Quantitation of Viral Genome Titer of Affinity Chromatography purified Adeno Associated Viral Particles by qPCR

Approvals:

Preparer: Robin M. Zuck Date: 25APR24
Reviewer: Dr. Maggie Bryans Date: 10MAY24

1. Purpose:

2. Determine the viral genome titer of chromatography purified adeno-associated viral particles by quantitative PCR.

3. Scope and Applicability:

3.1. This method applies to the determination of the viral genome titer of viral particle samples that have been purified by affinity chromatography, using quantitative PCR.

4. Summary of Method:

4.1. Review the programmed template to be used on the qPCR instrument and make any needed edits to the plate map.
4.2. Prepare the standard curve dilutions needed.
4.3. Prepare the samples for analysis.
4.4. Prepare the needed volume of 50X Primer Mix.
4.5. Prepare the needed volume of Master Mix.
4.6. Prepare the reaction wells needed.
4.7. Load the plate and initiate the qPCR run.

5. References:

5.1. Takara AAVpro Titration Kit (for Real Time PCR) Ver.2 Product Manual
5.2. QuantStudio Design and Analysis Software User Guide, Publication Number MAN0010408, Revision B.0, https://www.ffclrp.usp.br/divulgacao/emu/real_time/Manuais/QuantStudioDesign_Analysis_Desktop_Software_UG.pdf

6. Definitions:

7. Precautions:

7.1. Use proper PPE, including a lab coat, gloves, safety glasses. If working on the benchtop wear a facemask.
7.2. When possible, this assay is best performed using a BSC or Laminar flow hood.
7.3. Thoroughly clean the work area to be used with a 10% bleach solution and then a 70% ethanol solution before performing the assay and again after completing the assay.
7.4. Clean pipettes with 10% bleach solution and then 70% ethanol before performing the assay and again after completing the assay.
7.5. Treat waste materials with 10% bleach and then dispose of in Biohazard waste.
7.6. Use of barrier tips to prevent aerosolization of sample solutions.
7.7. Care should be exercised in the handling of buffers and samples of biological materials, which may have harmful biological activity in case of accidental ingestion, needle stick etc.

8. Responsibilities:

8.1. It is the responsibility of the course instructor /lab assistant to ensure that this SOP is performed as described and to update the procedure when necessary.

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8.2. It is the responsibility of the students/technician to follow the SOP as described and to inform the instructor about any deviations or problems that may occur while performing the procedure.

9. Equipment and Materials:

9.1. 10% Bleach solution, (prepared fresh weekly)
9.2. 70% Ethanol solution
9.3. Ice and ice bucket
9.4. Cool Caddy PCR workstation, chilled from freezer
9.5. Timer
9.6. Heating block preheated to 70°C
9.7. 50ml autoclaved MilliQ water, (RNase/DNase free)
9.8. Autoclaved 1.5ml and 2.0 ml microfuge tubes, (RNase/DNase free)
9.9. Microfuge tube rack.
9.10. qPCR MicroAmp Endura Plate Optical 96 well plate, Applied biosystems reference number 4483485
9.11. QuantStudio 3 Real Time qPCR instrument
9.12. Eppendorf 5804R centrifuge and Eppendorf A-2-DWP rotor (for 96 well plates)
9.13. Pipettors, P1000, P200, P20, and P10
9.14. Sterile pipette tips, barrier tips recommended
9.15. Quantabio PerfeCTa SYBR Green FastMix, low ROX, Quantabio catalog #95074-250
9.16. Takara AAVpro Titration Kit for Real Time PCR ver.2, Takara catalog #6233
  9.16.1. 10X DNA Lysis Buffer
  9.16.2. Lysis Buffer
  9.16.3. AAV Forward Primer and AAV Reverse Primer
  9.16.4. Positive Control solution
  9.16.5. EASY Dilution Buffer

10. Procedure:

10.1. **Preparation**
10.2. Set the heating block to 70°C and add water to the wells as needed.
10.3. Prepare the workspace by cleaning the work surface first with the 10% bleach solution and then with the 70% ethanol solution.
10.4. Clean the pipettors to be used with 10% bleach solution and then with the 70% ethanol solution.
10.5. Assemble all necessary reagents and materials, keep samples and reagents on ice. The SYBR green dye is light sensitive, protect the tube from light.
10.6. Prepare a plate map to be used for the assay, indicating the location of the standard curve wells, the positive and negative control wells, and the sample wells along with any additional dilutions.
10.7. Turn on the power for the Real Time PCR instrument using the power switch on the back lower right corner.
10.8. Login to the Instrument using the instrument display screen.
  10.8.1. Tap the “sign in” icon.

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10.8.2. Tap sign in, tap username and then enter the pin number.

10.9. Login to the dedicated computer connected to the Quant Studio3 instrument as Instrument Administrator.

10.10. Click on the QuantStudio Design and Analysis Software Icon on the lower left corner of the screen to open the Design and Analysis software.

10.11. On the software screen click on the "Open Existing Experiment" button, when using an existing template.

10.12. Navigate to the existing template file by choosing,

10.12.1. This PC, C: drive, and Template folder and the name of the template file to be used.
Note: Template files have a file type of ".edt".

10.13. Select the, "AAV2 Quantification SYBR-Takara qPCR.edt", template file and click on the OPEN button.

10.14. Review the Method and edit steps as needed. See **Attachment 1** for the method details.

10.15. Review the plate map add well details for the samples to be included in the assay and any additional controls

11. Prepare the Standard Curve by preparing dilutions of the positive control solution provided by kit.

11.1. Dilute the Positive Control, $2x10^7$ copies, using EASY Dilution buffer as follows in 5 1.5 ml microfuge tubes.

11.2. **NOTE:** the most concentrated standard is not diluted; it can be added directly to the qPCR plate later while preparing the plate.

11.3. Label 5 –1.5ml DNase/RNase free microfuge tubes as follows, 10^6, 10^5, 10^4, 10^3, 10^2 .

11.4. Add 45µl of Easy Dilution buffer to each of the 5 tubes.

11.5. **5 µl of 2 x 10^7 copies/µl (Positive Control solution) – Added directly to qPCR plate when preparing the plate**

11.5.1. (2) 2 x 10^6 copies/µl (5 µl of Positive Control solution + 45 µl of EASY Dilution buffer)

11.5.2. (3) 2 x 10^5 copies/µl (5 µl of (2) + 45 µl of EASY Dilution buffer)

11.5.3. (4) 2 x 10^4 copies/µl (5 µl of (3) + 45 µl of EASY Dilution buffer)

11.5.4. (5) 2 x 10^3 copies/µl (5 µl of (4) + 45 µl of EASY Dilution buffer)

11.5.5. (6) 2 x 10^2 copies/µl (5 µl of (5) + 45 µl of EASY Dilution buffer)

12. Sample Preparation, extraction of the viral genomes from the protein capsids

12.1. **All samples and reagents should be kept on ice or in the Cooled PCR station.**

12.2. For each unique viral particle sample, label a sterile, 2ml microfuge tube with sample identification.

12.3. For each sample prepare the following reaction mixture in the labeled 2 ml tube.

Solution	**Volume**
AAV2 particle sample solution	2 µl
10X DNase 1 Buffer	2 µl
DNase/RNase free water	16 µl
Lysis Buffer	20 µl
Total volume	40 µl

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12.4. Flick the tube gently to mix.
12.5. Incubate the sample tubes at 70°C for 10 minutes.
12.6. Dilute the vector genome solution 50-fold using Easy dilution Buffer. For a 50-fold dilution of the 40 µl of lysis reaction add 1960 µl of Easy Dilution to each sample reaction tube. This sample solution will be used directly as the sample template.
12.7. Place the prepared samples in the Cool Caddy or on ice.

13. Preparation of 50X Primer Mix
13.1. Label a 1.5 microfuge tube "50X Primer Mix".
13.2. Prepare immediately prior to use. Do not store.
13.3. Each well requires 0.5µl. Using the table below prepare enough 50X primer Mix for the number of wells to be used plus 10 extra wells.

	For 50 wells	For 25 wells
AAV2 Forward Titer Primer	5 µl	2.5 µl
AAV2 Reverse Titer Primer	5 µl	2.5 µl
TE or sterile dH_2O	15 µl	7.5 µl
Total volume	25 µl	12.5 µl

13.3.1. Mix the by gently flicking and place on ice.

14. Preparation of Reaction Master Mix.
14.1. Prepare enough volume for each sample, standard and control well plus 6 extra wells. Each well requires 20µl. **Prepare on ice.**
14.2. Label a 1.5 ml microtube, "Master Mix" and place on ice.
14.3. Add the appropriate volumes of the reagents listed in the table below to the labelled microfuge tube. Calculate the volume of Master Mix to prepare, by adding the number of sample wells, standard wells, and control wells together, to this number add an additional 6 wells. Multiply the calculated number of wells by the "Volume for 1 reaction" for each of the reagents listed in the table below and use the resulting volumes to prepare the Master Mix.
14.4. Once the reagents are combined, flick the tube to mix and place on ice.

Reagent	**Volume for 1 reaction**	**Number of wells + 6**	**Volumes needed**
SYBR Green	12.5µl		
50X Primer Mix	0.5µl		
dH_2O	7.0µl		
Total volume of Master Mix	20 µl		

15. Preparation of the reaction wells.
15.1. Place a 96 well qPCR plate on the plate holder of the Cool Caddy.
15.2. Using the prepared plate map, keeping the assay plate cold, and keeping as many of the wells that are not actively being prepared covered to prevent contamination.

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15.2.1. Add 20 µl of Master Mix to each of the wells that will contain a standard, control, or template sample.
15.2.2. Add 5 µl of the undiluted positive control to the designated $2X10^7$ Standard Curve well.
15.2.3. Add 5µl of each standard curve dilution to its designated well.
15.2.4. Add 5µl of each sample template to its designated well.
15.2.5. Add 5µl of DNase/RNase free water to each of the Negative control wells.
15.2.6. Cover the plate with the Adhesive plate cover, take care to seal the cover to the plate especially at the edges.
15.3. Centrifuge the plate at 750xg 10°C for 2minutes.
15.4. Visually confirm that the liquid in each well is at the bottom of the well and free of bubbles. If not, centrifuge the plate again.
15.5. Keep the reaction plate at 4°C and in the dark until ready to load the plate into the instrument.

16. Start the run.
16.1. Load the plate into the QuantStudio 3 drawer, confirm that well A1 is in the upper left corner. Tap the triangle icon in the upper right corner of the instrument display screen to open/close the drawer.
16.2. On the computer screen, click on the RUN tab in the "Workflow Bar", at the top of the screen.
16.3. On the RUN screen, click on the "Start RUN" button then double click on the box displaying the instrument serial number.
16.4. In the window that opens type a name for the run data file and click, "SAVE". The run should now start. The assay runs for 55minutes.The assay progress can be watched in real time on either the instrument display or the computer screen.

17. Transfer the data file.
17.1. Once the assay has finished the screen on the instrument will prompt you to transfer the data file to the PC. Tap on "TRANSFER", then choose a destination folder from the dropdown list, USB flashdrive
17.2. Remove the plate from the Instrument and close the plate holder.
17.3. Navigate to the results file on the computer (via USB), open the file name entered when starting the run with a file type of ".eds", stored in the folder chosen for the transfer.
17.4. Analyze data and print report. Go to file, select print report, select all, preview, save as PDF, select location.

18. Attachments/Figures

18.1. **qPCR method details**

STEP	Temperature °C	Duration	Number of Cycles
initial denaturation	95	2 minutes	
denaturation	95	5 seconds	35
anneal/extension	60	30 seconds detection of fluorescence	

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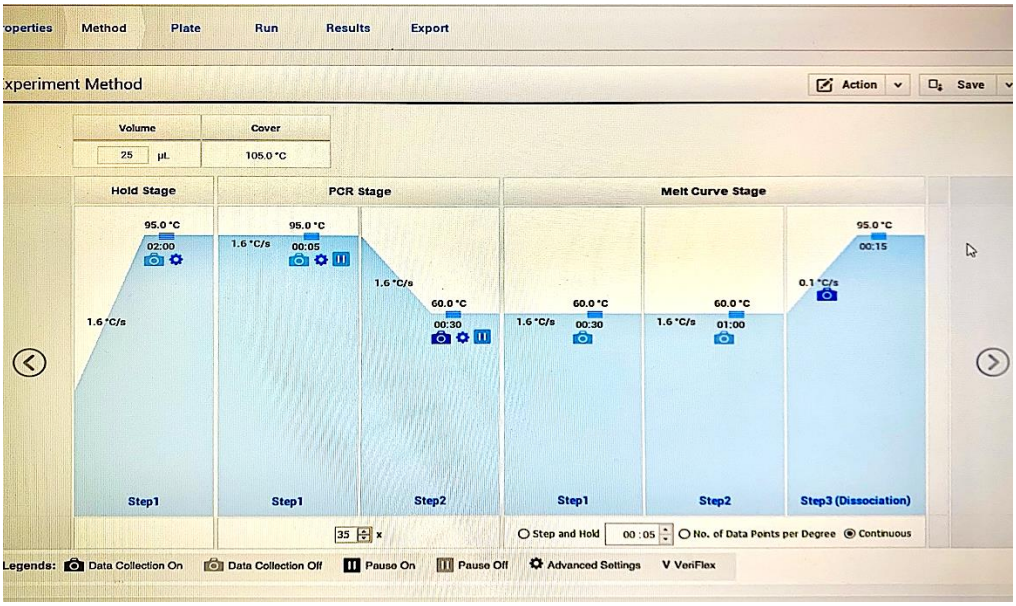
Fluorescence Melt curve (detection of fluorescence)



19. History:

Revision Number	Effective Date	Preparer	Description of Change
0	10MAY24	Robin Zuck	Initial Release