SHENTEK

rcAAV-5/N Quantitation Kit

User Guide

Version: A/2
For Research Use Only
Product No.: 1403445
Reagents for 2×100 Reactions

Huzhou Shenke Biotechnology Co., Ltd

(IMPORTANT: Please read this document carefully before experiment.)

1. Product information

■ Product description

SHENTEK® rcAAV Quantitation Kit is suitable for qPCR detection of replication-competent adeno-associated virus (rcAAV) from cell culture harvested bulk and purified stock. This Kit is designed for the quantification of rcAAV-5/N contamination in serotypes of rAAV-5/N (N stands for possible different capsid serotypes). The sample types include but are not limited to recombinant adeno-associated virus (rAAV) bulk and end-products, as well as harvested samples from cell culture desired for rcAAV detection.

Key information before using this kit:

- AAV serotypes
- The inverted terminal repeat (ITR) sequence of the test sample rAAV need to match the following sequences:

> ITR sequence of rAAV-5/N
CTCTCCCCCCTGTCGCGTTCGCTCGCTCGCTGGCTCGTTTGGGGGGGTGG
CAGCTCAAAGAGCTGCCAGACGACGGCCCTCTGGCCGTCGCCCCCCCAA
ACGAGCCAGCGAGCGAGCGAACGCGACAGGGGGGAGAGTGCCACACTC
TCAAGCAAGGGGGT

■ Kit contents and storage

WARNING: Please read the Material Safety Data Sheets (MSDSs) and follow the handling instructions. Wear appropriate protective eyewear, mask, clothing and gloves.

Table 1. Kit components and storage

Reagent	Part No.	Quantity	Storage
T&R DNA Control-5	NNA028	lyophilized powder × 1 tube	-20℃
rcAAV qPCR Reaction Buffer	NNB009	400 μL × 4 tubes	-20℃, protect from light
Target Primer&Probe MIX-5	NNC050	200 μL × 1 tube	
Reference Primer&Probe MIX-5	NNC051	200 μL × 1 tube	
100×ROX	NND007	20 μL × 1 tube	
DNA Dilution Buffer (DDB)	NND001	1.5 mL × 4 tubes	-20℃
ddH_2O	NND010	1 mL × 1 tube	

The kit components can be stored at appropriate conditions for up to 24 months. Please check the expiration date on the labels.

■ Applied instruments, including but not limited to the following

- 7500 Real-Time PCR system
- CFX96 Real-Time PCR system
- SHENTEK-96S Real-Time PCR system
- Roche 480 Real-Time PCR system

■ Required materials not included in the kit

- Nonstick, DNase-free & Low Retention Microfuge Tubes of 1.5 mL
- Nonstick, Low Retention Tips: 1000 μL, 100 μL and10 μL
- 96-well qPCR plates with sealing film or PCR 8-strip tubes with caps
- SHENTEK® Virus DNA & RNA Extraction Kit (Product No. 1506730)

■ Related equipments

- Benchtop microcentrifuge
- Real-Time PCR system
- Vortex mixer
- Micropipettes: 1000 μL, 100 μL and 10 μL

■ Instructions for extraction and test

1. Samples start from harvested bulk

1.1 Extraction

(1) Refer to the user guide of Virus DNA & RNA Extraction Kit for specific steps.

(2) Benzonase Nuclease treatment of recombinant adeno-associated virus prior to extraction of the stock or final product is required to protect from interference of nucleic acid fragments that are not protected by viral capsid. Benzonase treatment ensures more accurate results for the direct qPCR method.

1.2 Test

Depending on the gene being tested, different dilutions of purified sample solution

are required:

(1) When detecting the Reference gene, first dilute the purified sample solution into the range of standard curve (20 - 2×10^6 copies/uL).

(2) When detecting the Target gene, no dilution of the sample purification solution is required.

For example, if the concentration of rAAV virus stock solution is about 2×10^{12} vg*/mL, then purified sample solution can be directly used for Target gene detection, but need to be diluted at least 1000 times before Reference gene detection.*

2. Samples start from cultured cells

(1) Titer of virus stock or final product can be quantified prior to infection of host cells, the sample can be extracted and purified according to the extraction procedure in 1.1, and use Reference gene sets to determine the concentration of rAAV;

Note: Dilute the extracted and purified solution of the test sample into the range of standard curve (20-2×10^6 *copies/uL).*

(2) After cell culture, the extraction process of test samples refer to the instruction manual of Virus DNA & RNA Extraction Kit for specific steps. the concentration of rcAAV was determined in using Target gene alone.

2. Methods

■ Experiment preparation

1. Irradiate the tabletop, pipettes and tubes with UV for 30 minutes, and disinfect with 75% alcohol.

2. Thaw the kit completely at 2-8°C or melt on ice, vortex and spin briefly.

■ DNA Control serial dilutions for the standard curve

For the first use, Please check the concentration labeled on the tube containing the T&R DNA Control-5 prior to dilution. Centrifuge the T&R DNA Control-5 at 12000 rpm for 1 min. Then pipette precisely 55uL ddH_2O to the bottom of the tube to dissolve the lyophilized powder.

Note:To ensure that the lyophilized powder is fully dissolved, centrifuge briefly for 3-5 seconds, then gently invert to mix well, and repeat 3 times.If it stands for a long time, remix by repeating the above step once..

1. Thaw DDB completely at 2-8°C or melt on ice. Vortex to mix well and quickly spin down the reagents for 3-5 seconds in microcentrifuge, and repeat 3 times.
2. Label seven nonstick 1.5 mL centrifuge tubes: ST0, ST1, ST2, ST3, ST4, ST5 and ST6.
3. Dilute the DNA Control to 2×10^8 copies/uL with DDB in ST tube. Vortex to mix well and quickly spin down the reagents for 3-5 seconds in microcentrifuge, and repeat 3 times to mix thoroughly.
4. Add 90μL DDB to ST0 and 180 μL DDB to each tube of ST1, ST2, ST3, ST4, ST5 and ST6.
5. Perform the serial dilutions according to Table 2:

Table 2. Dilution for T&R DNA Control

Serial dilution tube	Dilution	Conc. (copies/μL)
ST0	10 μL ST + 90 μL DDB	2×10^7
ST1	20 μL ST0 + 180 μL DDB	2×10^6
ST2	20 μL ST1 + 180 μL DDB	2×10^5
ST3	20 μL ST2 + 180 μL DDB	2×10^4
ST4	20 μL ST3 + 180 μL DDB	2×10^3
ST5	20 μL ST4 + 180 μL DDB	2×10^2
ST6	20 μL ST5 + 180 μL DDB	2×10^1

- *The remaining unused DDB need to be stored at 2-8℃. If the solution is cloudy or contains precipitates, heat at 37℃ until it clear.*
- *At least five concentration of standard curve should be included. To select appropriate sample dilutions, we recommend to perform method validation before sample testing.*

■ qPCR MIX preparation

1. Determine the number of reaction wells based on your selected standard curve, with

the number of test samples and control samples. Generally, triplicates are tested for each sample.

- Number of Reference gene reaction wells = (6 standard points on the standard curve + 1 NTC + 1 NCS + 1 test samples) ×3
- Number of Target gene reaction wells = (6 standard points on the standard curve + 1 NTC + 1 NCS + 1 test samples) ×3

2. Prepare qPCR MIX referring to Table 3.

Table 3. qPCR MIX preparation

Reference gene		
Reagents	**Volume/reaction**	**Volume for 30 reaction (includes 10% overage)**
rcAAV qPCR Reaction Buffer	8 μL	264 μL
Reference Primer&Probe MIX-5	2 μL	66 μL
Total volume	10 μL	330 μL
Target gene		
Reagents	**Volume/reaction**	**Volume for 30 reaction (includes 10% overage)**
rcAAV qPCR Reaction Buffer	8 μL	264 μL
Target Primer&Probe MIX-5	2 μL	66 μL
100× ROX	0.04 μL*	1.32 μL
Total volume	10.04 μL	331.32

** For the use of ROX reference dye, whether to add 100× ROX for Target gene and its amount depend on the model of the quantitative PCR instrument you choose:*

- *For 7500 Real-Time PCR system, add 0.04μL ROX per reaction as indicated in Table 3;*
- *For SHENTEK-96S Real-Time PCR system, Roche 480 Real-Time PCR system and other models, no need to add ROX as a calibration fluorescence;*
- *For other models, the amount of ROX can be adjusted according to the instruction of instrument.*

3. Mix thoroughly and place on ice, aliquot 10 μL/well into 96-well qPCR plate or PCR 8-strip tubes.

■ qPCR Reaction MIX preparation

1. Prepare qPCR reactions following Table 4, and a 96-well plate layout template is shown in Table 5.

Table 4. qPCR Reaction MIX preparation

Tubes	ST	NTC	NCS	Test sample
qPCR MIX	10 μL	10 μL	10 μL	10 μL
Samples	20 μL ST1 - ST6	20 μL DDB	20 μL purified NCS	20 μL purified test sample
Total Volume	30 μL	30 μL	30 μL	30 μL

Table 5. Example of 96-well plate layout

NCS Reference	NCS Reference	NCS Reference										A
NTC Reference	NTC Reference	NTC Reference				ST6 Reference	ST6 Reference	ST6 Reference	ST6 Target	ST6 Target	ST6 Target	B
1/x S-Reference	1/x S-Reference	1/x S-Reference				ST5 Reference	ST5 Reference	ST5 Reference	ST5 Target	ST5 Target	ST5 Target	C
						ST4 Reference	ST4 Reference	ST4 Reference	ST4 Target	ST4 Target	ST4 Target	D
						ST3 Reference	ST3 Reference	ST3 Reference	ST3 Target	ST3 Target	ST3 Target	E
NCS Target	NCS Target	NCS Target				ST2 Reference	ST2 Reference	ST2 Reference	ST2 Target	ST2 Target	ST2 Target	F
NTC Target	NTC Target	NTC Target				ST1 Reference	ST1 Reference	ST1 Reference	ST1 Target	ST1 Target	ST1 Target	G
S-Target	S-Target	S-Target										H
1	2	3	4	5	6	7	8	9	10	11	12	

- *The example represents the detection of 6 concentration gradients of Reference gene standard curve (ST1-ST6) and Target gene standard curve (ST1-ST6), no template control NTC: NTC-Reference/NTC-Target, negative quality control NCS: NCS-Reference/NCS-Target, and diluted sample-Reference or Target, with 3 replicates for each sample.*
- *The plate layout for sample loading can be adjusted based on the sample quantity.*
- *For 1/x S-Reference wells, x refers to the number of dilution folds. The principle of dilution is that diluted test sample needs to be within the range of standard curve (20-2×10^6 copies/uL).*

■ qRT-PCR program setting

NOTE: The following instructions apply only to the ABI7500 instrument with SDS v1.4. If you use a different instrument or software, refer to the applicable instrument or software documentation.

1. Create a new document, then in the Assay drop-down list, select Standard Curve **(Absolute Quantitation)**.
2. In the Run Mode drop-down list, select **Standard 7500**, then click **Next.**
3. Click **New Detector:**

   a. Enter Target-5 in the Name field, Select **FAM** in the Reporter Dye drop-down list and select **none** in the Quencher Dye drop-down list, then click **OK**

   b. Enter Reference-5 in the Name field, Select **CY5** in the Reporter Dye drop-down list and select **none** in the Quencher Dye drop-down list, then click **OK**.

   c. Select a color for the detector.

   d. Select the detectors, then click **Add** to add the detectors to the document
4. Select **ROX** as the passive reference dye, then Click **Next**.
5. Select the applicable set of wells for the samples, then select Target-5 or Reference-5 detector for each well.
6. Select Finish, and then set thermal-cycling conditions:

   a. Set the thermal cycling reaction volume to 30 μL.

   b. Set the temperature and time as follows in Table 6:

Table 6. qRT-PCR running program

Step	Temp.	Time(mm:sec)	Cycles
Pre-mutability	95℃	10 :00	1
Activation	95℃	00 :15	40
Denature	60℃	00 :30	
Anneal/extend	72℃*	00 :30**	

* *Instrument will read the fluorescence signal during this step.*

*** For ABI 7500 instrument, please change this step from 72℃ 30s to 72℃ 34s, and keep 72℃ 30s for other qPCR instruments.*

■ Results analysis

1. Select **Set up** tab, then set tasks for each sample type by clicking on the Task Column drop-down list:
   a. NTC: target DNA detector task = **NTC**
   b. NCS, test samples: target DNA detector task = **Unknown**
2. Set up the standard curve as shown in the following table (Table 7):

Table 7. Settings for Standard curve

Tube label	Task	Quantity (copies/μL)
ST1	Standard	2×10^6
ST2	Standard	2×10^5
ST3	Standard	2×10^4
ST4	Standard	2×10^3
ST5	Standard	2×10^2
ST6	Standard	2×10^1

3. Select the **Results** tab, then select Amplification Plot.
4. In the Data drop-down list, select **Delta Rn vs Cycle.**
5. In the Analysis Settings window, enter the following settings:
   a. Select **Manual Ct**.
   b. In the Threshold field: Set the Threshold for the Reference-5 gene to 0.06; Set the Threshold for the Target-5 gene to 0.02
   c. Select **Automatic Baseline**.
6. Click the button ▶ in the toolbar, then wait the plate analyzing.
7. Select the **Result** tab**> >Standard curve** tab, then verify the Slope, Intercept and R^2 values.
8. Select the Report tab, then achieve the mean quantity and standard deviation for each sample.
9. Select **File > > Export > > Results**. In the Save as type drop-down list, select **Results Export Files**, then click **Save**.

10. In the Report panel of Results, the 'Mean Quantity' column shows the detection values of NTC, NCS, test sample, in copies/μL.

Note: The parameter settings of the result analysis should be based on the specific model and the software version, and generally can also be automatically interpreted by the instrument.

11. $Ct_{Reference}$ and Ct_{Target} values of NTC should be >35.00 or undetermined.
12. $Ct_{Reference}$ and Ct_{Target} values of NCS should be larger than the mean Ct value of the lowest standard curve.
13. The actual concentration of the test samples (copies/mL) need to be multiplied by its own dilution factors.
14. The contamination rate of rcAAV in rAAV is calculated according to the following equation:

Detection values of Target genes ÷ (Detection values of Reference genes × dilution factor ÷ 2).

Note: Each rAAV contains two copies of Reference gene.

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Support & Contact

SHENTEK

Huzhou Shenke Biotechnology Co., Ltd.
www.shentekbio.com
Address: No.1366 Hongfeng Road, Huzhou313000, Zhejiang Province, China
E-mail: info@shentekbio.com
Phone: +1 (908) 822-3199 / (+86) 400-878-2189