Video Article

A Quantitative Dot Blot Assay for AAV Titration and Its Use for Functional Assessment of the Adeno-associated Virus Assembly-activating Proteins

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URL: https://www.jove.com/video/56766
DOI: doi:10.3791/56766

Keywords: Biology, Issue 136, Adeno-associated virus (AAV), assembly-activating protein (AAP), cross-complementation, quantitative dot blot assay, viral titer, gene therapy

Date Published: 6/12/2018

Citation: Powers, J.M., Chang, X.L., Song, Z., Nakai, H. A Quantitative Dot Blot Assay for AAV Titration and Its Use for Functional Assessment of the Adeno-associated Virus Assembly-activating Proteins. *J. Vis. Exp.* (136), e56766, doi:10.3791/56766 (2018).

Abstract

While adeno-associated virus (AAV) is widely accepted as an attractive vector for gene therapy, it also serves as a model virus for understanding virus biology. In the latter respect, the recent discovery of a non-structural AAV protein, termed assembly-activating protein (AAP), has shed new light on the processes involved in assembly of the viral capsid VP proteins into a capsid. Although many AAV serotypes require AAP for assembly, we have recently reported that AAV4, 5, and 11 are exceptions to this rule. Furthermore, we demonstrated that AAPs and assembled capsids of different serotypes localize to different subcellular compartments. This unexpected heterogeneity in the biological properties and functional roles of AAPs among different AAV serotypes underscores the importance of studies on AAPs derived from diverse serotypes. This manuscript details a straightforward dot blot assay for AAV quantitation and its application to assess AAP dependency and serotype specificity in capsid assembly. To demonstrate the utility of this dot blot assay, we set out to characterize capsid assembly and AAP dependency of Snake AAV, a previously uncharacterized reptile AAV, as well as AAV5 and AAV9, which have previously been shown to be AAP-independent and AAP-dependent serotypes, respectively. The assay revealed that Snake AAV capsid assembly requires Snake AAP and cannot be promoted by AAPs from AAV5 and AAV9. The assay also showed that, unlike many of the common serotype AAPs that promote heterologous capsid assembly by cross-complementation, Snake AAP does not promote assembly of AAV9 capsids. In addition, we show that the choice of nuclease significantly affects the readout of the dot blot assay, and thus, choosing an optimal enzyme is critical for successful assessment of AAV titers.

Video Link

The video component of this article can be found at https://www.jove.com/video/56766/

Introduction

Adeno-associated virus (AAV) is a small, non-enveloped, single-stranded DNA virus with a genome of approximately 4.7 kilobases (kb). The AAV genome contains open-reading frames (ORFs) for the *rep* and *cap* genes. In 2010, a previously unidentified nonstructural protein encoded by a +1 frame-shifted ORF within the AAV2 *cap* gene was discovered by Sonntag *et al.* and found to play a critical role in the assembly of AAV2 capsid VP monomer proteins into a viral capsid[1]. This novel protein has been named assembly-activating protein (AAP) after the role it plays in promoting capsid assembly[1].

The ORFs for AAP have been identified bioinformatically in the genomes of all parvoviruses within the genus *Dependoparvovirus*, but not within the genomes of viruses of different genera of the parvovirus family[1,2]. Functional studies of this novel protein were initially focused on the AAP from the prototype AAV2 (AAP2), which has established the essential role of AAP2 in targeting unassembled VP proteins to the nucleolus for their accumulation and formation into fully assembled capsids[1,3,4]. The inability of the AAV2 capsids to assemble in the absence of AAP expression has been independently confirmed by multiple groups, including ours[1,2,3,4,5]. Subsequent studies on AAV serotypes 1, 8, and 9 corroborated the critical role of AAPs in capsid assembly, as VP3 monomer proteins of AAV1, 8, and 9 were unable to form a fully assembled capsid in the absence of co-expression of AAP[2].

Recently, through approaches that include the use of quantitative dot blot assays, we investigated the ability of AAV1 to 12 VP3 monomers to assemble into capsids in the absence of AAP expression and the ability of AAP1 to 12 to promote assembly of VP3 monomers from heterologous serotypes. This study has revealed that AAV4, 5, and 11 VP3 monomers can assemble without AAP. Additionally, it was found that eight out of the twelve AAP serotypes we examined (*i.e.*, all but AAP4, 5, 11, and 12) displayed a broad ability to support capsid assembly of heterologous AAV serotype capsids, while AAP4, 5, 11 and 12 displayed a substantially limited ability in this regard[6]. These four serotypes are phylogenetically distant from the other AAP serotypes[2,3]. Moreover, the study has uncovered significant heterogeneity in subcellular localizations of different AAPs[6]. Furthermore, the study has suggested that the tight association of AAP with assembled capsids and the nucleolus, the hallmark of AAV2 capsid assembly, cannot necessarily be extended to other serotypes including AAV5, 8, and 9, which display nucleolar exclusion of assembled capsids[6]. Thus, the information gained from the study of any particular serotype AAP is not broadly applicable to all AAP

biology. Such puzzling nature of AAP biology underscores the need to investigate the role and function of each AAP from both canonical and non-canonical AAV serotypes.

The biological role of AAP in capsid assembly can be assessed by determining the fully-packaged AAV viral particle titers produced in human embryonic kidney (HEK) 293 cells, the most commonly used cell line for AAV vector production, with or without AAP protein expression. The standard methods for AAV quantitation are quantitative PCR (qPCR)-based assays[7,8] and quantitative dot blot-based assays[9]. Other methods for AAV viral particle quantitation such as enzyme-linked immunosorbent assay[10,11] or optical density measurement[12] are not ideal for samples derived from many different AAV serotypes or samples contaminated with impurities (crude lysates or culture media), which are often the samples used for AAV research. Currently, qPCR is most widely used for AAV quantitation; however, it is necessary to acknowledge potential caveats of the qPCR-based assay, as the assay can result in systemic errors and significant titer variations[13,14]. PCR-based assays are affected by a number of potentially confounding factors, such as the presence of covalently closed terminal hairpins in PCR templates that inhibit amplification[13]. Even an experienced individual can introduce potential confounding factors into a qPCR-based assay unknowingly[13]. In contrast, quantitative dot blot assays are a classical molecular biology technique that does not involve genome amplification and uses a much simpler principle with a minimal risk of errors as compared to qPCR-based assays. The method is less technically challenging; therefore, the assay results are reasonably reproducible even by inexperienced individuals.

In this report, we describe the methodological details of a quantitative dot blot assay we routinely use for AAV vector quantitation and provide an example of how to apply the assay to study the assembly-promoting role of AAPs in common serotypes (AAV5 and AAV9) and a previously uncharacterized AAP from Snake AAV[14]. In nature, AAV VP proteins and AAP proteins are expressed in cis from a single gene (*i.e.*, VP-AAP cis-complementation), while in the assay described here, VP and AAP proteins are supplied in trans from two separate plasmids (*i.e.*, VP-AAP trans-complementation). Since each VP or AAP protein from different serotypes can be expressed from each independent plasmid, it becomes possible to test heterologous VP-AAP combinations for capsid assembly (*i.e.*, VP-AAP cross-complementation). Briefly, AAV VP3 from various serotypes is expressed in HEK 293 cells by plasmid DNA transfection to package an AAV vector genome in the presence or absence of the cognate serotype AAP, or in the presence of a heterologous serotype AAP. Following production, culture media and cell lysates are subjected to a dot blot assay to quantify the viral genome within the capsid shell. The first step of the dot blot assay is to treat samples with a nuclease to remove contaminating plasmid DNAs and unpackaged AAV genomes in samples. Failure to do so would increase the background signals in particular when unpurified samples are assayed. This is then followed by a protease treatment to break viral capsids and release nuclease-resistant viral genomes into sample solutions. Next, viral genomes are denatured, blotted on a membrane, and hybridized with a viral genome-specific DNA probe for quantitation. In the example assay reported here, we demonstrate that Snake AAV VP3 requires Snake AAP for capsid assembly and that Snake AAP does not promote the assembly of AAV9 capsids unlike many of the AAPs derived from AAP-dependent serotypes that can also promote assembly of heterologous serotype capsids. Lastly, we report an important caveat to qPCR or dot blot-based AAV quantitation assays that the choice of nuclease significantly affects the assay results.

Protocol

NOTE: Recipes for the solutions and buffers needed for this protocol are provided in **Table 1**. The protocol described below is for the VP-AAP cross-complementation dot blot assay to study the roles of the AAP proteins in capsid assembly. The method for the more generic quantitative dot blot assay for purified AAV vector titration is explained in the **Representative Results** section.

1. Construction of VP3, AAP, and AAV2 Rep Expressing Plasmids

1. **Construction of pCMV-AAVx-VP3 (x = serotypes)**
    1. PCR-amplify the entire VP3 ORF (1.6 kb) using a high-fidelity DNA polymerase and the following primer pair: VP3 forward, CTAA-RE1-CACC-N_{25} (the first 25 nucleotides of the VP3 ORF); VP3 reverse, TCTT-RE2-N_{25} (the last 25 nucleotides of the VP3 ORF).
    NOTE: RE1 and RE2 are sites for restriction enzymes (REs) for cloning. CTAA and TCTT are the terminal 5' and 3' tetranucleotides added to facilitate restriction enzyme digestion near the end of double-stranded DNA. CACC is a Kozak consensus sequence.
    2. Clone the RE-digested PCR products between the corresponding RE sites of a mammalian expression vector that uses the human cytomegalovirus immediately-early (CMV-IE) enhancer-promoter for high-level expression.
    NOTE: For molecular cloning, digest 5 µg of the backbone plasmid DNA with a restriction enzyme(s) at a concentration of 4 U/µg DNA for 1 h at an optimal temperature. For PCR fragments, increase the units of enzymes used (*e.g.*, 10 U/µg of the 1.6 kb VP3 ORF PCR product) and a longer incubation time (*e.g.*, 4 h) due to an increase of the number of restriction enzyme recognition sites per unit length. Helpful information in molecular biology enzymes and cloning procedures including bacterial transformation can be found elsewhere[15,16,17].

2. **Construction of pCMV-FLAG-AAPx (x = serotypes)**
    1. PCR-amplify the entire AAP ORF (0.6 kb) except for the first amino acid using a high-fidelity DNA polymerase and the following primer pair: AAP forward, CTAA-RE1-CACCATGGACTACAAGGACGACGATGACAAA-N_{25} (the 25 nucleotides from the 4th nucleotide in the AAP ORF); AAP reverse, TCTT-RE2-N_{25} (the last 25 nucleotides of the AAP ORF).
    NOTE: GACTACAAGGACGACGATGACAAA codes a FLAG tag, which has been shown to have no detrimental effects[4,5] but can be omitted if unnecessary.
    2. Clone the RE-digested PCR products between the corresponding sites of a mammalian expression vector with the CMV-IE enhancer-promoter[15,16,17].

3. **Construction of pHLP-Rep**
    1. Digest 5 µg of the pAAV-RC2 plasmid (7.3 kb) with 20 units each of Xho I and Xcm I, and purify the DNA using a commercial DNA purification kit or by phenol-chloroform extraction.
    NOTE: Removal of the 1.8 kb Xho I-Xcm I fragment from the 7.3 kb pAAV-RC2 plasmid results in a loss of capsid VP protein expression while preserving the Rep protein expression.

2. Blunt the DNA ends with 6 units of T4 DNA Polymerase, agarose gel-purify the 5.5 kb DNA fragment, and self-ligate the purified fragment using 50 to 100 ng of DNA and 400 units of T4 DNA ligase according to the manufacturer's recommendation[15].
3. Follow the standard bacterial transformation procedure referenced in step 1.1.2 Note.

4. Verify the plasmid constructs by restriction enzyme digestion and sequencing[15,18].
5. Perform plasmid minipreps or maxipreps using commercially available kits to obtain a sufficient amount of plasmid DNA for the downstream AAV production experiments.

2. Production of AAV in HEK 293 Cells by Plasmid DNA Transfection (Cross-complementation Assay)

1. Culture HEK 293 cells in Dulbecco's Modified Eagle Medium (DMEM)-high glucose (4.5 g/L) supplemented with 10% fetal bovine serum (FBS), 1% Penicillin & Streptomycin Mix, and 1 mM L-glutamine, in a 37 °C incubator with 5% carbon dioxide (CO_2).
   NOTE: AAV titers significantly vary depending on the source of HEK 293 cells.
   Caution: Although AAV can be handled at biosafety level 1 (BSL1) containment, BSL2 containment is recommended for HEK 293 cell work.
2. On Day -1 (24 h prior to transfection), plate 6–7 x 10^5 HEK 293 cells/well in 2 mL of the complete medium described in step 2.1 in a 6-well plate(s). This generally achieves ~90% confluency the next day.
3. **Day 0: Transfection**
   1. Preparation for DNA transfection
      1. Make sure that cells have reached ~90% confluency.
      2. Warm up DMEM supplemented with 1% Penicillin & Streptomycin Mix and 1 mM L-glutamine but without 10% FBS (*i.e.*, serum-free medium) in a 37 °C water bath.
      3. Allow the polyethylenimine (PEI) solution to reach room temperature.

   2. Preparation of plasmid DNA mixture
      1. Mix the plasmids in 96 µL of phosphate buffered saline (PBS) without $CaCl_2$ or $MgCl_2$ in sterile 1.5 mL microcentrifuge tubes as indicated in **Table 2**. The total amount of DNA is 2 µg/well.
         NOTE: The volumes for plasmid DNA solution can be disregarded if they are nominal. Volume adjustment is recommended if the total volume of the plasmid DNA solutions is ≥10 µL.

   3. PEI transfection
      1. Add 4 µL of PEI solution (1 mg/mL) to the PBS-plasmid DNA mix (prepared as above). The final volume is approximately 100 µL (5% volume of the culture medium). Vortex the tubes briefly and incubate the DNA:PEI mixture for 15 min at room temperature.
      2. While waiting for the 15 min incubation to complete, replace the culture medium with prewarmed serum-free medium described in step 2.3.1.2.
      3. Once the 15-min incubation of the DNA: PEI mixture is complete, briefly spin the tubes with a microcentrifuge to collect the liquid to the bottom of the tubes, and add the DNA:PEI mixture dropwise to the culture medium in each well of the HEK 293 culture plate. Gently agitate the plates and return them to a 37 °C incubator with 5% CO_2.
      4. Maintain the cells in this transfection medium until the harvest at Day 5 (no medium change is required).

4. On Day 1 and Day 2, observe cells transfected with pCMV-GFP plasmid under an inverted fluorescence microscope to assess transfection efficiency.
   NOTE: For fluorescence microscopy, here a 10X/0.25 numerical aperture objective in combination with a 10× eyepiece was used, and 450–490 nm excitation bandpass filter and 515–565 nm bandpass emission filter were employed. The above condition normally yields more than 70% transfection efficiency. Cells may exhibit some morphologic changes (*e.g.* cells have become slim) due to the serum-free condition.
5. On Days 3–5, continue to culture the transfected cells in a 37 °C incubator with 5% CO_2.
6. On Day 5, collect both cells and virus-containing medium into 15 mL polypropylene conical tubes by pipetting up and down or by scraping with a cell scraper.
7. Store the samples at -80 °C until use.

3. Dot Blot Assay for AAV Quantitation

1. **Recovery of viral particles**
   1. Quickly thaw the frozen tubes in a 37 °C water bath. Vortex the tubes vigorously for 1 min to maximize the recovery of AAV from cells.
   2. Pellet the cell debris by centrifuging at 3,700 x g at 4 °C for 10 min. Take 200 µL of the supernatant from each tube and transfer it into a labeled microcentrifuge tube with a screw cap for the dot blot assay.
      NOTE: Aliquot the remaining supernatant into microcentrifuge tubes and store them frozen at -80 °C for future use. Here, polypropylene microcentrifuge tubes attached with screw caps and an O-ring were used. Tight sealing is required to prevent spills of AAV and phenol-chloroform.

2. **Treatment with *Serratia marcescens* endonuclease**
   1. Prepare Mix A and Mix B reagents (**Table 3**). Add 10 µL of Mix A and 10 µL of Mix B into each tube. Vortex the tubes for 5 s, briefly spin the tubes to collect the liquid to the bottom of the tubes and incubate the tubes at 37 °C at least for 1 h.
      NOTE: Mix A contains NaOH and optimizes pH for treatment with *S. marcescens* endonuclease. Mix B supplements magnesium. Concentration of *S. marcescens* endonuclease in commercially available enzyme stocks may vary. The volume of *S. marcescens* endonuclease needs to be adjusted accordingly to make 200 U/mL after adding Mix A and Mix B into tubes in step 3.2.1.
      NOTE: A longer incubation time, up to 4 h, can decrease background signals.

2. At the end of the incubation, briefly spin the tubes with a benchtop centrifuge to collect condensation and solution from the top and sides of the tubes.
   NOTE: The protocol can be paused here. The samples can be stored frozen at -20 °C or -80 °C.

3. **Proteinase K treatment**
   1. Prepare the Mix C reagent (**Table 3**). Add 180 µL of Mix C into each tube. Vortex the tubes for 5 s, briefly spin the tubes and incubate the tubes at 55 °C for 1 h.
      NOTE: The EDTA in the Mix C reagent chelates free magnesium ions and inactivates *S. marcescens* endonuclease.
   2. At the end of the incubation, allow samples to reach room temperature, and briefly spin the tubes with a benchtop centrifuge to collect condensation and solution from the top and sides of the tubes.
      NOTE: The protocol can be paused here. The samples can be stored frozen at -20 °C or -80 °C. To resume the protocol, incubate the tubes at 55 °C for 5 to 10 min to dissolve SDS crystals contained in the buffer completely.

4. **Phenol-chloroform extraction and ethanol precipitation**
   1. Add 200 µL of phenol-chloroform to the samples and vortex them for 1 min. Spin the samples in a microcentrifuge at ≥16,100 x g for ≥5 min at room temperature.
      CAUTION: Phenol-chloroform should be handled in a chemical fume hood with appropriate personal protective equipment (PPE; *i.e.*, nitrile gloves, goggles or face shield, and lab coat with long sleeves).
   2. Transfer 320 µL of the aqueous layer (160 µL twice with a P200 pipette) to a new standard microcentrifuge tube (80% of aqueous fluid volume).
      NOTE: It is vitally important to take the same volume of aqueous solution between samples, otherwise the assay loses quantitative accuracy.
   3. Prepare the Mix D reagent (**Table 3**). Add 833 µL of Mix D into each tube. Vortex the tubes for 5 s, and incubate the tubes at -80 °C for ≥20 min.
      NOTE: Mix D is a mixture of ethanol, sodium acetate, and glycogen for convenient ethanol precipitation of DNA. Samples can be stored at -80 °C at this step and the protocol can be resumed later.
   4. Centrifuge samples with a microcentrifuge at ≥16,100 x g at 4 °C for ≥15 min. Pour off supernatant and blot once on a clean paper towel. Put approximately 500 µL of 70% ethanol into each tube, vortex the tubes for 5 s, and centrifuge the tubes with a benchtop microcentrifuge at ≥16,100 x g at 4 °C for ≥5 min.
   5. Pour off the supernatant and blot once on a clean paper towel. Dry pellets at 65 °C; pellets can be dried completely.
      NOTE: Do not use a pipette to remove excess ethanol that remains after blotting the tubes. Pellets can also be dried at room temperature overnight. The protocol can be paused here and dried DNA pellets can be stored at room temperature for several days with the tube lid closed.

5. **Resuspension of viral DNA in TE buffer**
   1. Dissolve the DNA pellets in 120 µL each of TE buffer by shaking each tube for 30 min to 1 h at room temperature.

6. **Dot blot**
   1. Preparation of plasmid DNA standards
      1. Dilute AAV vector genome plasmid DNA to 10 ng/µL in water or Tris-HCl buffer (10 mM, pH 8.0). Take 25 µL of this dilution and digest with an appropriate restriction enzyme for 1 h to linearize the plasmid DNA, in a reaction volume of 50 µL.
         NOTE: We make a duplicated set of digestion (see step 3.6.4.1). The appropriate enzyme should be one that cuts the plasmid DNA outside the dot blot probe-binding region. For convenience, the diluted plasmid DNA can be aliquoted (25 µL/tube) and stored frozen at -20 °C for future use. Digest the plasmid DNA while the tubes are shaking in step 3.5.1. Do not over-digest the plasmid DNA standard.
      2. Add 450 µL of water or TE to the tube containing the digested plasmid DNA standard and mix thoroughly. Transfer 70 µL of this mixture to a new 1.5 mL microcentrifuge tube with 1,330 µL of water or TE to make a diluted plasmid standard (25 pg/µL).
      3. Follow **Table 4** to create a set of two-fold serial dilutions (600 µL/tube). Mix the dilutions thoroughly by vortexing for 5 s.

   2. Denaturing of standards and viral DNA samples
      1. Add 600 µL of 2x Alkaline Solution to each standard dilution. Mix well by vortexing for 5 to 10 s. Incubate at room temperature for 10 min.
      2. Add 120 µL of 2x Alkaline Solution to each viral DNA sample. Mix well by vortexing for 5 to 10 s. Incubate at room temperature for 10 min.

   3. Setting up the dot blot apparatus
      1. Using scissors, cut a blotting (*e.g.*, zeta-probe) membrane to an appropriate size for the number of samples and standards. Soak the membrane with water for 10 min before placing it on a dot blot apparatus. Cover unused wells on the membrane apparatus.
         NOTE: Handle the membrane with clean tweezers. To cover empty wells, the light blue protection sheet that comes with the membrane can be used. Do not allow the membrane to dry prior to binding samples and standards. For more details on the assembly and use of the apparatus, please refer to the user manual[19].
      2. Add water to the wells to which samples will be loaded. Apply vacuum and pull water through the wells to check for errors and retest when fixed. Re-tighten the screws while applying vacuum to ensure tight sealing.
         NOTE: Incomplete sealing may cause sample leakage between the wells.
      3. Once water is completely pulled through, release the vacuum completely (*i.e.*, the vacuum manifold should be open to air pressure).

   4. Loading standards and samples to the dot blot apparatus
      1. Apply 400 µL of each diluted plasmid DNA standard to each well, and run four lanes of standard dilutions. Use two separate aliquots of the standard digest and load each in duplicate. Apply 200 µL/well of each viral DNA sample.

NOTE: Using the remaining ~40 µL of denatured samples, diluted samples can be prepared (*e.g.*, 10-fold diluted samples using 20 µL of sample plus 180 µL of 1x Alkaline Solution) and blotted if necessary.

2. Apply gentle vacuum to pull the DNA solutions through the well.
   NOTE: Vacuum pressure needs to be adjusted by partially opening a three-way valve so that the vacuum pressure is applied to the dot blot apparatus as well as the atmosphere (*i.e.*, with the stopcock arms positioned at an approximately 45° angle where it makes a louder suction noise).
3. Once all the wells have emptied, release the vacuum by adjusting the three-way valve. Add 400 µL of 1x Alkaline Solution to each well that contained standards and samples. Wait for 5 min before re-applying vacuum to empty the wells.
4. Re-apply the vacuum in the same way (see step 3.6.4.2).
5. Disassemble the dot blot apparatus under vacuum, remove the membrane, and rinse it with 2x SSC. Place the membrane on a clean paper towel with the DNA-bound side facing up to remove excess 2x SSC.
6. UV-crosslink the blotted DNA to the membrane with an appropriate UV crosslinker; the membrane is now ready for hybridization.
   NOTE: Wet membranes can be used for UV crosslinking. The dried, UV-crosslinked membranes can be stored at room temperature. Further information can be found in the **Table of Materials**.

7. **Hybridization and washing**
   1. Warm up the Hybridization Buffer in a 65 °C water bath.
   2. Enzymatically label a DNA probe with radioactive α-^{32}P dCTP and purify it using commercially available kits according to the manufacturer's recommendation.
      NOTE: We use a probe of 0.5–2.0 kb in length from an enhancer-promoter region or a protein-coding sequence in the viral genome. Although this protocol utilizes ^{32}P-labeled radioactive probes for signal detection, non-radioactive chemiluminescent or fluorescent probes can also be used (please refer to the **Discussion** section).
   3. Place the membrane in a hybridization bottle with the DNA-bound side up, rinse the membrane with 5 mL prewarmed Hybridization Buffer, and discard the buffer. Then add 10 mL of prewarmed Hybridization Buffer and place the bottle in a rotating hybridization oven set at 65 °C. Rotate for ≥5 min.
   4. Heat-denature 20 µL of 10 mg/mL sheared herring or salmon sperm DNA solution and the ^{32}P-labeled probe (≥10^7 cpm) for 5 min by placing the tubes on a heat block set at 100 °C. Then snap-chill them on ice for ≥2 min, spin briefly, and keep on ice until use.
      Caution: For radioactive DNA probes, 1.5 mL tubes with screw cap and O-ring must be used.
   5. Quickly add the denatured sperm DNA and radioactive probe to the Hybridization Buffer in the hybridization bottle and shake the bottle for 10 s to mix. Return the bottle to the 65 °C oven and incubate the bottle with rotation at 65 °C for ≥4 h.
   6. Once hybridization is complete, stop the rotation, remove the hybridization bottle, and then pour the radioactive probe solution into a 50 mL conical tube with a leak-proof plug seal cap. Store the probe in an appropriate container placed in a refrigerator designated for radioactive materials.
      NOTE: Used Hybridization Buffer with a probe that is stored at 4 °C can be re-used at least 5 times by placing the 50 mL conical tube with a leak-proof plug seal cap that contains Hybridization Buffer in a 100 °C water bath for 5 min.
   7. Wash the membrane with Wash Buffer heated to 65 °C. Add 20 to 30 mL of Wash Buffer to the hybridization bottle and rotate for 5 min. Repeat this wash 2 more times.
      NOTE: Measure radioactivity of wash solutions and record it if required by the local institute.
   8. While washing the membrane, place a phosphor imaging screen on an image eraser for 5 min.
   9. After the third wash, remove the membrane from the hybridization bottle, remove excess buffer on the membrane with paper towels, and place the membrane in a clear plastic paper holder. Check radioactive signals on the membrane using a Geiger counter. Expose the erased phosphor imaging screen to the membrane for 10 min to overnight depending on the signal intensity.
   10. Scan the screen using a phosphor image scanning system and obtain the data on signal intensity of each dot.

8. **Data analysis**
   1. Draw a standard curve using spreadsheet and data analysis software (*e.g.*, Excel).
      NOTE: Log-log linear regression was used to draw a standard curve.
   2. Determine nanogram-equivalent (ng-eq) for each of the viral DNA samples by interpolation. The ng-eq is the amount of the plasmid DNA used to draw a standard curve that is equivalent to the number of viral DNA molecules. When the length of the plasmid DNA is 8,000 bp, 1 ng-eq of single-stranded AAV viral DNA corresponds to 2.275 x 10^8 particles.
      NOTE: The ng-eq can be converted to the number of viral particles by the following equations:

      Equation 1: $$\text{Number of single-stranded AAV particles} = \frac{1.82 \times \text{ng-eq}}{\text{length of plasmid (bp)}} \times 10^{12}$$

      Equation 2: $$\text{Number of double-stranded AAV particles} = \frac{0.91 \times \text{ng-eq}}{\text{length of plasmid (bp)}} \times 10^{12}$$

      The number of AAV particles are conventionally expressed as "vg" (vector genomes) or "DRP" (DNase I-resistant particles).
   3. Calculate the AAV concentrations of the starting materials. Because the blotted viral DNA represents 66.7% $\left(\frac{320}{400} \times \frac{200}{240} \times 100\%\right)$ of the viral DNA in the starting materials, 1 ng-eq corresponds to 1.7 x 10^9 particles/mL $\left(\left[\left(\frac{2.275}{0.667}\right) \div (0.2)\right] \times 1 \times 10^8\right)$.
      NOTE: This correction is not needed if all the viral DNA contained in the starting material is blotted on a membrane without loss (see **Figure 1**).

Representative Results

A representative result of quantitative dot blots for quantitation of purified AAV vector stocks produced on a large scale is shown in **Figure 1**. With this dot blot assay, the titer of a double-stranded AAV2G9-CMV-GFP vector stock was determined. The vector was purified by two rounds of cesium chloride (CsCl) density-gradient ultracentrifugation followed by dialysis as previously described[20]. In general, for purified AAV vector stocks, three different volumes of each AAV vector stock (*e.g.*, 0.3 µL, 0.1 µL, and 0.03 µL) are subjected to the dot blot procedure described above in duplicate with a modification. DNase I Enzyme A is used in place of *S. marcescens* endonuclease in step 3.2, and a commercially available kit to extract and purify viral DNA is used in step 3.4 (see **Table of Materials**). In the example shown in **Figure 1**, signal intensity values (arbitrary unit) obtained from each plasmid DNA standard (**Figure 1A**) were plotted against the known DNA quantities to draw a standard curve (**Figure 1B**), showing a correlation coefficient of 0.998. Using this standard curve, it was determined by interpolation that 0.3, 0.1 and 0.03 µL aliquots of the AAV2G9 vector showed 1.487 and 1.522 ng-eq (0.3 µL), 0.487 and 0.507 ng-eq (0.1 µL), and 0.158 and 0.171 ng-eq (0.03 µL). This gave the following six values: 4.957, 5.073, 4.870, 5.070, 5.267, and 5.700 ng-eq/µL for this particular AAV2G9 vector stock, leading to an average of 5.16 ± 0.27 ng-eq/µL (mean ± SD). Since the length of the plasmid used as the standard (pEMBL-CMV-GFP) is 5,848 bp, the titer of this AAV2G9 vector was determined to be 8.0×10^{11} vg/mL according to **Equation 2** in step 3.8.2. This assay is repeated at least twice to determine the final titers of AAV vector stocks.

A representative result of cross-complementation assays to assess the AAP dependency in VP3 capsid assembly and the ability for AAPs to assemble VP3 proteins of heterologous origins is displayed in **Figure 2**. *In vitro* studies of AAV often do not require virus purification to make conclusions, and experiments using unpurified virus preparations such as crude cell lysates and virus-containing culture media are sufficient to yield meaningful results. In this experiment, AAV viral particle production was assessed from all possible AAV VP3-AAP combinations among AAV5, AAV9, and Snake AAV including VP3-no AAP combinations by a quantitative dot blot assay. The samples obtained in a duplicated set of an experiment were blotted at 1x and 10x dilutions (Set A and Set B in **Figure 2A**, respectively). The graph shown in **Figure 2B** summarizes the quantitative analysis of the dots. The results show that: (1) AAV5VP3 assembles regardless of whether AAP was provided in trans, (2) AAP5 and AAP9 can promote AAV9VP3 capsid assembly although AAP5 functions less effectively than AAP9, (3) neither AAP5 nor AAP9 exhibits an assembly promoting activity on Snake AAV VP3, and (4) Snake AAP only promotes assembly of Snake AAV VP3. (1) and (2) are in line with previous observations[6], but the uniquely specific AAV VP3-AAP interaction in Snake AAV is a novel discovery in this experiment. One weakness of the cross-complementation assay based on quantitative dot blot is that the negative control always shows appreciable levels of background signals that cannot be totally eliminated. Therefore, the dot blot assay by itself cannot exclude the possibility that capsid assembles to a level below the sensitivity of the assay. In this regard, it should be noted that, to generate the negative controls, a condition is used under which AAV viral genomes exponentially replicate in the absence of the capsid VP3 protein. Such negative controls can be generated by transfecting HEK 293 cells with pAAV-Reporter, pHLP-Rep, and pHelper (**Table 2**), and are used to reduce false positives.

DNase I has been widely used as a nuclease in dot blot and qPCR-based assays for AAV quantitation to remove residual plasmid DNAs and unpackaged viral genomes that have contaminated AAV preparations. These contaminants would otherwise lead to an overestimation of titers; therefore, the nuclease digestion is a very important step for accurately quantifying viral genome titers. DNase I enzymes are available from various manufacturers and commercial vendors; however, the importance of the selection of DNase I in AAV quantitation appears to have been underappreciated. To investigate how the choice of nuclease might affect the outcomes of the dot blot assay, the following three nucleases were compared for their ability to remove background signals from the AAV vector-containing culture media prepared as described above in steps 2 and 3: DNase I Enzyme A, DNase I Enzyme B, and *S. marcescens* endonuclease (**Table of Materials**). Published studies have used the former two DNase I enzymes[13,21,22,23,24] and we have been using *S. marcescens* endonuclease in previous and current studies. To our surprise, it was identified that DNase I Enzyme A is not at all an appropriate choice for the dot blot assay using the virus-containing media in the various conditions tested, resulting in high background signals from the negative control, while DNase I Enzyme B and *S. marcescens* endonuclease effectively reduced the background signals with DNase I Enzyme B being ~2-fold more effective than *S. marcescens* endonuclease (**Figure 3A, B**). DNase I Enzyme C was also found to be very effective to reduce the background signals (data not shown). These data demonstrate that there are significant differences in enzyme activities among commercially available nucleases when the enzyme reactions are performed in unpurified AAV preparations although nuclease digestion should be effective when a small quantity of purified viral preps is treated under an optimized condition. Thus, these data highlight the importance of the correct selection of nuclease in the assay when unpurified AAV preparations undergo a quantitative dot blot or qPCR assay.

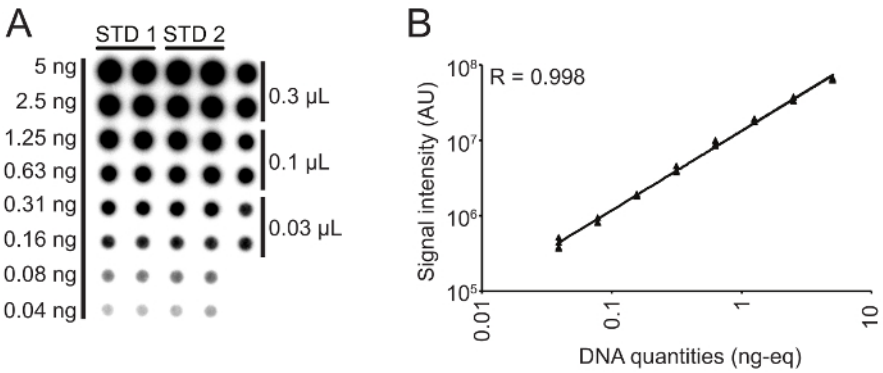

Figure 1: A representative dot blot analysis to determine the titer of a CsCl-purified AAV vector. (**A**) Double-stranded AAV2G9-CMV-GFP vector was produced in HEK 293 cells by a standard adenovirus-free three plasmid transfection method on a large scale, and purified by two rounds of CsCl gradient ultracentrifugation, followed by dialysis. 0.3, 0.1 and 0.03 µL of the purified AAV vector stock (blotted on the rightmost column) were subjected to the quantitative dot blot assay in duplicate with two sets of duplicated plasmid DNA standards (STDs). The blot was hybridized with a ^{32}P-labeled GFP probe (0.77 kb), and the image was obtained using a phosphor image scanning system. (**B**) A standard curve showing the relationship between known plasmid DNA quantities (ng-eq, X-axis) and dot intensities (arbitrary unit (AU), Y-axis). The numbers on the Y-axis were obtained with the phosphor image scanning system. R indicates Pearson's correlation coefficient. Please click here to view a larger version of this figure.

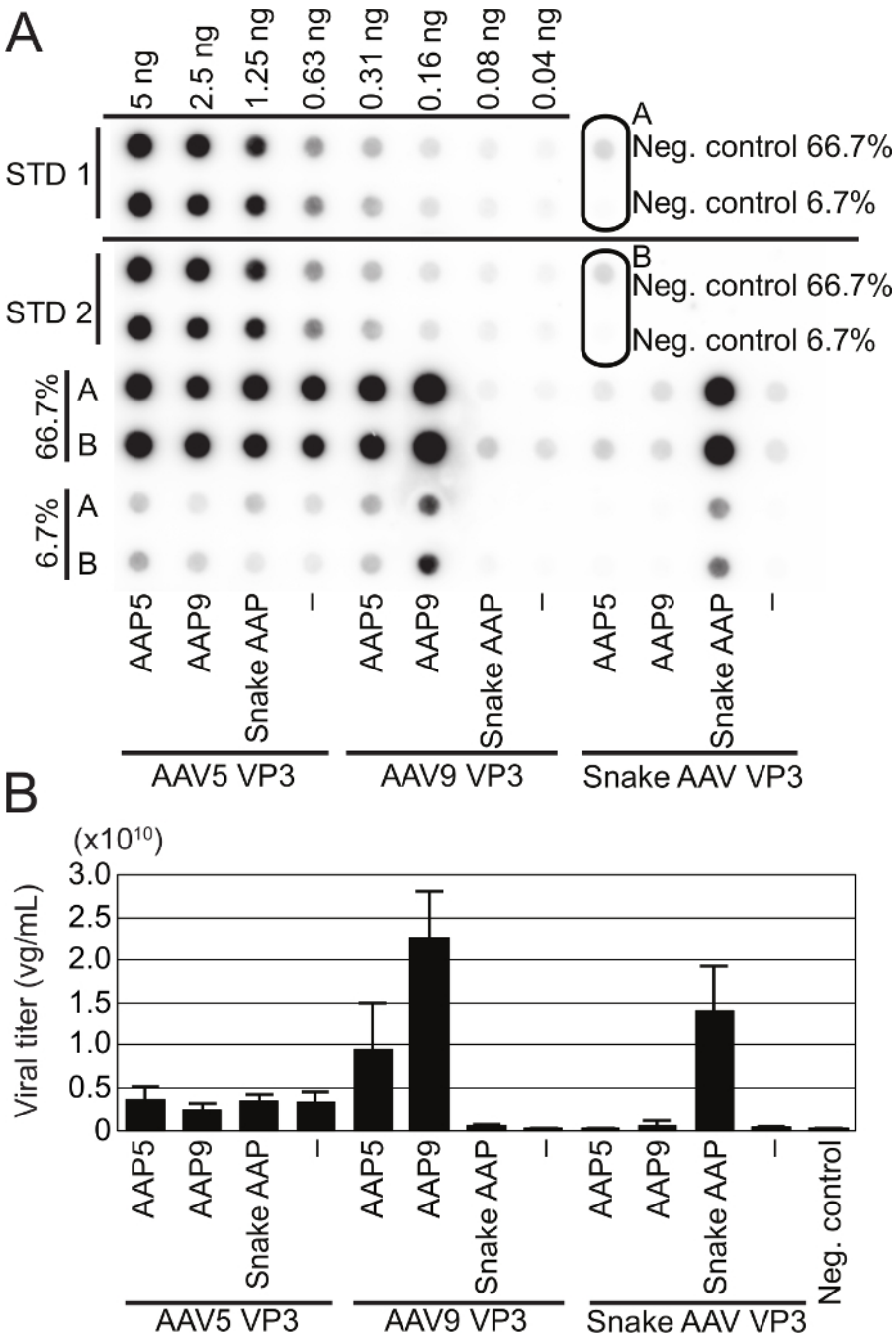

Figure 2: AAV VP3-AAP cross-complementation dot blot assay. Double-stranded AAV-CMV-GFP vector particle production was tested for the VP3 proteins from AAV5, AAV9, and Snake AAV in the presence or absence of their cognate AAPs or in the presence of AAPs of heterologous origins. (**A**) The assay was performed in a biologically duplicated set of experiments (Set A and Set B) with 4 h incubation time with *S. marcescens* endonuclease, and the AAV vector titer obtained from each combination was determined by a quantitative dot blot method described in the Protocol section. Each dot represents two-thirds (the 5th and 6th rows, 66.7%) or two-thirtieths (the 7th and 8th rows, 6.7%) of DNA recovered from each 200 µL of medium collected from the samples or the negative control. The top four rows (1st to 4th rows) represent two sets of plasmid DNA standards (STD 1 and STD 2) blotted in duplicate (*i.e.*, linearized pEMBL-CMV-GFP plasmid, which is the plasmid used for double-stranded AAV-CMV-GFP vector production). The dot blot membrane was probed with a ^{32}P-labeled GFP probe. The pair of dots indicated with rounded rectangles are negative controls. The top two rows (STD 1) are from the bottom of the original blot but have been cut and moved to the top without altering image intensity to display both plasmid standards (STD 1 and STD 2) side by side. This manipulation is indicated with a black line in the figure. (**B**) Viral titer was determined for the combinations of VP3 and AAP proteins by the dot blot assay. The graph represents a biologically quadruplicated set of data, two from Panel A and two from another dot blot that is not shown. Error bars indicates mean +/- SD (n = 4). Please click here to view a larger version of this figure.

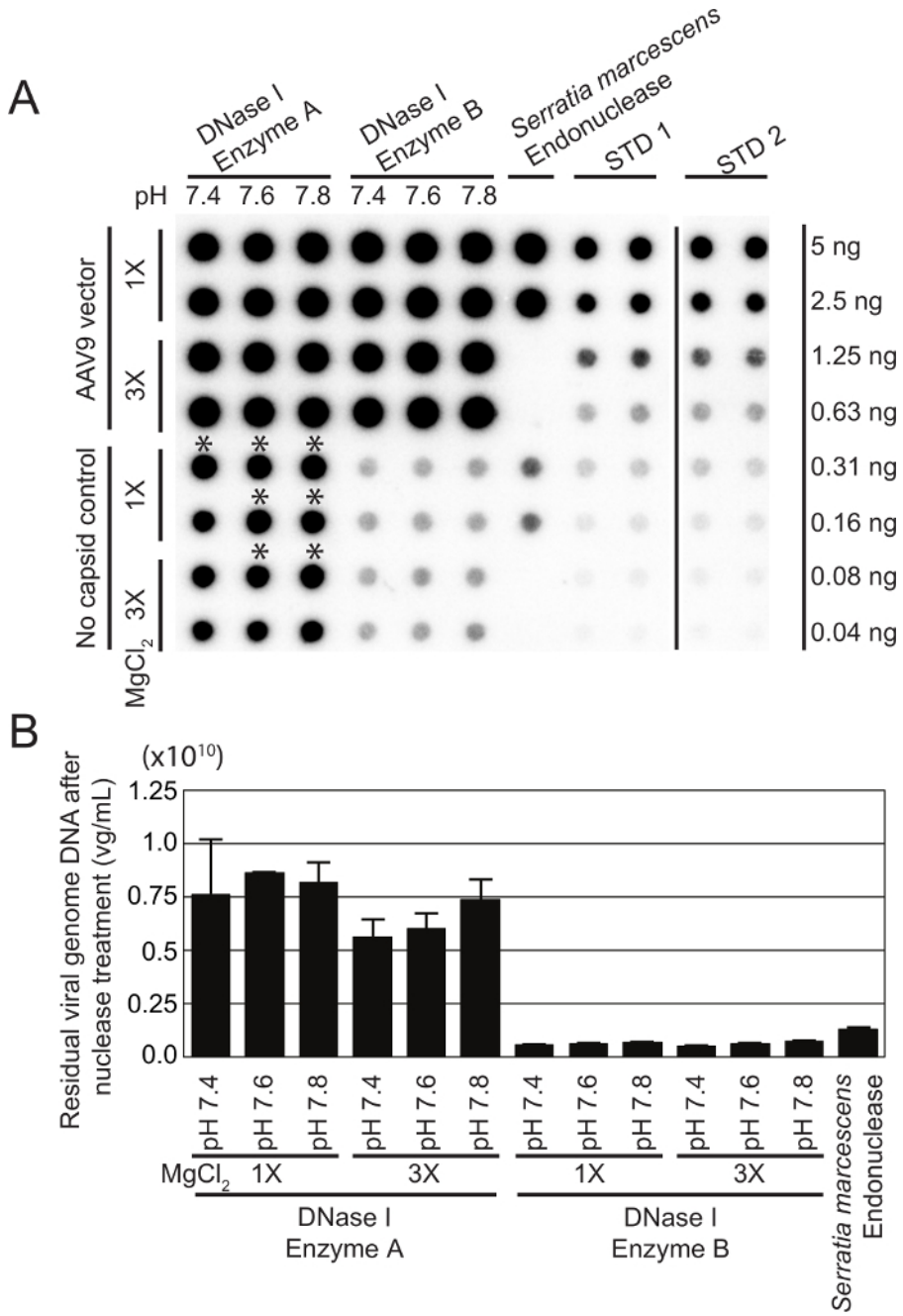

Figure 3: A comparison of enzymatic activities of different nucleases in unpurified AAV vector preparations. (**A**) A quantitative dot blot showing the efficacy of each nuclease treatment in eliminating background signals. A double-stranded AAV9-CMV-GFP vector-containing preparation ("AAV9 vector") and a "no-capsid control" were produced by HEK 293 cell transfection with plasmid DNAs, and subjected to the assay. The no-capsid control did not contain viral particles but contained exponentially amplified unpackaged CMV-GFP vector genomes. $MgCl_2$ was supplemented at one or three-times the recommended amounts (6 mM and 18 mM for DNase I Enzyme A; and 2.5 mM and 7.5 mM for DNase I Enzyme B) without taking into account the 0.8 mM $MgSO_4$ present in the medium. For the treatment with *S. marcescens* endonuclease, only one condition described in the Protocol section was tested. All the samples were treated with each nuclease for 1 h. The dot blot membrane was probed with a ^{32}P-labeled GFP probe. The right two columns (STD 2) are from the left of the original blot but have been cut and moved to the right without altering image intensity to display both plasmid standards (STD 1 and STD 2) side by side. This manipulation is indicated with a thin black line in the figure. (**B**) Dots in the no-capsid control samples in Panel A are quantified and displayed as mean ± |each value — mean value|. Seven out of the 12 samples treated with DNase I Enzyme A (indicated with an asterisk) show values only slightly higher than the highest standard; therefore, they are included in this graph for comparison. Please click here to view a larger version of this figure.

Serratia marcescens Endonuclease Buffer	
50 mM Tris-HCl	
2 mM $MgCl_2$	
Adjust to pH 8.5 with 4 M NaOH.	
10x Proteinase K Buffer	
100 mM Tris-HCl pH 8.0	
100 mM EDTA pH 8.0	
5% SDS	
2x Alkaline Solution	
800 mM NaOH	
20 mM EDTA	
20x SSC (1L)	
175.3 g NaCl	
88.2 g Sodium citrate tribasic ($Na_3C_6H_5O_7$)	
Denhardt's Solution 100x (50 mL)	
1 g Bovine serum albumin (fraction V)	NOTE: Filtering is critical in order to remove small particles as they cause background hybridization signals.
1 g Polysucrose 400	
1 g Polyvinylpyrrolidone	
Dissolve in 20 mL of water in a 55 °C water bath.	
Adjust final volume to 50 mL.	
Filter-sterilize with a 0.22 µm filter.	
Hybridization Buffer	
1% SDS	NOTE: Store Hybridization Buffer at 4 °C and heat to 65 °C in a water bath prior to use.
6x SSC	
5x Denhardt's Solution	
10 mM Tris-HCl pH 8.0	
Wash Buffer	
0.1% SDS	
0.1x SSC	
Polyethylenimine (PEI) Solution (1 mg/mL, 500 mL)	
Add 500 mg PEI in 450 mL of water and stir.	NOTE: For longer storage, -80 °C is recommended.
Add concentrated HCl to bring pH down to <2.0 to dissolve PEI (approximately 800 µL of HCl will be required).	
Add 10 M NaOH to bring pH up to 7.0 (approximately 500 µL of 10 M NaOH will be required).	
Adjust the volume to 500 mL of water.	
Filter-sterilize with a 0.22 µm filter.	
Aliquot and store at -20 °C.	
Phenol-chloroform (1:1 mix) for quantitative dot blotting	
Add buffer-saturated phenol (pH 8.0) and chloroform at a 1:1 ratio in a 50 mL polypropylene conical tube.	NOTE: The buffer covering the organic layer, if left in the tube, can be carried over into sample tubes through pipetting and may make the assay inaccurate.
Vortex the tube vigorously to mix.	
Allow for phase separation by centrifugation and then remove the aqueous layer completely.	
Store at 4 °C.	

Table 1: Solution and Buffer Recipes. Recipes for solutions and buffers needed to complete the quantitative dot blot protocol.

Plasmid	AAP(+) (µg)	AAP(-) (µg)	Negative control (µg)	Transfection control (µg)
pCMV-AAVx-VP3	0.4	0.4		
pCMV-FLAG-AAPx	0.4			
pAAV-Reporter	0.4	0.4	0.4	
pHLP-Rep	0.4	0.4	0.4	
pHelper	0.4	0.4	0.4	
pCMV (Empty)		0.4	0.8	
pCMV-GFP				2.0
Total	2.0	2.0	2.0	2.0

Table 2: Plasmid combinations for AAV VP3-AAP cross-complementation assay performed in a 6-well plate format. Combinations of the plasmid DNAs to be used for HEK 293 cell transfection in each experimental group are shown. pCMV-AAVx-VP3, a plasmid expressing AAV serotype x (x = 1, 2, 3, *etc.*) VP3 protein under the CMV-IE enhancer-promoter; pCMV-FLAG-AAPx, a plasmid expressing AAV serotype x (x = 1, 2, 3, *etc.*) AAP protein under the CMV-IE enhancer-promoter; pAAV-Reporter, a plasmid that has two AAV2 inverted terminal repeats (ITRs) and is designed for recombinant AAV vector production; pHLP-Rep, a plasmid expressing AAV2 Rep protein; pHelper, an adenovirus helper plasmid; pCMV (Empty), an empty plasmid added to control the experimental conditions; pCMV-GFP, a plasmid expressing a fluorescence marker gene (*e.g.*, GFP) to verify successful transfection. Transfections are conducted in 6-well plates, and use a total of 2 µg of plasmid DNAs.

Mix A	For 10 tubes (µL)
Serratia marcescens Endonuclease Buffer	91.2
0.1 M NaOH	8.8
Total	100
Mix B	For 10 tubes (µL)
Serratia marcescens Endonuclease Buffer	91.2
1 M $MgCl_2$	7.04
Serratia marcescens Endonuclease (250 units/µL)	0.176
Total	100
Mix C	For 10 tubes (µL)
10x Proteinase K Buffer	400
Proteinase K (20 mg/mL)	100
H_2O	1,300
Total	1,800
Mix D	For 10 tubes (µL)
100% Ethanol	8,000
3 M Sodium acetate (pH 5.2)	320
Oyster glycogen (20 mg/mL)	10
Total	8,330

Table 3: List of master mix reagents. Mix A and Mix B are used for the *S. marcescens* endonuclease treatment in step 3.2. These two mixtures should be made separately to prevent precipitation of magnesium hydroxide. Mix C is used for Proteinase K treatment in step 3.3. Mix D is used for ethanol precipitation in step 3.4.3. The volumes indicated in the table are for master mix reagents for 10 tubes.

Plasmid DNA standard (ng)	Volumes (µL) of 25 pg/µL plasmid DNA standard solution	Volumes (µL) of water	Total volume (µL)
5	600	0	600
2.5	300	300	600
1.25	150	450	600
0.625	75	525	600
0.313	37.5	562.5	600
0.156	18.8	581.2	600
0.078	9.4	590.6	600
0.039	4.7	595.3	600

Table 4: Quantitative dot blot plasmid DNA standards. Necessary volumes of 25 pg/µL plasmid DNA standard solution and water or TE to prepare plasmid DNA standards by two-fold serial dilutions (600 µL/tube) are shown. The numbers in the plasmid DNA standard column (0.039 to 5 ng) are the quantity of DNA in 200 µL of each plasmid DNA standard solution.

Discussion

In this report, the utility of quantitative dot blot assays to study AAV AAPs and their role in capsid assembly is described. Knowledge gained from these studies can provide detailed insights into the innate differences in the process of AAV capsid assembly and the functional role of AAPs between different serotypes. In this respect, the AAV VP3-AAP cross-complementation dot blot assay revealed that Snake AAV VP3 displayed a strict dependency on the co-expression of its cognate AAP for capsid assembly and that Snake AAP does not promote capsid assembly of heterologous serotypes. This observation is intriguing because the AAP-dependent AAV serotypes that were previously investigated (AAV1, 2, 3, 6, 7, 8, 9, and 12) are all able to process assembly at least to some degree by utilizing a heterologous AAP[6].

Quantitative dot or slot blot hybridization is a traditional method for quantitation of nucleic acids contained in multiple samples at the same time in a convenient manner[25]. The method had been widely used for DNA and RNA quantitation until qPCR became prevalent in the 1990s[26,27]. Although qPCR has advantages over quantitative dot blot and other hybridization-based assays in that qPCR exhibits a higher sensitivity and a wider dynamic range, it also carries an inherent risk of exponentially augmenting errors unknowingly, which was the case for titers of AAV vector stocks determined by qPCR[13,23]. Quantitative dot blot assays are easily set up with an inexpensive cost and easily carried out as long as researchers have access to a quantitative molecular imaging system that can acquire signals from dot blot membranes hybridized with either a radioactive probe or a non-radioactive chemiluminescent or fluorescent probe. Although this protocol utilizes ^{32}P-labeled radioactive probes for signal detection, others successfully use non-radioactive DNA probes directly labeled with a commercially available thermostable alkaline phosphatase[28]. Dot blot assays use a straightforward principle and the assay by itself does not pose a technical challenge to performers; therefore, the results are generally reproducible even by inexperienced individuals.

Besides the applications described here, we routinely use a simplified and expedited version of the dot blot procedure that can semi-quantify AAV particles quickly. Advantages of dot blot assays in this context include: (1) the assay does not require purification or enzymatic amplification of viral genomes which takes hours, (2) a combination of heat and alkaline denaturation is sufficient to break AAV particles in a sample solution and release denatured viral genomes into the solution that are ready to bind to a dot blot membrane, and (3) the presence of salt at high concentrations in samples does not significantly affect the assay results. For example, it is possible to semi-quantify AAV particles in CsCl-rich solutions (*e.g.*, fractions obtained by CsCl density-gradient ultracentrifugation) by putting a small aliquot (≤10 µL) of samples into 100 µL of 1x Alkaline Solution, heating at 100 °C for 10 min, and blotting onto a membrane with or without standards prepared in advance, followed by a 15 min hybridization, 3 x 3 min washes and exposure to a phosphor imaging screen for 15 min (or longer when using a probe with decreased radioactivity). The whole procedure can be completed in 1 h once the user becomes familiar with the procedure. We use this expedited method, which we customarily call "boiling dot blot method", to identify AAV particle-rich CsCl fractions during the vector purification process and roughly determine titers of purified AAV vector stocks before beginning the extensive processes for vector characterization. Thus, although dot blot assays might be viewed as an outmoded method to quantify nucleic acids and have already been replaced with various PCR-based methods in a wide range of scientific disciplines, there are still a number of advantages to this method that should make researchers consider employing it in their laboratories.

The most critical step in the protocol is the nuclease treatment of samples. If this step is not carried out in an optimal condition, it would cause high background signals. We use *S. marcescens* endonuclease while DNase I enzymes of different sources are widely used in other laboratories for viral DNA quantification. The DNase I of bovine pancreas origin has been most widely used by researchers. This is in part because the bovine pancreatic DNase I was first identified and most extensively characterized biochemically[29]. The DNase I enzymes currently available from commercial vendors are produced from several different biological sources such as *Pichia pastoris* (DNase I Enzyme A), the native form purified from the bovine pancreas (*e.g.*, DNase I Enzyme B), and recombinant enzymes produced in either a yeast species or *Escherichia coli* (DNase I Enzyme C). We have found that the DNase I enzymes from all three of these different sources have been used in published AAV vector quantitation studies; however, to our knowledge, none of the previous studies have investigated whether the enzymes from different sources are equally effective in digesting contaminating DNA molecules in AAV vector preparations. It should be noted that DNase I treatment for AAV vector assays is often carried out under non-optimized conditions due to the presence of impurities derived from culture medium and cells. The observation that DNase I Enzyme A is only partially active in the culture medium we used has significant implications in designing the assay for AAV vector quantitation by dot blot and qPCR, and alerts researchers to this previously unidentified issue. In this regard, *S. marcescens* endonuclease expressed in *E. coli*, is an ideal endonuclease not only for the purpose of manufacturing AAV vectors but also for AAV vector quantitation. This is because its enzymatic activity can be retained over a wide range of pH values and concentrations of magnesium ions and

monovalent cations. For this reason, and because *S. marcescens* endonuclease is approximately 2 times less expensive than DNase I Enzyme B on a per-unit basis, we prefer to use *S. marcescens* endonuclease in routine quantitative dot blot analyses.

In summary, quantitative dot blot assays are a relatively straightforward procedure that can readily provide information on the ability to produce viral particles under varying conditions. Compared to alternative titering approaches, such as qPCR, this approach requires little, if any, optimization. It can also be readily applied to vectors of any serotype and can be used to titer both single- and double-stranded vectors without modifying the protocol. Following transfections and harvest of AAV vector-containing samples (culture medium and/or cells), the whole protocol can be completed in a day, thus rapidly answering questions about the ability to produce viral particles from diverse conditions, including various combinations of AAV VP3 and AAP proteins. Quantitative dot blots offer an expedient method to address various unanswered questions as to VP-AAP interactions and their roles in capsid assembly in a wide variety of different AAV serotypes and isolates.

Disclosures

The authors have nothing to disclose.

Acknowledgements

We thank Xiao Xiao at the University of North Carolina at Chapel Hill for providing us with the pEMBL-CMV-GFP plasmid. We thank Christopher Cheng and Samuel J. Huang for critical reading of the manuscript. This work was supported by Public Health Service grants (NIH R01 NS088399 and T32 EY232113).

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