

ANIMAL CELL TECHNOLOGY AND DOWNSTREAM PROCESSING GROUP

DEVELOPMENTAL OPERATING PROCEDURE

Title: Quantification of Adenovirus Type 5 (Ad5) Total Particle Concentration by HPLC Assay	Page 1 of 32
Date: July 09, 2001	Version: 01
Written by: Julia Transfiguracion	

1.0 Objective

To measure the total Adenovirus Type 5 (Ad5) particle concentration (VP/mL) in cell lysate, semi-purified and purified samples using a UNO™Q Polishing anion exchange column.

2.0 Scope

This method allows the quantification of the intact virus particle which is eluted using a linear salt gradient. The method described herein has already been published (Klyusnichenko et. al., J. Chromatography B, Vol 755 Issues 1-2 pp 27-36) and has been fully validated according to industry and regulatory guidelines (manuscript submitted to J. Chromatography B, Transfiguracion et. al.,). The major advantage of this method is that quantification of total particles in cell lysate preparations does not require the pre-treatment of DNase prior to the analysis. The Ad5 particle is separated efficiently from the other peaks and can be quantified accurately. The virus is eluted at ~ 8 min specifically and quantification is done at OD260nm against a standard curve of known Ad5 concentrations. The Ad5 reference standard used to construct the standard curve is prepared in-house by a 2-step CsCl gradient purification of a recombinant Ad5 expressing the green fluorescent protein (GFP).

3.0 Responsibilities

3.1 It shall be the responsibility of the supervisor to:

3.1.1 Ensure that this procedure is followed by all Animal Cell Technology and Downstream Processing personnel.

3.1.2 Ensure that the personnel working on the analyses are trained to work in a Biosafety Level 2 Laboratory (BSL-2)

3.1.3 Ensure that the security rules are applied at the Biotechnology Research Institute (BRI) concerning the shipping and receiving of biorisk-2 agents.

4.0 Materials

4.1 Solutions (Refer to attachment # 1 for the preparation of solutions)

4.1.1 Solution A: 0.25 M HEPES, pH 7.5

4.1.2 Solution B: 2 M NaCl

4.1.3 Solution C: Milli Q H₂O

4.2 50 mM HEPES, pH 7.5 as Buffer Blank. Prepared from the stock of 0.25 M HEPES, pH 7.5 (Solution A). Filtered using a 0.8/0.2 µm Acrodisc[®] PF syringe filter (Pall Gelman, PN 4187).

4.3 Ad5 Standard: CsCl purified rAd5 , particle concentration determined by UV260nm/SDS (Maizel et. al., 1968)

4.4 Ad5 Assay Control: CsCl purified rAd at 5×10^{10} VP/mL, particle concentration determined by UV260nm/SDS (Maizel et. al., 1968)

4.5 0.45 µm GHP Acrodisc, 13 mm syringe filter, Pall Gelman , PN 4556

4.6 0.5 µm PEEK frit in a ferrule. S.P.E Ltd. P-275X

4.7 1mL syringe. B-D Biosciences. Cat # 309602

4.8 6 x 32 mm borosilicate, 0.3 mL HPLC insert vials . CSC. Cat # 03-CV.

- 4.9 1.5 mL microcentrifuge tubes
- 4.10 Pipette tips for the Microliter pipettes
- 4.11 4 mL HPLC vials. Waters Limited. Cat # WAT 072710
- 4.12 Black Phenolic Caps for HPLC vials. Waters Ltd. Cat # WAT 0727 11
- 4.13 Compression springs for HPLC vials. Waters Ltd. Cat #. WAT 072708
- 4.14 Self Sealing Septa for Black Phenolic Cap. Waters Ltd. Cat #. WAT 022861

5.0 Equipments

- 5.1 HPLC System (Waters, Milford, MA) equipped with a 996 Photodiode Array (PDA) Detector, 717 plus Autosampler, Waters 600 Controller, and Waters In-Line Degasser. A Millennium Chromatography Software version 3.05.01 is used for data acquisition and peak integration.
- 5.2 Conditioned UNO™Q Polishing column, 0.160 mL CV (Bio-Rad Laboratories, Cat # 720-0009). For a new column conditioning refer to attachment # 2 .
- 5.3 Microcentrifuge (Eppendorf 5415 C or equivalent).
- 5.4 Microliter pipettes: 1-20 µL, 20-200 µL, 200-1000 µL
- 5.5 Biohazardous waste container

6.0 Procedure

IMPORTANT: Samples containing Ad5 are classified as BSL 2 agents and should be handled as biohazardous material. Wear gloves, gowns, glasses when manipulating such samples. Properly dispose all waste accordingly.

- 6.1 HPLC system start-up (If system is stored in 20% EtoH solution over the

weekend or for a longer period of time, perform the start up according to attachment # 3)

- 6.1.1 Turn on PDA 996, 717 plus Autosampler, 600 Controller, and In-Line Degasser , Computer and Printer.
 - 6.1.2 Check to make sure that Lines A, B and C are immersed into their corresponding buffers. Line A = 0.25 M HEPES, pH 7.5 (Buffer A); Line B = 2 M NaCl in Milli Q H₂O (Buffer B) and Line C = Milli Q H₂O.
 - 6.1.3 If you are using freshly prepared solutions, follow steps 6.4.1 to 6.4.10 of attachment # 3 to prime the head pumps and fill all lines with the fresh solutions. Otherwise, continue to the next steps.
 - 6.1.4 Do the needle wash for 30 s.
 - 6.1.5 Login to Millenium³².
 - 6.1.6 Browse project. Select project. OK
 - 6.1.7 Connect the UNO™Q Polishing column (refer to attachment # 2, if it has not already been connected).
- 6.2 System Suitability and Assay Control
- 6.2.1 Equilibrate the column with 10 CV of 20% A (50 mM HEPES), 15% B (300 mM NaCl), 65 % C (Milli Q H₂O) at 0.2 mL/min and then increase slowly up to 1 mL/min. During column equilibration, check for the pressure. It should be less than 300 psi. Otherwise, replace the frit and verify the pressure again.

6.2.2 Run a buffer blank 3 consecutive times

6.2.3 Perform 3 consecutive 25 μ L injections of the assay control

6.3 New Standard Curve Generation.

For the generation of a new standard curve including preparation of the standards and processing of results go to attachment # 4.

6.4 Samples Preparation

6.4.1 Ad5 Lysate

6.4.1.1 Thaw at $37^{\circ}\text{C}\pm 1^{\circ}\text{C}$ using a water bath

6.4.1.2 Gently mix the lysate

6.4.1.3 Take out 1 mL of the sample

6.4.1.4 Centrifuge at 7500 rpm (4,650 x g) for 5 min.

6.4.1.5 Collect the supernatant and filter with 0.45 μ m GHP Acrodisc[®] syringe filter.

6.4.1.6 Pipet 200 μ L of filtered sample into the HPLC insert vial.

6.4.2 Semi-Pure and Pure Ad5

6.4.2.1 If the sample has been frozen, thaw at RT

6.4.2.2 If the expected concentration of Ad5 total particle is more than 1×10^{11} VP/mL, perform the appropriate dilution using a 50 mM HEPES buffer pH 7.5 to come up to a concentration that will lie in the range of the standard curve (1×10^{10} to 1×10^{11} VP/mL).

6.4.2.3 Pipet 200 µL volume into the HPLC insert vials.

Note: Semi-pure and pure Ad5 are not filtered prior to injection.

6.5 Running the Sample Set

6.5.1 Load the samples in the sample carousel according to the sample set design

6.5.2 Open the Quick Set Window (blue icon).

6.5.3 Select a sample set template for running a particular sample

Example: For a Lysate sample : Ad_LYS_UNK

6.5.4 Open the sample set template required

6.5.5 Replace the existing Method Set/Report Method with the Newly Created Method Set containing the new standard curve (refer to attachment # 4 for the creation of a new method set)

6.5.6 Save

6.5.7 Click the green button to run the sample set. Save with a filename.

6.6 Processing of Results

6.6.1 In the Project Window, go to Channels

6.6.2 Select the Channels that you want to Process. For example, look under the Sample Set Name or Date Acquired.

- 6.6.3 Process the Channels selected (Little Calculator Icon). Use Acquisition Method Set or the Newly Created Specified Method Set if re-Processing with a new method set containing a newly created standard curve. OK.
- 6.6.4 Go to Results. Select Results. Go to Review.
- 6.6.5 Highlight the results that you want to print (example: channel 260nm and 280nm for the same injection). Click on the print icon.
- 6.6.6 Select report method overlay 3a. OK

7.0 Acceptance Criteria and Validity of Results.

The results obtained for the unknowns are valid if the following criteria are met:

7.1 System Suitability

- 7.1.1 The 3 buffer blank runs should have a straight baseline monitored at both OD260nm and OD280nm.
- 7.1.2 The Ad5 assay control which was injected 3 times consecutively must have a rsd of $\leq 5\%$ for the retention time and $\leq 10\%$ for the peak area. And that Ad5 elution time should fall in the range of $8.0 \text{ min} \pm 2\text{sd}$.
- 7.1.3 The Ad5 assay control particle concentration should be within $\pm 10\%$ of the expected value or the reference concentration.

7.2 Standard Curve. (See attachment # 4 for the acceptance criteria)

8.0 Results

- 8.1 Calculate the mean of the replicate injections (if applicable) and record this as the final value for a particular sample analysed, otherwise just record the obtained value as is.

9.0 References

- 9.1 Vadim Klyushnichenko, Alice Bernier, Amine Kamen and Eef Harmsen. 2001. Improved high performance liquid chromatographic method in the analysis of Adenovirus particles. J. Chromatography B. Vol 755, Issues 1-2 pp 27-36
- 9.2 Julia Transfiguracion, Alice Bernier, Normand Arcand, Amine Kamen and Parminder Chahal. 2001. Validation of a HPLC Assay for the Quantification of Adenovirus Type 5 (Ad5) Particles. Manuscript Submitted to J. Chromatography B.
- 9.3 Jacob V. Maizel, Jr., David O. White and Matthew D. Scharff. 1968. The Polypeptides of Adenovirus. I. Evidence of Multiple Protein Components in the Virion and Comparison of Types 2, 7A and 12. Virology. 36 115-125.
- 9.4 Yumi, Kanegae, Miho Makimura, Isumu Saito. 1994. A simple and efficient method for the purification of infectious recombinant adenovirus. Jpn J Med. Sci. Biol. 47 157-166.

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ATTACHMENT # 1

ANIMAL CELL TECHNOLOGY AND DOWNSTREAM PROCESSING GROUP	
DEVELOPMENTAL OPERATING PROCEDURE	
Title: Solutions Preparation Used for the Quantification of Adenovirus Type 5 (Ad5) Total Particles	Page 1 of
Date: July 09, 2001	Version No: 01
Written by: Julia Transfiguracion	

1.0 Objective

To prepare the solutions required for the quantification of Adenovirus Type 5 (Ad5) total particles by the HPLC method.

2.0 Scope

This procedure describes the preparation of solutions used for the quantification of Ad5 total particles using the UNO™Q Polishing column

3.0 Responsibilities

3.1 It shall be the responsibility of the supervisor to:

- 3.1.1 Ensure that this procedure is followed by all Animal Cell Technology and Downstream Processing personnel.
- 3.1.2 Ensure that the personnel working on the analyses are trained to work in a Biosafety Level 2 Laboratory (BSL-2)

3.1.3 Ensure that the security rules are applied at the Biotechnology Research Institute (BRI) concerning the shipping and receiving of biorisk-2 agents.

4.0 Materials

- 4.1 HEPES, Free Acid. ULTROL[®] Grade. Calbiochem. Cat #:391338
- 4.2 NaCl, USP Grade, A & C. Cat # S240
- 4.3 Milli Q water
- 4.4 10 N NaOH
- 4.5 3 x 1 L pyrex bottles for the solutions
- 4.6 0.45 µm filter membrane, Type HATF, 47 mm. Millipore Cat # HATF 04700.
- 4.7 2 x 2 L Beakers
- 4.8 Weigh boats
- 4.9 Spatula
- 4.10 Magnetic Stirrer
- 4.11 pH calibration buffers (pH 7.0 and 4.0)
- 4.12 2 x 1 L volumetric flasks
- 4.13 1 x 2 L Vacuum flask

5.0 Equipments

- 5.1 Balance
- 5.2 Stirring plate
- 5.3 pH meter
- 5.4 Vacuum pump or vacuum water system

6.0 Procedure

- 6.1 Calibrate the pH meter according to the manufacturer's instructions
- 6.2 Solutions Preparation
 - 6.2.1 Solution A: 0.25 M HEPES pH 7.5, 1 L Volume
 - 6.2.1.1 Weigh 59.57 g of HEPES
 - 6.2.1.2 Dissolve in 900 mL of Milli Q H₂O
 - 6.2.1.3 Check pH and note in the logbook
 - 6.2.1.4 Adjust pH with 10 N NaOH slowly
 - 6.2.1.5 Complete the volume up to 1 L with Milli Q H₂O using a volumetric flask
 - 6.2.1.6 Check for the final pH and note in the logbook
 - 6.2.1.7 Filter with 0.45 µm membrane and degas by vacuum for 20 min.

- 6.2.1.8 Transfer into a 1L buffer bottle and cover the bottle with aluminum foil (HEPES is light sensitive).
- 6.2.1.9 Store at RT.
- 6.2.2 Solution B: 2 M NaCl in Milli Q H₂O, 1 L Volume
 - 6.2.2.1 Weigh 116.88 g of NaCl
 - 6.2.2.2 Dissolve in 900 mL of Milli Q water
 - 6.2.2.3 Complete the volume up to 1 L with Milli Q H₂O using a volumetric flask
 - 6.2.2.4 Filter with 0.45 µm membrane and degas by vacuum for 20 min
 - 6.2.2.5 Store at RT.
- 6.2.3 Solution C: Milli Q H₂O
 - 6.2.3.1 Filter 1 L of fresh Milli Q H₂O with 0.45 µm membrane
 - 6.2.3.2 Degas for 20 min. Transfer into a 1 L buffer bottle
 - 6.2.3.3 Keep at RT.

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ATTACHMENT # 2

<u>ANIMAL CELL TECHNOLOGY AND DOWNSTREAM PROCESSING GROUP</u>	
DEVELOPMENTAL OPERATING PROCEDURE	
Title: Preparation of a New UNO™Q Polishing Column	Page 1 of
Date: July 09, 2001	Version No: 01
Written by: Julia Transfiguracion	

1.0 Objective

To equilibrate and condition a new UNO™Q Polishing column before use

2.0 Scope

This procedure describes how to prepare a new column that is used for the quantification of total Adenovirus Type 5 (Ad5) particles in crude lysate, semi-pure and pure samples

3.0 Responsibilities

3.1 It shall be the responsibility of the supervisor to:

3.1.1 Ensure that this procedure is followed by all Animal Cell Technology and Downstream Processing personnel.

3.1.2 Ensure that the personnel working on the analyses are trained to work in a Biosafety Level 2 Laboratory (BSL-2)

- 3.1.3 Ensure that the security rules are applied at the Biotechnology Research Institute (BRI) concerning the shipping and receiving of biorisk-2 agent

4.0 Materials

- 4.1 Solutions (Refer to Attachment # 1 for the Solutions Preparation)
- 4.1.1 Solution A: 0.25 M HEPES, pH 7.5
 - 4.1.2 Solution B: 2 M NaCl in Milli Q H₂O
 - 4.1.3 Solution C: Milli Q H₂O
- 4.2 Bovine Serum Albumin (BSA) Standard. Chromatographic Specialties. Cat # 23209.
- 4.3 Ad Lysate Sample
- 4.4 0.45 µm GHP Acrodisc® syringe membrane. Pall Gelman Lab. Cat #. PN 4556.
- 4.5 1 mL syringe. B-D Biosciences. Cat # 309602.
- 4.6 1.5 mL eppendorf vials
- 4.7 0.5 µm PEEK frit in a ferrule. S.P.E. Ltd. Cat #. P-275X.
- 4.8 HPLC vials with inserts, springs, and self sealing septums

5.0 Equipments

- 5.1 HPLC System (Waters Ltd) equipped with a 996 PDA detector , 717^{plus} Autosampler, 600 Controller, In-Line Degasser and a Millennium³² version 3.05.01 software for data acquisition and peak integration.

5.2 UNO™Q Polishing column, 0.16 mL CV (BioRad Lab. Cat # 720-0009).

5.3 Centrifuge, Eppendorf 5415 C or equivalent

5.4 Microliter Pipettes and pipette tips: P20, P200, P1000

6.0 Procedure

6.1 BSA Standard Preparation: Stock Concentration: 2 mg/mL.

6.1.1 Pipet 300 µL into the HPLC sample vials

6.2 Lysate Sample Preparation

Note: Samples containing Ad are classified as BSL 2 agents and should be handled as biohazardous material. Work according to the BSL-2 guidelines. Properly dispose all waste accordingly.

6.2.1 Thaw the lysate preparation at $37^{\circ}\text{C} \pm 1^{\circ}\text{C}$.

6.2.2 Centrifuge 1 mL at 7,500 rpm (4610 xg) for 5 min

6.2.3 Collect the supernatant

6.2.4 Filter with 0.45 µm GHP Acrodisc® syringe filter

6.2.5 Pipet 100 µL into two HPLC vial inserts with self sealing septums

6.3 Turn on the HPLC system, 996 PDA, 717plus Autosampler, 600 Controller, In-Line Degasser, Computer, Printer. If column has been stored over the weekend or for a longer period of time, follow attachment # 3 on how to start up the system).

- 6.4 Connect the column as follows: (follow the arrow indicated on the column that indicates flow going downwards).
 - 6.4.1 Change the inlet frit prior to the installation of a new column.
 - 6.4.2 Remove the old ferrule and compressing ring from the inlet.
 - 6.4.3 Slide the nut, compressing ring and ferrule, in that order onto the tubing. The flattened end of the ring should face towards the nut.
 - 6.4.4 Manually in the 600 Controller, set gradient at 0% A, 0% B, 100 % C and 0% D. Set flow rate at 0.5 mL/min. Enter. Liquid should be coming out of the inlet tubing.
 - 6.4.5 Unscrew the upper part of the column and fill the inlet of the column with the eluent.
 - 6.4.6 Insert the nut and the ferrule assembly into the inlet of the column. Ensure to hold the tubing against the ferrule while tightening down the nut.
 - 6.4.7 Stop the flow
 - 6.4.8 Unscrew the bottom part of the column
 - 6.4.9 Set flow rate again at 0.5 mL/min at 100 % C.
 - 6.4.10 Visually inspect that the eluent flow is continuous
 - 6.4.11 Connect the column to the detector and run for 20 min.
 - 6.4.12 Check for any leakage. If there is no leakage, proceed to the next step, otherwise tighten the connectors.

- 6.5 Login to Millenium
- 6.6 Browse project. Select project. If project is new or create a new project by going to Configure System, File, New, Project, Name of Project etc..... Finish.
- 6.7 Open the project that you have selected
- 6.8 Open Quick Set (blue icon like a carousel)
- 6.9 Load samples (first button on left) from a previously created sample set. Click on **Column StartUp**. OK
- 6.10 Click on the green button (Run the sample set) The column equilibration will run for 90 min. Check for the pressure when the column is being equilibrated with 50 mM HEPES, pH 7.5, no salt at 1mL/min.
- 6.11 BSA Column Conditioning: 1 injection of 150 μ L of 2mg/mL BSA
 - 6.11.1 Load samples (first button on left) from a previously created sample set. Click on UNOQ_BSA_8. Make sure BSA standard is in the sample carousel.
 - 6.11.2 Click on the green button. Save with a filename
- 6.12 After the BSA conditioning is done, equilibrate the column with 20% A, 15% B and 65% C and monitor the baseline. Also check the pressure at 1 mL/min.
- 6.13 Run a buffer blank, 50 mM HEPES, pH 7.5 to make sure that the baseline is flat

6.14 Lysate Injection

6.14.1 Load samples (first button on the left) from previously created sample set. Click on Ad5_UNK_Lys.

6.14.2 Click on the green button. Save with a filename

6.15 Processing of Results

6.15.1 Go to Channels Tab

6.15.2 Select BSA 2mg/mL.150 µL (Sample Name).

6.15.3 Click on the Process button (fourth button on the left). Process using the acquisition method set. OK

6.15.4 Go to Results. Highlight on BSA 2mg/mL. 150 µL. Click the mouse button 2x to review. If review looks fine, close the review window.

6.15.5 Click on the printer icon for a hard copy of the results. Print using acquisition method set. OK

6.15.6 Do the same procedure for the processing of the lysate injection except that when you print the results you highlight both channels, one for OD260nm and the other OD280nm and select the report method Ad5 overlay 03a.

7.0 Acceptance Criteria

7.1 The BSA injected should elute as a very large peak between 6.8 to 7.0 min with an AU of > 0.25 with a shoulder at ~ 8 min.

- 7.2 The Ad5 peak in the lysate sample should resolve nicely from the adjacent DNA peak. The difference in retention time (RT) between the two peaks must be between 0.9 – 1.0 min.
- 7.3 The pressure for a new column when being equilibrated with 50 mM HEPES, pH 7.5 without salt as well as with 50 mM HEPES, pH 7.5, 300 mM NaCl and with a new frit at 1 mL/min should be < 300 psi.

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ATTACHMENT # 3

<u>ANIMAL CELL TECHNOLOGY AND DOWNSTREAM PROCESSING GROUP</u>	
DEVELOPMENTAL OPERATING PROCEDURE	
Title: HPLC System Start Up (F-65)	Page 1 of
Date: July 09, 2001	Version No: 01
Written by: Julia Transfiguracion	

1.0 Objective

To start the HPLC system used for the quantification of Adenovirus Type 5 (Ad5) total particles

2.0 Scope

This procedure applies only to the system that is used for the quantification of Ad5 total particles.

3.0 Responsibilities

3.1 It shall be the responsibility of the supervisor to:

- 3.1.1 Ensure that this procedure is followed by all Animal Cell Technology and Downstream Processing personnel.
- 3.1.2 Ensure that the personnel working on the analyses are trained to work in a Biosafety Level 2 Laboratory (BSL-2)

- 3.1.3 Ensure that the security rules are applied at the Biotechnology Research Institute (BRI) concerning the shipping and receiving of biorisk-2 agents.

4.0 Materials

4.1 Milli Q H₂O

4.2 Solutions (See attachment # 1 for the Solutions Preparation))

4.2.1 Solution A: 0.25 M HEPES, pH 7.5 (pH adjusted with 10 N NaOH)

4.2.2 Solution B: 2 M NaCl in Milli Q H₂O

4.2.3 Solution C: Milli Q H₂O

4.3 Priming Syringe (KHLOEN brand, 10 mL)

4.4 Polypropylene 250 mL beaker for liquid waste

5.0 Equipments

5.1 HPLC System (Waters, Milford, MA) equipped with a 996 Photodiode Array (PDA) Detector, 717 plus Autosampler, Waters 600 Controller, and Waters In-Line Degasser.

6.0 Procedure

6.1 Turn on the 996 PDA detector, 717^{plus} Autosampler, 600 Controller, In-Line Degasser, Computer.

6.2 Immerse Lines A, B and C into their respective solution reservoirs

6.3 Verify that the needle wash and plunger seal wash reservoirs are full. If not, fill them up.

- 6.4 Prime the pumps and fill the lines with their respective solutions as follows:
- 6.4.1 Place the beaker under the open tubing of the inlet manifold valve.
 - 6.4.2 Switch the reference valve to the right position (column bypass)
 - 6.4.3 In the 600 Controller, press on the "Direct" button. Type 100 % A, 0 % B, 0 % C and 0 % D to fill line A and a flow rate of 1.0 mL/min. Enter.
 - 6.4.4 Attach the priming syringe to the Luer fitting of the inlet manifold valve.
 - 6.4.5 Rotate the inlet manifold valve handle fully counterclockwise to the "draw" position.
 - 6.4.6 Draw off 10 mL of buffer A, rotate the handle to the "run" position, remove the syringe and discard the eluent into the beaker.
 - 6.4.7 Repeat step 6.4.6 two more times. At the 2nd eluent withdrawal, rotate the valve handle to the "Inj" position (fully clockwise) and apply slight pressure to force eluent through the pump heads.
 - 6.4.8 Rotate valve handle to the "Run" position and observe the flow. If flow is not constant, repeat this step, otherwise if flow is constant repeat this procedure using solution B for Line B and solution C for Line C.
 - 6.4.9 Switch the reference valve handle to the left hand position (no column attached yet).

- 6.5 In the 600 controller, type 0% A, 0% B, 100% C and 0% D, at 1 mL/min. ENTER. Run for 10 min. This is flushing the system fluid lines with Milli Q water.
- 6.5.1 Reduce flow rate to 0.5 mL/min
- 6.6 Compressibility Check and Needle Wash
 - 6.6.1 On the 717 Autosampler, go to "Diagnostic Page".
 - 6.6.2 Press on the "Purge Page", then on the "Start Purge".
 - 6.6.3 After the purging, check to see if compressibility check passed ("Yes" on the screen). If it is, go to the next step. If not, check to see if there is a leak somewhere.
 - 6.6.4 Still on the 717 Autosampler, and on the "Diagnostic Page" press on the "Usermaint Page", then on the "Needlewash Page" and on to the "Start Needlewash" button.
 - 6.6.5 After the needle wash, stop the flow and connect the column.

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ATTACHMENT # 4

<u>ANIMAL CELL TECHNOLOGY AND DOWNSTREAM PROCESSING GROUP</u>	
DEVELOPMENTAL OPERATING PROCEDURE	
Title: New Standard Curve Generation for the Quantification of Adenovirus Type 5(Ad) Total Particles	Page 1 of
Date: July 09, 2001	Version No: 01
Written by: Julia Transfiguracion	

1.0 Objective

To generate a new standard curve used for the quantification of Adenovirus Type 5 (Ad5) total particles using the UNO™Q polishing column

2.0 Scope

A recombinant Adenovirus Type 5 (rAd5) expressing the GFP protein which is purified by a 2-step CsCl density gradient centrifugation (Yumi Kanegae et. al., 1994) is used to generate the standard curve. The total particle concentration of the freshly purified virus is quantified according to the method of Maizel et. al., (1968) where 1 OD260 is multiplied by a factor of 1.1×10^{12} VP/mL. The particle concentration obtained by this method is then used as the reference concentration for the generation of the standard curve.

An assay control which comes from the same preparation is diluted to obtain a concentration of 5×10^{10} VP/mL (according to OD260) is quantified by HPLC using the newly created standard curve. The variation between the values obtained between the two different methods is evaluated. The stability of each of the CsCl

purified Ad5 standard is followed by the HPLC and the Gene Transfer Assays for total and infectious particles, respectively.

3.0 Responsibilities

3.1 It shall be the responsibility of the supervisor to:

3.1.1 Ensure that this procedure is followed by all Animal Cell Technology and Downstream Processing personnel.

3.1.2 Ensure that the personnel working on the analyses are trained to work in a Biosafety Level 2 Laboratory (BSL-2)

3.1.3 Ensure that the security rules are applied at the Biotechnology Research Institute (BRI) concerning the shipping and receiving of biorisk-2 agents.

4.0 Materials

4.1 Solutions (refer attachment # 1 for the Solutions Preparation)

4.1.1 Solution A: 0.25 M HEPES, pH 7.5

4.1.2 Solution B: 2 M NaCl in Milli Q H₂O

4.1.3 Solution C: Milli Q H₂O

4.2 50 mM HEPES, pH 7.5: Buffer blank

4.3 CsCl purified Ad5 stock (concentration of $\geq 1 \times 10^{12}$ VP/mL)

4.4 Ad5 Assay Control. 5×10^{10} VP/mL of a CsCl purified Ad5 standard

4.5 Microliter Pipettes and Tips: P20, P200 and P1000

4.6 1.5 mL microcentrifuge tubes

- 4.7 0.5 µm frit in a ferrule. S.P.E. Ltd. Cat #. P-275X
- 4.8 6 x32 mm borosilicate insert vials. CSC. Cat #. 03-CV, sample vials, self sealing septum, compression springs.

5.0 Equipments

- 5.1 HPLC System (Waters Ltd) equipped with a 996 PDA, 717^{plus} Autosampler 600 Controller, In-Line Degasser and a Millennium³² version 3.05.01 software for data acquisition and peak integration.
- 5.2 Conditioned UNO™Q Polishing column, 0.160 mL CV (BioRad Lab. Cat # 720-0009).

6.0 Procedure

- 6.1 Instrument Start Up (see attachment # 3 for procedure)
- 6.2 Login to Millenium³²
- 6.3 Browse Project. Select Project. If creating a New Project, go to Configure System, File, New, Project, Name of Project..... Finish
- 6.4 Go to Project Window. Create a New Method Set (from previously created method)
 - 6.4.1 Select a previously created instrument method, for example: UNOQ_Ad5_070200. Open this method and save it under a different filename, example: UNOQ_Ad5_yyddmm (year, day, month).

- 6.4.2 Select a previously created processing method, for example: UNOQ_Ad5_070200. Open this method and save it under a different filename, example: UNOQ_Ad5_yyddmm (year, day, month).

Note: If you are saving the processing method under a new filename but in the same project, a message will appear that says clear curve, copy curve or save curve. In the case where this message appears, just click on clear curve and resave the method under a new filename. This message though does not appear if you are resaving the method in a new project.

- 6.4.3 Select a previously created method set, for example: UNOQ_Ad5_070200. Open this method and replace the instrument method and the processing method with the newly saved instrument and processing methods and save it under a different filename, example: UNOQ_Ad5_yyddmm (year, day, month). For the report method, select Default _Amount_ Concentration. So now this is your new method set which you will replace with in the STD_ UNOQ_ Ad5_ Set Method in the Quick Set Window when you are ready to run the samples

- 6.5 Open the Quick Set Window
- 6.6 Equilibrate the column with the newly saved instrument method for 10 CV or more until baseline is straight .
- 6.7 Standards Preparation (to be performed in BSL2 Biological Hood)

Note: Samples containing Ad are classified as BSL 2 agents and should be handled as biohazardous material. Wear gloves, gowns, glasses when manipulating such samples. Properly dispose all waste accordingly.

- 6.7.1 Thaw 1 vial of CsCl purified Ad5 preparation at RT

6.7.2 Prepare the working solution (WS) as 1×10^{11} VP/mL . For

Note: Not all CsCl purified Ad5 standard preparations have the same concentrations. The concentration varies, so perform the dilution accordingly in such a way that the WS is 1×10^{11} VP/mL.

6.7.3 Dilute stock (example: Ad5 stock concentration is 1×10^{12} VP/mL) 1:10. Take 100 μ L of the stock and add 900 μ L of dilution buffer (50 mM HEPES, pH 7.5)

6.7.4 Mix the solution gently

6.7.5 Follow the table below

Ad5 Concentration (VP/mL)	Working Solution (μ L)	Dilution Buffer (μ L)	Total Volume (μ L)
1×10^{10}	20	180	200
3×10^{10}	60	140	200
5×10^{10}	100	100	200
7×10^{10}	140	60	200
1×10^{11}	200	0	200

6.7.6 Pipet all the 200 μ L volume for each of the standard into the HPLC sample vials .

6.7.7 Prepare 1 vial of the dilution buffer.

6.7.8 Put samples into the carousel. Follow the sequence of the sample set method: STD_UNOQ_Ad5 below when putting the samples in the carousel.

Vial #	Sample Name
1	Buffer Blank
2	Ad5_CsCl preparation_ 5 x 10 ¹⁰ VP/mL
1	Buffer Blank
3	Ad5_1x10 ¹⁰ VP/mL
1	Buffer Blank
4	Ad5_3x10 ¹⁰ VP/mL
1	Buffer Blank
5	Ad5_5x10 ¹⁰ VP/mL
1	Buffer Blank
6	Ad5_7x10 ¹⁰ VP/mL
1	Buffer Blank
7	Ad5_1x10 ¹¹ VP/mL
1	Buffer Blank

- 6.8 Stop the column equilibration and baseline monitoring.
- 6.9 Go to File, Load Sample, Load Using a Previously Created Sample Set method, OK.
- 6.10 Click on STD_UNOQ_Ad5, Open.
- 6.11 Change the existing Method Set/Report Method with the newly created Method Set/Report Method.
- 6.12 File, Save
- 6.13 Click on the green button to run the sample set.
- 6.14 Processing of Results
 - 6.14.1.1 Go to Project Window

- 6.14.1.2 Go to Channels
- 6.14.1.3 Filter by Standard Descending
- 6.14.1.4 Highlight from Ad5_1x10¹⁰ to Ad5 1 x 10¹¹ VP/mL
- 6.14.1.5 Click on Process acquisition method set
- 6.14.1.6 Go to Results
- 6.14.1.7 Highlight from Ad5_1x10¹⁰ to Ad5 1 x 10¹¹ VP/mL
- 6.14.1.8 Click on Review
- 6.14.1.9 Click on Window, Calibration Curve
- 6.14.1.10 Close the Window
- 6.14.1.11 Go to Curves
- 6.14.1.12 Select the newly created curve
- 6.14.1.13 Click on the print icon. OK.

7.0 Acceptance Criteria

- 7.1 The Ad5 assay control particle concentration obtained must be within $\pm 10\%$ of the particle concentration obtained with the OD260 method.
Range: 4.5×10^{10} to 5.5×10^{10} VP/mL.
- 7.2 The peak area of the duplicate injections of the assay performed before and after the standards injections must a rsd of $\leq 10\%$.

- 7.3 The correlation coefficient (r) = ≥ 0.9900
- 7.4 The relative standard deviation (rsd) of the triplicate injections for each of the standard must be $\leq 5\%$.
- 7.5 The slope of the curve must lie within the range of $\text{mean} \pm 2\text{sd}$. For a preparation whose stability has already been determined. For a new preparation, slope must be within 20% variation from a preparation whose stability has already been determined.

8.0 Results

- 8.1 Calculate the % variation of the obtained Ad5 assay control concentration with the reference concentration. Within or Out of Range.
- 8.2 Calculate the % rsd of the peak area of the replicate injection for each of the standards using the following formula:
- 8.3 $\% \text{ rsd} = \text{sd} / \text{mean} * 100$
where: sd = standard deviation
mean = summation of the peak area of the replicate injections divided by the # of injections.
- 8.4 Calculate the variation of the Ad5 assay control injected before and after the standards injections using the same formula as in 8.2
- 8.5 Correlation coefficient and slope can be found in the hard copy of the results
- 8.6 Record all results obtained in the HPLC of Ad5 red book.

9.0 References

- 9.1 Jacob V. Maizel, Jr, David O. White and Matthew D. Scharff. The Polypeptides of Adenovirus. I. Evidence for Multiple Protein Components in the Virion and a Comparison of Types 2, 7A and 12. *Virology* 36 115-125
- 9.2 Yumi, Kanegae, Miho Makimura, Isumu Saito. 1994. A simple and efficient method for the purification of infectious recombinant adenovirus. *Jpn J Med. Sci. Biol.* 47 157-166.