qiagen Cat #120001)
  - 2.2. Prepare work space in Biosafety cabinet
  - 2.3. Make diluent
    - 2.3.1. Dilute the H5 adenovirus into serum free media (SFM) to make a final concentration of 3.2x10<sup>8</sup> adenovirus particles per milliliter. Prepare 15 mL of diluent per 96 well plate.
  - 2.4. Vector dilutions
    - 2.4.1. Thaw vector on ice and mix by pipette before making dilutions
    - 2.4.2. Seven serial dilutions are made in cryovials for each vector in a total of 1ml
      - 2.4.2.1. AAV8 RSM vector dilution series:  
1x10<sup>-2</sup> = 990µl diluent + 10µl stock  
1x10<sup>-4</sup> = 990µl diluent + 10µl previous dilution

- $1 \times 10^{-5} = 900\mu\text{l diluent} + 100\mu\text{l previous dilution}$
- $1 \times 10^{-6} = 900\mu\text{l diluent} + 100\mu\text{l previous dilution}$
- $1 \times 10^{-7} = 900\mu\text{l diluent} + 100\mu\text{l previous dilution}$
- $1 \times 10^{-8} = 900\mu\text{l diluent} + 100\mu\text{l previous dilution}$
- $1 \times 10^{-9} = 900\mu\text{l diluent} + 100\mu\text{l previous dilution}$

2.5. Infect cells with vector

2.5.1. Infection scheme

2.5.1.1. Twelve replicates of the six most diluted samples are used on each plate of cells. Twelve replicates of a negative control (diluent only) are used on each plate of cells below the highest dilution of vector. The final row of wells is left blank

Plate layout

	12 Replicates →											
	1	2	3	4	5	6	7	8	9	10	11	12
Dilution 1 (-4) →	A											
Dilution 2 (-5) →	B											
Dilution 3 (-6) →	C											
Dilution 4 (-7) →	D											
Dilution 5 (-8) →	E											
Dilution 6 (-9) →	F											
Negative Control →	G											
Uninfected	H											

2.5.2. Pipette vector dilutions and diluent to appropriate wells of the multi-well basin

Note: Open the cryovial of vector dilution away from the multi-well basin to minimize contamination. When pipetting vector dilutions, enter the plate with the pipette from the side adjacent to the appropriate well, such that pipette tips cross only wells designated for that dilution.

2.5.3. Aspirate the complete media off cells in the 96 well plate

2.5.3.1. Use a 200µl unplugged pipette tip on the end of an unplugged aspiration pipette

2.5.3.2. Hold the plate at an angle and aspirate from the side of well rather than the bottom of the well

2.5.4. Align the multi-well basin and 96-well plate such that the vector dilution in the well of the basin is in line with the row of wells to which the dilution will be added. Pipette 50µl of virus dilution (or diluent for the negative control) into the first column of the 96-well plate, repeat for columns 2 to 12. Move the pipette during transfer such that each tip passes over only wells in which it will be used. Cover the plate with it's lid

2.5.5. Label the plate with experiment number, sample name, lot number, vector dilutions used, and time of infection

2.5.6. Incubate the plate for two (2) hours in a 37°C/5% CO<sub>2</sub> incubator

- 2.5.7. Warm complete media to 37°C in a water bath
- 2.5.8. Pipette 12 ml complete media for each plate of infected cells into a 50ml conical tube. Pipette 1ml complete media into each well of a multi-well basin (Use a new multi-well basin for each plate of infected cells)
- 2.5.9. Using a 12-channel pipette add 50µl complete media to each well of the 96-well plate containing infected cells- try to avoid contact with the infection medium.
- 2.5.10. Seal the plate with adhesive air-pore film
- 2.5.11. Cover the plate with it's lid
- 2.5.12. Incubate the plate for 72 hours in 37°C /5%CO<sub>2</sub> incubator

### 3. DNA Extraction

#### 3.1. Materials

- 3.1.1. 96-well plates containing cells from which DNA is to be extracted
- 3.1.2. Deoxycholate – 10% (DOC)
- 3.1.3. 10X Tween – 8.9%
  - 3.1.3.1. Combine 8.9ml 50% Tween, 11.1ml 1M HEPES pH 8, and 30ml sterile water to make 50ml stock solution
- 3.1.4. Proteinase K – 1.2mg/ ml (Qiagen 20mg/mL stock; cat #19133)
- 3.1.5. 10X Proteinase K buffer (10mM Tris-HCL pH 8.0, 10mM EDTA, 0.01% SDS)
- 3.1.6. Adhesive sealing film (Fisher Scientific Cat #550032 or Corning Cat #6524)

#### 3.2. Prepare extraction solution by combining for each plate to be extracted:

<u>Reagent</u>	<u>[Initial]</u>	<u>[Final]</u>	<u>Volume</u>
DOC	10%	0.062 %	0.125ml
Tween Solution	8.9%	0.45%	1.000ml
Proteinase K Buffer	10X	1X	2.000ml
Proteinase K	10mg/ml	0.2mg/ml	0.400ml
Water			5.675ml
<b>Total</b>			<b>9.200ml</b>

#### 3.3. Prepare work space in the Tissue Culture Hood

#### 3.4. Centrifuge the 96-well plate of cells to collect all liquid in the bottom of the wells

- 3.4.1. Use a centrifuge with a swinging bucket rotor for 96-well plates
- 3.4.2. Centrifuge at 1500 rpm for 30 seconds

#### 3.5. Carefully remove the adhesive air-pore film from the plate (peel from the bottom row upwards) and inspect the cells under a microscope. Close to 100% CPE should be observed.

3.6. Add 85µl extraction solution to each well of the 96-well plate. Mix the reaction by pipetting 5 times upon addition. Exercise caution not to cross-contaminate adjacent columns.

3.7. Seal the 96-well plate with adhesive sealing film.

3.8. Incubate the plates in a hybridization oven

3.8.1. 37°C for 1 hour

3.8.2. 55°C for 2 hour

3.8.3. 95°C for 30 minutes (to heat inactivate Proteinase K)

3.9. The 96-well plates containing the DNA samples can be used immediately for PCR or stored at 4°C for up to 7 days

## 4. Real-time PCR

### 4.1. Materials

4.1.1. 96-well PCR plate and adhesive plate cover (ABI Cat #4314320)

4.1.2. 96-well plate holder

4.1.3. 96-well plate containing cell lysate prepared in Section 3 (above)

### 4.2. Reagents

4.2.1. Sterile Water

4.2.2. Taqman masterMix (ABI Cat # 4326614)

4.2.3. PCR Standards (linearized pTRUF-11 plasmid DNA containing the AAV reference standard genome) – set of 8 from 10<sup>1</sup> copies to 10<sup>8</sup> copies. Please refer to appendix B for instructions on preparing PCR standards.

4.2.4. SV40 polyA Primer/ Probe set

4.2.4.1. Forward primer (10µM) AGC AAT AGC ATC ACA AAT TTC ACA A

4.2.4.2. Reverse Primer (10µM) CCA GAC ATG ATA AGA TAC ATT GAT GAG TT

4.2.4.3. Probe (10µM) 6FAM- AGC ATT TTT TTC ACT GCA TTC TAG TTG TGG TTT GTC TAMRA

4.2.5. Working in a dedicated PCR facility or hood, set up the PCR plate in the same format as the infection plate, ie twelve (12) replicates of each sample dilution and a negative control. Standards and No Template Control (water) are loaded in row H of the 96-well PCR plate (see diagram below)

		1	2	3	4	5	6	7	8	9	10	11	12
Dilution 1 (-4) →	A												
Dilution 2 (-5) →	B												
Dilution 3 (-6) →	C												
Dilution 4 (-7) →	D												
Dilution 5 (-8) →	E												
Dilution 6 (-9) →	F												
Negative Control →	G												
Standards →	H	10 <sup>8</sup>	10 <sup>7</sup>	10 <sup>6</sup>	10 <sup>5</sup>	10 <sup>4</sup>	10 <sup>3</sup>	10 <sup>2</sup>	10 <sup>1</sup>	H <sub>2</sub> O			

4.3. Prepare Master Mix

4.3.1. For each plate make a Master Mix for 100 reactions as per the table below:

Reagent	[Stock]	1 Rxn ( $\mu$ l)	100 Rxns ( $\mu$ l)	[Rxn]
PCR Mix	2X	25	2500	1X
Forward Primer	10 $\mu$ M	1	100	200nM
Reverse Primer	10 $\mu$ M	1	100	200nM
Probe	10 $\mu$ M	0.5	50	100nM
Water	NA	17.5	1750	NA
Total		45	4500	

4.3.2. Combine appropriate volumes of reagents in a 15ml conical tube

4.4. Add 45 $\mu$ l Master Mix into each well of the 96-well PCR plate being used

4.5. Pipette and mix 5 $\mu$ l water and standards into the Master Mix in the appropriate wells of row H of the 96-well PCR plate

4.6. Add 2.5 $\mu$ l water to the rest of the wells with the exception of the no template control where 5 $\mu$ l should be added.

4.7. Add and mix 2.5 $\mu$ l cell lysate prepared in section 3 (above) into the Master Mix in the corresponding wells of the 96-well PCR plate. Proceed from the top of the plate towards the bottom.

4.8. Cover the PCR plate with an adhesive plate cover and ensure a tight seal

4.9. Perform PCR reactions in an ABI 7500 Real-time PCR Machine (or similar) following manufacturer directions

95°C for 10 minutes

40 cycles of 95°C for 15 seconds / 60°C for 1 minute

5. Data Collection

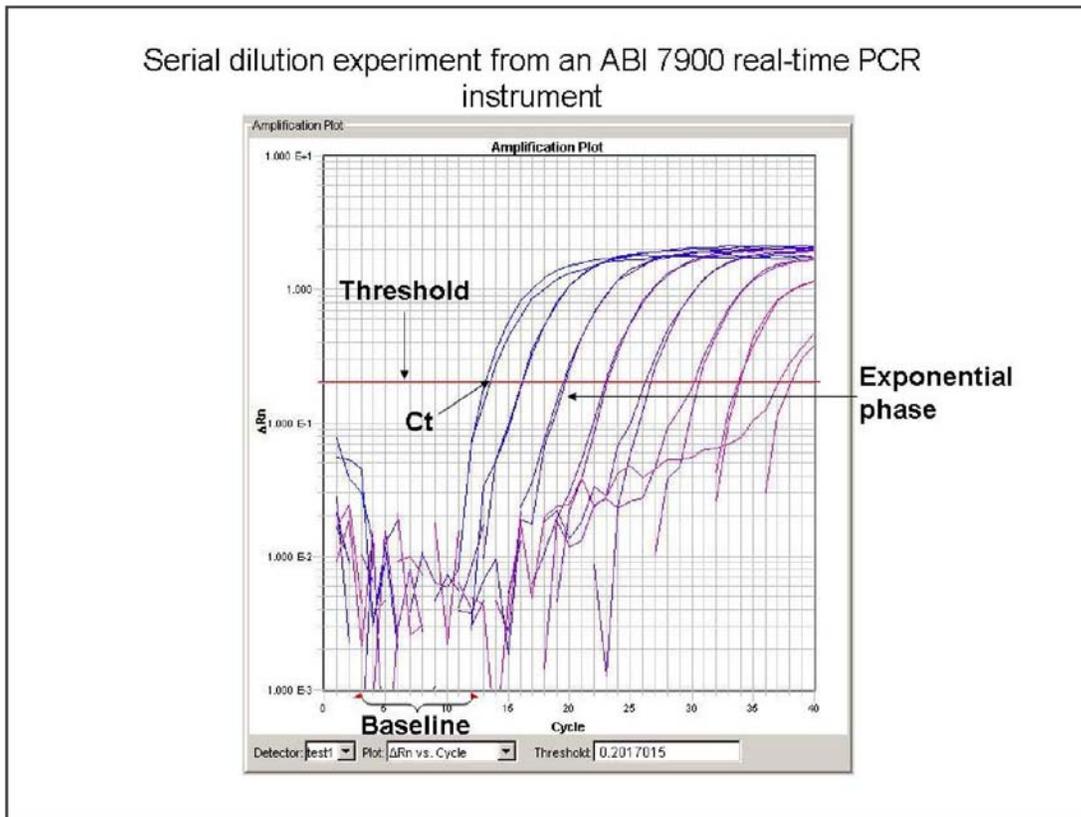
Refer to the appropriate instrument user guide for instructions on how to analyze your data.

The general process for analyzing the data involves the following procedures:

1-View the amplification plots

2-Set the baseline and threshold values

During early PCR cycles, the background signal in all wells is used to determine the baseline fluorescence. The threshold should be placed in the region of the exponential phase. For ABI 7500 machines or similar the baseline is routinely set as cycles 3 to 12 and the threshold adjusted such that the 10<sup>8</sup> standard reads 13.09 Ct.



## 6. Data Analysis

- 6.1. Calculate vector copies used to infect for each dilution level (vector input value)
  - 6.1.1. Multiply the input vector concentration (pt/ml) by the dilution factor
  - 6.1.2. Divide the result by 20 to get particles per 50 $\mu$ l
- 6.2. Calculate input subtracted vector copies per well
  - 6.2.1. Multiply the PCR determined quantity by a dilution factor of 74 to get a total number of vector copies in the infection assay well
  - 6.2.2. Subtract the vector input value for that dilution from the result
- 6.3. Determine the number of positive replicates
  - 6.3.1. For the purposes of this assay the PCR detection limit is set at 10 vector copies which translates to 740 vector copies in the infection assay well
  - 6.3.2. Input-subtracted vector copies greater than 740 are designated positive for infectious particles measured by replicated vector DNA
- 6.4. Calculate TCID<sub>50</sub> by Karber's method using the appropriate worksheet in the results packet.

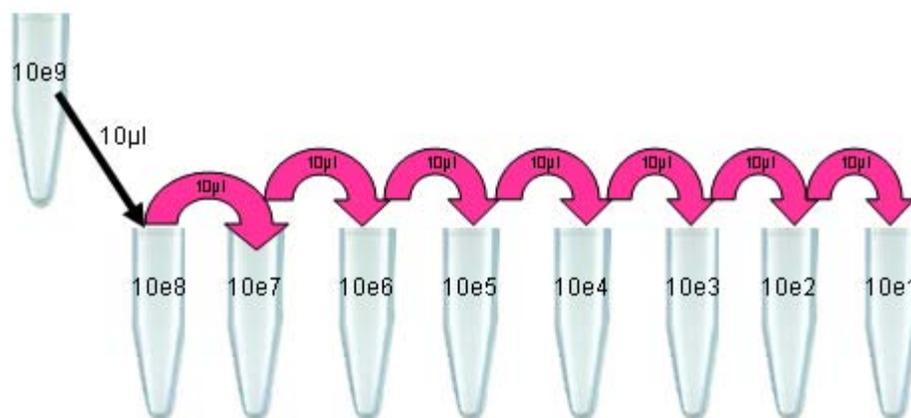
## Appendices:

### A. Preparation of real time PCR standards protocol

#### Procedure

*Note: Work inside the biosafety cabinet to avoid potential contamination from the laboratory environment.*

- Clean the biosafety cabinet work surface and pipette shafts with 20% Lysol, followed by 70% Ethanol. Irradiate with UV light for 10-15 minutes.
- Linearize the standard plasmid with a restriction enzyme that cuts outside your intended PCR target.
- Purify the linearized plasmid using the QIAquick PCR purification kit or similar if it is less than 10kb in size. For larger plasmids, use the QIAmp DNA mini kit or similar. In both cases, pre-warm the elution buffer to 70°C before use. Also, pre-warm the columns loaded with elution buffer at 70°C for 5 minutes. Carry out two elutions in a minimum volume to maximize recovery and concentration of the plasmid.  
*Note: Do not carry out a phenol:chloroform: isoamyl purification of the digest as any contamination with phenol will affect the accuracy of the spectrophotometric reading.*
- Determine the concentration of the linearized plasmid by spectrophotometry and convert the concentration readout to grams per Liter (g/L).  
*Note: Make sure the spec reading falls within the linear range of the instrument. Dilute the sample less if necessary. Blank the reading against an equal dilution of elution buffer and use the background correction feature of the instrument (read at 320nm).*
- Calculate the formula weight (F.W.) of your standard plasmid: F.W. = Plasmid size (in base pairs) x 662 grams/mole.bp **(For TRUF11, this is 4766400)**
- Calculate the molar concentration (M) of your linearized plasmid.  $M = \text{mole/L} = (\text{mass (in grams)}/\text{F.W.})/1\text{L}$ .
- Determine copy number per microliter of your linearized plasmid based on molar concentration. 1M is equivalent to about  $6.0221415 \times 10^{23}$  copy numbers.
- Make the first dilution, typically with a final concentration of  $1 \times 10^{10}$  copies per  $20\mu\text{L}$  ( $5 \times 10^8$  copies/ $\mu\text{L}$ ). Use the  $C_1V_1=C_2V_2$  relationship, where  $V_1$  is the unknown volume,  $C_1$  is the stock linearized plasmid concentration (copies/ $\mu\text{L}$ ),  $V_2$  is the final volume (typically  $100\mu\text{L}$ ) and  $C_2$  is the final concentration ( $5 \times 10^8$  copies/ $\mu\text{L}$ ). Add  $V_1$  volume of stock linearized plasmid to enough Sample dilution buffer for  $100\mu\text{L}$  of solution.
- Carry out a serial dilution of  $1 \times 10^{10}$  copies/ $20\mu\text{L}$  of the linearized standard using sample dilution buffer. A volume of  $90\mu\text{L}$  of diluent is used per dilution



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## Sample dilution buffer:

	<u>[Final]</u>
1mL 1X GeneAmp PCR Buffer	1X
0.1mL 100X SSS DNA 200µg/mL	2µg/mL
0.1mL 100X pluronic F68 (5%)	0.05%
8.8mL NF H <sub>2</sub> O	

## **B. TCID50 test reagents**

### **Media, Complete**

Reagent	[Stock]	Volume	[Final]
DMEM		1 L	
FBS	100%	100 ml	10%
Pen/Strep	100%	10 ml	1%
<b>Total</b>		<b>1.11 L</b>	

### **Media, Serum Free**

Reagent	[Stock]	Volume	[Final]
DMEM		1 L	
Hepes	1M	25 ml	25mM
Pen/Strep	100%	10 ml	1%
<b>Total</b>		<b>1.04 L</b>	

### **SDS solution (10% w/v)**

Reagent	[Stock]	Volume	[Final]
SDS	solid	25 g	10%
Sterile Water	to	250 ml	
<b>Total</b>		<b>250 ml</b>	

### **EDTA solution (0.5M)**

Reagent	[Stock]	Volume	[Final]
EDTA	solid	46.5 g	0.5M%
NaOH*	solid	5 g	
Sterile Water	to	250 ml	
<b>Total</b>		<b>250 ml</b>	

\*solution needs to be ~pH 8 for EDTA to dissolve. Adjust to final pH8

### **Tris HCl (1M, pH 8)**

Reagent	[Stock]	Volume	[Final]
Tris	solid	30.3 g	1M
Sterile Water	to	250 ml	
<b>Total</b>		<b>250 ml</b>	

adjust to pH8 with HCl

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## Proteinase K Buffer (10X)

Reagent	[Stock]	Volume	[Final]
Tris HCl, pH 8	1M	0.5 ml	10mM
EDTA	0.5M	1 ml	10mM
SDS	10%	0.05 ml	0.01%
Sterile Water		48.45 ml	
Total		50 ml	

## Deoxycholate (10%)

Reagent	[Stock]	Volume	[Final]
Deoxycholic Acid	solid	5 g	10%
Sterile Water		50 ml	
Total		50 ml	

## Tween Solution (10X)

Reagent	[Stock]	Volume	[Final]
Tween 20	50% (v/v)	8.9 ml	8.90%
Hepes pH 8	1M	11.1 ml	10mM
Sterile Water		30 ml	
Total		50 ml	

c) AAV8 Reference Standard Material Test Record Packet.