NIST Stability Study Results for Ad5 RM

Introduction

NIST was asked to perform the final stability study measurements on the Adenovirus Serotype 5 (Ad5) Reference Material distributed by ATCC. The measurements include particle count by OD260 nm in the presence of 0.1% SDS and TCID 50 assay to determine infectious particles/mL. Detailed protocols were provided by Keith Carson from ISBiotech for both assays.

Materials were provided by ATCC (courtesy of Liz Kerrigan) at no charge to NIST to complete the stability study. The items received are listed below.

1) Two vials of HEK293 cells deposited by the Adenovirus Reference Material Working Group (ATCC# DS-CELLS1, batch# 3553)

2) Two vials of Ad5 RM (ATCC# VR-1516, lot# 001506)

Ad5 RM PARTICLE COUNT MEASUREMENT

Materials

Ultra-pure water (Thermo Fisher# 10977015)

1 M Tris-HCL pH8 (Millipore Sigma# T3038)

NaCl (Millipore Sigma#71376-1KG)

Glycerol (Thermo Fisher# 15514011)

SDS, 10% (Millipore Sigma# 71736-500ml)

Hellmanex III (Millipore Sigma# Z805939)

Methanol (Fisher Scientific# A542-1)

Agilent Cary 3500 UV-Vis Spectrophotometer, double beam

Quartz cuvettes, 1cm pathlength

Procedure (provided by ISBiotech)

Title: Adenovirus reference material standard operating procedure for determination of particle concentration via spectrophotometric analysis.

Version 4 (Nov 7, 2001)

Date of analysis: Oct 19, 2021

Name of analyst: Jamie Almeida

Deviations made to SOP:

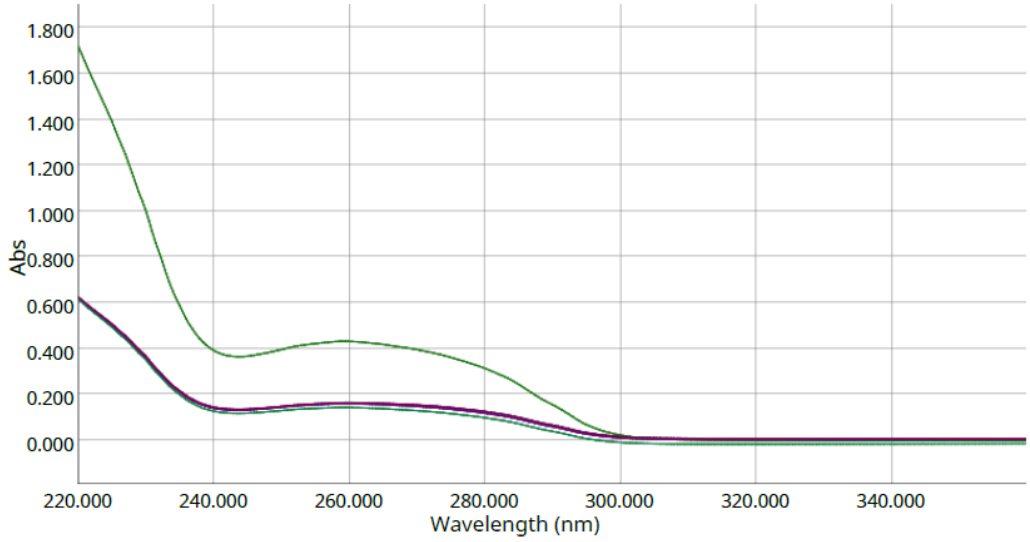
- The Particle Count SOP requires 0.85 mL of Ad5 RM to perform the measurements; however, there is only 0.5 mL of Ad5 RM in a single vial. The particle count and infectious titer should be performed on the same vial. Therefore, some of the volumes had to be reduced in the SOP.
    - Ad5 volumes for the 80% and 30% samples were cut in half. Ultimately, 200 µL of Ad5 RM was added to the 80% tube with 25 µL of excipient, and 75 µL of Ad5 RM was added to each of the 30% tubes with 150 µL of excipient.
- 100 µL (instead of 200 µL) of the blank and Ad5 RM dilutions (80% and 30% samples) were added to the quartz cuvettes for OD260 nm measurements.

Results

Acceptability of the assay parameters as stated in the SOP were all met.

JA AD5 Oct2021

X mode	Y mode	Averaging time (s)	Spectral bandwidth (nm)
nm	Absorbance	0.048	1.00
Data interval (nm)	**Scan range start (nm)**	**Scan range stop (nm)**	
0.50	360.00	220.00	
Detector module	**Multiple experiments**	**Experimental temperature (°C)**	
Multicell Peltier UV-Vis	1 zone	Zone 1: N/A	



Wavelength scan (2021-10-19 11:12:27 (-04:00))

Sample	260.00 nm
	Abs
80% AD5 repA	0.432
30% AD5 #1a	0.160
30% AD5 #2a	0.144
30% AD5 #3a	0.165
80% AD5 repB	0.432
30% AD5 #1b	0.160
30% AD5 #2b	0.144
30% AD5 #3b	0.165
80% AD5 repC	0.432
30% AD5 #1c	0.160
30% AD5 #2c	0.144
30% AD5 #3c	0.165

Sample	OD260nm	AVG A260	Std Dev	Dil Fact	virus particle/mL	AVG particle/mL	Std Dev particle/mL
80% AD5 repA	0.43211496						
80% AD5 repB	0.43181982	0.432	0.000197	0.8	5.93851E+11		
80% AD5 repC	0.43174031						
30% AD5 #1a	0.16026363						
30% AD5 #1b	0.1600499	0.160	0.000148	0.3	5.87025E+11		
30% AD5 #1c	0.15997997					**5.781E+11**	**3.509E+10**
30% AD5 #2a	0.14378244						
30% AD5 #2b	0.14353283	0.144	0.000137	0.3	5.26624E+11		
30% AD5 #2c	0.14355877						
30% AD5 #3a	0.16513694						
30% AD5 #3b	0.16489912	0.165	0.000151	0.3	6.0487E+11		
30% AD5 #3c	0.16485761						

The assigned particle concentration of the Ad5 RM in 2001 was 5.8 E11 particles/mL with a 95% certainty range of 5.6 E11 to 6.0 E11 particles/mL. The NIST measured value of 5.781 E11 +/- 3.509 E10 particles/mL falls within the 95% certainty range.

Ad5 RM INFECTIOUS TITER MEASUREMENT

Materials

HEK 293 cells deposited by the ARMWG (ATCC# DS-CELLS1, batch# 3553)

FBS, US origin (Thermo Fisher # 26140-079)

DPBS, -Ca, -Mg (Thermo Fisher# 14190144)

DMEM high glucose (Thermo Fisher# 11995065)

DMEM low glucose (Thermo Fisher# 11054020)

Sodium pyruvate, 100 mM (Thermo Fisher# 11360-070)

Glutamax, 100X ((Thermo Fisher# 35050061)

Bovine calf serum (Thermo Fisher# 10371-029)

Trypsin-EDTA, 0.05% (Thermo Fisher# 25300-054)

96 well plates (Costar# 3598)

T225 cm tissue culture flasks (Corning# 431082)

T75 cm tissue culture flasks (Corning# 430641)

Incubator, humidified (37°C, 5% CO_2)

Isoton solution (Beckman Coulter# 8546719)

Beckman Multisizer 3 coulter counter

Olympus CKX53 inverted microscope

Laminar flow biosafety cabinet

Hermle Tabletop Centrifuge

Procedure (provided by ISBiotech)

Title: Adenovirus reference material standard operating procedure for determination of infectious titer in 293 cells in a 96-well format

Version 3 (Nov 7, 2001)

Date of analysis: Oct 19, 2021 (Assay A) and Oct 20, 2021 (Assay B)

Name of analyst: Jamie Almeida

Assay A: HEK 293 cells were at passage 7

Assay B: HEK 293 cells were at passage 8

Deviations made to SOP:

- An initial plating of 40,000 cells per well of a 96 well plate resulted in HEK 293 cells that were over confluent with cells stacking on top of one another. A lower dilution was necessary to obtain a confluency of 60-80% at the time of infection. Cells were seeded at a density of 10,000 cells per well the day before infection and resulted in approximately 60% confluency on Day 0 for both A and B assay plates.
- The Particle Count SOP requires 0.85 mL of Ad5 RM to perform the measurements; however, there is only 0.5 mL of Ad5 RM in a single vial. The particle count and infectious titer should be performed on the same vial. Therefore, the initial volume in the Ad5 dilution series had to be reduced in the SOP.
    - The volume for the 1:2 dilution was reduced to 70 µL so that there would be enough material for both the A and B assays.

Results

Acceptability of the assay parameters as stated in the SOP were all met except for one condition.

- There was at least one sample dilution where CPE is evident in all 12 wells.
    - At the highest dilution in Assay A, 11 out of 12 wells were positive for CPE.
    - At the highest dilution in Assay B, 7 out of 12 wells were positive for CPE.

TCID50 Results for Assay A

Calculation for infectious titer per mL (IU/mL)

V=-([ln(1-(p/n))]*D/[A*C*I*√t]

Protocol section 6.2.5.3: Optimally, the number of positive wells should be between 20-80% of the total wells in the dilution (>2 and <10)

Assay A

Sample Dilution	Positive Wells	Confluency	Time (sec)	last part of the formula	first part of formula	Pos wells/total	Full formula	AVG	SD
5.00E+07	11	0.6	3600	0.00274176	2.48E+00	0.083333333	4.53E+10	**3.72E+10**	**6.63E+09**
1.00E+08	8	0.6	3600	0.00274176	1.10E+00	0.333333333	4.01E+10		
2.00E+08	4	0.6	3600	0.00274176	4.05E-01	0.666666667	2.96E+10		
2.83E+08	4	0.6	3600	0.00274176	4.05E-01	0.666666667	4.19E+10		
4.00E+08	2	0.6	3600	0.00274176	1.82E-01	0.833333333	2.66E+10		
5.66E+08	2	0.6	3600	0.00274176	1.82E-01	0.833333333	3.76E+10		
8.00E+08	3	0.6	3600	0.00274176	2.88E-01	0.75	8.39E+10		
1.13E+09	1	0.6	3600	0.00274176	8.70E-02	0.916666667	3.59E+10		
1.60E+09	2	0.6	3600	0.00274176	1.82E-01	0.833333333	1.06E+11		
2.26E+09	1	0.6	3600	0.00274176	8.70E-02	0.916666667	7.17E+10		
3.20E+09	1	0.6	3600	0.00274176	8.70E-02	0.916666667	1.02E+11		
4.53E+09	0	0.6	3600	0.00274176	0.00E+00	1	0.00E+00		
6.40E+09	0	0.6	3600	0.00274176	0.00E+00	1	0.00E+00		
1.28E+10	0	0.6	3600	0.00274176	0.00E+00	1	0.00E+00		

TCID50 Results for Assay B

Assay B

Sample Dilution	Positive Wells	Confluency	Time (sec)	last part of the formula	first part of formula	Pos wells/total	Full formula	AVG	SD
5.00E+07	7	0.6	3600	0.00274176	8.75E-01	0.416666667	1.60E+10	**2.01E+10**	**8.22E+09**
1.00E+08	4	0.6	3600	0.00274176	4.05E-01	0.666666667	1.48E+10		
2.00E+08	4	0.6	3600	0.00274176	4.05E-01	0.666666667	2.96E+10		
2.83E+08	1	0.6	3600	0.00274176	8.70E-02	0.916666667	8.98E+09		
4.00E+08	1	0.6	3600	0.00274176	8.70E-02	0.916666667	1.27E+10		
5.66E+08	2	0.6	3600	0.00274176	1.82E-01	0.833333333	3.76E+10		
8.00E+08	1	0.6	3600	0.00274176	8.70E-02	0.916666667	2.54E+10		
1.13E+09	0	0.6	3600	0.00274176	0.00E+00	1	0.00E+00		
1.60E+09	0	0.6	3600	0.00274176	0.00E+00	1	0.00E+00		
2.26E+09	0	0.6	3600	0.00274176	0.00E+00	1	0.00E+00		
3.20E+09	0	0.6	3600	0.00274176	0.00E+00	1	0.00E+00		
4.53E+09	0	0.6	3600	0.00274176	0.00E+00	1	0.00E+00		
6.40E+09	0	0.6	3600	0.00274176	0.00E+00	1	0.00E+00		
1.28E+10	0	0.6	3600	0.00274176	0.00E+00	1	0.00E+00		

Average of plate A and B concentrations = 2.86 E10 IU/mL

Standard deviation of plate A and B = 1.15 E10 IU/mL

The assigned infectious titer for the Ad5 RM on HEK 293 cells in 2001 was 7 E10 IU/mL with a 95% certainty range of 7 E10 to 8 E10 IU/mL. Previous data from the 17 laboratories that conducted initial TCID 50 assays for the Ad5 RM is also described in the RM product sheet and states that 99.7% of the time the result would fall within the range of 3 E10 and 18 E10 IU/mL (within 3 SD of the mean). The NIST measured value of 2.86 E10 +/- 1.15 E10 IU/mL falls within the 99.7% range that the previous 17 laboratories measured.

STR analysis on HEK 293 cells deposited by the ARMWG

STR analysis was performed on HEK 293 cells at passage 6 using the following protocol.

One million cells were deposited into a sterile 1.5 mL microfuge tube and washed with DPBS and centrifuged (200 x g, 3 min) to pellet. The cell pellet was resuspended in 150 µL of DPBS and frozen at -80°C until ready for testing.

Cells were thawed, mixed well, and 15 µL was added to pre-cut sterile FTA punches. These punches were allowed to dry overnight in a 96 well plate in a laminar flow biosafety cabinet. Once dry, the FTA punches were added to tubes and given to Becky Steffen (NIST, DIV 645) to perform STR genotyping.

Promega PowerPlex Fusion 6C kit was used (based on the manufacturers protocol) to determine STR allele calls for the HEK 293 cells. PCR products were separated on the Applied Biosystems Genetic Analyzer 3500xl and results analyzed using Applied Biosystems GeneMapper ID-X software.

Results

HEK 293 cells (passage 6) deposited by the ARMWG have the following STR profile determined by Becky Steffen at NIST (see table below). Alleles in green show 100% concordance between NIST, ATCC, and Cellosaurus STR data for HEK 293 cells. Discrepancies between the STR profile of these cells and those from ATCC and Cellosaurus are highlighted in blue for human markers CSF1PO and D5S818. NIST data shows loss of heterozygosity (LOH) at these two loci which is not uncommon in cell lines after passaging. I was unable to locate STR profile data from the original characterization of these cells back in 2001. It is unclear if these cells obtained the LOH at these two loci while being adapted for adherence, or from passaging the cells at NIST. Either way, these cells are HEK 293 derived.

STR Marker	Allele 1	Allele 2	Notes
CSF1PO	12	12	11,12 (ATCC and Cellosaurus)
Penta D	9	10	
TH01	7	9.3	
vWA	16	19	
D21S11	28	30.2	
D7S820	11	12	
D5S818	8	8	8,9 (Cellosaurus)
TPOX	11	11	
D8S1179	12	14	
D12S391	19	21	
D19S433	15	18	
SE33	17	20	
D22S1045	16	16	
FGA	23	23	

ATCC STR profile for HEK 293

STR profiling	
	Amelogenin: X
	CSF1PO: 11,12
	D13S317: 12,14
	D16S539: 9,13
	D5S818: 8,9
	D7S820: 11,12
	THO1: 7,9.3
	TPOX: 11
	vWA: 16,19

Cellosaurus STR profile for HEK 293

Markers:

Amelogenin	X
CSF1PO	11,12
D2S1338	19
D3S1358	15,17
D5S818	8,9
D7S820	11,12
D8S1179	12,14
D13S317	12 (JCRB; TKG) 12,14 (ATCC; CCRID; CLS; DSMZ; ECACC; KCLB; PubMed=15218237; RCB)
D16S539	9 (DSMZ) 9,13 (ATCC; CCRID; CLS; ECACC; JCRB; RCB; TKG)
D18S51	17 (PubMed=15218237) 17,18 (CCRID; PubMed=11416159) 18 (CLS)
D19S433	15,18 (CCRID) 18 (CLS)
D21S11	28,30.2
FGA	23
Penta D	9,10
Penta E	7,15
TH01	7,9.3
TPOX	11
vWA	16,19