APPLICATION NOTE

AAV capsid detection: a new simple, no-wash, reliable, and quantitative assay platform based on AlphaLISA technology.

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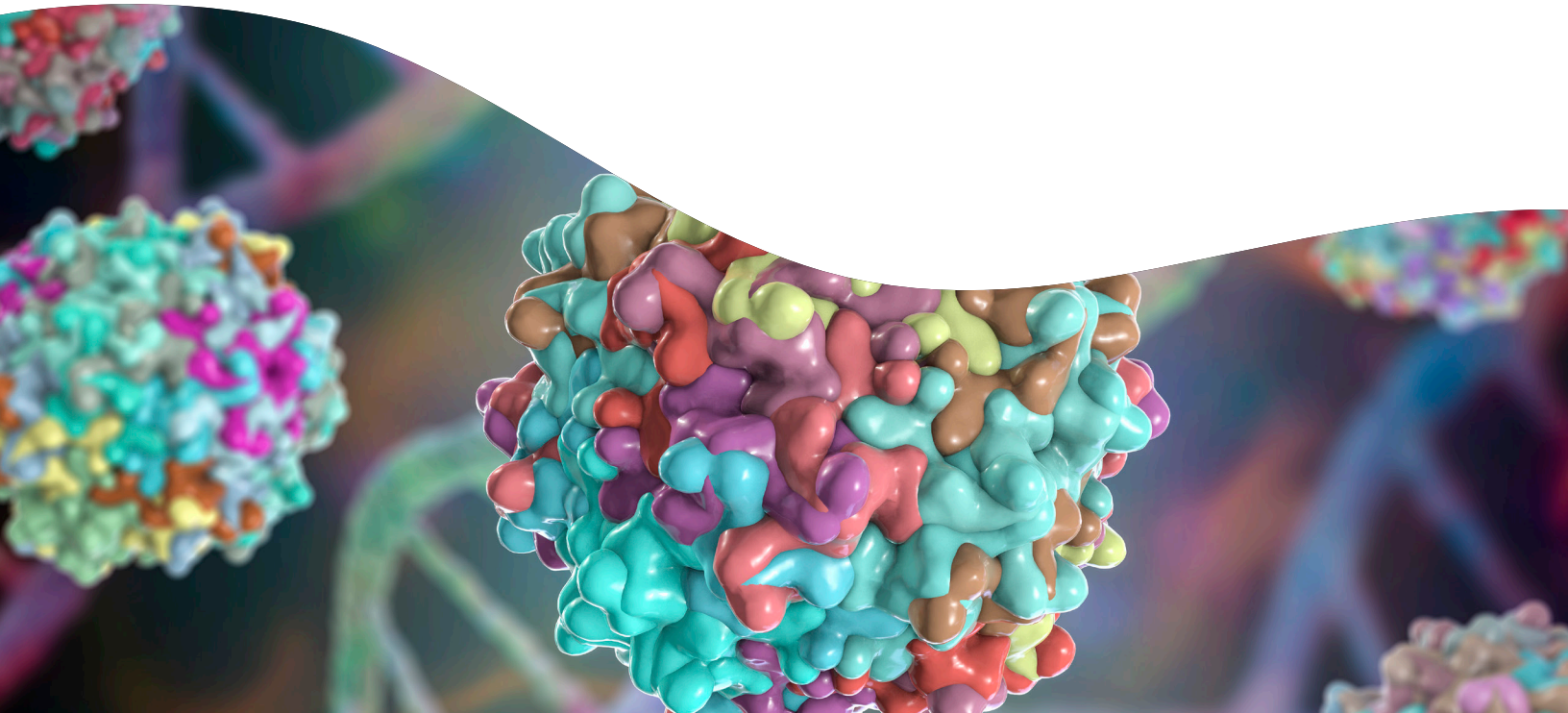
Introduction

AAV (Adeno-associated virus) is a relatively simple, small (~25 nm), non-enveloped parvovirus with a ~4.7 kb single stranded genome.[1] It was initially identified as a viral contaminant in purified adenovirus preparations.[2] AAVs are not capable of replicating on their own. They require helper viruses (e.g., adenovirus) to propagate in host cells and are not known to cause disease in either humans or animals.[3] The AAV genome comprises three genes, each transcriptionally and translationally regulated by individual promoters, alternate splicing mechanisms and differential translational start sites. The genes encode non-structural, regulatory proteins involved in replication (*rep*), capsid assembly (*aap*) and the structural building blocks that form the viral capsid (*cap*).[4] The cap gene is translated into three overlapping gene-products VP1, VP2, and VP3, with respective monomeric molecular weights ranging from 73 - 82 kDa, 64 - 67 kDa and 59 - 61 kDa. These proteins assemble into a 60-subunit viral capsid with T=1 icosahedral symmetry and a VP1:VP2:VP3 ratio of approximately 5:5:50.5 An illustration depicting the genetic and structural organization of an AAV is provided in Figure 1.



AAVs are highly studied viruses and partly due to their replication-defective nature, widely used as viral vectors in human gene therapy utilizing recombinant DNA technology.[6] Recombinant AAVs (rAAV) are essentially viral DNA-free, bioengineered nanoparticles used to deliver recombinant genes to target cells to treat monogenic diseases.[7] rAAVs are often produced by transfecting HEK293 or SF9 cells with plasmids carrying AAV serotype specific rep/cap genes, a gene of interest (or gene therapy) and genes from a helper virus.[8] The rAAVs are then purified from cell culture and used to infect target cells. Several biochemical and biophysical methods are needed to fully characterize AAVs. General methods for AAV analysis include real-time qPCR or ddPCR for genomic content, immunoassays for capsid quantification and microfluidics (LabChip), electron microscopy and dynamic light scattering for viral particle visualization and characterization. Accurately measuring the AAV concentration in complex biological matrices (such as cell culture medium and cell lysate) is critical for the safe and effective manufacturing and clinical utilization of AAV gene therapies.[9] There are 12 known naturally occurring, human specific AAV serotypes. Differences in the capsid of each AAV serotype gives rise to tropism, resulting in varying cell/tissue-specific viral transduction efficiencies.[10] This reflects the need for the development of analytical tools that cover multiple AAV serotypes used in development of human gene therapies.

Revvity has developed and manufactured a line of immunoassays that detect and quantify AAV capsid (in Viral Particles per milliliter - VP/mL) utilizing AlphaLISA technology. The AlphaLISA AAV Capsid Detection Kits are available for AAV1, AAV2, AAV3B, AAV5, AAV6, AAV8 and AAV9 serotypes, and can measure AAV particles present in cell culture media, lysis buffer and cell lysate. Each AlphaLISA kit was thoroughly tested and data regarding the detection limit, assay reproducibility, variability, specificity, and recovery from multiple analyte matrices is provided in the technical data sheet (TDS) for each AlphaLISA AAV capsid detection kit. A summary outlining the testing of each AlphaLISA AAV capsid detection kit is provided in Table 1.

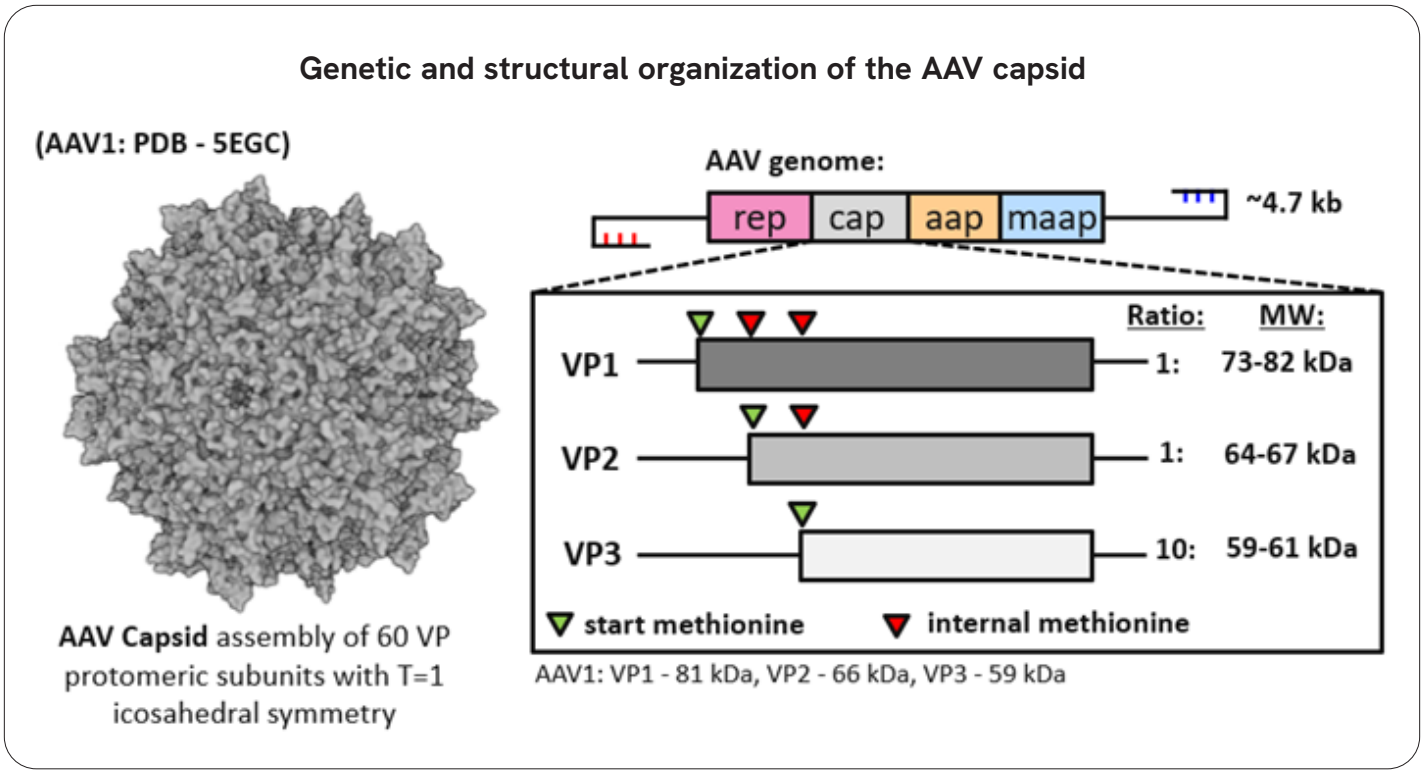


Figure 1: 3D structure was rendered using * MOL 3D viewer (www.rcsb.org/3d-view)[11] using the x-ray diffraction coordinates for PDB structure: 5EGC[12] www.rcsb.org with surface density enabled. Position of start and internal methionine shown for overlapping VP gene products. Figure adapted from Wörner, Tobias P. et al. (Ref. 5).

Table 1. Summary for testing of AlphaLISA AAV Capsid Detection Kit.

Parameter	Matrix	AAV1	AAV2	AAV3B	AAV5	AAV6	AAV8	AAV9
LDL	*Assay buffer	✓	✓	✓	✓	✓	✓	✓
	AlphaLISA lysis buffer	✓	✓	✓	✓	✓	✓	✓
	DMEM	✓	✓	✓	✓	✓	✓	✓
	RPMI (containing free biotin)	✓	✓	✓	✓	✓	✓	✓
CV (% intra-assay and inter-assay)	*Assay buffer	✓	✓	✓	✓	✓	✓	✓
	AlphaLISA lysis buffer	✓	✓	✓	✓	✓	✓	✓
	DMEM	✓	✓	✓	✓	✓	✓	✓
	RPMI (containing free biotin)	✓	✓	✓	✓	✓	✓	✓
AAV spiked recovery (%)	*Assay buffer	✓	✓	✓	✓	✓	✓	✓
	AlphaLISA lysis buffer	✓	✓	✓	✓	✓	✓	✓
	DMEM	✓	✓	✓	✓	✓	✓	✓
	RPMI (containing free biotin)	✓	✓	✓	✓	✓	✓	✓
	SF9 & HEK293 cell lysate	✓	✓	✓	✓	✓	✓	✓
Cross-reactivity of AAV1 AlphaLISA kit	AAV1	N/A	✓	✓	✓	✓	✓	✓
	AAV2	✓	N/A	✓	✓	✓	✓	✓
	AAV3B	✓	✓	N/A	✓	✓	✓	✓
	AAV5	✓	✓	✓	N/A	✓	✓	✓
	AAV6	✓	✓	✓	✓	N/A	✓	✓
	AAV8	✓	✓	✓	✓	✓	N/A	✓
	AAV9	✓	✓	✓	✓	✓	✓	N/A
Empty and loaded AAV detection	*Assay buffer	✓	✓	✓	✓	✓	✓	✓

*Assay buffer: HiBlock buffer - AAV1 and AAV9; NaCl buffer - AAV2, AAV3B and AAV5 and AAV6; immunoassay buffer (IAB) - AAV8. N/A = not applicable.

AlphaLISA AAV capsid detection kit technology.

AlphaLISA technology allows for simple, sensitive, quantitative, and reproducible detection of analytes of interest in complex matrices such as cell culture medium and cell lysate. The AlphaLISA AAV capsid detection kits for AAV2, AAV3B, AAV5, AAV8 and AAV9 all use similar chemistry in both design and function. A schematic representing the chemistry for these kits is provided in figure 2A. In these assays, biotinylated AAV specific antibodies bind to streptavidin coated AlphaLISA donor beads with high affinity and AlphaLISA acceptor beads are conjugated to anti-AAV antibodies. In the presence of AAV capsid, the beads come into proximity. The excitation of donor beads triggers the release of singlet oxygen, which facilitates an energy transfer cascade within the acceptor beads resulting in an emission spectrum with a λ_{max} of 615 nm.

The AlphaLISA AAV1 and AAV6 capsid detection kit donor bead chemistry is identical to the other AAV AlphaLISA kits. However, the acceptor beads are conjugated to an Anti-HRP antibody, which binds an HRP-conjugated Anti-AAV antibody. An illustration depicting the bead chemistry for this kit is shown in figure 2B. Lyophilized empty serotype specific AAV capsid is provided with each AlphaLISA AAV capsid detection kit to be reconstituted, diluted, and run as a standard curve to quantify the amount of AAV in unknown samples in VP/mL.

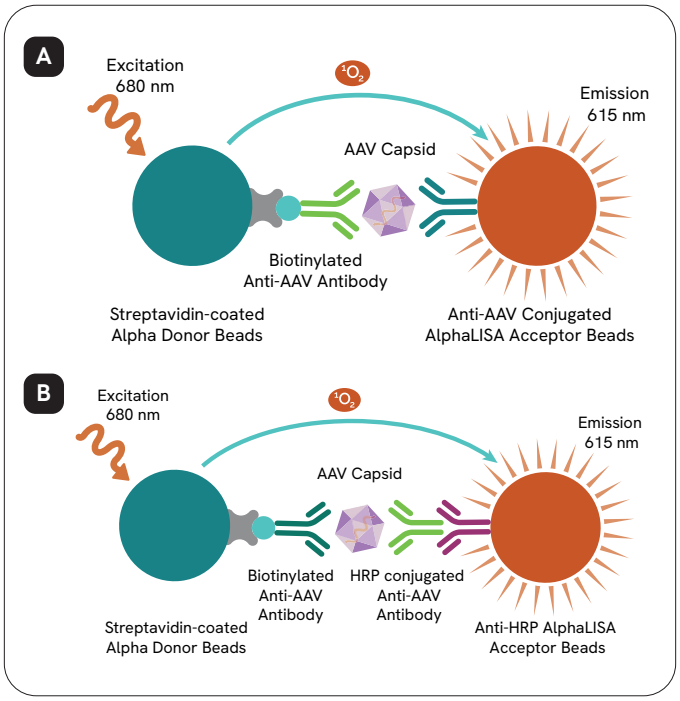
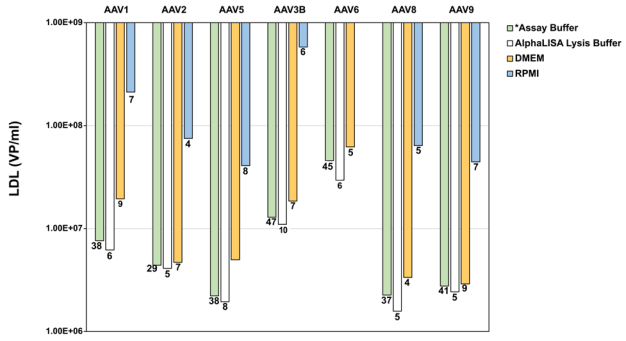


Figure 2: (A) Canonical AlphaLISA AAV capsid detection kit bead chemistry. (B) AlphaLISA AAV1 & AAV6 capsid detection kit bead chemistry.

Sensitivity of AlphaLISA AAV capsid detection kits.

The lower detection limit (LDL) for all AlphaLISA AAV capsid detection kits was calculated from 12 wells containing 10 µl of the following analyte matrices: assay buffer (HiBlock Buffer - AAV1 and AAV9; NaCl Buffer - AAV2, AAV3B, AAV5 and AAV6; immunoassay buffer (IAB) – AAV8, AlphaLISA lysis buffer, DMEM and RPMI cell culture medium. These analyte matrix (background only) samples contained no AAV. The LDL was calculated by taking the average AlphaLISA Signal (counts) for the 12 background wells for each matrix plus 3 x the standard deviation (SD) for those wells. This value was interpolated using the AAV standard curve for the respective analyte matrix to find the equivalent concentration in VP/mL (Table 2). All other kit components were diluted in kit-specific assay buffer. Details related to setting up AAV capsid standard dilutions and other technical information can be found in the TDS provided for each AlphaLISA AAV capsid detection kit. The LDL for each AAV serotype is plotted in figure 3. The number below each histogram represents the number of experimental replicates for each sample-type. Analyte matrices with higher LDL values have lower assay sensitivity.

The data shows that overall, the AlphaLISA AAV capsid detection kit sensitivity is highest in assay buffer and AlphaLISA lysis buffer (figure 3). Assay sensitivity in DMEM is shown to be either as sensitive, or slightly less sensitive than assay buffer and lysis buffer. RPMI (containing free biotin) negatively affects assay sensitivity, as biotin is known to interfere with the donor bead chemistry described above. Assay interference from free biotin in RPMI is the leading contributor to values missing from the data tables throughout this document. Nonetheless, AlphaLISA AAV capsid detection kits can quantify AAV in RPMI and the TDS for each kit contains an alternate protocol using a 5 µL instead of 10 µL sample to limit the effect of biotin on the assay (data not provided). The overall relative sensitivity of each kit was assessed by averaging the LDL across assay buffer, AlphaLISA Lysis Buffer and DMEM analyte matrices, omitting the RPMI data. The ranking for each AlphaLISA AAV capsid detection kit Is listed from highest to lowest relative sensitivity: AAV8 > AAV9 > AAV5 > AAV2 > AAV3B > AAV1 > AAV6 based on overall average LDL values of 2.40E+06, 2.70E+06, 3.05E+06, 4.40E+06, 1.41E+07, 1.11E+07 and 4.58E+07 VP/mL, respectively. It should be noted that the LDL is not reported for the AlphaLISA AAV6 capsid detection kit in an RPMI background. The AAV6 kit is the least sensitive overall when the LDL values reported for AAV in RPMI are omitted from the analysis for all other AlphaLISA kits. With current techniques in molecular and cellular biology, AAV yields after purification are often reported to be >1.0E+12 VG/mL (Viral Genome/milliliter).[13] This demonstrates that AlphaLISA AAV capsid detection kits provide the sensitivity required to quantify the concentration of AAV or rAAV produced during the manufacturing process.



Higher LDL values indicate analyte matrices with lower assay sensitivity. Numbers below individual data points represent the number of experimental replicates for each sample. *Assay buffer: HiBlock buffer - AAV1 and AAV9; NaCl buffer - AAV2, AAV3B and AAV5 and AAV6; immunoassay buffer (IAB) - AAV8.

Figure 3: Histogram of AlphaLISA AAV capsid detection kit matrix-specific LDL.

Table 2. Analyte matrix-specific LDL for each AlphaLISA AAV capsid detection kits.

	LDL (VP/mL)						
Matrix	**AAV1**	**AAV2**	**AAV5**	**AAV3B**	**AAV6**	**AAV8**	**AAV9**
*Assay Buffer	7.61E+06	4.40E+06	2.22E+06	1.29E+07	4.56E+07	2.26E+06	2.77E+06
AlphaLISA Lysis Buffer	6.19E+06	4.10E+06	1.95E+06	1.10E+07	2.95E+07	1.58E+06	2.43E+06
DMEM	1.95E+07	4.70E+06	4.98E+06	1.85E+07	6.23E+07	3.36E+06	2.90E+06
RPMI	2.12E+08	7.50E+07	4.09E+07	5.80E+08	N/A	6.42E+07	4.44E+07

LDL = (average of background wells + (3 x SD)). Values interpolated using standard curve from empty AAV capsid provided with each AlphaLISA kit. LDL is given in VP/mL.
*Assay buffer: HiBlock buffer - AAV1 and AAV9; NaCl Buffer - AAV2, AAV3B, AAV5 and AAV6; Immunoassay Buffer (IAB) - AAV8. N/A, data not available.

Precision of AlphaLISA AAV capsid detection kits.

Both intra- and inter-assay precision was determined for each AlphaLISA AAV capsid detection kit. Empty AAV capsid analyte standards (provided with the kit) were diluted in assay buffer, AlphaLISA lysis buffer, DMEM and RPMI medium to known concentrations and used as samples in the assay. A 12-point standard curve in triplicate was also set up for each assay, in addition to a total of 12 background wells for each analyte matrix (no AAV). The intra-assay precision for each kit was calculated using no less than 12 wells from the same plate (tables 3A - D). Inter-assay precision was calculated from 3 independent assays using triplicate samples covering two different lots of AlphaLISA AAV capsid detection kits (tables 3E - H). The measured values were interpolated using the standard curve to find the concentration in VP/mL. The variability between replicate samples was calculated and provided in tables 3A - H as the coefficient of variation or CV (standard deviation ÷ average x 100 = CV %).

Tables 3 - D. Intra-assay precision of AlphaLISA AAV capsid detection kits.

3A

	[AAV1] (VP/mL)		[AAV2] (VP/mL)			[AAV3B] (VP/mL)		
	2.00E+09	2.00E+10	5.00E+07	1.00E+09	2.00E+10	5.00E+08	2.00E+09	2.00E+10
Matrix	**CV (%) Intra-assay variability**							
*Assay Buffer	5%	10%	8%	6%	5%	9%	6%	8%
AlphaLISA Lysis Buffer	3%	8%	7%	5%	7%	7%	5%	9%
DMEM	7%	7%	7%	5%	9%	9%	7%	8%
RPMI	16%	13%	N/A	7%	8%	8%	10%	9%

Intra-assay CV (%) for AAV1, AAV2 and AAV3B AlphaLISA kits. CV values >10% or data not available (N/A) are shown in red.

3B

	[AAV5] (VP/mL)		[AAV6] (VP/mL)			[AAV8] (VP/mL)		
	5.00E+07	1.00E+09	5.00E+08	2.00E+09	2.00E+10	5.00E+08	2.00E+09	2.00E+10
Matrix	**CV (%) Inter-assay variability**							
*Assay Buffer	8%	3%	9%	5%	8%	5%	7%	9%
AlphaLISA Lysis Buffer	7%	8%	6%	5%	5%	8%	5%	8%
DMEM	8%	4%	6%	4%	3%	3%	6%	9%
RPMI	N/A	9%	N/A	N/A	N/A	8%	8%	14%

Intra-assay CV (%) for AAV5, AAV6 and AAV8. CV values >10% or data not available (N/A) are shown in red.

3C

	[AAV9] (VP/mL)	
	5.00E+07	2.00E+09
Matrix	**CV (%)**	
HiBlock buffer	11%	8%
AlphaLISA lysis buffer	8%	4%
DMEM	14%	4%
RPMI	N/A	8%

Intra-assay CV (%) for AAV9. CV values >10% or data not available (N/A) are shown in red.

Tables 3 - H. Inter-assay precision of AlphaLISA AAV capsid detection kits.

3D

	Matrix-specific average intra-assay CV (%)							
Matrix	**AAV1**	**AAV2**	**AAV3B**	**AAV5**	**AAV6**	**AAV8**	**AAV9**	**Overall**
*Assay buffer	8%	6%	8%	6%	7%	7%	7%	7%
AlphaLISA lysis buffer	6%	6%	7%	8%	5%	7%	6%	6%
DMEM	7%	7%	8%	6%	4%	6%	5%	7%
RPMI	15%	8%	9%	9%	N/A	10%	10%	10%
							Average inter-assay CV (%) for all kits and conditions:	7%

Matrix-specific average intra-assay CV (%) for all AlphaLISA AAV capsid detection kits. CV values >10% or data not available (N/A) are shown in red.

3E

	[AAV1] (VP/mL)			[AAV2] (VP/mL)			[AAV3B] (VP/mL)		
	8.00E+07	**2.00E+09**	**2.00E+10**	**1.00E+08**	**1.00E+09**	**1.00E+10**	**2.00E+08**	**2.00E+09**	**2.00E+10**
Matrix	**CV (%) Inter-assay variability**								
*Assay buffer	11%	7%	4%	4%	2%	2%	9%	11%	2%
AlphaLISA lysis buffer	4%	3%	2%	10%	1%	5%	8%	1%	7%
DMEM	N/A	5%	4%	2%	7%	7%	14%	12%	1%
RPMI	N/A	6%	6%	N/A	2%	10%	15%	13%	14%

Inter-assay CV (%) for AAV1, AAV2 and AAV3B. CV values >10% or data not available (N/A) are shown in red.

3F

	[AAV5] (VP/mL)			[AAV6] (VP/mL)			[AAV8] (VP/mL)		
	1.00E+08	**1.00E+09**	**1.00E+10**	**2.00E+09**	**2.00E+10**	**5.00E+10**	**2.00E+08**	**2.00E+09**	**2.00E+10**
Matrix	**CV (%) Intra-assay variability**								
*Assay buffer	4%	5%	6%	5%	2%	7%	8%	9%	12%
AlphaLISA lysis buffer	10%	3%	6%	2%	2%	4%	5%	5%	8%
DMEM	5%	6%	7%	5%	2%	4%	9%	4%	5%
RPMI	N/A	7%	9%	N/A	N/A	N/A	N/A	6%	15%

Inter-assay CV (%) for AAV5, AAV6 and AAV8 CV values >10% or data not available (N/A) are shown in red.

3G

	[AAV9] (VP/mL)		
	1.00E+08	**1.00E+09**	**1.00E+10**
Matrix	**CV (%) Inter-assay variability**		
*Assay Buffer	2%	4%	6%
AlphaLISA lysis buffer	8%	3%	5%
DMEM	13%	3%	4%
RPMI	N/A	7%	5%

Inter-assay CV (%) for AAV9 CV values >10% or data not available (N/A) are shown in red.

3H

	Matrix-specific average Inter-assay CV(%)							
Matrix	**AAV1**	**AAV2**	**AAV3B**	**AAV5**	**AAV5**	**AAV8**	**AAV9**	**Overall**
*Assay buffer	7%	3%	7%	5%	5%	10%	4%	6%
AlphaLISA lysis buffer	3%	5%	5%	3%	6%	6%	5%	5%
DMEM	5%	5%	9%	4%	6%	6%	7%	6%
RPMI	6%	6%	14%	N/A	8%	11%	6%	8%
							Average inter-assay CV (%) for all kits and conditions:	6%

Matrix-specific average inter-assay CV (%) for all AlphaLISA AAV Capsid Detection Kits. CV values >10% or data not available (N/A) are shown in red.

The overall average intra-assay variability in CV (%) for all AAV kits and matrices tested is 7% (Table 3D). Tables 3A - C contain individual intra-assay CV values at known AAV capsid concentrations measured in the given analyte matrices and Table 3D contains the matrix-specific average intra-assay variability for each AlphaLISA AAV kit. Individual or averaged intra-assay CV values >10% and data not available (N/A), are labeled red (Tables 3A - D). Most AlphaLISA AAV kits have intra-assay CV values <10%. However, the CV for the AAV1 AlphaLISA Kit is >10% in RPMI at both analyte concentrations tested (Table 3A).The intra-assay variability was not measured for AAV2 or AAV5 kit in RPMI at the lowest capsid concentration (Table 3A and B). An intra-assay CV is not reported for AAV6 in RPMI at any of the capsid concentrations tested (Table 3B). AAV8 has >10% CV in RPMI at the highest concentration and AAV9 had >10% CV in IAB and DMEM at the lowest analyte concentration (Table 3C). No intra-assay CV was obtained for the AAV9 kit in RPMI at the lowest analyte concentration.

The average inter-assay variability across all AlphaLISA AAV capsid detection kits is 6% (Table 3H). AAV1 has >10% inter-assay CV in HiBlock buffer and no CV is provided for AAV1 measured in DMEM or RPMI medium at the lowest concentrations (Table 3E). An inter-assay CV value is not provided for AAV2 detected RPMI at the lowest capsid concentration (Table 3E). The AAV3B kit has >10% CV in IAB at the middle capsid concentration, DMEM at the two lower concentrations and in RPMI at all concentrations tested (Table 3E) and results in the highest matrix-specific average inter-assay CV reported for any kit at 14% (Table 3H). An inter-assay CV for the AAV6 kit in RPMI is not reported at any concentration (Table 3F). The AAV8 kit has >10% CV in IAB and RPMI medium at the highest capsid concentration and no CV is given for the AAV8 kit in RPMI at the lowest capsid concentration (Table 3F). The AAV9 kit has >10% CV in DMEM at the lowest concentration tested, and no CV is given for AAV9 at the lowest analyte concentration in RPMI (Table 3G). These results demonstrate that while the AlphaLISA AAV capsid Detection kits have different intra- and inter-assay variability at specific analyte concentrations and backgrounds, in most cases, AlphaLISA AAV kit variability is <10%. However, care should be taken when working with RPMI cell culture medium or an analyte matrix containing free biotin as intra- or inter-assay CV values are often either >10%, or not obtained at the lowest analyte concentrations tested. Furthermore, no intra- or inter-assay CV is reported for AAV6 at any concentration in RPMI (Table 3B and 3F).

Recovery of AAV from different analyte matrices using AlphaLISA AAV capsid detection kits.

To determine if a particular analyte matrix influences the detection of AAV by AlphaLISA kits, known concentrations of empty AAV capsid (analyte standard provided with each kit) were spiked into assay buffer, AlphaLISA lysis buffer, DMEM and RPMI cell culture medium. All other AlphaLISA components were diluted in assay buffer. AAV standard curves were set up following the dilution matrix provided in the AlphaLISA AAV capsid detection kit TDS. The resulting AlphaLISA signal (counts) for known samples were interpolated using the respective analyte standard curve and given in VP/mL. The experimentally derived concentrations were divided by the known concentrations and multiplied by 100 to calculate recovery (%). The known concentrations, recovery, and average matrix-specific recovery (%) for each AlphaLISA AAV kit and analyte matrix is provided in Tables 4A - 4G. The average recovery (%) and standard deviation across all AlphaLISA AAV capsid detection kits is provided for each analyte matrix in Table 4H.

Tables 4 - H. Percent Recovery of AAV from buffer and cell culture medium.

4A

	[AAV1] (VP/mL)						
	1.02E+08	2.56E+08	1.60E+09	4.00E+09	1.00E+10		
Matrix	**Spike recovery (%)**					**Average**	**St. Dev.**
HiBlock buffer	94%	94%	94%	95%	100%	95%	3%
AlphaLISA lysis buffer	103%	82%	94%	97%	100%	95%	8%
DMEM	91%	99%	94%	96%	100%	96%	4%
RPMI	N/A	90%	85%	90%	100%	91%	6%

AAV1 recovery from buffer and cell culture medium. AAV1 assay buffer: HiBlock buffer.

Tables 4 - H. Percent Recovery of AAV from buffer and cell culture medium.

4B

	[AAV2] (VP/mL)				
	1.00E+08	1.00E+09	1.00E+10		
Matrix	**Spike recovery (%)**			**Average**	**St. Dev.**
NaCl buffer	94%	112%	99%	102%	9%
AlphaLISA lysis buffer	118%	93%	104%	105%	13%
DMEM	104%	92%	115%	104%	12%
RPMI	92%	84%	86%	87%	4%

AAV2 recovery from buffer and cell culture medium. AAV2 assay buffer: NaCl buffer.

4C

	[AAV3B] (VP/mL)						
	4.10E+07	1.02E+08	1.60E+09	4.00E+09	1.00E+10		
Matrix	**Spike recovery (%)**					**Average**	**St. Dev.**
NaCl buffer	94%	94%	94%	95%	100%	95%	3%
AlphaLISA lysis buffer	103%	82%	94%	97%	100%	95%	8%
DMEM	91%	99%	94%	96%	100%	96%	4%
Matrix	2.50E+09	5.00E+09	2.50E+10	5.00E+10	7.50E+10	**Average**	**St. Dev.**
RPMI	107%	106%	100%	107%	106%	105%	3%

AAV3B recovery from buffer and cell culture medium. AAV3B assay buffer: NaCl buffer.

4D

	[AAV5] (VP/mL)						
	1.02E+08	2.56E+08	6.40E+08	1.60E+09	4.00E+09		
Matrix	**Spike recovery (%)**					**Average**	**St. Dev.**
NaCl buffer	101%	94%	95%	94%	97%	96%	3%
AlphaLISA lysis buffer	84%	91%	84%	90%	100%	90%	7%
DMEM	94%	88%	92%	101%	92%	93%	5%
RPMI	98%	110%	108%	100%	109%	105%	6%

AAV5 recovery from buffer and cell culture medium. AAV5 assay buffer: NaCl Buffer.

4E

	[AAV6] (VP/mL)					
	2.56E+08	6.40E+08	1.60E+09	4.00E+09		
Matrix	**Spike recovery (%)**				**Average**	**St. Dev.**
Immunoassay buffer	103%	104%	117%	100%	106%	8%
AlphaLISA lysis buffer	97%	94%	98%	91%	95%	3%
DMEM	112%	97%	93%	89%	98%	10%

AAV6 recovery from buffer and cell culture medium. AAV6 assay buffer: immunoassay buffer.

4F

	[AAV8] (VP/mL)						
	1.02E+08	2.56E+08	1.60E+09	4.00E+09	1.00E+10		
Matrix	**Spike recovery (%)**					**Average**	**St. Dev.**
Immunoassay Buffer	99%	92%	84%	N/A	N/A	92%	8%
AlphaLISA Lysis Buffer	115%	111%	113%	N/A	N/A	113%	2%
DMEM	80%	80%	80%	N/A	N/A	80%	0%
RPMI	N/A	122%	101%	106%	110%	110%	9%

AAV8 recovery from buffer and cell culture medium. AAV8 assay buffer: immunoassay buffer. Data that is not available is listed as "N/A".

Tables 4 - H. Percent Recovery of AAV from buffer and cell culture medium.

4G

	[AAV9] (VP/mL)						
	1.02E+08	6.40E+08	1.00E+09	1.60E+09	4.00E+09		
Matrix	Spike recovery (%)					Average	St. Dev.
HiBlock buffer	108%	88%	100%	91%	93%	96%	8%
AlphaLISA lysis buffer	108%	81%	104%	89%	98%	96%	11%
DMEM	81%	104%	87%	95%	103%	94%	10%
RPMI	94%	90%	104%	94%	100%	96%	6%

AAV9 recovery from buffer and cell culture medium. AAV9 assay buffer: HiBlock buffer.

4H

Overall average recovery for all AAVs	St.Dev.
*Assay buffer	97%	7%
AlphaLISA lysis buffer	97%	10%
DMEM	95%	9%
RPMI	100%	9%

Overall average matrix-specific recovery across all AlphaLISA AAV Capsid Detection Kits.

The average matrix-specific recovery and respective standard deviation for all AlphaLISA capsid detection kits are listed in Table 4H. On average, analyte recovery from each matrix deviated at maximum 14% from 100% recovery. This is well within the ±20% - 30% recovery threshold listed in the AlphaLISA AAV capsid detection kit TDS.

Strikingly, the AAV1 AlphaLISA kit had recovery values of 100% across all analyte matrices tested at a capsid concentration of 1.00E+10 (Table 4A). However, no recovery is reported for the AAV1 kit from RPMI at the lowest concentration of AAV1 capsid. The AAV2 kit had average recovery values >100% in NaCl Buffer, AlphaLISA Lysis Buffer and DMEM cell culture medium (Table 4B). The AAV3B AlphaLISA kit had relatively comparable analyte recovery in NaCl buffer, AlphaLISA lysis buffer and DMEM. However, to obtain recovery data from an RPMI analyte background, the concentration range of AAV3B capsid was reduced to 2.50E+09 - 7.50E+10 VP/mL and the average recovery is reported to be >100% (Table 4C). The AAV5 kit has recovery values close the AAV3B kit, including >100% recovery from RPMI medium (Table 4D). The AAV6 AlphaLISA kit has an average recovery greater than 100% in IAB and there is no reported recovery from RPMI at any concentration (Table 4E). There is also no analyte recovery reported for the AAV8 AlphaLISA kit from immunoassay buffer, AlphaLISA lysis buffer and DMEM at the two highest capsid concentrations tested, or from RPMI at the lowest concentration (Table 4F). The AAV9 kit has consistent capsid recovery from all analyte matrices tested (Table 4G). Based on these results AAV capsids were detected in all media or buffer except in RPMI when concentration is close to the lower limit of quantification (LLOQ) or LDL with a recovery around ±20%, which is considered acceptable.

Recovery of AAV from cell lysate.

Recombinant AAVs are primarily produced in HEK293 and SF9 in human cell lines. AAVs are often isolated from either crude cell lysate or supernatant. Therefore, it is important for AlphaLISA AAV capsid detection kits to detect and quantify AAV capsid concentrations in a complex biological matrix with a high protein background, like cell culture medium and cell lysate. To test this, cell lysate was prepared from non-transfected HEK293 and SF9 cells using AlphaLISA lysis buffer. The total protein concentration was determined using a BCA assay and the lysates were diluted accordingly. The total protein concentration of the lysate tested is listed in Tables 5A - E below. Known concentrations of empty AAV capsid (provided with each kit) were spiked into each lysate dilution and recovery (%) was determined as described in the previous section. Initially, a range of cell lysate dilutions were used with a single known concentration of AAV capsid to determine the optimal total protein concentration (dilution of lysate) for AAV recovery. AAV analyte standards and cell lysates were prepared in AlphaLISA lysis buffer, all other reagents were prepared in assay buffer. the analyte recovery results from lysate with a range of total protein concentration using each AlphaLISA AAV capsid detection kit are provided in Tables 5A - E.

Empty AAV capsid at a concentration of 1.00E+10 VP/mL was spiked into SF9 and HEK293 cell lysate at the given total protein concentrations (Table 5A). The recovery of empty AAV1, AAV5 and AAV9 capsids were measured in both SF9 and HEK293 cell lysates at the same total protein concentration using the appropriate AlphaLISA kits. The spiked-in analyte recovery (%) for all AlphaLISA AAV Capsid detection kits tested was better in cell lysate dilutions with lower total protein concentrations (Tables 5A - E). Lysate specific recovery was determined for each AlphaLISA AAV capsid detection kit by averaging the analyte recovery across the range of total protein concentrations of the SF9 and HEK293 lysate dilutions provided in Tables 5A - E. Average recovery from SF9 and HEK293 cell lysate ≥80% are highlighted yellow in the tables above. The AAV1 kit has similar recovery in both SF9 and HEK293 lysates. However,

Tables 5 - E. Effect of lysate total protein concentration on recovery of AAV capsid.

5A

Spiked recovery (%)				Spiked recovery (%)			
SF9 Lysate				HEK293 lysate			
[Total Protein] (mg/mL)	AAV1	AAV5	AAV9	[Total Protein] (mg/mL)	AAV1	AAV5	AAV9
2	72%	43%	59%	2	66%	85%	88%
1.5	78%	48%	65%	1.5	75%	77%	91%
1	83%	52%	72%	1	88%	74%	97%
0.5	95%	74%	83%	0.5	97%	80%	98%
0.25	100%	95%	82%	0.25	102%	88%	91%
Average:	86%	62%	72%	Average:	86%	81%	93%
St. Dev.:	12%	22%	10%	St. Dev.:	15%	6%	4%

Recovery of AAV1, AAV5 & AAV9 from SF9 and HEK293 cell lysate at different total protein concentrations. Lysates with ≥80% recovery are highlighted yellow. All AAVs were tested at a fixed concentration of 1.00E+10 VP/mL.

5B

Spiked recovery (%)			
mL	AAV2 (SF9 Lysate)	[Total Protein] (mg/mL)	AAV2 (HEK293 lysate)
1.3	46%	1	115%
1	65%	0.5	84%
0.5	80%	0.2	85%
0.3	102%	0.02	86%
Average:	73%	Average:	93%
St.Dev.:	24%	St.Dev.:	15%

Recovery of AAV2 from SF9 and HEK293 cell lysate at different total protein concentrations. Lysates with ≥80% recovery are highlighted yellow. AAV2 was tested at a fixed concentration of 1.00E+10 VP/mL.

5C

Spiked recovery (%)			
[Total Protein] (mg/mL)	AAV3B (SF9 lysate)	[Total Protein] (mg/mL)	AAV3B (HEK293 lysate)
1.5	30%	1.75	83%
1	63%	1.5	87%
0.5	65%	1	87%
0.25	76%	0.5	90%
0.125	84%	N/A	N/A
Average:	64%	Average:	87%
St.Dev.:	21%	St. Dev.:	3%

Recovery of AAV3B from SF9 and HEK293 cell lysate at different total protein concentrations. Lysates with ≥80% recovery are highlighted yellow. AAV3B was tested at a fixed concentration of 1.00E+10 VP/mL.

5D

Spiked recovery (%)			
[Total Protein] (mg/mL)	AAV6 (SF9 Lysate)	[Total Protein] (mg/mL)	AAV6 (HEK293 lysate)
1.75	25%	1.75	88%
0.5	75%	0.5	86%
0.25	88%	0.25	94%
Average:	63%	Average:	89%
St.Dev.:	33%	St. Dev.:	4%

Recovery of AAV6 from SF9 and HEK293 cell lysate at different total protein concentrations. Lysates with ≥80% recovery are highlighted yellow. AAV6 was tested at a fixed concentration of 1.00E+10 VP/mL.

5E

Spiked recovery (%)			
[Total Protein] (mg/mL)	AAV8 (SF9 Lysate)	[Total Protein] (mg/mL)	AAV8 (HEK293 lysate)
0.5	42%	2	94%
0.25	65%	1.5	91%
0.125	78%	1	91%
Average:	62%	Average:	92%
St.Dev.:	18%	St. Dev.:	2%

Recovery of AAV8 from SF9 and HEK293 cell lysate at different total protein concentrations. Lysates with ≥80% recovery are highlighted yellow. AAV8 was tested at a fixed concentration of 1.00E+10 VP/mL.

AAV5 and AAV9 kits have a much higher analyte recovery from HEK293 cell lysate, with average recovery >80% for both AlphaLISA AAV kits (Table 5A). The AAV2, AAV3B, AAV6 and AAV8 AlphaLISA kits all have higher average analyte recovery from HEK293 cell lysate versus SF9 cell lysate, with lower standard deviation (Tables 5B - E). These results suggest that cell lysate with a high total protein concentration can interfere with the detection and accurate quantification of AAVs using AlphaLISA AAV capsid detection kits. Furthermore, the AAV detection accuracy in HEK293 cell lysate may be greater than in SF9 lysate. Although, the kits work with lysate from either cell type.

Based on the results above, the total protein concentration of cell lysate samples was kept constant and each AlphaLISA AAV kit was used to determine if the AAV capsid concentration impacts recovery from SF9 and HEK293 cell lysate (Tables 6A - G). Empty AAV capsid at 2 - 3 known concentrations were spiked into the cell lysates and the recovery (%) was calculated by interpolating the AAV concentration using the respective AAV standard curves and dividing the measured values by the known concentration in VP/mL. The cell lysate total protein concentration, analyte concentrations and spiked-in recovery (%) for each AlphaLISA AAV kit is provided in Tables 6A - G, along with the calculated average recovery across the analyte concentration range and standard deviation. AAV analyte standard dilutions and cell lysates were made using AlphaLISA lysis buffer, all other reagents were prepared in kit-specific assay buffer.

Table 6 - G. Effect of AAV capsid concentration on recovery from cell lysate.

The percent recovery at AAV capsid concentrations in VP/mL in SF9 and HEK293 cell lysate is provided in the above tables. The total protein concentration of the lysates is also provided. The average recovery (%) across the AAV concentrations from each cell lysate and standard deviation is listed below each table. Average recovery values ≥80% are highlighted yellow.

6A

Spiked recovery (%)		
AAV1		
[AAV capsid] (VP/mL)	[SF9 lysate] (0.5 mg/mL)	[HEK293 lysate] (0.5 mg/mL)
1.00E+10	92%	93%
1.00E+09	93%	87%
1.00E+08	101%	86%
Average:	95%	89%
St. Dev.:	5%	4%

6B

Spiked recovery (%)		
AAV1		
[AAV capsid] (VP/mL)	[SF9 lysate] (0.3 mg/mL)	[HEK293 lysate] (0.2 mg/mL)
1.00E+10	91%	107%
1.00E+09	94%	98%
3.00E+07	90%	92%
Average:	92%	99%
St. Dev.:	2%	8%

6C

Spiked recovery (%)		
AAV3B		
[AAV capsid] (VP/mL)	[SF9 lysate] (0.125 mg/mL)	[HEK293 lysate] (1.75 mg/mL)
1.00E+10	84%	83%
2.00E+09	95%	88%
Average:	90%	86%
St. Dev.:	8%	4%

6D

Spiked recovery (%)		
AAV5		
[AAV capsid] (VP/mL)	[SF9 lysate] (0.25 mg/mL)	[HEK293 lysate] (0.5 mg/mL)
3.00E+09	92%	78%
3.00E+08	92%	84%
3.00E+07	87%	81%
Average:	90%	81%
St. Dev.:	3%	3%

Table 6E - F. Effect of AAV capsid concentration on recovery from cell lysate.

The percent recovery at AAV capsid concentrations in VP/mL in SF9 and HEK293 cell lysate is provided in the above tables. The total protein concentration of the lysates is also provided. The average recovery (%) across the AAV concentrations from each cell lysate and standard deviation is listed below each table. Average recovery values ≥80% are highlighted yellow.

6E

Spiked recovery (%)		
AAV6		
[AAV capsid] (VP/mL)	[SF9 lysate] (0.25 mg/mL)	[HEK293 lysate] (1.75 mg/mL)
1.00E+10	85%	99%
2.00E+09	86%	96%
Average:	86%	98%
St. Dev.:	1%	2%

6F

Spiked recovery (%)		
AAV8		
[AAV capsid] (VP/mL)	[SF9 lysate] (0.125 mg/mL)	[HEK293 lysate] (2.0 mg /mL)
1.00E+10	91%	107%
1.00E+09	94%	98%
Average:	79%	91%
St. Dev.:	1%	5%

6G

Spiked recovery (%)		
AAV9		
[AAV Capsid] (VP/mL)	[SF9 Lysate] (0.5 mg/mL)	[HEK293 Lysate] (1.5 mg/mL)
1.00E+10	96%	102%
1.00E+09	91%	95%
1.00E+08	93%	95%
Average:	93%	97%
St. Dev.:	3%	4%

The average analyte recovery for all AAV AlphaLISA kits is 90%, ±6% from SF9 cell lysate and 91%, ±8% from HEK293 lysate. All AlphaLISA AAV capsid detection kits have average recovery of >80% and standard deviation of ≤ 8% (Tables 6A - E and 6G) except for the AAV8 kit, which has an average analyte recovery of 79%, ±1% from SF9 cells (Table 6F). The average spiked-in recovery using a range of analyte concentrations for each AlphaLISA AAV kit from both SF9 and HEK293 cell lysates with a single total protein concentration are much closer than what was observed when using lysate with a range of total protein concentrations (Tables 5A - E vs. Tables 6A - G). The AAV1, AAV3B and AAV5 kits all have higher average recovery from SF9 cell lysate at the analyte concentrations tested (Tables 4A, 4C and 4D). The AAV2, AAV6, AAV8 and AAV9 kits have higher recovery from HEK293 cell lysate than from SF9 cells (Table 4B, 4E, 4F and 4D). Overall, changes in the analyte concentration do not drastically alter the spiked-in recovery from either lysate. Taken together, these results demonstrate that the accurate measurement of AAV capsid is influenced by the lysate total protein concentration when analyte is present at concentrations within the dynamic range of the assay. Therefore, it is advisable to check the total protein concentration of any cell lysate sample containing AAV to be quantified with an AlphaLISA AAV kit using a standardized method (e.g., BCA Assay). The sample should then be diluted to the respective total protein concentrations listed in Tables 6A - G, according to the AlphaLISA AAV kit being used. Overall, these results show that AlphaLISA AAV capsid detection kits can measure AAV capsid concentration accurately and reproducibly in biological matrices with high protein background like AAV production cell lysates.

Specificity of AlphaLISA AAV capsid detection kits.

The specificity of each AlphaLISA AAV capsid detection kit was determined by using AAV capsid from the serotypes listed in Table 7, with capsid concentrations ranging from 0 to 1.0E+11 VP/mL diluted in Assay Buffer. Empty AlphaLISA kit specific AAV capsid standards and the capsid from all other AAV serotypes were prepared using the standard curve dilution table provided in the TDS with each kit. The AlphaLISA signal of the other (non-kit) AAV serotypes were interpolated using the standard curve set up with the kit specific AAV capsid standard. The concentration of AAV in VP/mL was compared between the kit specific AAV and other AAVs at each point on the standard curve to determine cross-reactivity, given as

a percentage based on the kit specific AAV capsid reactivity. The average of the cross-reactivity values on the linear portion of the standard curve was calculated and presented as the overall cross-reactivity listed in Table 7. The AAV2 AlphaLISA kit was only tested on AAV3B, which has 100% cross-reactivity (Table 7), however, the antibody used to develop the AAV2 AlphaLISA kit is reported to have no cross-reactivity with AAV serotypes: AAV1, AAV4, AAV5, AAV6, AAV8, AAV9, AAVrh10 and AAVDJ. A complete table containing AlphaLISA AAV1 and AAV6 Capsid Detection Kit cross-reactivity (%) for each non-kit AAV capsid concentration is provided in the TDS for both kits and is presented as a range in the table below. A singular cross-reactivity percentage is listed for the remaining AAV serotypes in Table 7.

AAV capsid analyte specificity is highest for AlphaLISA AAV5 and AAV9 Capsid Detection Kits, which only interact with their canonical AAV capsids. The AAV8 AlphaLISA kit is also relatively specific, only displaying 41% cross-reactivity with AAV3B. AAV2 also has 100% cross-reactivity with AAV3B. Interestingly, the AAV3B kit cross-reacted with AAV8 capsids, but not AAV2 capsids. The AlphaLISA AAV1 and AAV6 Capsid Detection Kits are by far the least specific, with measurable cross-reactivity for AAV2, AAV3B, AAV5, AAV6, and AAV8. The AAV1 AlphaLISA kit has higher reactivity with AAV5 capsids than it does with AAV1 at all concentrations tested. The AAV6 kit is by far the least specific, with cross-reactivity >100% for AAV1, AAV2, AAV3B and AAV5 capsids. This is likely due to differences in the specificity of the antibody used to develop the assay. Assay specificity is important and must be documented to demonstrate that material to be used in clinical trials is not cross contaminated with other AAV serotypes. However, AlphaLISA AAV Capsid Detection Kits are intended to be used to measure the concentration of AAV in VP/mL, not identify the capsid of unknown AAV serotypes in samples.

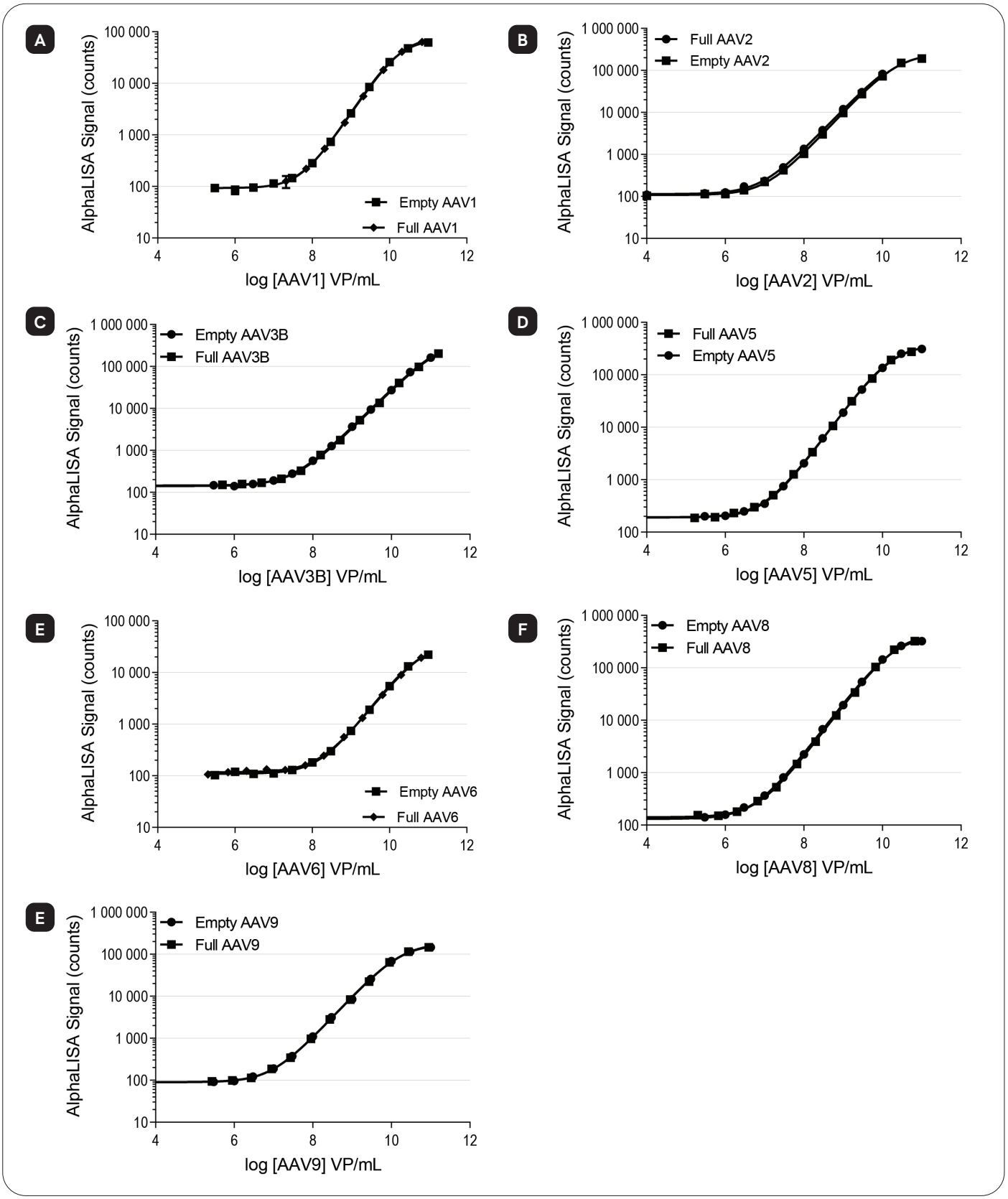
Detection of empty vs DNA cargo loaded AAV using AlphaLISA AAV capsid detection kits.

Current literature on AAVs suggest the loading of AAV with DNA cargo can alter the conformation of the VP proteins in the viral capsid and result in changes in antigenicity.12, 13 Both, serotype specific empty AAV capsids and capsids loaded with DNA containing an eGFP gene and CMV promoter (SIRION Biotech) were quantified using AlphaLISA AAV Capsid Detection Kits. The concentration of the loaded AAVs is given in VG or GC/mL (Viral Genome or Genome Content/mL). Since the concentration of empty AAV capsids is given in VP/mL, an orthogonal and independent method, a standard ELISA assay was used to independently determine a VP/GC ratio. The stock of loaded AAV in GC/mL concentration was converted to VP/mL using this ratio. The loaded capsids were then diluted in Assay Buffer to a range of concentrations that fall on the standard curve for the empty AAV standards provided with the AlphaLISA AAV kits (1.00E+11 - 3.00E+05 VP/mL). The data for empty and loaded AAV were then plotted on the same graph, resulting in nearly identical standard curves demonstrating that the AlphaLISA kits can detect and quantify empty and loaded AAV equally. Empty and full AAV capsid standard curves are provided in Figures 4A - G for each AlphaLISA AAV Capsid Detection Kit.

Table 7. Summary of AlphaLISA AAV Capsid Detection Kit cross-reactivity

AlphaLISA AAV capsid detection kit	AAV1	AAV2	AAV3B	AAV5	AAV6	AAV8	AAV9
*AAV1	100%	9 - 20%	20 - 37%	140 - 771%	2 - 5%	3 - 10%	0 - 1%
AAV2	0%	100%	100%	0%	0%	0%	0%
AAV3B	0%	0%	100%	0%	0%	120%	0%
AAV5	0%	0%	0%	100%	0%	0%	0%
*AAV6	167 - 178%	95 - 105%	153 - 220%	549 - 723%	100%	2 - 3%	0%
AAV8	0%	0%	41%	0%	0%	100%	0%
AAV9	0%	0%	0%	0%	0%	0%	100%

*AAV capsids were tested at a concentration ranging from 0 to 1.0E+11 VP/mL.



Figures 4A - G. Both empty and loaded capsids were diluted to concentrations in the range of the AAV standard curve, provided for each AlphaLISA kit. The counts at each concentration were averaged and fitted using non-linear regression 4-parameter logistic equation (sigmoidal dose-response curve with variable slope and 1/Y2 data weighting) and plotted on the same graph.

Conclusion

AlphaLISA AAV Capsid Detection Kits are simple, no wash, quantitative assays that can accurately detect and quantify the concentration of both empty and cargo loaded AAV (Figures 4A - G) in biological matrices with a relatively high protein background, such as cell culture medium and cell lysate. The AlphaLISA AAV kits are sensitive, with LDL values between 5.8E+08 to 1.9E+06 VP/mL depending on the analyte matrix. RPMI cell culture medium (containing free biotin) resulted in the lowest overall assay sensitivity (Table 2 and Figure 3). Furthermore, information related to assay sensitivity and precision in RPMI medium is not available for the AlphaLISA AAV6 kit (Tables 2, 3B and 3F). Therefore, AlphaLISA AAV capsid detection kits should be used cautiously when measuring AAV concentrations in an RPMI background, especially when using the AlphaLISA AAV6 kit. The TDS for each AlphaLISA AAV kit provides an alternate assay protocol using 5 µL of AAV in RPMI instead of a 10 µL sample to reduce the interference of free-biotin in solution. It is highly recommended that this protocol be followed when measuring AAV in RPMI or other matrices containing biotin. The AlphaLISA AAV kits are precise, with intra- and inter-assay variability <10% in most cases with CV (%) as low as 1% under certain conditions (Tables 3A - 3H). On average, spiked recovery (%) of AAV capsid from Assay Buffer, AlphaLISA Lysis Buffer, DMEM and RPMI cell culture medium is ~97%, ranging from 80% to 110% depending on the matrix used and concentration of analyte (Tables 5A - E). AlphaLISA AAV Capsid Detection Kits can optimally detect AAV in cell lysates with a total protein concentration up to 2 mg/mL depending on the concentration of AAV capsid and cell lysate tested (Tables 6A - G). Lysate samples containing AAV should be diluted to the total protein concentration thresholds listed for each AlphaLISA AAV kit in Tables 6A - E and the dilution factor should be accounted for when calculating the AAV concentration from the standard curve. Most AAV AlphaLISA kits are highly specific, with AAV5 and AAV9 kits displaying no detectable cross-reactivity with capsids from other AAV serotypes. Both AAV2 and AAV8 cross-react only with AAV3B. The AAV1 and AAV6 AlphaLISA kits cross-react with each other, in addition to AAV2, AAV3B and AAV5 capsids (Table 7). The information provided in this document and the TDS for each AlphaLISA AAV Capsid Detection Kit should be used as a guide to set up and optimize the detection and quantification of AAV in analogous analyte matrices.

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