

COLLECTION OF ANIMAL VIRUSES, CHLAMYDIAE and RICKETTSIAE

ATCC® Catalog No. VR-1616

Recombinant Adeno-associated Virus 2 Reference Standard Stock (rAAV2-RSS)

Produced by the AAV Reference Standard Working Group (AAVRSWG)

Description and Background

The recombinant Adeno-associated Virus 2 Reference Standard Stock (rAAV2 RSS) consists of highly purified preparation formulated as a sterile liquid in PBS + 135 mM NaCl at room temperature, and stored frozen at -80°C. The configuration is 0.5 ml per vial in virgin polypropylene cryogenic vials with screw caps, external threads.

The purpose of the rAAV2 RSS is to define the particle, vector genome, and infectious units for adeno-associated virus 2 based gene vectors and establish a reference point for comparisons. It was developed at the recommendation of the U.S. Food and Drug Administration (FDA) and the National Institutes of Health (NIH) Recombinant DNA Advisory Committee (RAC) [1], under the guidance of the AAV Reference Standard Working Group (AAVRSWG) and the U.S. Food and Drug Administration (FDA). It was realized through the donation of services and supplies by a large number of laboratories and organizations from the United States, Canada, France, Spain, Italy, Belgium, the Netherlands, Germany, the United Kingdom, and Japan [2].

More information regarding the development and characterization of the rAAV2 RSS can be found in the Reference Standards section of the website of the Bioprocessing Journal (<http://www.bioprocessingjournal.com/>).

Characterization

rAAV2 RSS lot 58051221 was characterized for particles per ml, vector genomes per ml, transducing units 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 Reference Standards section of the Bioprocessing Journal web site. Results are summarized in Tables 1 and 2, and Figure 1.

The particle concentration in the AAV2 RSS lot 58051221 is 9.18×10^{11} particles/ml, with 95% certainty that the true particle concentration lies within the range of 7.89×10^{11} to 1.05×10^{12} particles/ml. The particle concentration was

determined by each laboratory, using four separate dilution series from a single vial in the Progen AAV2 Titration ELISA (Progen Biotechnik GMBH, Article number PRATV), against a standard curve prepared from a previously titered rAAV2 preparation. See posted protocol: http://www.bioprocessingjournal.com/ReferenceMaterials/pdf/s/AAV2_capsid_titer_assay_V2.pdf

The number of vector genomes per ml is 3.28×10^{10} vector genomes/ml, with 95% certainty that the true particle concentration lies within the range of 2.70×10^{10} to 4.75×10^{10} vector genomes/ml. The vector genome concentration was determined in duplicate, testing one replicate from each of three vials, by quantitative PCR of serial dilutions of rAAV2 RSS against a standard curve of plasmid pTR-UF-11 (ATCC MBA-331™). [3] See posted protocol: http://www.bioprocessingjournal.com/ReferenceMaterials/pdf/s/AAV2_RSS_genome_copy_titration_QPCR.pdf

The number of transducing units per ml is 5.09×10^8 transducing units/ml, with 95% certainty that the true value lies within the range of 2.00×10^8 to 9.60×10^8 transducing units/ml. The transducing titer is based on data from twelve replicate tests. Serial ten fold dilutions of rAAV2 RSS were made on HeLa RC32 cells (ATCC CRL-2972™) [4] and co-infected with Adenovirus type 5 (ATCC VR-1516™). Fluorescence microscopy was used to detect GFP-expressing cells. See posted protocol: http://www.bioprocessingjournal.com/ReferenceMaterials/pdf/s/AAV2_RSS_infectious_titer_assays_V2.pdf

The infectious titer on HeLa RC32 cells is 4.37×10^9 TCID₅₀/ml, with 95% certainty that the infectious titer on HeLa RC32 cells lies within the range of 2.06×10^9 and 9.26×10^9 TCID₅₀/ml. Values are based on independent testing of twelve replicates. Serial ten fold dilutions of rAAV2 RSS were made on HeLa RC32 cells (ATCC CRL-2972™) and co-infected with Adenovirus type 5 (ATCC VR-1516™). Replicated vector DNA was analyzed by qPCR. See posted protocol: http://www.bioprocessingjournal.com/ReferenceMaterials/pdf/s/AAV2_RSS_infectious_titer_assays_V2.pdf

The purity and identity of the vector in rAAV2 RSS lot 58051221 were evaluated by SDS polyacrylamide gel electrophoresis (SDS-PAGE), using SYPRO ruby or silver staining. The AAV2 VP1, VP2, and VP3 capsid protein bands were evaluated for their stoichiometry and size. Purity relative to non-vector impurities visible on stained gels was determined to be greater than 94%. Vector identity was verified by observation of the electrophoretic banding pattern expected for AAV2. See posted protocol: <http://www.bioprocessingjournal.com/ReferenceMaterials/pdf>

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[s/AAV2 RSS Identity-Purity assay.pdf](#)

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Table 1. Testing results for rAAV2 RSS Lot 58051221.

Titer units (Method)	Transformation*	Mean (95% confidence interval)
Particles per ml (ELISA)	untransformed	9.18E11 (7.89E11, 1.05E12)
Vector genomes per ml (qPCR)	square root	3.28E10 (2.70E10, 4.75E10)
Transducing units per ml (Green Cells)	square root	5.09E08 (2.00E08, 9.60E08)
Infectious Units per ml (TCID50)	log(10)	4.37E9 (2.06E9, 9.26E09)

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

Table 2. Physical parameters of rAAV2 RSS Lot 58051221.

Parameters Compared	RATIO
Particles : Vector Genomes**	27.99
Vector Genomes : Infectious Units	7.51
Vector Genomes : Transducing Units	64.44
Particles : Infectious Units	210.07

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

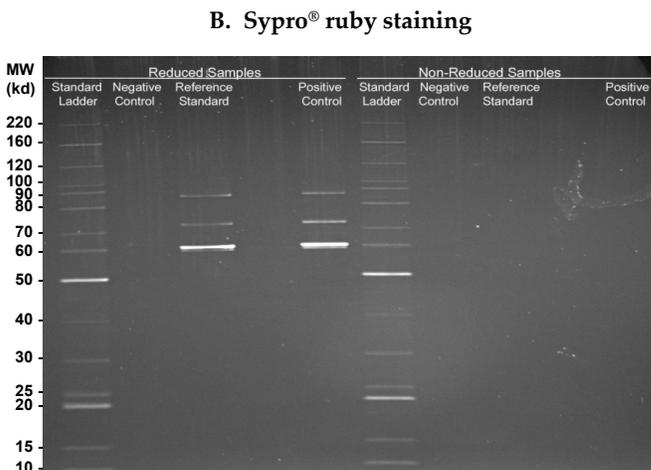
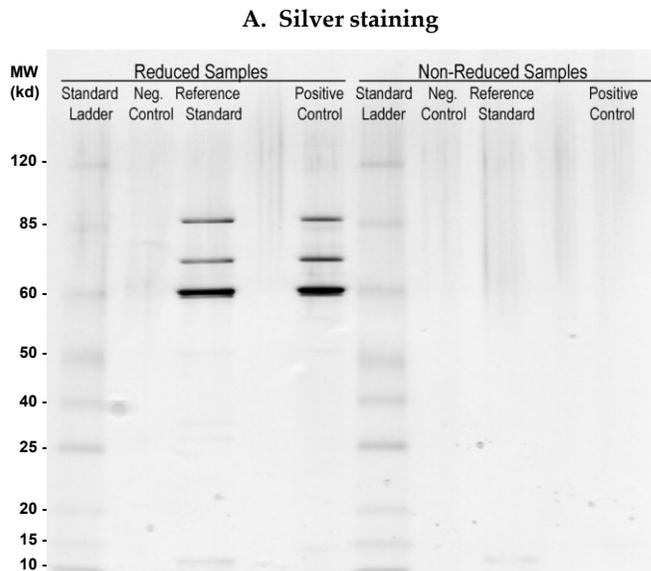
Other Available Characterization Information

Prior to freezing, the filtered formulated bulk was sampled (5 ml) and tested for bioburden by AppTec (now WuXi AppTec, Inc.). It was found negative for aerobes, fungi, spores, and obligate anaerobes, with an assay sensitivity of <5 CFU/sample. The filtered formulated bulk was tested for endotoxin and found negative, with an assay limit of detection of 0.06EU/mL.

The viald rAAV2 RSS (ATCC VR-1616 lot 58051221) was tested for sterility and endotoxin at the Indiana University Vector Production Facility according to GLP guidelines. No aerobes, anaerobes or fungi were detected following direct inoculation onto Thioglycollate Broth, Trypticase Soy Broth and Sabouraud Dextrose Agar and incubation for 14 days at the appropriate temperature. Negative and positive controls (*Bacillus subtilis*, *Candida albicans*, *Bacteroides vulgatus*) were included in the test. Absence of endotoxin was verified using the limulus amoebocyte lysate gel clotting assay. Test samples were assayed in duplicate. Negative, positive and spiked controls were included. The sensitivity of the assay was

0.06EU/mL

Figure 1. Purity evaluation of rAAV2-RSS Lot 58051221 by SDS-PAGE



C. Lane descriptions

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

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9	blank lane
10	Positive Control - Native

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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.

For use in assays of infectivity (e. g. TCID₅₀ or GFP transduction assays), thaw vial(s) at room temperature while mixing gently. DO NOT VORTEX THE rAAV2 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.

Stability data

Prior to filling the rAAV2 RSS, a study was conducted to evaluate the short term stability of vector at room temperature (the filling condition) and -80°C (the storage condition). A rAAV2-GFP vector preparation at 2x10¹¹ vg/ml, in the same formulation as the rAAV2 RSS (PBS + 135 mM NaCl), was filled into polypropylene and glass vials, 0.5 ml per vial. Vials of each type were stored at both temperatures. Vials held at room temperature were assayed for infectious titer after 1 hour, 1 day, 3 days and 7 days; vials stored at -80°C were assayed for infectious titer at 1 hour, 1 day, 14 days, 35 days and 124 days. In all scenarios, a 30 – 40% drop was observed between the initial titer and the average of all samples taken during the time course, most likely indicating absorption to the container surfaces at this low vector concentration. [5]

After filling, a limited study was conducted to evaluate the stability of the rAAV2 RSS to storage post thaw and refreezing. Upon receipt, the rAAV2 RSS was thawed on ice and aliquoted into siliconized plastic vials. Aliquots were either tested immediately, or stored at 4°C or -80°C and then tested for transducing titer, infectious titer and physical titer using the protocols posted on the Reference Standards section of the Bioprocessing Journal web site. Results are provided in Table 3.

Additional stability data for rAAV2 RSS Lot 58051221 will be collected by yearly testing of vials for capsid protein integrity, infectious titer, transducing titer and vector genomes per ml. Data will be reported by the AAVRSWG through the Reference Standards section of the Bioprocessing Journal web site.

Table 3. Testing results for rAAV2 RSS Lot 58051221 at thaw and three days post-thawed under different storage conditions.

Parameter	At thaw	4°C	-80°C
Transducing titer (GFU/ml)	1.89E+09	1.23E+09	8.38E+08
Infectious titer (TCID ₅₀ /ml)	1.65E+10	3.56E+09	6.23E+09
Physical titer (Vector genomes/ml)	3.39E+10	3.39E+10	3.41E+10
Vector genomes : Infectious units	2.05	9.53	5.39
Vector genomes : Transducing units	17.90	27.50	40.70

Recommended Use of the Recombinant Adeno-associated Virus 2 Reference Standard Stock

The rAAV2 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 2 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 rAAV2 RSS. Extensive validation work should be performed in each laboratory using the laboratory’s qualified reference material, as the availability of the rAAV2 RSS is limited.

The FDA recommends use of the rAAV2 RSS as a common Reference Material as described. This recommendation does not imply any intent to standardize assay methods across the field or to require that the values assigned to the rAAV2 RSS be duplicated during validation studies.

Recommended Vectors and Host Cells

Plasmid pTR-UF-11 [3], 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

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gene under the control of the mutant polyoma virus enhancer/promoter (PYF441) and the human bovine growth hormone poly(A) site, flanked by AAV2 TRs. The HEK293 cell line was co-transfected with vector plasmid pTR-UF-11 and the helper plasmid pDG-KanR (6) to generate the rAAV2-RSS. pTR-UF-11 is available from ATCC, distributed as product number MBA-331™.

The cell line used for titration of infectivity of the rAAV2 RSS, HeLa RC32 [4], 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 2 Reference Standard Stock

Production and purification of the rAAV2 RSS were carried out at the Vector Core of the University of Florida's Powell Gene Therapy Center between February, 2006, and January, 2007, using helper virus-free transient transfection and chromatographic purification.[5]

Production was initiated by co-transfection of HEK293 cells in ten 10-layer Nunc cell factories with AAV vector plasmid pTR-UF-11 [3] and the pDG-KanR helper plasmid, a kanamycin-resistant version of pDG [6], into HEK293 cells at a 1:1 molar ratio using a calcium phosphate precipitation method. After 60 hours incubation with precipitate at 37°C, 5% CO₂, transfected cells were washed with PBS and harvested using PBS containing 5 mM EDTA. Samples of cells were combined with spent tissue culture media for mycoplasma and *in vitro* adventitious agent testing. Cells were collected by centrifugation and stored at -20°C until purified.

For vector purification, cells were thawed, lysed with 0.5% sodium deoxycholate, treated with Benzonase® (Merck KgaA), and then disrupted by microfluidization. Virions were then purified by STREAMLINE™ (GE Healthcare Life Sciences) heparin affinity chromatography. Peak fractions were pooled and applied to a Phenyl Sepharose™ (GE Healthcare Life Sciences) chromatography column. The flow through collected from this second purification step was finally purified and concentrated by sulfopropyl cation exchange chromatography. Vector was eluted with 5–10 ml of 135 mM NaCl in phosphate buffered saline (PBS) (equivalent to 285 mM ionic strength) and stored at -80°C.

Eighteen batches were prepared in this way and pooled, yielding a total of ~150 ml of purified vector at a concentration of $\sim 3.79 \times 10^{12}$ vector genomes (vg)/ml. The purified bulk was

diluted to $\sim 2 \times 10^{11}$ vg/ml with 135 mM NaCl in phosphate buffered saline (PBS), and sterile filtered into two 1.3 l portions. This filtered formulated bulk was stored frozen (-80°C) until vialled. [5]

In March, 2008, one of the 1.3 l portions of the bulk rAAV2 RSS was thawed, re-filtered and dispensed into 2,087 vials at ATCC to produce VR-1616™, the rAAV2 RSS. Each vial contains 0.5 ml of the final purified vector preparation. The vials, frozen in the repository at ATCC, are available for distribution. The other 1.3 l portion remains frozen at ATCC, to be dispensed at a later date if demand warrants.

Comments

While the final rAAV2 RSS preparation was found to be pure and free of adventitious agents, the cell harvest from which it was made tested positive for *Mycoplasma arginini*. The rAAV2 RSS has therefore been exposed to mycoplasma, but the purified rAAV2 RSS was found by extensive testing to be free of viable mycoplasma and mycoplasma DNA. The AAVRSWG has recommended distribution of the purified reference material because the purification process includes several steps likely to be inactivating for mycoplasma. Since the material is not intended for human use, the exposure to mycoplasma does not preclude its use for control and comparison of *in vitro* tests.

References:

All information regarding the development and characterization of the recombinant Adeno-associated Virus 2 Reference Standard Stock can be found on the Bioprocessing Journal's web site at <http://www.bioprocessingjournal.com/ReferenceMaterials/aaav2.htm>. Additional relevant publications are listed below.

- 1 Flotte, T.R., Burd, P., and Snyder, R.O. Utility of a recombinant adeno-associated viral vector reference standard. *BioProcessing*, 1:75, 2002
- 2 Moullier, P., and Snyder, R.O. International efforts for recombinant adeno-associated viral vector reference standards. *Mol. Ther.* 16:1185-1188, 2008.
- 3 Burger, C., Gorbatyuk, O. S., Velardo, M. J., Peden, C. S., Williams, P., Zolotukhin, S., Reier, P. J., Mandel, R. J., Muzyczka, N. 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(2):302-17, 2004 CELLS, pTR-UF-11

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- 4 Chadeuf G, Favre, D., Tessier, J., Provost, N., Nony, P., Kleinschmidt, J., Moullier, P., Salvetti, A.. Efficient recombinant adeno-associated virus production by a stable rep-cap HeLa cell line correlates with adenovirus-induced amplification of the integrated rep-cap genome. *J. Gene Med.* 2(4):260-268, 2000.
- 5 Potter, M., Phillipsberg, G., Phillipsberg, T., Pettersen, M., Sanders, D., Korytov, I., Fife, J., Zolotukhin, S., Byrne, B.J., Muzyczka, N., Flotte, T., and Snyder, R.O. Manufacture and stability study of the recombinant adeno-associated virus serotype 2 vector reference standard. *BioProcessing J.* 7:8-14, 2008.
http://www.biotechnoblog.net/public_html/tech-blasts/103009/172_01_Snyder.pdf
- 6 Grimm, D., Kern, A., Rittner K, Kleinschmidt, J. A. Novel tools for production and purification of recombinant adeno-associated virus vectors. *Hum. Gene Ther.* 9:2745-2760, 1998.

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

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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)

Aldevron (Fargo, ND)
Amsterdam Molecular Therapeutics (Amsterdam, The Netherlands)
Applied Genetic Technologies Corporation (Alachua, FL)
ATCC (Manassas, VA)
Bayer Corporation Bayer HealthCare (Berkeley, CA)
Bioprocessing Journal (Virginia Beach, VA)
Ceregene, Inc. (San Diego, CA)
Childrens Hospital of Philadelphia (Philadelphia, PA)
Clean Cells (Bouféré, France)
Cobra Biomanufacturing (Stoke-on-Trent, UK)
Columbus Children's Research Institute (Columbus, OH)
Corning (Corning, NY)
Food and Drug Administration / Center for Biologics

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Evaluation and Research (FDA / CBER; Rockville, MD)
Food and Drug Administration Office of Cellular, Tissue, and Gene Therapies (FDA; Rockville, MD)
Genethon (Évry, France)
Genzyme Corporation (Cambridge, MA)
German Cancer Research Center (Heidelberg, Germany)
Federico II University (Naples, Italy)
Hôpital Necker-Enfants Malades (Paris, France)
HyClone (Logan, UT)
Indiana University–Purdue University (Indianapolis, IN)
Indiana University Vector Production Facility (Indianapolis, IN)
International Centre for Genetic Engineering and Biotechnology (Trieste, Italy)
Introgen Therapeutics (Austin, TX)
Jichi Medical University (Tochigi-ken, Japan)
Laboratoire de Therapie Genique, Institute Nationale de la Sante et de la Recherche Médicale (INSERM) (Nantes, France)
Mediatech (Manassas, VA)
Netherlands Institute for Neuroscience (Amsterdam, The Netherlands)
National Institutes of Health / National Institute of Dental and Craniofacial Research (NIH / NIDCR; Bethesda, MD)
National Institutes of Health / Division for Clinical Research Resources—Islet Cell Resource Centers (NIH / DCRR; Bethesda, MD)
National Institutes of Health / National Center for Research Resources (NIH / NCRR; Bethesda, MD)
National Institutes of Health / National Heart, Lung, and Blood Institute (NIH / NHLBI; Bethesda, MD)
National Institutes of Health / National Institute of Diabetes and Digestive and Kidney Diseases (NIH / NIDDK; Bethesda, MD)
Nunc (Rochester, NY)
Philip J. Cross & Associates, Inc. (Wilmington, DE)
Progen Biotechnik GmbH (Heidelberg, Germany)
Royal Holloway University of London (Egham, Surrey, England, UK)
Sangamo BioSciences (Richmond, CA)
Seqwright (Houston, TX)
Targeted Genetics Corporation, (Seattle, WA)
Thermo Fisher Scientific (Waltham, MA)
Universitat Autònoma de Barcelona (Barcelona, Spain)
University College London (London, England, UK)
University of Florida (Gainesville, FL)
University of Leuven (Leuven, Belgium)
Université Libre de Bruxelles (Brussels, Belgium)
University of Massachusetts Medical School (Worcester, MA)

University of North Carolina (Chapel Hill, NC)
University of North Carolina Vector Laboratories (Chapel Hill, NC)
University of Pennsylvania (Philadelphia, PA)
The Williamsburg BioProcessing Foundation (Virginia Beach, VA)

COLLECTION OF ANIMAL VIRUSES, CHLAMYDIAE and RICKETTSIAE**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

IND – Investigational New Drug (application to FDA)

NIH – National Institutes of Health, U.S.

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