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AAV Quantification Kit

Product Insert

Product# 63800

Intended Use

Norgen's AAV Quantification Kit is designed for the detection of adeno-associated virus (AAV) inverted terminal repeat (ITR) sequences in a real-time PCR based on the use of TaqMan® technology. A purified standard based solely on the AAV2 ITR sequence purified using Norgen's proprietary technology simplifies the generation of a reliable standard curve for AAV quantification. Avoidance of a plasmid-based standard eliminates problems associated with efficient melting of the ITR sequence due to coupling of the ITR to the much longer plasmid sequence, as well as variability due to rearrangements/duplications/deletions of the recombination prone ITR. An easy and rapid method for viral DNA extraction simplifies the step of obtaining AAV DNA while simultaneously eliminating contaminating non-encapsidated DNA. Norgen's AAV Quantification Kit can facilitate pre-clinical studies that require accurate vector titration as well as interlab comparisons of vector quantities. This kit is designed for research use only and not for use in diagnostic procedures.

Background Information

AAV is a highly popular gene therapy vector. However, one major problem associated with its use is inter-lab and inter-assay variability with respect to accurate quantification due to differences in AAV standards, quantification techniques, extraction methods, etc. The Norgen AAV Quantification Kit aims to solve these problems by introducing a standard method for extraction, generation of a standard curve, and real time PCR setup. The PCR reaction is based on a previously reported universal assay specific for the AAV2 ITR, described by Aurnhammer *et al* (1) in 2012. Although the majority of recombinant AAV vectors utilize the AAV2 ITR, the qPCR assay can also cross-react with ITRs from AAV1, AAV3, AAV6, and AAV7 (1). As put forth by D'Costa *et al* in 2016 (2), one limitation of the plasmid based standard curve utilized by Aurnhammer might be that plasmid sequences adjacent to the ITR inhibit the complete melting of the palindromic ITR, resulting in an artificial increase in apparent vector titer. The standard used in the Norgen AAV Quantification Kit represents a 62 bp amplicon that covers only the region amplified in the qPCR assay, thereby eliminating the possibility of adjacent sequences affecting amplification. The standard is purified using Norgen's proprietary methods, ensuring a high degree of purity.

Product Description

Norgen's AAV Quantification Kit comprises Master Mix for the target and PCR control detection, Primer & Probe Mix, as well as a purified and quantified AAV standard that can be used to generate a standard curve through serial dilution. A negative control (nuclease-free water) is also included to confirm the integrity of the kit reagents.

Norgen's AAV Quantification Kit also contains reagents necessary for DNAse digestion of non-encapsidated DNA, including a digestion buffer and DNAse, as well as DNAse stop solution. A heating step facilitates rapid AAV vector DNA extraction without any losses due to excessive handling. The full protocol consisting of DNAse digestion, AAV extraction, and qPCR measurement can be done in approximately 2 hours.

Kit Components

Component	Cat. 63800 (24 rxns)
Enzyme Incubation Buffer A	500 µL
DNAse I	25 µL
DNAse Stop Solution	100 µL
2X PCR Mastermix	350 µL
AAV Primer & Probe Mix	90 µL
AAV DNA Standard	6 µL
Nuclease-Free Water (Negative control)	1.25 mL
Product Insert	1

Storage Conditions and Product Stability

- All kit components should be stored at -20°C upon arrival
- Repeated thawing and freezing (> 2 x) of the Master Mix, Primer/Probe Mix, and AAV Standard should be avoided, as this may affect the performance of the assay. If the reagents are to be used only intermittently, they should be frozen in aliquots.
- All reagents can be stored for 1 year at -20°C without showing any reduction in performance.

Customer-Supplied Reagents and Equipment

- Appropriate Real-Time PCR Instrument with FAM or SYBR channel
- Disposable powder-free gloves
- Micropipettors
- Sterile pipette tips with filters
- PCR tubes
- PCR reaction preparation station (Optional)

Quality Control

In accordance with Norgen's ISO 9001 and ISO 13485-certified Quality Management System, each lot of Norgen's AAV Quantification Kit is tested against predetermined specifications to ensure consistent product quality.

Warnings and Precautions

- Norgen's AAV Quantification Kit is intended for research purposes only. It is not intended for diagnostic use.
- Follow universal precautions. All specimens should be considered as potentially infectious and handled accordingly.
- Ensure that a suitable lab coat, disposable gloves and protective goggles are worn when handling specimens and kit reagents.
- Use sterile pipette tips with filters. Use proper pipetting techniques and maintain the same pipetting pattern throughout the procedure to ensure optimal and reproducible values.

- As contamination of specimens or reagents can produce erroneous results, it is essential to use aseptic techniques. Pipette and handle reagents carefully to avoid mixing of the samples.
- Do not use supplies and equipment across the dedicated areas of i) specimen extraction, ii) reaction set-up and iii) amplification/detection. No cross-movement should be allowed between the different areas. Personal protective equipment, such as laboratory coats and disposable gloves, should be area specific.
- Store and extract positive material (specimens, controls and amplicons) separately from all other reagents and add it to the reaction mix in a spatially separated facility.
- Dispose of unused kit reagents and specimens according to local, provincial or federal regulations.
- Do not substitute or mix reagents from different kit lots or from other manufacturers. Do not use components of the kit that have been stored for more than 1 year.
- The presence of PCR inhibitors may cause false negative or invalid results.
- Potential mutations within the target regions of the AAV genome covered by the primers in this kit may result in failure to detect the presence of the vector.
- Good laboratory practice is essential for the proper performance of this kit. Ensure that the purity of the kit and reactions is maintained at all times, and closely monitor all reagents for contamination. Do not use any reagents that appear to be contaminated.
- Ensure that appropriate specimen collection, transport, storage and processing techniques are followed for optimal performance of this test.

Instructions for Use

A. Sample Preparation

Purified AAV is the ideal starting material for the Norgen AAV Quantification Kit. We recommend the use of **Norgen's AAV Purification Kits (Cat No. 63200 and 63300)** for AAV purification applications. However, a crude input of cells or supernatant from producer cells transfected with AAV production plasmids can also be used as starting material. This type of input may be contaminated with plasmid DNA containing the ITR sequence that is the target of the qPCR reaction, which may decrease the Ct value and inflate the apparent AAV titer. For this reason, we recommend an increase in the DNAse incubation time to at least 2 hours for crude samples containing large amounts of non-encapsidated DNA. Alternately, additional units of DNAse may be added.

AAV DNA Extraction

a. Add 5-13 µL of purified AAV sample or crude AAV sample (remember to account for the volume added in subsequent calculations), 4 µL Enzyme Incubation Buffer A, and 1 µL Norgen DNAse I (2 U) to a PCR tube. For very high titer samples (>10^{12} gc/mL), it may be necessary to run a dilution series of the sample to ensure that the Ct values fall within the range of the standard curve.

b. Bring the final volume to 18 µL with nuclease free water (See Table 1 below).

Table 1. AAV DNA Extraction

Reagent	Volume added
AAV Sample	5 to 13 µL
Enzyme incubation buffer	4 µL
DNAse I	1 µL
Nuclease free water	0 to 8 µL
Final Volume	**18 µL**

c. Incubate for 30 minutes at 37°C in an incubator or thermocycler.
d. Add 2 µL of DNAse Stop Solution after incubation.
e. Heat at 90°C for 20 minutes in a thermocycler.
f. For best results, use DNA immediately in the qPCR assay.
g. DNA can be stored at 4°C or -20°C for short term storage (1-3 days), or at -80°C for long term storage.

B. TaqMan PCR Assay Preparation

Notes:

- **The 2X PCR Mastermix provided with this kit does not contain a ROX passive reference dye. Should your instrument utilize ROX to normalize PCR fluorescence please supplement the PCR reaction with ROX to the final concentration based on the instrument manufacturers' recommendations**
- Before use, suitable amounts of all TaqMan PCR components should be completely thawed at room temperature, mixed by gentle vortexing or by pipetting, and centrifuged briefly.
- Work quickly on ice.
- The amount of 2X PCR Master Mix provided is enough for up to 33 PCR reactions (24 sample PCRs, 8 AAV Standard curve PCRs and 1 no template control PCR).
- For best results, one reaction should serve as a no template control (negative control) and at least 4-8 reactions should serve as standards for generation of a standard curve.
- The recommended minimum number of DNA samples tested per TaqMan PCR run is 6.
- To avoid any contamination while preparing the TaqMan PCR assay, follow the order outlined in Tables 1, 2 and 3 below to prepare the Negative Control, Detection Assay and Positive Control:
    1. Prepare the PCR Negative Control (Table 2)
    2. Prepare the PCR AAV Samples (Table 3)
    3. Prepare the AAV PCR Standards (Table 5)
- To avoid cross-contamination, add the components to the PCR tubes in the order shown in the tables below (ie: 1) Nuclease-free water; 2) Master Mix; 3) Primer & Probe Mix; and 4) the Sample DNA or Positive Control).

1. For each TaqMan PCR run, prepare a no template control reaction as shown in Table 2 below:

Table 2. TaqMan PCR Negative Control Preparation

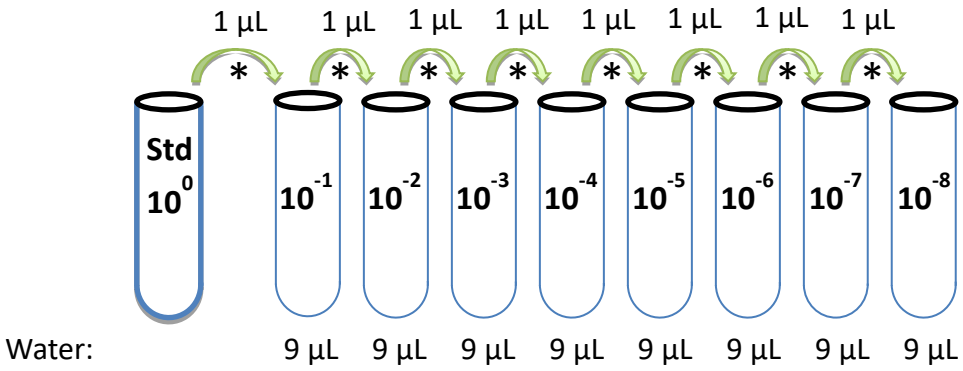
PCR Components	Target detection (with 2x PCR Master Mix)
Nuclease-Free Water	7.6 µL
2X PCR Master Mix	10 µL
AAV Primer & Probe Mix	2.4 µL
Total Volume	20 µL

2. Prepare the PCR reaction for sample quantification as shown in Table 3 below.

Table 3. TaqMan PCR AAV Assay Preparation

PCR Components	Target detection (with 2x PCR Master Mix)
Nuclease-Free Water	6.6 µL
2X PCR Master Mix	10 µL
AAV Primer & Probe Mix	2.4 µL
AAV Sample DNA	1 µL
Total Volume	20 µL

3. For each run, we recommend preparing a fresh dilution series using the provided AAV standard and nuclease free water. Make 10-fold serial dilutions ranging from 1 in 10 (10^{-1}) to 1 in 100000000 (10^{-8}) to use as template for the generation of a standard curve (see Figure 1 below). Using the appropriate micropipette, add 1 µL of AAV Standard to 9 µL of nuclease free water to make a 10 fold dilution of AAV Standard. Mix well by pipetting up and down repeatedly or by flicking tube. Obtain a new pipette tip and add 1 µL of the 10 fold dilution to the next tube containing 9 µL of nuclease free water. Repeat this process until serial dilutions of up to eight 10-fold dilutions (up to 1 X 10^{-8}) have been made. The standard curve is most linear over a range of 10^{-1} to 10^{-8}, ranging from an approximate copy number of 2 X 10^{3} to 2 X 10^{10}. Set up standard qPCR reactions as shown in Table 5, by adding 1 µL of diluted standard to each reaction.



* Remember to mix well and change to a new pipette tip with every dilution

Figure 1. Procedure for dilution of AAV standard into serial dilutions ranging from 10^{-1} to 10^{-8}

Table 4. Approximate Copy Numbers for AAV Stds After Dilution

AAV Std Dilution	Copy Number (per 1 µL)
1 X 10^{0}	2 X 10^{11}
1 X 10^{-1}	2 X 10^{10}
1 X 10^{-2}	2 X 10^{9}
1 X 10^{-3}	2 X 10^{8}
1 X 10^{-4}	2 X 10^{7}
1 X 10^{-5}	2 X 10^{6}
1 X 10^{-6}	2 X 10^{5}
1 X 10^{-7}	2 X 10^{4}
1 X 10^{-8}	2 X 10^{3}

C. AAV PCR Standards Preparation

Table 5. TaqMan PCR Positive Control Preparation

PCR Components	Target detection (with 2x PCR Master Mix)
Nuclease-Free Water	6.6 µL
2X PCR Master Mix	10 µL
AAV Primer & Probe Mix	2.4 µL
AAV Diluted Standard	1 µL
Total Volume	20 µL

D. AAV TaqMan PCR Assay Programming

1. Program the thermocycler according to the program shown in Table 6 below.

Table 6. AAV TaqMan PCR Program

Fluorophore: FAM/SYBR

PCR Cycle	PCR Step	Step	Temperature	Duration
Cycle 1 (x1)	Initial denaturation	Step 1	95°C	2 min
Cycle 2 (x40)	Denaturation	Step 1	95°C	10 sec
	Extension	Step 2	60°C	30 sec
	Plate Read	Step 3		

E. AAV TaqMan PCR Assay Interpretation

a. Plot an X-Y scatter plot, with Ct values for the diluted AAV standards on the Y axis and copy number on the X axis. A logarithmic trendline can be used to approximate the curve and determine an equation and R^2 value. An R^2 value of 1 or approaching 1 indicates a good fit of the trend line to the data, and suggests accurate pipetting for the dilution series. The equation can be rearranged to predict the copy number for the AAV samples by substituting y for the Ct value derived from the sample qPCR reaction. Depending on the initial volume of sample added, determine the dilution factor to multiply by to get the copy number for the entire sample.

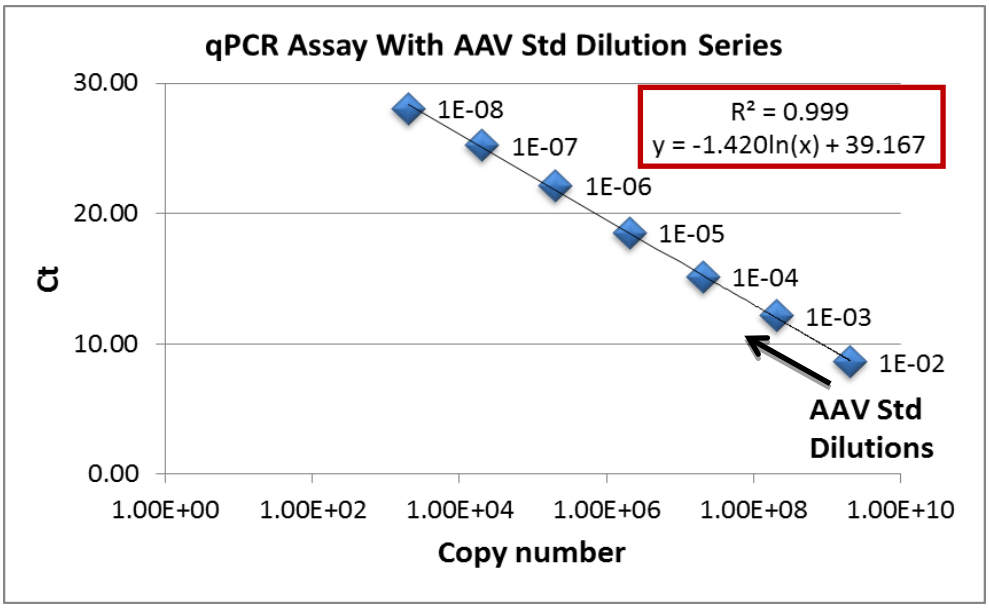


Figure 2 – AAV qPCR Assay on diluted AAV Standards from 10^{-2} to 10^{-8} dilutions. Ct values are plotted on the y-axis versus copy number on the x-axis. A logarithmic regression was applied to the data points to create a line of best fit that could be represented by an equation. An R-squared value close to one indicates that the trend line is a good fit for the data.

b. For example, for 5 µL of sample DNA added to 20 µL final volume during the extraction stage, one would multiply by 4 since the original AAV sample is being diluted 4 fold, and then divide by 5 to get a per µL amount. This can be multiplied by the number of µL of the original sample, for example, X 200 for a 200 µL sample to get the total amount within the original sample. This value can then be multiplied by 2 since AAV vector genomes tend to exist as a mixed population of positive and negative sense single stranded copies and will anneal together during the extraction and initial PCR steps.

In this particular case, based on the AAV standard dilution series data, the equation was:

$$Ct = -1.420\ ln(Copy\ number) + 39.167$$

Rearranging the equation yields the relationship below:

$$Copy\ number = e^{\frac{Ct - 39.167}{-1.420}}$$

For example, substituting a Ct value of 15 into the equation:

$$Copy\ number = e^{\frac{15-39.167}{-1.420}}$$
$$Copy\ number = e^{17.019}$$
$$Copy\ number = 2.46 \times 10^{7}$$

If 5 µL of AAV vector was originally added during the extraction step, then one would multiply by 4 since there was a 4-fold dilution into the 20 µL final volume. To get to a per µL amount for the original sample, divide by the amount of µL sample added, in this case 5. Since the original sample was eluted AAV from the Norgen AAV Purification Mini Kit (Cat No. 63200), it was eluted into 200 µL. Therefore, to obtain the total sample titer, multiply by 200. Lastly, one could multiply by 2 since positive and negative single stranded AAV genomes will anneal together during extraction and initial PCR steps and thus artificially decrease the AAV ss genome copy number estimation.

$$AAV_{\ ss\ genomes} = \frac{2.46 \times 10^{7} \times 4}{5\ \mu L} \times 200\ \mu L \times 2\ (dsDNA\ to\ ssDNA)$$

$$AAV\ ss\ genomes = 7.87 \times 10^{9}$$

Therefore, according to the standard curve and subsequent calculations, a sample with a Ct value of 15 would have a total copy number of approximately 7.87 X 10^9 ss genomes.

Related Products	Product #
AAV Purification Mini Kit	63200
AAV Purification Midi Kit	63300

References

(1) Aurnhammer C, Haase M, Muether N, Hausl M, Rauschhuber C, Huber I, Nitschko H, Busch U, Sing A, Ehrhardt A, Baiker A. 2012. Universal real-time PCR for the detection and quantification of adeno-associated virus serotype 2-derived inverted terminal repeat sequences. Hum. Gene Ther. Methods 23:18–28.

(2) D'Costa S, Blouin V, Broucque F, Penaud-Budloo M, François A, Perez IC, Le Bec C, Moullier P, Snyder RO, Ayuso E. 2016. Practical utilization of recombinant AAV vector reference standards: focus on vector genomes titration by free ITR qPCR. Mol Ther Methods Clin Dev. 16019.

Technical Support

Contact our Technical Support Team between the hours of 8:30 and 5:30 (Eastern Standard Time) at (905) 227-8848 or Toll Free at 1-866-667-4362.

Technical support can also be obtained from our website (www.norgenbiotek.com) or through email at techsupport@norgenbiotek.com.

Product Use Restriction

Norgen's AAV Quantification Kit is designed for the detection of AAV specific DNA in a real-time PCR based on the use of TaqMan technology. This kit is designed for research use only and not for use in diagnostic procedures.

Norgen's AAV Quantification Kit is intended for use by professional users such as technicians and biologists experienced and trained in molecular biological techniques including PCR.

Good laboratory practice is essential for the proper performance of this kit. Ensure that the purity of the kit and reactions is maintained at all times, and closely monitor all reagents for contamination. Do not use any reagents that appear to be contaminated.

Ensure that appropriate sample collection, transport, storage and processing techniques are followed for optimal performance of this test.

The presence of PCR inhibitors may cause false negative or invalid results.

The respective user is liable for any and all damages resulting from application of Norgen's AAV Quantification Kit for use deviating from the intended use as specified in the user manual.

All products sold by Norgen Biotek are subjected to extensive quality control procedures and are warranted to perform as described when used correctly. Any problems should be reported immediately. The kit contents are for laboratory use only, and they must be stored in the laboratory and must not be used for purposes other than intended. The kit contents are unfit for consumption.

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