1.0 PURPOSE

To describe a method for measuring the concentration of adenoviral particles in purified preparation of virus using UV spectrophotometry at 260 nm. The method can be used as long as adenovirus concentrations are greater than or equal to 1×10^{11} particles/mL.

2.0 SCOPE

The method described uses a spectrophotometer to determine the concentration of viral particles in a sample. Absorbance at 260 nm is measured in the presence of an ionic detergent. Particle concentration is proportional to the A_{260} value in the presence of detergent.

The theoretical composition of Adenovirus 5 WT is 87% protein and 13% DNA. The concentration of adenoviral particles may be measured directly by UV-spectroscopy, since proteins have a UV absorbance at 277 nm due to their tryptophan and tyrosine content and double-stranded linear DNA has an absorbance maximum at 260 nm. In this assay, 0.1% SDS (w/v) disassembles the virus capsid into its component proteins and DNA. The absorbance of the lysed virus in SDS is measured at 260 nm. The viral particle concentration is calculated using a method described by Maizel, *et al*. In this method an absorbance of 1.00 AU (1-cm pathlength) at 260 nm corresponds to a concentration of 1.1×10^{12} viral particles/mL.

3.0 REFERENCES

3.1 Maizel, J.V., White, D.O., Scharff, M.D., "The Polypeptides of Adenovirus," (1968) Virology 36: 115-125.

3.2 ARMWG Particle Concentration Worksheet

4.0 MATERIALS

4.1 Water, ultra-pure (Milli-Q water, or, Sterile distilled water, Gibco Invitrogen cat. no. 523452, or equivalent)

4.2 Ad5 WT Reference Material Excipient ("Excipient"), 20 mM TRIS, 25 mM NaCl, 2.5% Glycerol (w/v), pH 8.0, prepared as:

4.2.1 A 20 mM Tris solution is prepared using USP grade Tris(hydroxymethyl)aminomethane, FW 121.14 [also known as TRIS base or Tromethamine] (Sigma cat. no. T6687, or equivalent). For a 1.0 L buffer, use 2.42 g.

4.2.2 Add TRIS base to ultra-pure water (approximately 900 mL).

4.2.3 Adjust the pH to 8.0 by dropwise addition of concentrated Hydrochloric Acid (HCl, VWR cat. no. JT9544-2, or equivalent). Determine the pH at room temperature (25°C).

4.2.4 Add sodium chloride (NaCl, ultra-pure or USP grade, FW 58.44, VWR cat. no. JT3628-1, or equivalent) to bring the concentration to 25 mM.

4.2.5 Add 19.8 mL of glycerol (Glycerin, anhydrous, ultra-pure or USP grade, EM Science, cat. no. GX0195-2, or equivalent) to adjust the solution to 2.5% (w/v) at 1.26 g/mL density.

4.2.6 Q.S. to 1.0 L with ultra-pure water

4.3 Sample tubes with caps for preparing dilutions of the test article

4.4 Sterile microliter pipette tips, with aerosol filters for 1-200 µL and 200-1000 µL volumes

NOTE: Adenovirus 5 WT Reference Material samples should be handled as biohazardous, BL-2. Wear gloves, safety glasses, and laboratory coat. Properly dispose of waste, including cuvette wash solutions.

4.5 Ad5 WT Reference Material, prepared as:

4.5.1 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.

4.5.1.1 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.

4.6 Sodium dodecyl sulfate (SDS), 10% (w/v) (Sigma, cat. no. L-4522, or equivalent)

4.7 1% SDS (w/v) in Ad5 WT Reference Material Excipient ("1% SDS/Excipient solution"), prepared as:

4.7.1 Add 1 mL of 10% (w/v) SDS for every 9 mL of Ad5 WT Reference Material Excipient

4.7.2 Make at least 30 mL

4.8 Microcentrifuge tubes, 1.5 or 0.5 mL capacity

4.9 Bleach, 5% (v/v), prepared as:

4.9.1 5 mL Bleach (standard household chlorine bleach)

4.9.2 Q.S. to 100 mL with ultra-pure water

4.10 Cuvette Cleaning Solution, equivalent or prepared from:

4.10.1 2 mL Hellmanex II® (Hellma GMBH & Co.)

4.10.2 Q.S. to 100 mL with ultra-pure water

4.11 Methanol, HPLC grade (Fisher Scientific, cat. no. A452-1, or equivalent)

4.12 Biohazard bags and containers for liquid waste and pipette tips

5.0 EQUIPMENT

5.1 Biosafety cabinet (laminar flow hood), suitable for BL-2 containment

5.2 pH meter and pH 7.0 and pH 10.0 standard solutions, to make the Ad5 WT Reference Material Excipient

5.3 Balance, for weighing materials to make the Ad5 WT Reference Material Excipient

5.4 Graduated cylinder, 1000 mL, to make the Ad5 WT Reference Material Excipient

5.5 Pipette, 25 mL, to make the Ad5 WT Reference Material Excipient

5.6 Adjustable microliter pipettes: 1-20 µL, 20-200 µL, 200-1000 µL

5.7 UV (or UV-VIS) Spectrophotometer, which includes 260 nm wavelength, single or dual beam instrument

5.8 Quartz cuvette, 1-cm path length, requires a minimum of 100 µL (Hellma GBMH & Co., or equivalent). Other quartz cuvette sizes may be used as long as they have a 1-cm path length and require no more than a minimum of 500 µL for accurate readings. The method asks that 200 µL of each sample be placed in the cuvette but will make 500 µL of the dilution for assay. Therefore cuvettes requiring minimum volumes greater than 500 µL for accurate readings are not acceptable.

5.9 Vacuum operated cuvette washer or equivalent

5.10 Pipettes (to transfer samples and/or blank into/out of the cuvette)

6.0 PROCEDURE

NOTE: Adenovirus 5 WT Reference Material samples should be handled as biohazardous, BL-2. Wear gloves, safety glasses, and laboratory coat. Properly dispose of waste, including cuvette wash solutions.

6.1 Turn on the power to the UV spectrophotometer. Allow the instrument to warm up for the time recommended by the equipment manufacturer.

6.2 Perform the instrument-specific diagnostic checks recommended by the manufacturer to ensure that the instrument is operating properly.

6.3 Ensure that the spectrophotometer is within calibration.

6.4 Ensure that the cuvette is clean by:

6.4.1 Rinsing with Cuvette Cleaning Solution.

6.4.2 Rinsing with ultra-pure water until the foaming stops.

6.4.3 Rinsing with methanol.

6.4.4 Allow the cuvette to remain on vacuum until the cuvette appears dry.

6.5 Make a 0.1% SDS (w/v) blank solution using the 1% SDS/Excipient solution and the Ad5 WT Reference Material Excipient ("0.1% SDS/Excipient solution"):

6.5.1 Add 100 µL of 1% SDS/Excipient solution to 900 µL of Ad5 WT Reference Material Excipient.

6.5.2 Add 200 µL of 0.1% SDS/Excipient solution to the clean and dry cuvette.

NOTE: If the cuvette you are using requires more than 100 µL minimum volume to ensure accuracy, place the minimum volume required (i.e., up to 500 µL) into the cuvette for steps 5 and 10.

6.6 Sample preparation:

6.6.1 Mark sample tubes as follows:

6.6.1.1 "80% Ad5"

6.6.1.2 "30% Ad5 #1"

6.6.1.3 “30% Ad5 #2”

6.6.1.4 “30% Ad5 #3”

6.6.2 Pipette thawed Ad5 WT Reference Material into each marked sample tube as follows:

6.6.2.1 Add 400 µL into the “80% Ad5” tube

6.6.2.2 Add 150 µL into each of the tubes marked “30% Ad5 . . .”

6.6.3 Pipette Ad5 WT Reference Material Excipient into each marked sample tube as follows:

6.6.3.1 Add 50 µL into the “80% Ad5” tube

6.6.3.2 Add 300 µL into each of the tubes marked “30% Ad5 . . .”

6.6.4 Pipette 50 µL of 1% SDS/Excipient solution into each of the four marked sample tubes. The final concentration of SDS in each sample tube should be 0.1%.

6.6.5 Mix each sample tube thoroughly by trituration (pipetting the volume of the tube up and down several times), changing pipette tips between samples and being careful so that foaming does not occur. Foaming should not occur if not quite all of the volume is drawn into the pipette tip each time.

6.6.6 Allow the samples containing SDS to sit for at least 15 minutes (at room temperature) prior to taking their absorbance reading.

6.7 Insert the cuvette into the holder and blank the spectrophotometer against the 0.1% SDS/Excipient solution. If the spectrophotometer is a dual beam instrument, leave the 0.1% SDS/Excipient solution-containing “blank” cuvette in place for steps 9 through 14.

6.8 Remove and discard the 0.1% SDS/Excipient solution from the cuvette.

6.9 Rinse the cuvette with ultra-pure water and methanol using the cuvette washer. Allow the cuvette to remain on vacuum until the cuvette appears dry.

6.10 Transfer 200 µL of the sample marked “30% Ad5 #1” into the quartz cuvette.

6.11 Insert the cuvette into the holder and record the absorbance value at 260 nm. Take and record 3 consecutive readings.

6.12 Remove the cuvette. Remove the virus sample from the cuvette and discard as biohazardous liquid waste.

6.13 Rinse the cuvette thoroughly with (in order), 0.1% SDS/Excipient solution, ultra-pure water, and methanol using the cuvette washer. Allow the cuvette to remain on vacuum until the cuvette appears dry.

6.14 Process other adenovirus samples the same way (steps 8 through 13) one sample at a time, continuing in order with "30% Ad5 #2", "30% Ad5 #3", and, lastly, "80% Ad5".

6.15 After completing the analysis of all samples, rinse the cuvette(s) as follows:

6.15.1 Rinse with 5% chlorine bleach solution.

6.15.2 Rinse with Cuvette Cleaning Solution.

6.15.3 Rinse with ultra-pure water until the foaming stops.

6.15.4 Rinse with methanol.

6.15.5 Allow the cuvette to remain on vacuum until the cuvette appears dry.

NOTE: Disinfect and properly dispose of the cuvette rinses per local biohazard regulations.

6.16 Turn off the power to the spectrophotometer unless your institution has a different procedure.

7.0 DETERMINING THE RESULT

7.1 Record the raw data to 1/1000ths place onto the data collection form provided by the Ad5 WT Reference Material Working Group. Correct the sample A260 readings for the blank A260 reading if your spectrophotometer does not do this automatically. See worksheet.

7.2 Viral particle concentration can be determined using the following formula:

Concentration (particles/mL) = $[A_{260nm \text{ corrected for blank}}]$ x [1.1 x 10^{12} particles/mL] x [1/Dilution factor]

The following dilution factors should be used:

"30% Ad5 . . ." = 0.30

"80% Ad5" = 0.80

7.3 Record all calculations on SW033, ARMWG Particle Concentration Worksheet.

7.4 Report results:

7.4.1 Acceptability of the assay

7.4.1.1 The UV spectrophotometer was operating properly.

7.4.1.2 A_{260nm} values are greater than 0.100 and less than 1.000 AU. Do not include the data from sample dilutions whose values were not within this range in the calculation of the average particle concentration. Indicate if a dilution value was not included in the calculation.

7.4.1.3 The standard deviation for the three readings for each sample was <10% of the Average A260 sample reading. If sample readings are greater than 10% different for a single sample, check the equipment manufacturer's instructions regarding spectrophotometer stability. These readings may not be valid.

7.4.2 Report the particle concentration for each dilution to the nearest thousandth.

7.4.3 Report the average particle concentration for the Ad5 Reference Material in particles/mL to the nearest thousandth.