ATCC® Catalog No. VR-1816™

Recombinant Adeno-associated Virus 8 Reference Standard Stock (rAAV8-RSS)

Produced by the AAV Reference Standard Working Group (AAVRSWG)

Description and Background

The recombinant Adeno-associated Virus 8 Reference Standard Stock (rAAV8 RSS) consists of highly purified preparation formulated as a sterile liquid in dPBS at room temperature, and stored frozen at –80°C. The configuration is 0.125 ml per vial in polypropylene cryogenic vials with screw caps, external threads.

The purpose of the rAAV8 RSS is to define the particle, vector genome, and infectious units for adeno-associated virus 8 based gene vectors and establish a reference point for comparisons. It was developed under the guidance of the AAV Reference Standard Working Group (AAVRSWG), the U.S. Food and Drug Administration (FDA) and the European Directorate for the Quality of Medicines & HealthCare (EDQM); It was realized in the frame of the European Clinigene Network of Excellence (www.clinigene.eu) and through the donation of services and supplies by a large number of laboratories and organizations (see below).

More information regarding the development and characterization of the rAAV8 RSS can be found in the Virus Reference Standard section of the ATCC webpage and in several publications [1-3]

http://www.atcc.org/Standards/Standards_Programs/ATCC_Virus_Reference_Materials/AAV8_Additional_Information.aspx

Characterization

rAAV8 RSS lot #03112010SP2pcg was characterized for particles per ml, vector genomes per ml and infectious units per ml, and identity and purity by 16 laboratories worldwide, according to procedures established by the AAVRSWG, using frozen vials shipped to each laboratory on dry ice by ATCC and its distributors. Procedures used can be found in the Virus Reference Standards section of the ATCC web site. Results are summarized in Tables 1 and 2, and Figure 1.

The particle concentration in the AAV8 RSS lot #03112010SP2pcg is 5.50×10^{11} particles/ml, with 95% certainty that the true particle concentration lies within the range of 4.26×10^{11} to 6.75×10^{11} particles/ml The particle concentration was determined by each laboratory, using four separate dilution series from two rAAV8RSS vials in the Progen AAV8 Titration ELISA (Progen Biotechnik GMBH, Article number PRAAV8), against a standard curve prepared from a previously titered rAAV8 preparation. See posted protocol:

http://www.lgcstandards-atcc.org/~/media/AAV8_Information/AAV8%20RSM%20particle%20titer%20by%20ELISA.ashx

The number of vector genomes per ml is 5.75×10^{11} vector genomes/ml, with 95% certainty that the true particle concentration lies within the range of 3.05×10^{11} to 1.09×10^{12} vector genomes/ml. The vector genome concentration was determined in duplicate, testing one replicate from each of two vials, by quantitative PCR of serial dilutions of rAAV8 RSS against a standard curve of plasmid pTR-UF-11 (ATCC MBA-331™) [4]. See posted protocol:

http://www.lgcstandards-atcc.org/~/media/AAV8_Information/AAV8%20RSM%20genome%20copy%20titration%20by%20QPCR.ashx

The infectious titer on HeLa RC32 cells 1.26×10^{9} TCID50, with 95% certainty that the infectious titer on HeLa RC32 cells lies within the range of 6.46×10^{8} to 2.51×10^{9} TCID50 IU/ml . Values are based on independent testing of twelve replicates. Serial ten-fold dilutions of rAAV8 RSS were made on HeLa RC32 cells (ATCC CRL-2972™) and co-infected with Adenovirus type 5 (ATCC VR-1516™)[5]. Replicated vector DNA was analyzed by qPCR. See posted protocol:

http://www.lgcstandards-atcc.org/~/media/AAV8_Information/AAV8%20RSM%20Infectious%20titer%20assay.ashx

The purity and identity of the vector in rAAV8 RSS lot #03112010SP2pcg were evaluated by SDS polyacrylamide gel electrophoresis (SDS-PAGE), using SYPRO ruby or silver staining. The AAV8 VP1, VP2, and VP3 capsid protein bands were evaluated for their stoichoimetry and size. Purity relative to non-vector impurities visible on stained gels was determined to be greater than 99%. Vector identity was verified by observation of the electrophoretic banding pattern expected for AAV8. See posted protocol:

http://www.lgcstandards-atcc.org/~/media/AAV8_Information/AAV8_%20RSM_Identity-Purity_Assay.ashx

Table 1. Testing results for rAAV8 RSS Lot #03112010SP2pcg.

Titer units (Method)	Transformation*	Mean (*95% confidence interval*)
Particles per ml (ELISA)	untransformed	5.5E11 (4.26E11,6.75E11)
Vector genomes per ml (qPCR)	log(10)	5.75E11 (3.05E11 to 1.09E12)
Infectious Units per ml (TCID50)	log(10)	1.26E9 (6.46E8 ,2.51E9)

* used to better qualify the assumption of normal distribution for the purpose of determining mean values

Table 2. Physical parameters of rAAV8 RSS Lot #03112010SP2pcg.

Parameters Compared	RATIO
Particles : Vector Genomes**	0.95
Vector Genomes : Infectious Units	456.3
Particles : Infectious Units	436.5

** a measure of the ratio of total particles : full particles

Other Available Characterization Information

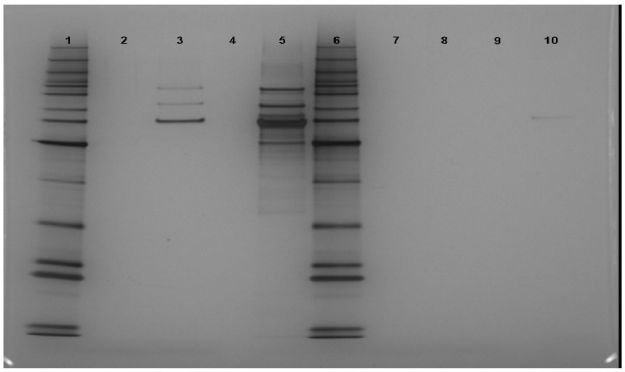
Prior to freezing, the filtered formulated bulk was sampled and tested for bioburden by SGS-Vitrology (Scotland, UK). The Total Aerobic Microbial Count (TAMC) was determined to be 0 cfu/ml and the Total Yeast/Mould Count (TYMC) was determined to be 0 cfu/ml. No inhibitory factors were detected in the test Material. The filtered formulated bulk was also tested for endotoxin and the content of endotoxin was less than 0.03 EU/ml when testing in a 1 in 5 dilution. No interference was detected at any dilution tested.

Growth of Mycoplasma species was not detected in the filtered purified bulk by either the culture method assay or the indicator cell culture method; no inhibitory factors should be present in the test article. Thus, the filtered purified bulk complies with the test for *Mycoplasma* as described Eu Pharm 6th Edition; section 2.6.7

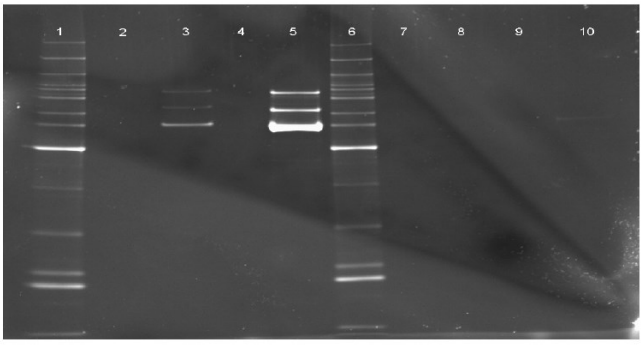
The vialed rAAV8 RSS (ATCC VR-1816 lot #03112010SP2pcg.) was tested for sterility at SGS-Vitrology. Growth of bacteria or fungi was not detected after 14 days incubation; no inhibitory factors were present. The rAAV8RSM complies with the test for sterility according to Eu Pharm 6th Edition; section 2.6.1 Sterility.

Figure 1. Purity evaluation of rAAV8-RSS Lot #03112010SP2pcg by SDS-PAGE

A. Silver staining



B. Sypro® ruby staining



C. Lane descriptions

Lane	Sample
1	Benchmark Ladder - denaturated
2	Negative Control - denaturated
3	AAV Reference Material - denaturated
4	blank lane
5	Positive Control - denaturated
6	Benchmark Ladder - Native
7	Negative Control - Native
8	AAV Reference Material - Native
9	blank lane
10	Positive Control - Native

Recommended Storage and Handling Information

Vials are shipped from ATCC on sufficient dry ice to maintain the product in frozen condition until received by the end user. Immediately upon receipt, store vials frozen at -70°C to -90°C.

It is important NOT to aliquot the RSM since this can result in significant loss of titer due to binding to the plastic ware.

For use in assays of infectivity (e. g. $TCID_{50}$), thaw vial(s) at room temperature while mixing gently. DO NOT VORTEX THE rAAV8 RSS. Keep thawed vial(s) on wet ice until use. Conduct infectivity assay(s) within 1 hour of thawing. Store the remainder of the thawed vial at 4°C and mix gently immediately prior to use.

Conduct physical assays (e. g. SDS-PAGE, capsid ELISA, or vector genome PCR assays) within 5 days of vial thaw.

Recommended Use of the Recombinant Adeno-associated Virus 8 Reference Standard Stock

The rAAV8 RSS is intended for use as a benchmarking tool to qualify and validate "in house" reference materials and assays used in basic, pre-clinical and clinical research employing recombinant adeno-associated virus 8 vectors, to support comparison of data across laboratories. Due to the wide variety of methods applied to vector characterization, and even the variability of data between laboratories using the same method, correlation of results from different sources is difficult, clouding interpretation of aggregated information. It is anticipated that introduction of a common Reference Material to characterize calibration reagents and to bridge assays will facilitate comparison. Each laboratory should characterize and qualify its own "in house" reference material using the rAAV8 RSS. Extensive validation work should be performed in each laboratory using the laboratory's qualified reference material, as the availability of the rAAV8 RSS is limited.

The FDA and EDQM recommends use of the rAAV8 RSS and the rAAV2 RSS (ATCC VR1616) [6] as common Reference Materials. This recommendation does not imply any intent to standardize assay methods across the field or to require that the values assigned to the rAAV8 RSS or rAAV2 RSS be duplicated during validation studies.

Recommended Vectors and Host Cells

Plasmid pTR-UF-11 [4], the recombinant AAV genome, contains the coding sequence for humanized GFP under the control of the synthetic CBA promoter and the SV40 polyadenylation signal, followed by the neomycin-resistance gene under the control of the mutant polyoma virus enhancer/promoter (PYF441) and the human bovine growth hormone poly(A) site, flanked by AAV2 ITRs. The HEK293 Master Cell Bank from EFS-ABG (Nantes, France) was co-transfected with vector plasmid pTR-UF-11 and the helper plasmid pDP8 to generate the rAAV8-RSS. pTR-UF-11 is available from ATCC, distributed as product number MBA-331™. The pDP8 helper plasmid harbors the rep gene from AAV2, the cap gene from AAV8, the adenovirus helper genes E2A, E4, VA-RNA and the ampicillin resistance gene. pD8 is available through Plasmidfactory (PlasmidFactory GmbH & Co, Biefield, Germany) as a product number PF478.

The cell line used for titration of infectivity of the rAAV8 RSS, HeLa RC32 [5], is a HeLa derivative which expresses the rep and cap genes for recombinant adeno-associated virus type 2 (rAAV-2). This cell line is distributed by ATCC as product number CRL-2972™.

Manufacture of the Adeno-Associated Virus 8 Reference Standard Stock

Production and purification of the rAAV8RSM were carried out at Atlantic Gene Therapies –UMR 1089 in Nantes (France) and the Center of Animal Biotechnology and Gene Therapy (CBATEG) at the Universitat Autonoma de Barcelona (UAB)(Spain) following a previously described method [7].

Production was initiated by co-transfection of a certified master cell bank of HEK293 cells in 5-chambers Corning Cellstacks (at Nantes) or Corning Roller Bottles (at Barcelone) with plasmid pTR-UF-11, [4] and the pDP8 helper plasmid (N° PF478, Plasmidfactory, Bielefeld, Germany) using a calcium phosphate precipitation method. Same lots of FBS (Hyclone-Thermo scientific, Waltham, MA), trypsin (PAA Laboratories GmbH, Linz , Austria), DMEM (PAA), Pen/Strep (PAA), PBS (PAA) and DPBS (PAA) were split between the two manufacturing sites.

For the transfection, the complete medium (DMEM, 10% FBS, 1% Pen/Strep) was removed and replaced by transfection medium (DMEM, 2% FBS, 1% Pen/Strep) including the transfection mixture. After 6 to 15 hours at 37°C and 5% CO2, the transfection medium was then removed and replaced by fresh exchange medium (DMEM, 1% Pen/Strep) prior to a 3 days incubation at 37°C and 5% CO2. The cells were harvested and centrifuged. The cell pellet was discarded and the supernatant was precipitated with PEG at a final concentration of 8% for a period of 15 hours to 3 days at 5 +/- 3°C. The PEG-precipitated supernatant was then centrifuged at 4°C. The supernatant was discarded and the PEG-pellet was resuspended in Tris Buffer Saline (TBS) before benzonase digestion.

Following benzonase digestion, the viral suspension was centrifuged at 4°C and the vector-containing supernatant was loaded on a step density CsCl gradient (1.5g/cm^3 at the bottom and 1.3g/cm^3 on top) in UltraClear tube for SW28 rotor (Beckman coulter, Brea, CA). The full particles band was collected with a 10mL syringe and transfered to a new UltraClear tube for SW41 rotor filled with 1.375 g/cm^3 density CsCl. The enriched-full particles band was then collected with a 10mL syringe. The viral suspension was then subjected to 4 successive rounds of dialysis against DPBS (containing Ca and Mg). Each purified vector sublot was finally collected, sampled for vg titer and purity assay, and stored at <-70°C in polypropylene low-binding cryovials.

Fill finish and final quality control were carried out at EFS-ABG (Nantes, France). Twenty five sublots produced at Nantes and Barcelona were combined and diluted to 200mL in DPBS. This purified bulk was subsequently tested for endotoxin (EP 2.6.14), Mycoplasma (EP 2.6.7) and Bioburden (EP 2.6.12) and the vector genome titer was determined by Q-PCR prior to final formulation, sterile filtration and filling in an ISO5 cleanroom at EFS-ABG with environmental monitoring. The purified bulk was diluted with 325mL of DPBS and was then sterile filtered. The filtered formulated bulk was then vialed in 14 sterile 50mL conical tubes and stored at < -70°C. The sterile bulk was then filled into 4088 cryovials with a volume of 0.125 mL/vial. Prior to storage at < -70°C, the vials were labeled as follows: ATCC®, VR-1816™, rAAV8-RSS– Reference Material, ABG[date] - 0.125mL, Store at < -70°C – For research use only. Lot #03112010SP2pcg. Due to the number of vials (> 4000 vials), seven fill days were required. Each fill date is indicated on the label

Stability data

A study was conducted to evaluate the stability of AAV8 vectors after dilution and repeated cycles of freeze and thaw, resembling the process that was used to combine the AAV8RSM sublots, the generation of the bulk and the final product.

Samples from 4 sublots of the AAV8RSM were thawed, diluted 1/20 in dPBS and aliquoted (T0, T1, T2, T3, T4). Sample T1 underwent to one additional cycle of freeze and thaw, while T2, T3 and T4, were frozen and thaw two, three and four times, respectively. Vector genome titration by qPCR and infectivity (GFU/ml) was measured in all samples (table 3).

As shown in table 3, no significant loss in vector genome titer or transducing units was observed under these conditions. Titers shown in the table are referred to the original samples and take into account the dilution factor 1/20.

Additional stability data for rAAV8 RSS Lot #03112010SP2pcg will be collected by yearly testing of vials for capsid protein integrity, infectious titer, and vector genomes per ml. Data will be reported by the AAVRSWG.

Table 3. Testing results for rAAV8 RSS subLots at thaw and under different storage conditions.

Sample ID	Cycles Freeze/thaw	Q PCR titration Vg/ml	GFU titer (HEK293 cells) GFU/ml
Sublot N1 - T0	0	4,64E+13	3 E+9
T1	1	2,77E+13	3 E+9
T2	2	2,77E+13	2,7 E+9
T3	3	2,63E+13	2,3 E+9
T4	4	2,59E+13	2,3 E+9
Sublot N5 - T0	0	5,65E+13	2,9 E+9
T1	1	ND	2,3 E+9
T2	2	ND	2,8 E+9
T3	3	4,34E+13	2,6 E+9
T4	4	3,63E+13	2,3 E+9
Sublot N9 - T0	0	4,60E+13	2,8 E+9
T1	1	2,43E+13	2,6 E+9
T2	2	1,79E+13	2,4 E+9
T3	3	2,70E+13	1,8 E+9
T4	4	1,79E+13	2,1 E+9
Sublot N11 - T0	0	5,07E+13	2,8 E+9
T1	1	ND	2,5 E+9
T2	2	ND	2,5 E+9
T3	3	3,67 E+13	2,7 E+9
T4	4	2,76 E+13	3,2 E+9

ND, not determined

References:

All information regarding the development and characterization of the recombinant Adeno-associated Virus 8 Reference Standard Stock can be found on the ATCC web site at http://www.lgcstandards-atcc.org/en/Standards/Standards_Programs/ATCC_Virus_Reference_Materials.aspx

Additional relevant publications are listed below.

1. Ayuso, E, Blouin, V, Darmon, C, Bosch, F, Lock, M, Snyder, R, et al. (2012). Reference materials for the characterization of adeno-associated viral vectors. In:

Cohen-haguenauer, O (ed). The Clinibook: clinical gene transfer state of the art. EDK: Paris. pp 83-90.
2. Moullier, P, and Snyder, RO (2012). Recombinant adeno-associated viral vector reference standards. Methods in enzymology 507: 297-311.
3. Moullier, P, and Snyder, RO (2008). International efforts for recombinant adeno-associated viral vector reference standards. Mol Ther 16: 1185-1188.
4. Burger, C, Gorbatyuk, OS, Velardo, MJ, Peden, CS, Williams, P, Zolotukhin, S, et al. (2004). Recombinant AAV viral vectors pseudotyped with viral capsids from serotypes 1, 2, and 5 display differential efficiency and cell tropism after delivery to different regions of the central nervous system. Mol Ther 10: 302-317.
5. Tessier, J, Chadeuf, G, Nony, P, Avet-Loiseau, H, Moullier, P, and Salvetti, A (2001). Characterization of adenovirus-induced inverted terminal repeat-independent amplification of integrated adeno-associated virus rep-cap sequences. J Virol 75: 375-383.
6. Lock, M, McGorray, S, Auricchio, A, Ayuso, E, Beecham, EJ, Blouin-Tavel, V, et al. (2010). Characterization of a recombinant adeno-associated virus type 2 Reference Standard Material. Hum Gene Ther 21: 1273-1285.
7. Ayuso, E, Mingozzi, F, Montane, J, Leon, X, Anguela, XM, Haurigot, V, et al. (2010). High AAV vector purity results in serotype- and tissue-independent enhancement of transduction efficiency. Gene Ther 17: 503-510.

Biosafety Level 2

Handle as a potentially biohazardous material under at least Biosafety Level 2 containment. Appropriate safety procedures should always be used with this material. See the National Institutes of Health publication, **Guidelines for Research Involving Recombinant DNA Molecules**. Detailed discussions of laboratory safety procedures are provided in the U.S. Government Publication **Biosafety in Microbiological and Biomedical Laboratories**, U.S. Department of Health and Human Services Centers for Disease Control and Prevention and National Institutes of Health , U.S. Dept. of Health and Human Services, 5th Edition, U.S. Government Printing Office, Washington, D.C: 2007. This information is available in its entirety on the Center for Disease Control Office of Health and Safety's website at http://www.cdc.gov/OD/ohs/biosfty/bmbl5/bmbl5toc.htm/.

Use Restrictions

This material is for laboratory research use only, and was sent with the condition that you are responsible for its safe storage, handling, and use. ATCC and AAVRSWG (including its members and their respective institutions) are not liable for damages or injuries resulting from receipt and/or use of the rAAV8 RSS material. The user assumes all risks associated with use.

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ATCC Replacement Policy

Please refer to the enclosed Material Transfer Agreement for ATCC warranty information. The Material Transfer Agreement is also available on our website http://www.atcc.org/.

Organizations and Laboratories That Donated Services and/or Materials, Participated in the Characterization Phase, or Otherwise Made Substantial Contributions Through Their Participation in the AAVRSWG

(Listed alphabetically)

ATCC (Manassas, VA)
Bioprocessing Journal (Virginia Beach, VA)
Childrens Hospital of Philadelphia (Philadelphia, PA)
Clean Cells (Bouféré, France)
Clinigene Network of excellence

Columbus Children's Research Institute (Columbus, OH)
Corning (Corning, NY)
Food and Drug Administration / Center for Biologics Evaluation and Research (FDA / CBER; Rockville, MD)
Etablissement français du sang-Atlantic BioGMP (Nantes, France)
Food and Drug Administration Office of Cellular, Tissue, and Gene Therapies (FDA; Rockville, MD)
GE Healthcare Life sciences (PAA Cell Culture Company, Germany)
Genethon (Évry, France)
German Cancer Research Center (Heidelberg, Germany)
HyClone (Logan, UT)
International Centre for Genetic Engineering and Biotechnology (Trieste, Italy)
Jichi Medical University (Tochigi-ken, Japan)
Labclinics (Barcelona, Spain)
Laboratoire de Therapie Genique, Institute Nationale de la Sante et de la Recherche Médicale (INSERM) (Nantes, France)
Lausanne University Hospital (Lausanne, Switzerland)
PlasmidFactory GmbH & Co (Biefield, Germany)
Progen Biotechnik GmbH (Heidelberg, Germany)
Sangamo BioSciences (Richmond, CA)
SGS-Vitrology (Scotland, UK)
TIGEM (Naples, Italy)
uniQure (Amsterdam, The Netherlands)
Universitat Autònoma de Barcelona (Barcelona, Spain)
University of Florida (Gainesville, FL)
Université Libre de Bruxelles (Brussels, Belgium)
University of Massachusetts Medical School (Worcester, MA)
University of Pennsylvania (Philadelphia, PA)

Key Abbreviations Used on this Product Sheet

AAVRSWG – Adeno-associated Virus Reference Standard Working Group
ATCC – American Type Culture Collection
FDA - CBER – Food and Drug Administration - Center for Biologics Evaluation and Research
EDQM- European Directorate for the Quality of Medicines & HealthCare

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