APPLICATION NOTE

A simple, reliable, no-wash assay platform for AAV capsid titer quantification based on HTRF technology.

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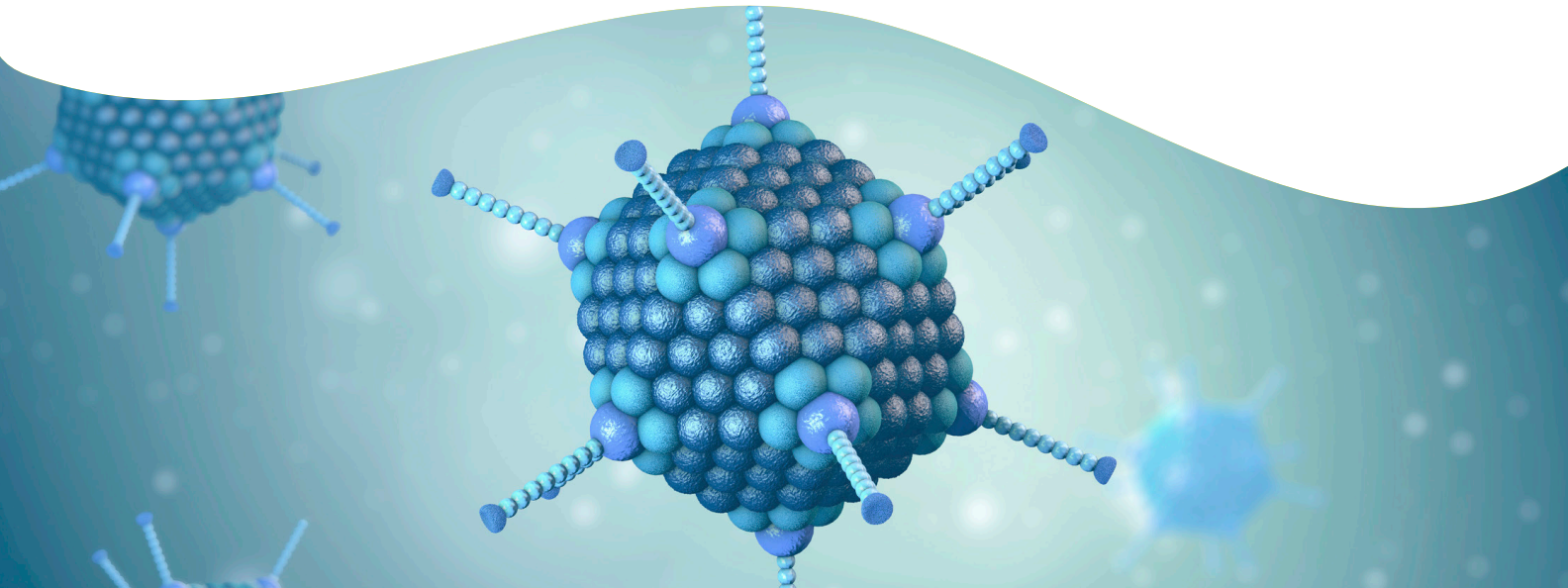
Introduction

This application note includes the performance-based assay specifications for all HTRF® AAV Capsid Detection Kits manufactured and marketed by Revvity. The assay parameters that are important for understanding how to efficiently use the HTRF kits for the quantification of AAV titers in gene therapy R&D and manufacturing pipelines are listed in **Table 1**. These no-wash, FRET-based immunoassay kits are available for AAV1, AAV2, AAV3B, AAV5, AAV6, AAV8, and AAV9 serotypes and measure the concentration of the AAV capsid in viral particles per mL (VP/mL) in buffer, cell culture medium, and cell lysate. The following assay performance parameters were determined for each HTRF AAV Capsid Detection Kit: assay sensitivity (LOD, LOQ, dynamic range), intra-assay precision, inter-assay precision, spike-recovery from samples diluted in buffer, cell culture medium, and cell lysate, as well as AAV capsid cross-reactivity, and the detection of both empty and full AAV capsids.

AAV (adeno-associated virus) is a relatively simple, small (~25 nm), non-enveloped parvovirus with an ~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 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 3 overlapping gene products, VP1, VP2, and VP3, with respective monomeric molecular weights ranging from 73 – 82 kDa, 64 – 67 kDa, and 59 – 61 kDa, respectively. 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 of 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. Methods for AAV analysis include real-time qPCR or ddPCR for genomic content, immunoassays for capsid quantification and microfluidic electrophoresis (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] Differences in the AAV capsid of each serotype give 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 the development of human gene therapies.

Table 1: Summary of work used to evaluate HTRF AAV Capsid Detection Kit performance.

Parameter	Matrix/Analyte	HTRF AAV Capsid Detection Kits						
		AAV1	AAV2	AAV3B	AAV5	AAV6	AAV8	AAV9
Assay Sensitivity (LOD, LOQ, dynamic range)	Diluent #5 (HTRF assay buffer)	✓	✓	✓	✓	✓	✓	✓
	Lysis Buffer #3	✓	✓	✓	✓	✓	✓	✓
	DMEM	✓	✓	✓	✓	✓	✓	✓
	RPMI (containing free biotin)	✓	✓	✓	✓	✓	✓	✓
CV (% intra-assay & inter-assay)	Diluent #5 (HTRF assay buffer)	✓	✓	✓	✓	✓	✓	✓
	Lysis Buffer #3	✓	✓	✓	✓	✓	✓	✓
	DMEM	✓	✓	✓	✓	✓	✓	✓
	RPMI (containing free biotin)	✓	✓	✓	✓	✓	✓	✓
AAV Spiked Recovery (%)	Diluent #5 (HTRF assay buffer)	✓	✓	✓	✓	✓	✓	✓
	Lysis Buffer #3	✓	✓	✓	✓	✓	✓	✓
	DMEM	✓	✓	✓	✓	✓	✓	✓
	RPMI (containing free biotin)	✓	✓	✓	✓	✓	✓	✓
	SF9 & HEK293 cell lysate	✓	✓	✓	✓	✓	✓	✓
Cross-reactivity of AAV HTRF kit	AAV1 capsids	N/A	✓	✓	✓	✓	✓	✓
	AAV2 capsids	✓	N/A	✓	✓	✓	✓	✓
	AAV3B capsids	✓	✓	N/A	✓	✓	✓	✓
	AAV5 capsids	✓	✓	✓	N/A	✓	✓	✓
	AAV6 capsids	✓	✓	✓	✓	N/A	✓	✓
	AAV8 capsids	✓	✓	✓	✓	✓	N/A	✓
	AAV9 capsids	✓	✓	✓	✓	✓	✓	N/A
Empty & Loaded AAV Detection	Diluent #5 (HTRF assay buffer)	✓	✓	✓	✓	✓	✓	✓
Compound Interference vs ELISA	Listed chemical additives	✓	✓	✓	✓	✓	✓	✓

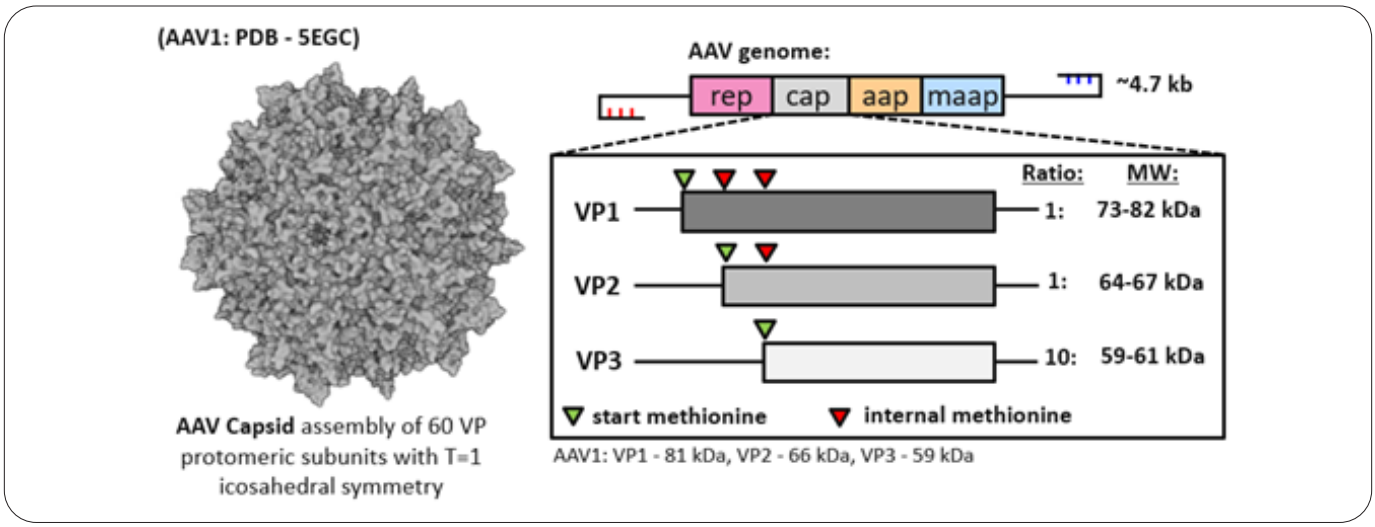


Figure 1: 3D structure was rendered using MOL* 3D viewer (www.rcsb.org/3d-view) using the x-ray diffraction coordinates for PDB structure: 5EGC with surface density enabled. Position of start and internal methionine shown for overlapping VP gene products. Figure adapted from Wörner, Tobias P. et al.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

Methods

HTRF AAV Capsid Detection Kits

HTRF AAV Capsid Detection Kits allow for the simple and rapid quantification of AAV particles (in VP/mL) in both cell lysates and supernatants and offer a fast, no-wash alternative to ELISA immunoassays. The detection principle of these kits is based on HTRF technology (Homogeneous Time-Resolved Fluorescence). As shown in **Figure 2**, the AAV capsid is detected in a sandwich assay by using a pre-mixture of biotinylated anti-AAV antibody bound to the streptavidin europium cryptate (donor), and a second pre-mixture of the HRP anti-AAV antibody bound to an anti-HRP labeled with d2 (acceptor). When the dyes are in proximity, the excitation of the donor with a light source (laser or flash lamp) triggers a Fluorescence Resonance Energy Transfer (FRET) towards the acceptor, which in turn fluoresces at a specific wavelength (665 nm). Signal intensity is proportional to the number of antigen-antibody complexes formed and therefore to the AAV capsid concentration. AAV samples and HTRF detection reagents were dispensed into a 384-well HTRF plate (ProxiPlate™-384 Plus, White 384-shallow well microplate, #6008280/9, Revvity) and read on an HTRF-compatible plate reader. The technical details associated with setting up the HTRF AAV Capsid Detection Kits and analyzing the resulting data can be found in each kit's respective manual. HTRF standard curves were fitted in GraphPad Prism using nonlinear regression with a 4-Parameter Logistic equation (4PL with $1/Y^2$ data weighting). Additional data reduction and calculations are provided in **Appendix I**.

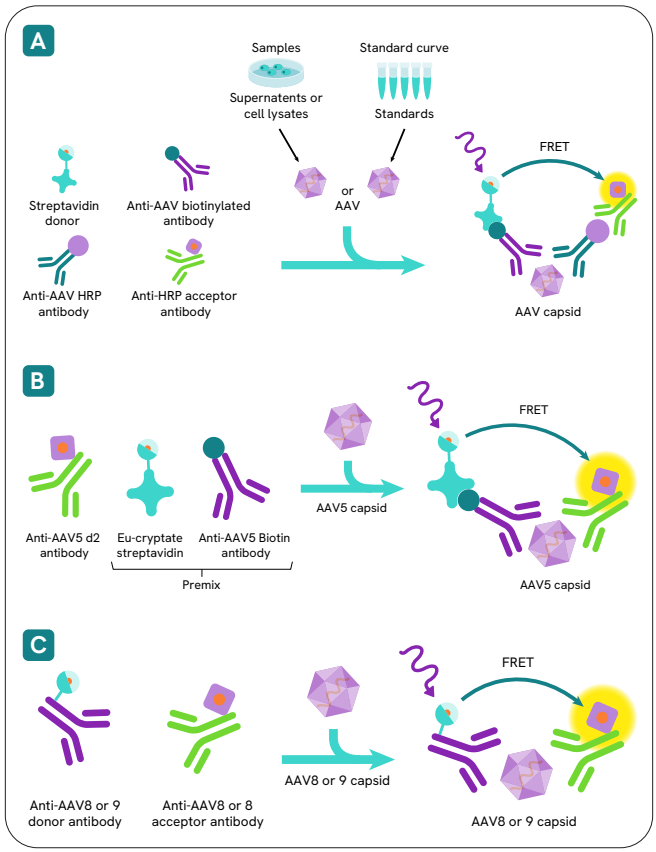


Figure 2: Detection of the AAV capsid using HTRF AAV Capsid Detection Kits. A. Assay scheme for HTRF AAV1, AAV2, AAV3B, and AAV6 Capsid Detection Kits. B. Assay scheme for HTRF AAV5 Capsid Detection Kit. C. Assay scheme for HTRF AAV8 and AAV9 Capsid Detection Kits.

SF9 and HEK293 cell culture, lysis, and quantification of total protein levels

T175 flasks were seeded with either SF9 or HEK293 cells representing the AAV producer cell lines used in the AAV manufacturing process. The cells were grown at 37 °C with 5% CO_2 in either DMEM or RPMI culture medium supplemented with 10% Fetal Bovine Serum (FBS) and 1% Penicillin/Streptomycin (PS) until fully confluent. The cell culture medium was aspirated from the flask and the cells were lysed in 3 mL of 1X HTRF lysis buffer #3 for 30 minutes at room temperature with gentle shaking. The total protein concentration of each cell lysate sample was determined using a Bicinchoninic Acid (BCA) assay using a BSA standard curve (Pierce™ BCA Protein Assay Kits - #23225, ThermoFisher Scientific). The cell lysate samples were diluted in Lysis Buffer #3 to the total protein concentrations listed in **Appendix V**.

Results

Assay sensitivity

Assay sensitivity was measured by determining the Limit of Detection (LOD) and Limit of Quantification (LOQ) for each HTRF AAV Capsid Detection Kit in Diluent #5, Lysis Buffer #3, DMEM, and RPMI analyte matrices. HTRF kit-specific detection reagents were diluted in each analyte matrix without analyte, plated in a 384-well HTRF plate, and read on an HTRF-compatible plate reader. A total of 24 wells were used per kit and analyte matrix. The LOD and LOQ were determined for each kit using the mean HTRF Ratio and standard deviation for the 24 wells following the calculations provided in **Appendix I**. The resulting LOD and LOQ were interpolated in VP/mL using an AAV capsid standard curve prepared from the empty AAV capsid standard provided with each kit, diluted in each analyte matrix. The LDL and LOQ (in VP/mL) for each HTRF assay kit is listed in **Figures 3 and 4** and shows the lowest concentration in AAV capsid that can be detected and quantitatively measured in each of the analyte matrices tested. The dynamic range represents the range of AAV capsids that can be accurately detected using the HTRF AAV Capsid Detection Kits and is provided in **Figure 5**.

Matrix	LOD (VP/mL)						
	AAV1	AAV2	AAV3B	AAV5	AAV6	AAV8	AAV9
Diluent #5	2.76E+08	1.11E+09	3.94E+08	4.h48E+08	1.42E+08	2.51E+08	1.78E+08
Lysis Buffer #3	1.46E+08	8.93E+08	4.76E+08	3.06E+07	1.53E+08	2.81E+08	7.79E+07
DMEM	1.30E+09	1.06E+09	6.70E+08	1.07E+08	7.87E+08	2.39E+08	5.71E+07
RPMI	1.31E+09	1.15E+09	9.35E+08	2.36E+08	7.44E+08	3.12E+08	9.82E+07

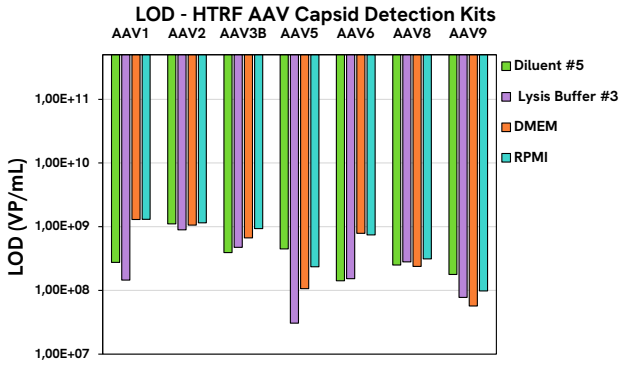


Figure 3: Assay Sensitivity – Limit of Detection (LOD).

Matrix	LOQ (VP/mL)						
	AAV1	AAV2	AAV3B	AAV5	AAV6	AAV8	AAV9
Diluent #5	9.73E+08	4.35E+09	2.62E+09	1.49E+09	2.47E+09	3.90E+09	1.06E+09
Lysis Buffer #3	1.02E+09	3.55E+09	1.94E+09	1.64E+09	3.96E+09	3.85E+09	9.34E+08
DMEM	5.08E+09	3.73E+09	2.70E+09	1.36E+09	2.24E+09	3.61E+09	9.76E+08
RPMI	5.16E+09	5.04E+09	4.64E+09	1.43E+09	1.69E+09	3.82E+09	9.11E+08

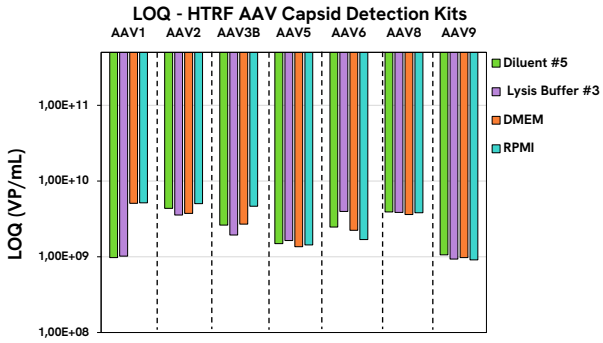


Figure 4: Assay Sensitivity – Limit of Quantification (LOQ).

Matrix	[AAV1] VP/mL		[AAV2] VP/mL		[AAV3B] VP/mL		[AAV5] VP/mL	
	Low	High	Low	High	Low	High	Low	High
RPMI	5.16E+09	2.50E+11	5.04E+09	6.00E+11	4.64E+09	6.00E+11	1.43E+09	2.50E+11
Lysis Buffer #3	5.08E+09	2.50E+11	3.73E+09	6.00E+11	2.70E+09	6.00E+11	1.36E+09	2.50E+11
DMEM	1.02E+09	2.50E+11	3.55E+09	6.00E+11	1.94E+09	6.00E+11	1.64E+09	2.50E+11
Diluent #5	9.73E+08	2.50E+11	4.35E+09	6.00E+11	2.62E+09	6.00E+11	1.49E+09	2.50E+11

Matrix	[AAV6] VP/mL		[AAV8] VP/mL		[AAV9] VP/mL	
	Low	High	Low	High	Low	High
RPMI	1.69E+09	5.00E+11	3.82E+09	2.50E+11	9.11E+08	2.00E+11
Lysis Buffer #3	2.24E+09	5.00E+11	3.61E+09	2.50E+11	9.76E+08	2.00E+11
DMEM	3.96E+09	5.00E+11	3.85E+09	2.50E+11	9.34E+08	2.00E+11
Diluent #5	2.47E+09	5.00E+11	3.90E+09	2.50E+11	1.06E+09	2.00E+11

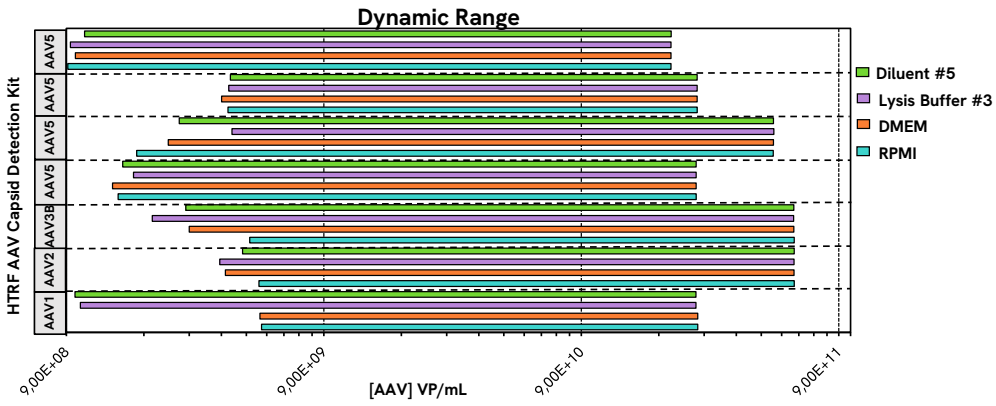


Figure 5: Analytical Performance – Dynamic Range.

Analytical performance

The analytical performance of each HTRF AAV Capsid Detection Kit was assessed using a variety of metrics including intra- and inter-assay precision, dilution linearity, antigen spike and recovery, the ability to detect empty and full AAV capsids, capsid cross-reactivity as well as, cell lysate and chemical-specific assay interference. This information can serve as a benchmark for assay performance and may potentially help with troubleshooting. The technical details associated with each of the analytical parameters tested are discussed in the subsequent sections below.

Intra-assay precision

The coefficient of variation (%CV) was calculated to determine the variability in the intra- and inter-assay measurements obtained with each HTRF AAV Capsid Detection Kit using AAV standards diluted in Diluent #5, Lysis Buffer #3, DMEM, and RPMI analyte matrices. The calculation for %CV is provided in **Appendix I**. An acceptable intra- and inter-assay %CV for HTRF is ≤20%. The intra-assay CV% was calculated from 24 wells plated for each AAV capsid concentration and are listed in the tables in **Appendix II**. The corresponding box plots in **Figure 6** show the range of intra-assay %CV for each kit diluted in the respective analyte matrices. The mean intra-assay %CV for each HTRF AAV Capsid Detection kit was <10% and was consistent across all analyte matrices.

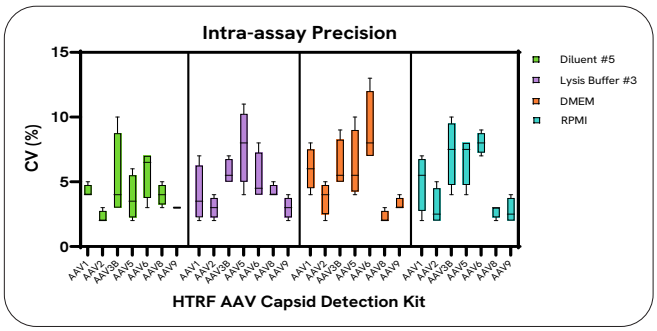


Figure 6: Analytical Performance – Intra-assay Precision. Numerical data related to intra-assay %CV is provided in the tables in **Appendix II.**

Inter-assay precision

The inter-assay %CV was calculated from 3 separate experiments prepared using the AAV standards from each kit, diluted to the concentrations listed in the tables in **Appendix III**. The corresponding box plots in **Figure 7** show the range of inter-assay %CV for each kit in the listed analyte matrices. The mean intra-assay %CV for each HTRF AAV Capsid Detection kit was consistently ≤ 13% in all analyte matrices tested, well within the acceptable threshold for HTRF.

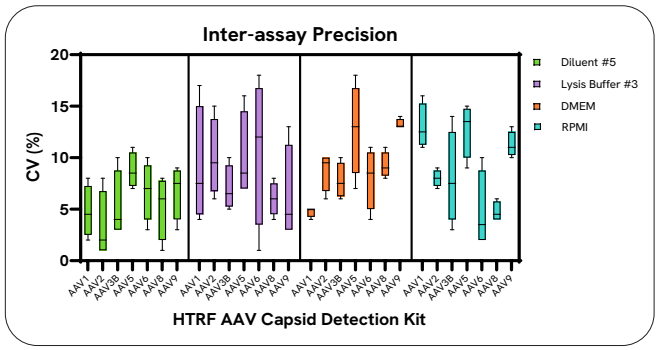


Figure 7: Analytical Performance – Inter-assay Precision. Numerical data related to inter-assay %CV is provided in the tables in **Appendix III**.

Dilution recovery

The accuracy in measuring the AAV capsid over a range of concentrations with each HTRF AAV Capsid Detection Kit was examined by calculating the %Recovery of analyte in each of the matrices listed below. Initially, the concentration of an AAV capsid stock solution in VP/mL was determined for AAV capsid standards diluted in Diluent #5, Lysis Buffer #3, DMEM, and RPMI analyte matrices using an AAV capsid standard curve set up in the same matrix. The AAV standards from AAV2 and AAV6 capsid detection kits were further diluted using 3 - 2x serial dilutions (up to 8-fold). The AAV5 kit standards were diluted using 4 - 2.5x serial dilutions (up to 39.1-fold) and the standards from all other AAV kits were further diluted using 4 - 2x serial dilutions (up to 16-fold) in triplicate. The resulting HTRF Ratio was interpolated to VP/mL using the analyte standard curves and the %Recovery was then calculated for each kit at each dilution following the calculation listed in **Appendix I**. A box plot showing the range of %Recovery for each kit diluted in each analyte matrix is provided in **Figure 8**. The data used to generate the box plots can be found in **Appendix IV**. The mean %Recovery over the dilution range for each kit was 100% ± 15%. However, the %Recovery at higher dilutions (e.g., 8-fold, 16-fold) was sometimes greater than this threshold for some of the HTRF kits and analyte matrices tested.

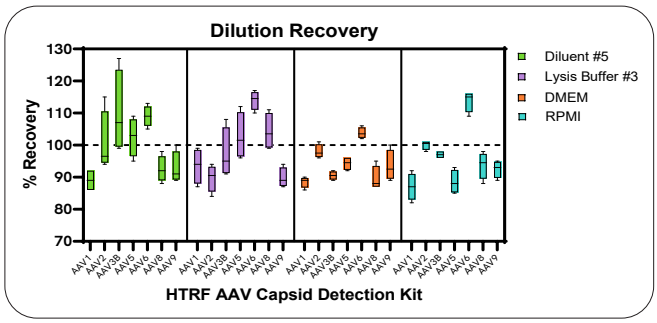


Figure 8: Analytical Performance – Dilution Recovery. Numerical data related to Dilution Linearity is provided in the tables in **Appendix IV**.

Assay interference – cell lysate

Recombinant AAVs are primarily produced in HEK293 and SF9 human cell lines and are often isolated from crude cell lysate. Therefore, it is important for HTRF AAV Capsid Detection Kits to detect and quantify the AAV capsid in complex biological matrices with a high protein background, like cell culture medium and cell lysate. SF9 and HEK293 cells were grown and lysed as described in the method section above to examine the potential for assay interference with cell lysate. The total protein concentration was determined using a BCA assay. The cell lysates were serially diluted, spiked with a single concentration of AAV capsid, and measured using the HTRF AAV Capsid Detection Kits. The lysate total protein concentration in mg/mL, AAV concentration in VP/mL, and %Recovery are listed for each cell lysate sample in the tables in **Appendix V**. The scatter plots in **Figure 9** show the relationship between the lysate total protein concentration and %Recovery of AAV capsid. On average, samples with lower lysate total protein concentrations had %Recovery closer to 100%. Both AAV8 and AAV9 kits had %Recovery values close to 100% for all the lysate total protein concentrations tested for both SF9 and HEK293 cell lysates. The AAV6 kit also had a %Recovery of around 100% for all the tested HEK293 cell lysate total protein concentrations. These results demonstrate that the HTRF Capsid Detection Kits can be used to detect AAV capsid in cell culture medium and cell lysates with a tolerable protein concentration.

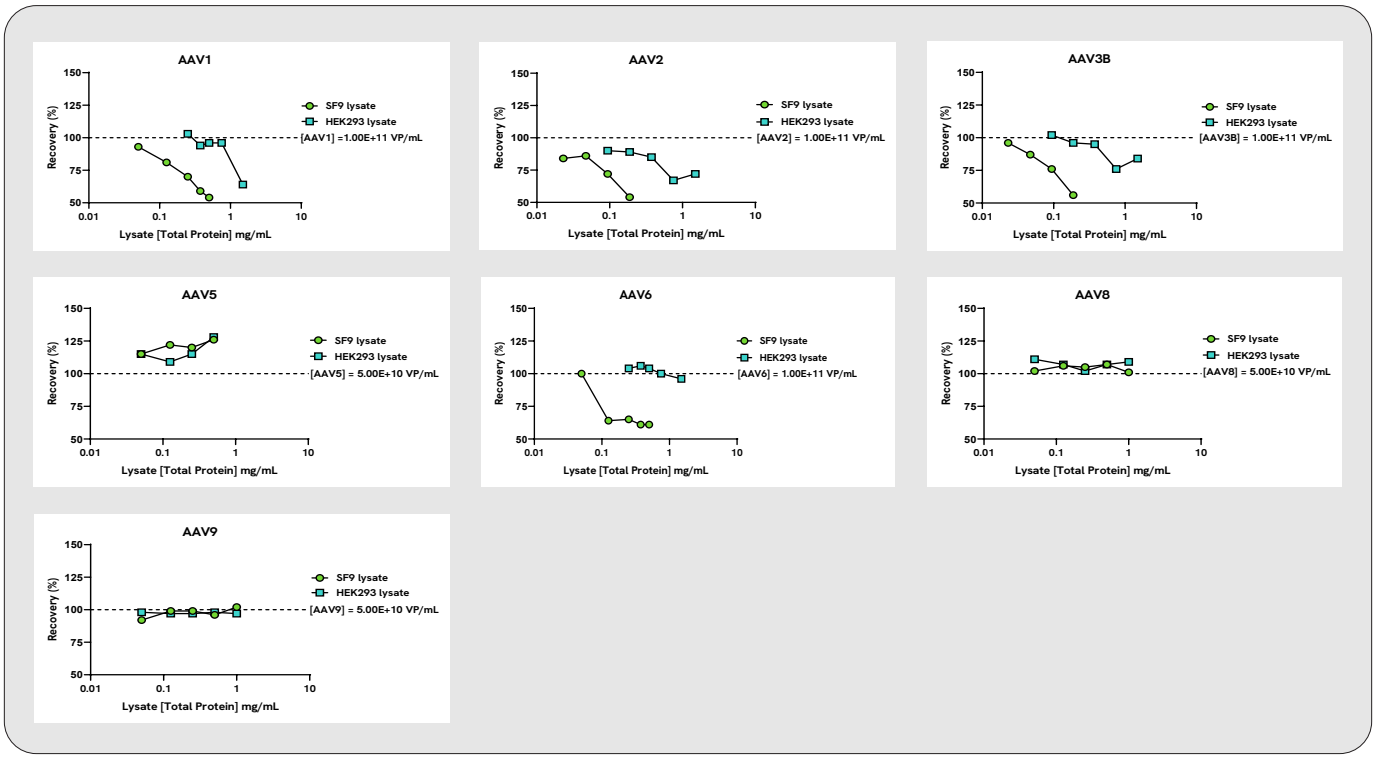


Figure 9: Analytical Performance – Assay Interference – Cell Lysate.

Antigen spike and recovery from cell lysate

AAV capsid at three different concentrations were used to determine the concentration range at which the AAV capsid can be accurately detected in SF9 and HEK293 cell lysates with each AAV kit. The resulting HTRF ratios were interpolated to VP/mL using an AAV capsid standard curve and the %Recovery was calculated for each sample. Data tables containing the %Recovery for each HTRF AAV Capsid Detection Kit from SF9 and HEK293 cell lysates at each AAV capsid concentration are provided in **Appendix VI**. The scatter plots in **Figure 10** show the effect of AAV capsid concentration on the %Recovery from both cell lysates. The data points that fall closest to 100% represent AAV and lysate total protein concentrations that resulted in the optimal quantification of the AAV capsid from cell lysate.

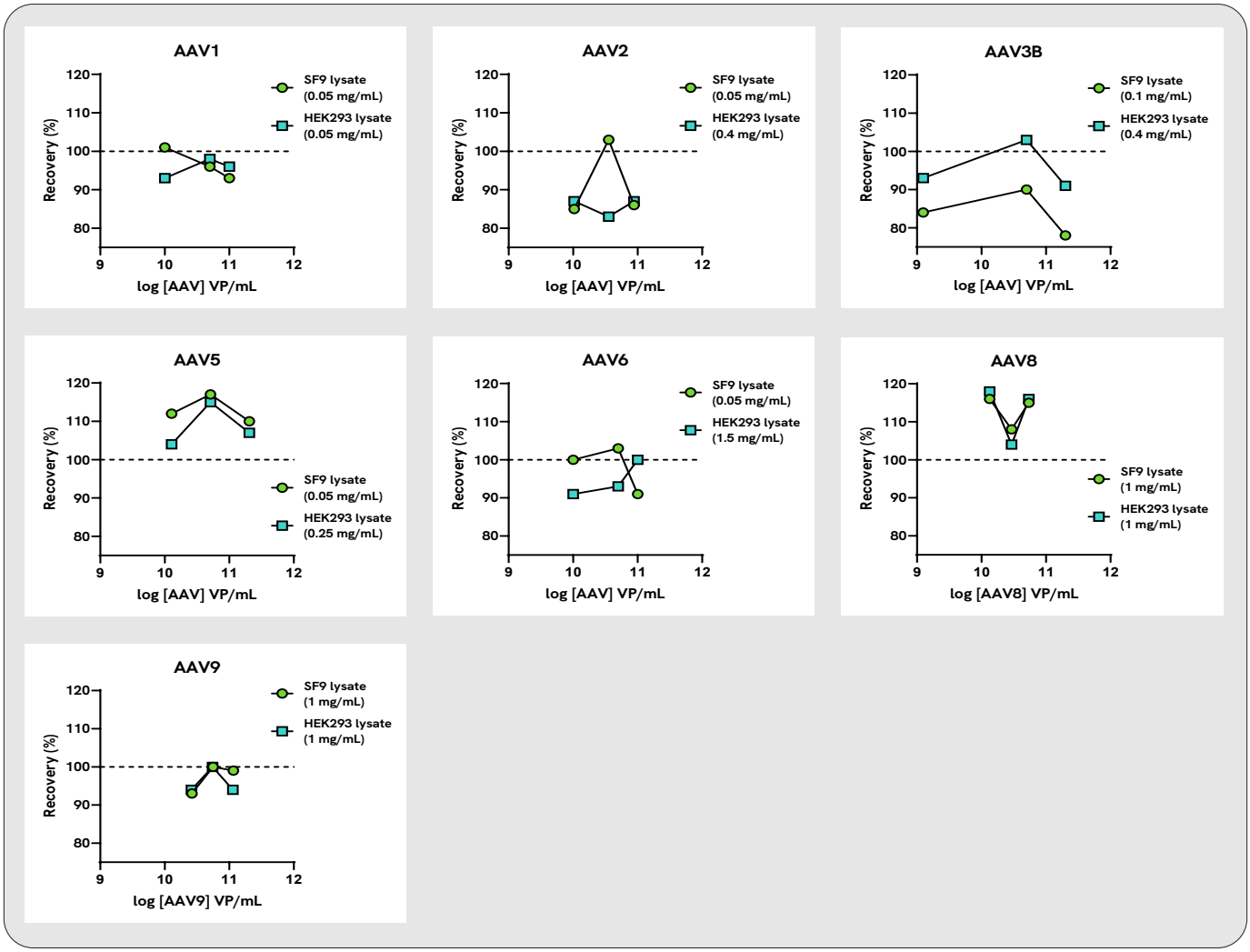


Figure 10: Analytical Performance – Spike and Recovery – Cell Lysate.

Detection of empty vs full AAV capsids

Current literature on AAVs suggests 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.[11,12] Both empty AAV capsids and capsids loaded with DNA containing an eGFP gene and CMV promoter (SIRION Biotech) were quantified using HTRF AAV Capsid Detection Kits. The concentration of the loaded AAV 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 concentration of AAV in GC/mL was converted to VP/mL using this ratio. The loaded capsids were then diluted in Diluent #5 to a range of concentrations that fall on the standard curve for the empty AAV standards provided with the HTRF AAV kits. The Delta Ratio for empty and loaded AAV were then plotted on the same graph, resulting in nearly identical standard curves, demonstrating that the HTRF kits can detect and quantify empty and loaded AAV equally. The calculation for the HTRF Delta Ratio is given in **Appendix I**. Empty and full AAV capsid standard curves are provided in **Figure 11** for each HTRF AAV Capsid Detection Kit.

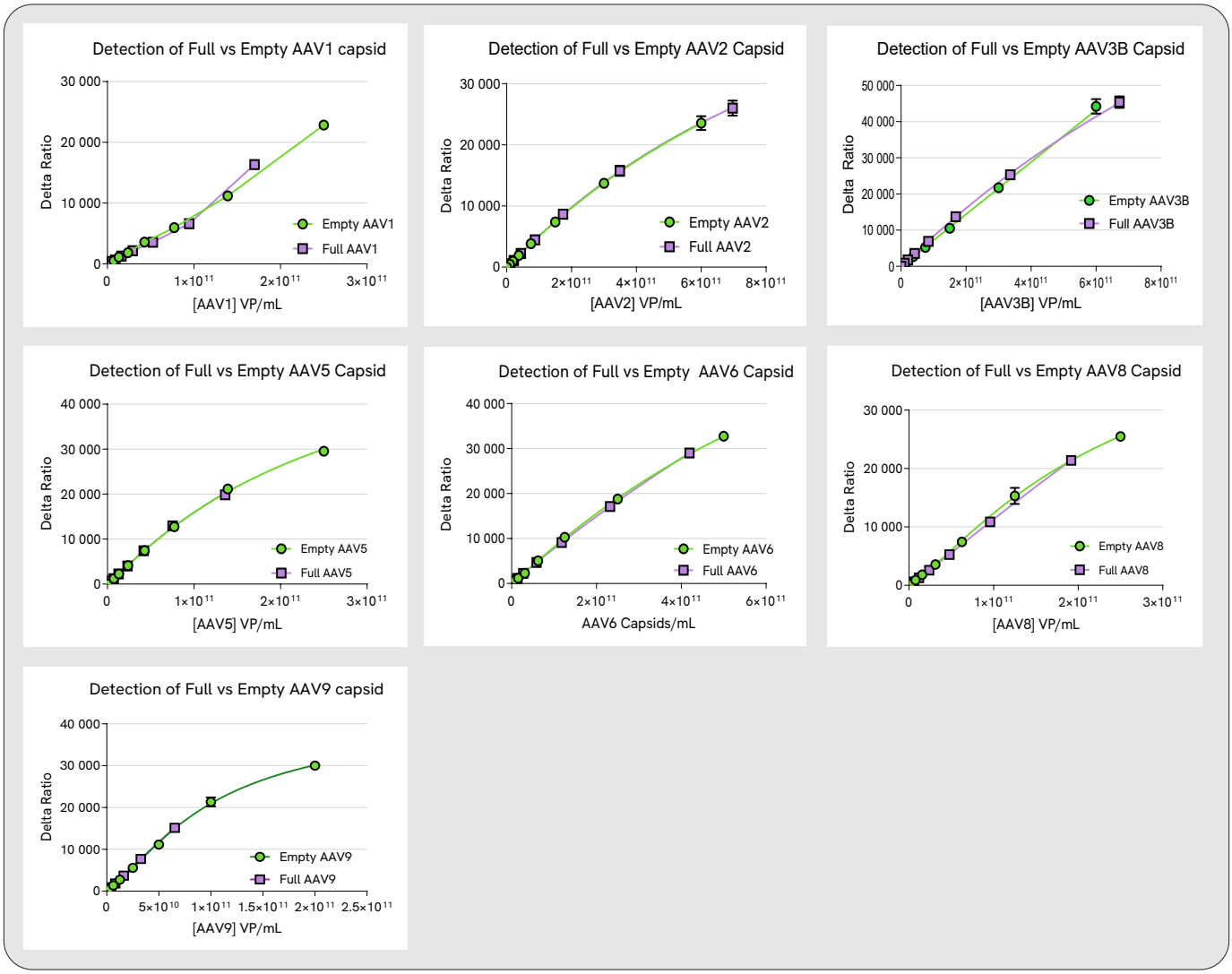


Figure 11: Analytical Performance – Detection of Empty vs Full AAV Capsid.

AAV capsid cross-reactivity

The reactivity of the HTRF AAV capsid detection reagents for each kit was used to measure the capsids of the other AAV serotypes to establish a cross-reactivity profile. Empty AAV standard capsids were diluted following the "standard dilution procedure" found in the manual for each kit. The resulting HTRF Ratios were converted to Delta Ratios and plotted on the same graph, shown in **Figure 12**. The Delta Ratio for each kit run against non-canonical AAV capsids at a concentration of 2.50E+11 VP/mL was used to construct a table listing the relative cross-reactivity of each kit with each AAV capsid and is provided in **Appendix VII**. AAV5, AAV8, and AAV9 HTRF Capsid Detection Kits are highly selective and only detect a single serotype. However, all other HTRF AAV Capsid Detection Kits have measurable cross-reactivity with capsids from every AAV serotype, except for AAV9.

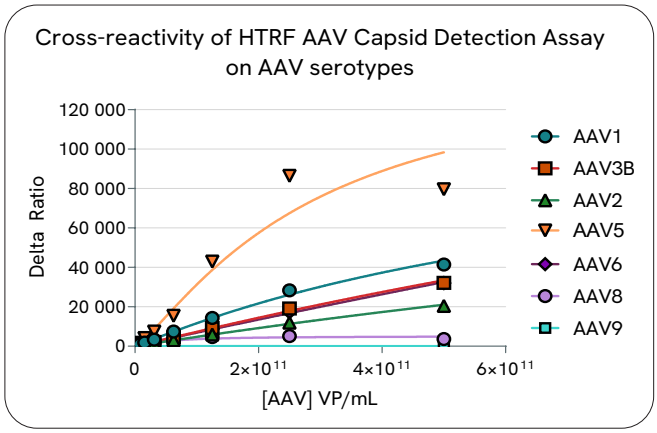


Figure 12: Cross-reactivity of AAV Capsids with HTRF AAV Capsid Detection Kits.

Assay interference - chemical

HTRF AAV Capsid Detection Kits are designed to measure the AAV capsid concentration in VP/mL in many different types of analyte matrices. These kits were designed to be used to quantify the AAV capsid at any point in a manufacturing process and are compatible with samples containing varying concentrations of chemical components. To assess the potential for assay interference, empty AAV capsids from each serotype were combined with chemicals often found in buffers used in the manufacturing of AAVs to determine the maximum tolerated concentration that does not lead to assay interference. Each AAV capsid at a concentration of 5.0E+10 VP/mL was combined with Diluent #5 supplemented with varying levels of the chemical compounds. The maximum tolerated concentration was the highest concentration that resulted in <10% change in %CV when compared to samples diluted in Diluent #5 without added chemicals. This data was compared to analogous information from ELISA AAV capsid detection kits available from a competitor company and is provided in **Table 2**. In most cases, the maximum tolerated concentration of each chemical is higher for HTRF kits compared to the ELISA kits.

Table 2: Analytical Performance – Assay Interference – Chemical.

Chemical	Maximum tolerated compound concentration resulting in ΔCV < 10%							
	AAV1		AAV2		AAV3B		AAV5	
	ELISA	HTRF	ELISA	HTRF	ELISA	HTRF	ELISA	HTRF
Pluronic F-68	0.10%	4%	0.025 - 0.1%	4%	0.10%	1%	0.05%	1%
$MgCl_2$	50 mM	200 mM	6.25 - 50 mM	125 mM	50 mM	125 mM	50 mM	50 mM
Triton™ X-100	0.50%	10%	0.50%	5%	0.50%	< 10 %	0.50%	5%
Deoxycholate	0.25%	10%	0.06 - 0.5%	0.30%*	0.50%	< 0.16%*	0.25%	10%
TWEEN 20	0.50%	2%	0.50%	0.60%	0.50%	0.62%	0.50%	10%
EDTA	10 mM	200 mM	2.5 - 10 mM	200 mM	10 mM	100 mM	10 mM	30 mM
NaCl	1 M	2 M	0.03 to 1 M	2.5 M	1 M	10M	1 M	2.5M
Iodixanol	0.30%	4%	0.16 - 0.63%	2.5%	< 0.08%	2.5%	0.16%	5%

Table 2: Analytical Performance – Assay Interference – Chemical (continued).

Chemical	Maximum tolerated [compound] (ΔCV < 10%)					
	AAV6		AAV8		AAV9	
	ELISA	HTRF	ELISA	HTRF	ELISA	HTRF
Pluronic F-68	0.10%	0.50%	0.10%	4%	0.50%	2%
MgCl2	50 mM	60 mM	50 mM	125 mM	6.25 mM	15.6 mM
Triton™ X-100	0.50%	0.62%†	0.50%	5%	0.50%	10%
Deoxycholate	0.25%	< 0.08%*	0.25%	5%	0.50%	2.5%
TWEEN 20	0.50%	0.08%†*	0.50%	10%	0.50%	10%
EDTA	10 mM	200 mM	10 mM	200 mM	10 mM	50 mM
NaCl	0.25 M	2.5 M	1 M	2 M	0.5 M	0.5 M
Iodixanol	0.16%	0.08%†*	0.16%	0.32%	0.63%	1.25%

**Maximum tolerated compound concentration where ΔCV < 10% is lower than ELISA.*
† Tolerated concentration significantly increases if ΔCV threshold is set to < 20%.

Summary

HTRF Capsid Detection Kits are no-wash, TR-FRET-based immunoassays that can accurately detect and measure AAV capsids in multiple types of analyte matrices, including assay and lysis buffer, cell culture medium, and cell lysate. These kits are precise with consistently low intra- and inter-assay %CV. Each kit can detect AAV capsids in cell lysate samples over a range of total protein concentrations and can be used to measure AAV capsids in test samples at any point in a manufacturing pipeline. The assays can measure both empty and full AAV capsids equally and cannot distinguish between the two. The AAV5, AAV8, and AAV9 kits are highly specific, only detecting AAV capsids of the same serotype. However, the other HTRF AAV kits had mild to moderate cross-reactivity with capsids from other serotypes. The kits can tolerate higher concentrations of chemicals in buffers commonly used in AAV preparations compared to ELISA AAV capsid immunoassays currently on the market. Collectively, these products offer a simple, versatile, and accurate method for rapidly quantifying AAV capsid titers in many different types of analyte matrices and can easily and advantageously replace ELISA assays when measuring the concentration of the AAV capsid in R&D or manufacturing workflows.

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Appendix I

HTRF Data Analysis - Data Reduction and Additional Calculations

1. ***Ratio:*** the donor and acceptor emission signal ratio for each well.

$$Ratio = \frac{signal\ @\ 665\ nm}{signal\ @\ 620\ nm} \times 10^4$$

2. ***Delta Ratio:*** the ratio from Standard 0 (negative control) subtracted from the acceptor and donor emission signal ratio for each well.

$$\Delta Ratio\ (delta\ ratio) = Ratio\ of\ standard\ or\ sample - Ratio\ of\ standard\ 0$$

3. ***Coefficient of variation (%CV):*** the ratio of the standard deviation to the mean given as a percent.

$$CV(\%) = \frac{standard\ deviation}{Mean\ Ratio} \times 100$$

4. ***%Recovery:*** the ratio of the interpolated concentration of AAV capsid in VP/mL to the expected concentration (based on dilution) in VP/mL given as a percent.

$$Recovery\ (\%) = \frac{measured\ [AAV\ capsid]\ VP/mL}{expected\ [AAV\ capsid]\ VP/mL} \times 100$$

5. ***Limit of Detection (LOD):*** the lowest concentration of analyte in VP/mL that can be consistently measured with the HTRF AAV Capsid Detection Kits in each analyte matrix. The LOD is calculated using 24 data points (wells) loaded with detection reagents and no analyte.

$$LOD = mean\ Ratio + (2 \times standard\ deviation)$$

The resulting values were interpolated on an AAV standard curve prepared in the same analyte matrix to convert the LOD in VP/mL.

6. ***Limit of Quantification (LOQ):*** the lowest concentration of analyte in VP/mL that can be quantified with acceptable precision and accuracy. The LOQ is calculated using 24 data points (wells) loaded with detection reagents and no analyte.

$$LOQ = mean\ Ratio + (10 \times standard\ deviation)$$

The resulting values were interpolated on an AAV standard curve prepared in the same analyte matrix to convert the LOQ in VP/mL.

Appendix II

Analytical Performance – Intra-assay Precision (%CV)

Diluent #5

Inter-assay CV				Inter-assay CV			Inter-assay CV		Inter-assay CV	
[AAV] (VP/mL)	AAV1	AAV5	AAV6	[AAV] (VP/mL)	AAV2	AAV3B	[AAV] (VP/mL)	AAV8	[AAV] (VP/mL)	AAV9
2.00E+11	4%	6%	7%	4.00E+11	3%	3%	1.60E+11	3%	1.60E+11	3%
5.00E+10	5%	2%	7%	1.00E+11	2%	3%	1.00E+10	4%	4.00E+10	3%
1.25E+10	4%	3%	3%	2.50E+10	2%	10%	5.00E+10	5%	1.00E+10	3%
Mean CV	4%	4%	6%	Mean CV	2%	5%	Mean CV	4%	Mean CV	3%

Lysis Buffer #3

Inter-assay CV				Inter-assay CV			Inter-assay CV		Inter-assay CV	
[AAV] (VP/mL)	AAV1	AAV5	AAV6	[AAV] (VP/mL)	AAV2	AAV3B	[AAV] (VP/mL)	AAV8	[AAV] (VP/mL)	AAV9
2.00E+11	2%	8%	8%	4.00E+11	2%	5%	1.00E+10	4%	1.60E+11	2%
5.00E+10	7%	4%	4%	1.00E+11	4%	5%	4.00E+11	4%	4.00E+10	4%
1.25E+10	3%	11%	4%	2.50E+10	3%	7%	1.60E+11	5%	1.00E+10	3%
Mean CV	4%	8%	5%	Mean CV	3%	6%	Mean CV	4%	Mean CV	3%

DMEM (supplemented with 10% FBS and 1% PS)

Inter-assay CV				Inter-assay CV			Inter-assay CV		Inter-assay CV	
[AAV] (VP/mL)	AAV1	AAV5	AAV6	[AAV] (VP/mL)	AAV2	AAV3B	[AAV] (VP/mL)	AAV8	[AAV] (VP/mL)	AAV9
2.00E+11	4%	5%	7%	4.00E+11	2%	5%	1.00E+10	2%	1.60E+11	3%
5.00E+10	8%	4%	13%	1.00E+11	4%	5%	4.00E+11	2%	4.00E+10	3%
1.25E+10	6%	10%	7%	2.50E+10	5%	9%	1.60E+11	3%	1.00E+10	4%
Mean CV	6%	6%	9%	Mean CV	4%	6%	Mean CV	2%	Mean CV	3%

RPMI (supplemented with 10% FBS and 1% PS)

Inter-assay CV				Inter-assay CV			Inter-assay CV		Inter-assay CV	
[AAV] (VP/mL)	AAV1	AAV5	AAV6	[AAV] (VP/mL)	AAV2	AAV3B	[AAV] (VP/mL)	AAV8	[AAV] (VP/mL)	AAV9
2.00E+11	2%	9%	7%	4.00E+11	2%	4%	1.00E+10	3%	1.60E+11	2%
5.00E+10	6%	8%	8%	1.00E+11	5%	8%	4.00E+11	2%	4.00E+10	3%
1.25E+10	7%	4%	9%	2.50E+10	2%	10%	1.60E+11	3%	1.00E+10	2%
Mean CV	5%	7%	8%	Mean CV	3%	7%	Mean CV	3%	Mean CV	4%

Appendix III

Analytical Performance – Inter-assay Precision (%CV)

Diluent #5

Inter-assay CV			Inter-assay CV			Inter-assay CV		Inter-assay CV		Inter-assay CV	
[AAV] (VP/mL)	AAV1	AAV5	[AAV] (VP/mL)	AAV2	AAV3B	[AAV] (VP/mL)	AAV5	[AAV] (VP/mL)	AAV8	[AAV] (VP/mL)	AAV9
2.00E+11	2%	10%	4.00E+11	1%	3%	2.00E+11	7%	2.00E+11	1%	1.60E+11	3%
5.00E+10	4%	7%	1.00E+10	8%	3%	3.20E+10	8%	1.00E+11	7%	4.00E+10	8%
1.25E+10	8%	3%	2.50E+10	1%	10%	1.28E+10	11%	5.00E+10	8%	1.00E+10	9%
Mean CV	5%	7%	Mean CV	3%	5%	Mean CV	9%	Mean CV	5%	Mean CV	7%

Lysis Buffer #3

Inter-assay CV			Inter-assay CV			Inter-assay CV		Inter-assay CV		Inter-assay CV	
[AAV] (VP/mL)	AAV1	AAV5	[AAV] (VP/mL)	AAV2	AAV3B	[AAV] VP/mL	AAV5	[AAV] (VP/mL)	AAV8	[AAV] (VP/mL)	AAV9
2.00E+11	4%	18%	4.00E+11	6%	6%	2.00E+11	16%	2.00E+11	8%	1.60E+11	13%
5.00E+10	6%	13%	1.00E+10	9%	5%	3.20E+10	7%	1.00E+11	6%	4.00E+10	3%
1.25E+10	17%	1%	2.50E+10	15%	10%	1.28E+10	7%	5.00E+10	4%	1.00E+10	3%
Mean CV	9%	11%	Mean CV	10%	7%	Mean CV	10%	Mean CV	6%	Mean CV	6%

DMEM (supplemented with 10% FBS and 1% PS)

Inter-assay CV			Inter-assay CV			Inter-assay CV		Inter-assay CV		Inter-assay CV	
[AAV] (VP/mL)	AAV1	AAV5	[AAV] (VP/mL)	AAV2	AAV3B	[AAV] VP/mL	AAV5	[AAV] (VP/mL)	AAV8	[AAV] (VP/mL)	AAV9
2.00E+11	5%	11%	4.00E+11	10%	7%	2.00E+11	7%	2.00E+11	8%	1.60E+11	14%
5.00E+10	5%	9%	1.00E+10	10%	10%	3.20E+10	13%	1.00E+11	9%	4.00E+10	13%
1.25E+10	4%	4%	2.50E+10	6%	6%	1.28E+10	18%	5.00E+10	11%	1.00E+10	13%
Mean CV	5%	8%	Mean CV	9%	8%	Mean CV	13%	Mean CV	9%	Mean CV	13%

RPMI (supplemented with 10% FBS and 1% PS)

Inter-assay CV			Inter-assay CV			Inter-assay CV		Inter-assay CV		Inter-assay CV	
[AAV] (VP/mL)	AAV1	AAV5	[AAV] (VP/mL)	AAV2	AAV3B	[AAV] VP/mL	AAV5	[AAV] (VP/mL)	AAV8	[AAV] (VP/mL)	AAV9
2.00E+11	11%	10%	4.00E+11	8%	3%	2.00E+11	9%	2.00E+11	4%	1.60E+11	11%
5.00E+10	16%	2%	1.00E+10	7%	7%	3.20E+10	14%	1.00E+11	6%	4.00E+10	10%
1.25E+10	12%	2%	2.50E+10	9%	14%	1.28E+10	15%	5.00E+10	4%	1.00E+10	13%
Mean CV	13%	5%	Mean CV	8%	8%	Mean CV	13%	Mean CV	5%	Mean CV	11%

Appendix IV

Analytical Performance – Dilution Recovery

Diluent #5

AAV1			
Dilution	Measured [AAV] (VP/mL)	Expected [AAV] (VP/mL)	Dilution Recovery
-	1.61E+11	-	-
2-fold	7.38E+10	8.05E+10	92%
4-fold	3.46E+10	4.03E+10	86%
8-fold	1.73E+10	2.01E+10	86%
16-fold	9.26E+09	1.01E+10	92%
		Mean Recovery	89%

AAV2			
Dilution	Measured [AAV] (VP/mL)	Expected [AAV] (VP/mL)	Dilution Recovery
-	3.76E+11	-	-
2-fold	1.82E+11	1.88E+11	97%
4-fold	8.84E+10	9.40E+10	94%
8-fold	4.50E+10	4.70E+10	96%
16-fold	2.71E+10	2.35E+10	115%
		Mean Recovery	100%

AAV3B			
Dilution	Measured [AAV] (VP/mL)	Expected [AAV] (VP/mL)	Dilution Recovery
-	3.92E+11	-	-
2-fold	1.94E+11	1.96E+11	99%
4-fold	9.90E+10	9.80E+10	101%
8-fold	5.54E+10	4.90E+10	113%
16-fold	3.10E+10	2.45E+10	127%
		Mean Recovery	110%

AAV5			
Dilution	Measured [AAV] (VP/mL)	Expected [AAV] (VP/mL)	Dilution Recovery
-	2.33E+11	-	-
2.5-fold	8.89E+10	9.32E+10	95%
6.3-fold	3.76E+10	3.73E+10	101%
15.6-fold	1.56E+10	1.49E+10	105%
39.1-fold	6.52E+09	5.96E+09	109%
		Mean Recovery	103%

AAV6			
Dilution	Measured [AAV] (VP/mL)	Expected [AAV] (VP/mL)	Dilution Recovery
-	3.87E+11	-	-
2-fold	2.03E+11	1.94E+11	105%
4-fold	1.05E+11	9.68E+10	109%
8-fold	5.49E+10	4.84E+10	113%
		Mean Recovery	109%

AAV8			
Dilution	Measured [AAV] (VP/mL)	Expected [AAV] (VP/mL)	Dilution Recovery
-	2.10E+11	-	-
2-fold	1.03E+11	1.05E+11	98%
4-fold	4.82E+10	5.25E+10	92%
8-fold	2.31E+10	2.63E+10	88%
16-fold	1.21E+10	1.31E+10	92%
		Mean Recovery	93%

AAV9			
Dilution	Measured [AAV] (VP/mL)	Expected [AAV] (VP/mL)	Dilution Recovery
-	1.63E+11	-	-
2-fold	8.11E+10	8.15E+10	100%
4-fold	3.64E+10	4.08E+10	89%
8-fold	1.84E+10	2.04E+10	90%
16-fold	9.39E+09	1.02E+10	92%
		Mean Recovery	93%

Lysis Buffer #3

AAV1			
Dilution	Measured [AAV] (VP/mL)	Expected [AAV] (VP/mL)	Dilution Recovery
-	1.77E+11	-	-
2-fold	8.72E+10	8.85E+10	99%
4-fold	3.84E+10	4.43E+10	87%
8-fold	2.02E+10	2.21E+10	91%
16-fold	1.07E+10	1.11E+10	97%
		Mean Recovery	93%

AAV2			
Dilution	Measured [AAV] (VP/mL)	Expected [AAV] (VP/mL)	Dilution Recovery
-	3.55E+11	-	-
2-fold	1.61E+11	1.78E+11	91%
4-fold	7.47E+10	8.88E+10	84%
8-fold	4.18E+10	4.44E+10	94%
		Mean Recovery	90%

AAV3B			
Dilution	Measured [AAV] (VP/mL)	Expected [AAV] (VP/mL)	Dilution Recovery
-	3.90E+11	-	-
2-fold	1.77E+11	1.95E+11	91%
4-fold	8.93E+10	9.75E+10	92%
8-fold	5.25E+10	4.88E+10	108%
16-fold	2.40E+10	2.44E+10	98%
		Mean Recovery	97%

AAV5			
Dilution	Measured [AAV] (VP/mL)	Expected [AAV] (VP/mL)	Dilution Recovery
-	2.07E+11	-	-
2.5-fold	7.96E+10	8.28E+10	96%
6.3-fold	3.42E+10	3.25E+10	105%
15.6-fold	1.42E+10	1.27E+10	112%
39.1-fold	4.89E+09	4.99E+09	98%
		Mean Recovery	103%

AAV6			
Dilution	Measured [AAV] (VP/mL)	Expected [AAV] (VP/mL)	Dilution Recovery
-	3.47E+11	-	-
2-fold	1.90E+11	1.74E+11	110%
4-fold	1.00E+11	8.68E+10	115%
8-fold	5.08E+10	4.34E+10	117%
		Mean Recovery	114%

AAV8			
Dilution	Measured [AAV] (VP/mL)	Expected [AAV] (VP/mL)	Dilution Recovery
-	1.93E+11	-	-
2-fold	1.07E+11	9.65E+10	111%
4-fold	5.16E+10	4.83E+10	107%
8-fold	2.41E+10	2.41E+10	100%
16-fold	1.19E+10	1.21E+10	99%
		Mean Recovery	104%

AAV9			
Dilution	Measured [AAV] (VP/mL)	Expected [AAV] (VP/mL)	Dilution Recovery
-	1.50E+11	-	-
2-fold	7.06E+10	7.50E+10	94%
4-fold	3.27E+10	3.75E+10	87%
8-fold	1.65E+10	1.88E+10	88%
16-fold	8.40E+09	9.38E+09	90%
		Mean Recovery	90%

DMEM (supplemented with 10% FBS and 1% PS)

AAV1			
Dilution	Measured [AAV] (VP/mL)	Expected [AAV] (VP/mL)	Dilution Recovery
-	1.68E+11	-	-
2-fold	7.53E+10	8.42E+10	89%
4-fold	3.74E+10	4.21E+10	89%
8-fold	1.90E+10	2.10E+10	90%
16-fold	9.01E+09	1.05E+10	86%
		Mean Recovery	89%

AAV2			
Dilution	Measured [AAV] (VP/mL)	Expected [AAV] (VP/mL)	Dilution Recovery
-	3.74E+11	-	-
2-fold	1.88E+11	1.87E+11	101%
4-fold	9.05E+10	9.35E+10	97%
8-fold	4.51E+10	4.68E+10	96%
		Mean Recovery	98%

AAV3B			
Dilution	Measured [AAV] (VP/mL)	Expected [AAV] (VP/mL)	Dilution Recovery
-	4.14E+11	-	-
2-fold	1.85E+11	2.07E+11	89%
4-fold	9.42E+10	1.04E+11	91%
8-fold	4.75E+10	5.18E+10	92%
16-fold	2.34E+10	2.59E+10	90%
		Mean Recovery	91%

AAV5			
Dilution	Measured [AAV] (VP/mL)	Expected [AAV] (VP/mL)	Dilution Recovery
-	1.95E+11	-	-
2.5-fold	8.37E+10	7.80E+10	93%
6.3-fold	3.25E+10	3.12E+10	96%
15.6-fold	1.30E+10	1.25E+10	96%
39.1-fold	5.43E+09	4.99E+09	92%
		Mean Recovery	94%

AAV6			
Dilution	Measured [AAV] (VP/mL)	Expected [AAV] (VP/mL)	Dilution Recovery
-	3.84E+11	-	-
2-fold	1.96E+11	1.92E+11	102%
4-fold	1.04E+11	9.80E+10	106%
8-fold	5.38E+10	5.20E+10	103%
		Mean Recovery	104%

AAV8			
Dilution	Measured [AAV] (VP/mL)	Expected [AAV] (VP/mL)	Dilution Recovery
-	2.12E+11	-	-
2-fold	1.01E+11	1.06E+11	95%
4-fold	4.71E+10	5.30E+10	89%
8-fold	2.30E+10	2.65E+10	87%
16-fold	1.15E+10	1.33E+10	87%
		Mean Recovery	89%

AAV9			
Dilution	Measured [AAV] (VP/mL)	Expected [AAV] (VP/mL)	Dilution Recovery
-	1.50E+11	-	-
2-fold	7.52E+10	7.50E+10	100%
4-fold	3.40E+10	3.75E+10	91%
8-fold	1.67E+10	1.88E+10	89%
16-fold	8.81E+09	9.38E+09	94%
		Mean Recovery	93%

RPMI (supplemented with 10% FBS and 1% PS)

AAV1			
Dilution	Measured [AAV] (VP/mL)	Expected [AAV] (VP/mL)	Dilution Recovery
-	1.85E+11	-	-
2-fold	8.52E+10	9.25E+10	92%
4-fold	3.97E+10	4.63E+10	86%
8-fold	1.89E+10	2.31E+10	82%
16-fold	1.02E+10	1.16E+10	88%
		Mean Recovery	87%

AAV2			
Dilution	Measured [AAV] (VP/mL)	Expected [AAV] (VP/mL)	Dilution Recovery
-	3.21E+11	-	-
2-fold	1.62E+11	1.61E+11	101%
4-fold	7.87E+10	8.03E+10	98%
8-fold	4.07E+10	4.01E+10	101%
		Mean Recovery	100%

AAV3B			
Dilution	Measured [AAV] (VP/mL)	Expected [AAV] (VP/mL)	Dilution Recovery
-	3.90E+11	-	-
2-fold	1.88E+11	1.95E+11	96%
4-fold	9.56E+10	9.75E+10	98%
8-fold	4.67E+10	4.88E+10	96%
16-fold	2.40E+10	2.44E+10	98%
		Mean Recovery	97%

AAV5			
Dilution	Measured [AAV] (VP/mL)	Expected [AAV] (VP/mL)	Dilution Recovery
-	2.38E+11	-	-
2.5-fold	8.89E+10	9.52E+10	93%
6.3-fold	3.27E+10	3.81E+10	86%
15.6-fold	1.30E+10	1.52E+10	85%
39.1-fold	5.48E+09	6.09E+09	90%
		Mean Recovery	89%

AAV6			
Dilution	Measured [AAV] (VP/mL)	Expected [AAV] (VP/mL)	Dilution Recovery
-	3.76E+11	-	-
2-fold	2.05E+11	1.88E+11	109%
4-fold	1.09E+11	9.40E+10	116%
8-fold	5.43E+10	4.70E+10	116%
		Mean Recovery	114%

AAV8			
Dilution	Measured [AAV] (VP/mL)	Expected [AAV] (VP/mL)	Dilution Recovery
-	2.12E+11	-	-
2-fold	1.04E+11	1.06E+11	98%
4-fold	5.01E+10	5.30E+10	95%
8-fold	2.34E+10	2.65E+10	88%
16-fold	1.25E+10	1.33E+10	94%
		Mean Recovery	94%

AAV9			
Dilution	Measured [AAV] (VP/mL)	Expected [AAV] (VP/mL)	Dilution Recovery
-	1.48E+11	-	-
2-fold	7.06E+10	7.40E+10	95%
4-fold	3.30E+10	3.70E+10	89%
8-fold	1.70E+10	1.85E+10	92%
16-fold	8.65E+09	9.25E+09	94%
		Mean Recovery	93%

Appendix V.

Analytical Performance – Assay Interference – Cell Lysate

SF9 cell lysate			
[AAV] (VP/mL)	[Total Protein] (mg/mL)	AAV1 Recovery	AAV6 Recovery
1.00E+11	0.500	54%	61%
	0.375	59%	61%
	0.250	70%	65%
	0.125	81%	64%
	0.050	93%	100%

SF9 cell lysate			
[AAV] (VP/mL)	[Total Protein] (mg/mL)	AAV2 Recovery	AAV3B Recovery
1.00E+11	0.188	54%	56%
	0.094	72%	76%
	0.047	86%	87%
	0.023	84%	96%

SF9 cell lysate				
[AAV] (VP/mL)	[Total Protein] (mg/mL)	AAV5 Recovery	AAV8 Recovery	AAV9 Recovery
5.00E+10	1.000	-	101%	102%
	0.500	126%	107%	96%
	0.250	120%	105%	99%
	0.125	122%	106%	99%
	0.050	115%	102%	92%

HEK293 cell lysate			
[AAV] (VP/mL)	[Total Protein] (mg/mL)	AAV1 Recovery	AAV6 Recovery
1.00E+11	1.500	64%	96%
	0.750	96%	100%
	0.500	96%	104%
	0.375	94%	106%
	0.250	103%	104%

HEK293 cell lysate			
[AAV] (VP/mL)	[Total Protein] (mg/mL)	AAV2 Recovery	AAV3B Recovery
1.00E+11	1.500	72%	84%
	0.750	67%	76%
	0.375	85%	95%
	0.188	89%	96%
	0.094	90%	102%

HEK293 cell lysate				
[AAV] (VP/mL)	[Total Protein] (mg/mL)	AAV5 Recovery	AAV8 Recovery	AAV9 Recovery
5.00E+10	1.000	-	109%	97%
	0.500	128%	107%	98%
	0.250	115%	102%	97%
	0.125	109%	107%	97%
	0.050	115%	111%	98%

Appendix VI.

Analytical Performance – Spike and Recovery from Cell Lysate

SF9 Cell Lysate

SF9 cell lysate (0.05 mg/mL)	
[AAV1] (VP/mL)	Recovery
1.00E+11	93%
5.00E+10	96%
1.00E+10	101%
Mean Recovery	97%

SF9 cell lysate (0.05 mg/mL)	
[AAV2] (VP/mL)	Recovery
8.77E+10	86%
3.56E+10	103%
1.04E+10	85%
Mean Recovery	91%

SF9 cell lysate (0.1 mg/mL)	
[AAV3B] (VP/mL)	Recovery
2.00E+11	78%
5.00E+10	90%
1.25E+09	84%
Mean Recovery	84%

SF9 cell lysate (0.05 mg/mL)	
[AAV5] (VP/mL)	Recovery
2.05E+11	110%
5.12E+10	117%
1.28E+10	112%
Mean Recovery	113%

SF9 cell lysate (0.05 mg/mL)	
[AAV6] (VP/mL)	Recovery
1.00E+11	91%
5.00E+10	103%
1.00E+10	100%
Mean Recovery	98%

SF9 cell lysate (1 mg/mL)	
[AAV8] (VP/mL)	Recovery
1.34E+10	116%
2.93E+10	108%
5.41E+10	115%
Mean Recovery	113%

SF9 cell lysate (1 mg/mL)	
[AAV9] (VP/mL)	Recovery
1.16E+11	99%
5.61E+10	100%
2.64E+10	93%
Mean Recovery	97%

HEK293 Cell Lysate

HEK293 cell lysate (0.05 mg/mL)	
[AAV1] (VP/mL)	Recovery
1.00E+11	96%
5.00E+10	98%
5.00E+09	93%
Mean Recovery	96%

HEK293 cell lysate (0.4 mg/mL)	
[AAV2] (VP/mL)	Recovery
8.77E+10	87%
3.56E+10	83%
1.04E+10	87%
Mean Recovery	86%

HEK293 cell lysate (0.4 mg/mL)	
[AAV3B] (VP/mL)	Recovery
2.00E+11	91%
5.00E+10	103%
1.25E+09	93%
Mean Recovery	96%

HEK293 cell lysate (0.25 mg/mL)	
[AAV5] (VP/mL)	Recovery
2.05E+11	107%
5.12E+10	115%
1.28E+10	104%
Mean Recovery	109%

HEK293 cell lysate (1.5 mg/mL)	
[AAV6] (VP/mL)	Recovery
1.00E+11	100%
5.00E+10	93%
5.00E+09	91%
Mean Recovery	95%

HEK293 cell lysate (1 mg/mL)	
[AAV8] (VP/mL)	Recovery
1.34E+10	118%
2.93E+10	104%
5.41E+10	116%
Mean Recovery	113%

HEK293 cell lysate (1 mg/mL)	
[AAV9] (VP/mL)	Recovery
1.14E+11	94%
5.51E+10	100%
2.60E+10	94%
Mean Recovery	96%

Appendix VII.

Analytical Performance – Cross-reactivity

HTRF AAV Capsid Detection Kit	AAV Capsid Serotype Cross-reactivity (%)						
	AAV Capsids						
	AAV1	AAV2	AAV3B	AAV5	AAV6	AAV8	AAV9
AAV1	100%	42%	68%	305%	61%	18%	0%
AAV2	238%	100%	161%	727%	145%	43%	0%
AAV3B	148%	62%	100%	450%	90%	27%	0%
AAV5	0%	0%	0%	100%	0%	0%	0%
AAV6	164%	69%	111%	500%	100%	30%	0%
AAV8	0%	0%	0%	0%	0%	100%	0%
AAV9	0%	0%	0%	0%	0%	0%	100%

Relative cross-reactivity was calculated at an AAV capsid concentration of 2.50E+11 VP/mL.

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