MANUAL

AlphaLISA® AAV1 Capsid Detection Kit

Product number: AL3181

Research Use Only. Not for use in diagnostic procedures.

Product Information

Application: This kit is designed for the quantitative determination of **AAV1 capsid** using a homogeneous no wash AlphaLISA assay. The assay cross-reacts with other AAV serotypes (see page 13).

Kit contents: The kit contains 7 components: anti-HRP AlphaLISA Acceptor beads, Streptavidin-coated Donor beads, HRP conjugated anti-AAV1, Biotinylated anti-AAV1 antibody, Lyophilized AAV1 empty capsid, 10X HiBlock Buffer and 5X AlphaLISA Lysis buffer.

Sensitivity: Lower Detection Limit (LDL): 7.6E+06 VP/mL

Lower Limit of Quantification (LLOQ): 2.6E+06 VP/mL
EC_{50}: 2.5E+10 VP/mL

Dynamic Range: 7.6E+06 – 1.0E+11 VP/mL

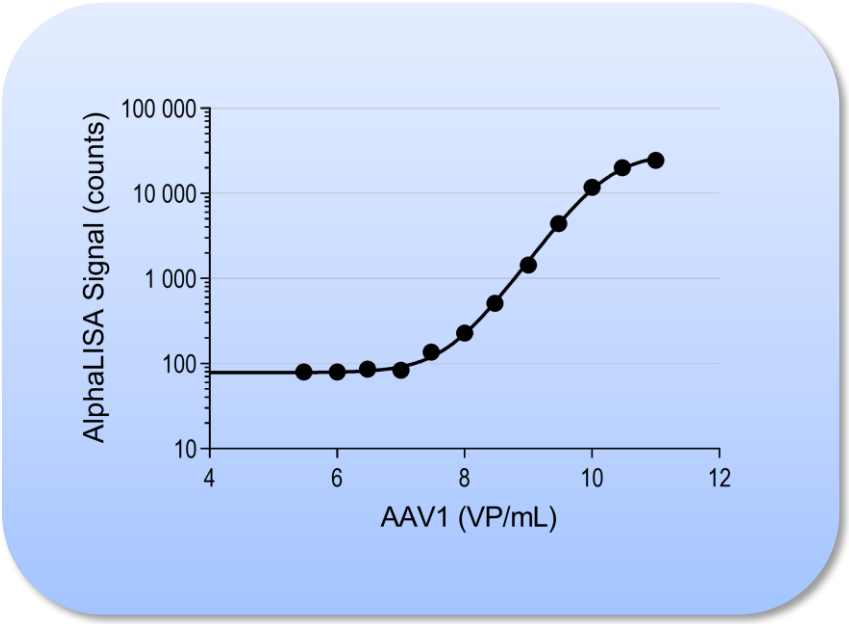


Figure 1. Typical sensitivity curve in AlphaLISA HiBlock Buffer. The data was generated using a gray AlphaPlate™-384 microplate and the EnVision® 2102 Multilabel Plate Reader with Alpha option.

Storage: Store kit in the dark at 4 ºC. For reconstituted analyte, aliquot and store at -80 ºC for up to 1 month. Avoid freeze-thaw cycles.

Stability: This kit is stable for at least 12 months from the date of manufacture when stored in its original packaging and the recommended storage conditions.

Analyte of Interest

Adeno-associated virus serotype 1 (AAV1) is a single-stranded DNA virus with a genome of approximately 4.7 kilobases. This virus needs co-infection with helper viruses such as adenovirus or herpes virus, to replicate. AAVs are used as vector for gene delivery mainly because of its ability to infect cells without pathogenicity, its ease of engineering and production. Serotype 1 is an efficient vector for gene delivery to skeletal muscle or central nervous system. The optimization of AAV vectors for their use in clinical trials places AAVs as a promising gene therapy tool. The AlphaLISA AAV1 capsid Detection Kits designed to detect and quantify AAV1 particles (being expressed in Viral Particles/mL or VP/mL) in both cell lysates and cell culture media.

Description of the AlphaLISA Assay

AlphaLISA technology allows the detection of molecules of interest in cell culture media (such as DMEM) and cell lysates in a highly sensitive, quantitative, reproducible and user-friendly mode. In this AlphaLISA assay, a biotinylated anti-AAV1 antibody binds to the streptavidin coated AlphaLISA Donor beads, while the HRP anti-AAV1 antibody is captured by anti-HRP AlphaLISA Acceptor beads. In the presence of AAV1 capsid, the beads come into proximity. The excitation of the Donor beads provokes the release of singlet oxygen molecules that triggers a cascade of energy transfer within the Acceptor beads, resulting in emission with λ_{max} at 615 nm (Figure 2). The assay can also cross react with several serotypes (see page 13).

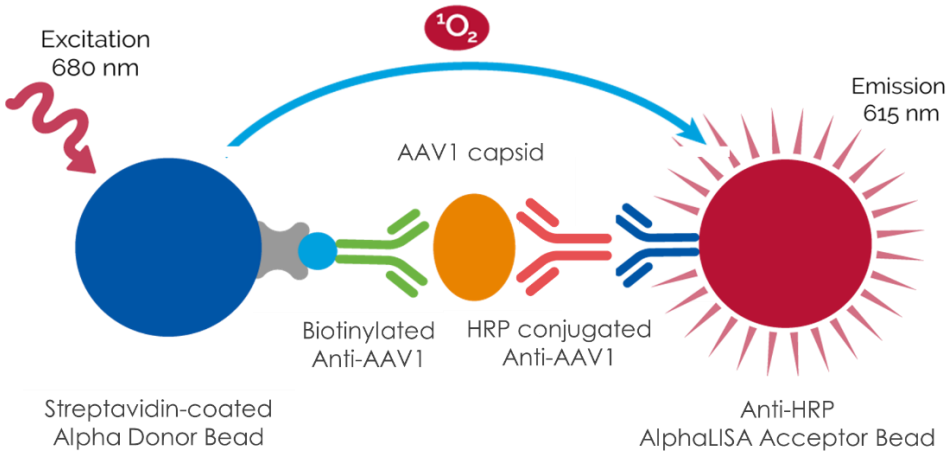


Figure 2. AlphaLISA AAV1 Capsid Detection Assay Principle.

Precautions

- The Alpha Donor beads are light-sensitive. All the other assay reagents can be used under normal light conditions. All Alpha assays using the Donor beads should be performed under subdued laboratory lighting (< 100 lux). Green filters (LEE 090 filters (preferred) or Roscolux filters #389 from Rosco) can be applied to light fixtures.
- Take precautionary measures to avoid contamination of the reagent solutions.

Kit Content: Reagents and Materials

Kit components	AL3181HV 100 assay points****	AL3181C 500 assay points****	AL3181F 5000 assay points****
AlphaLISA Anti-HRP Acceptor beads stored in PBS, 0.05% Kathon CG/ICP, pH 7.2	10 µL @ 5 mg/mL (1 brown tube, white cap)	25 µL @ 5 mg/mL (1 brown tube, white cap)	250 µL @ 5 mg/mL (1 brown tube, white cap)
HRP Anti-AAV1 Antibody stored in StabilZyme™ NOBLE Stabilizer (BSA-Free) - CMIT/MIT	1.5 µL @ 500 nM (1 tube, white cap)	6.5 µL @ 500 nM (1 tube, white cap)	65 µL @ 500 nM (1 tube, white cap)
Streptavidin (SA)-coated Donor beads stored in 25 mM HEPES, 100 mM NaCl, 0.05% Kathon CG/ICP, pH 7.4	40 µL @ 5 mg/mL (1 brown tube, black cap)	200 µL @ 5 mg/mL (1 brown tube, black cap)	1 000 µL @ 5 mg/mL (2 brown tubes, black cap)
Biotinylated Anti-AAV1 Antibody stored in StabilZyme™ NOBLE Stabilizer (BSA-Free) - CMIT/MIT	20 µL @ 500 nM (1 tube, black cap)	100 µL @ 500 nM (1 tube, black cap)	1 000 µL @ 500 nM (1 tube, black cap)
Lyophilized AAV1 empty capsids	1E+11 VP (1 tube, clear cap)	1E+11 VP (1 tube, clear cap)	1E+11 VP (1 tube, clear cap)
AlphaLISA HiBlock Buffer (10X)	2 mL, 1 small bottle	10 mL, 1 small bottle	100 mL, 1 large bottle
AlphaLISA Lysis Buffer (5X)	2 mL, 1 small bottle	10 mL, 1 small bottle	100 mL, 1 large bottle

* Reconstitute lyophilized analyte in 100 µL Milli-Q® grade H_2O. The reconstituted analyte should be used within 60 minutes or aliquoted into screw-capped 0.5 mL polypropylene vials and stored at **-80 C** for future experiments. The aliquoted analyte at -80 C is stable up to 1 month. Avoid freeze-thaw cycles. One vial contains an amount of analyte sufficient for performing 10 standard curves. Additional vials can be ordered separately (cat # AL3181S).

** Extra buffer can be ordered separately (cat # AL004C: 10 mL, cat # AL004F: 100 mL).

*** Extra buffer can be ordered separately (cat # AL003C: 10 mL, cat # AL003F: 100 mL)

**** The number of assay points is based on an assay volume of 100 µL in 96-well plates or 50 µL in 384-well assay plates using the kit components at the recommended concentrations.

Sodium azide should **not** be added to the stock reagents. High concentrations of sodium azide (> 0.001% final in the assay) might decrease the AlphaLISA signal.

Specific additional required reagents and materials:

The following materials are recommended:

Item	Suggested source	Catalog #
TopSeal™-A Plus Adhesive Sealing Film	Revvity Inc.	6050185
EnVision®-Alpha Reader	Revvity Inc.	-

Recommendations

IMPORTANT: PLEASE READ THE RECOMMENDATIONS BELOW BEFORE USE

- The volume indicated on each tube is guaranteed for single pipetting. Multiple pipetting of the reagents may reduce the theoretical amount left in the tube. To minimize loss when pipetting beads, it is preferable not to pre-wet the tip.
- Centrifuge all tubes (including lyophilized analyte) before use to improve recovery of content (2000*g*, 10-15 sec). Re-suspend all reagents by vortexing before use.
- Use Milli-Q® grade H_2O to dilute 10X AlphaLISA HiBlock Buffer, 5X AlphaLISA Lysis buffer and to reconstitute the lyophilized analyte.
- When diluting the standard or samples, change tips between each standard or sample dilution. When loading reagents in the assay microplate, change tips between each standard or sample addition and after each set of reagents.
- When reagents are added to the microplate, make sure the liquids are at the bottom of the well.
- Small volumes may be prone to evaporation. It is recommended to cover microplates with TopSeal-A Adhesive Sealing Films to reduce evaporation during incubation. Microplates can be read with the TopSeal-A Film in place.
- The AlphaLISA signal is detected with an EnVision Multilabel Plate Reader equipped with the Alpha option using the AlphaScreen standard settings (e.g. Total Measurement Time: 550 ms, Laser 680 nm Excitation Time: 180 ms, Mirror: D640as, Emission Filter: M570w, Center Wavelength 570 nm, Bandwidth 100 nm, Transmittance 75%).
- AlphaLISA signal will vary with temperature and incubation time. For consistent results, identical incubation times and temperature should be used for each plate.
- The standard curves shown in this technical data sheet are provided for information only. A standard curve must be generated for each experiment.

Assay Procedure

- Two different manuals can be utilized:
  - Manual 1: Default Manual (3 incubation steps) with **10 µL** of sample volume: Recommended to obtain the highest assay sensitivity
  - Manual 2: Alternative Manual (3 incubation steps) with **5 µL** of sample volume: Recommended when using culture media containing free biotin
- The manual described below is an example for generating one standard curve in a 50 µL final assay volume (48 wells, triplicate determinations). The manuals also include testing samples in 452 wells. If different amount of samples are tested, the volumes of all reagents have to be adjusted accordingly, as shown in the table below. These calculations do not include excess reagent to account for losses during transfer of solutions or dead volumes.
- The standard dilution manual is provided for information only. As needed, the number of replicates or the range of concentrations covered can be modified.
- Use of four background points in triplicate (12 wells) is recommended when LDL/LLOQ is calculated. One background point in triplicate (3 wells) can be used when LDL/LLOQ is not calculated.

		Volume					
Format	**# of data points**	**Final**	**Sample**	**AlphaLISA Acceptor Beads + HRP anti-AAV1**	**Biotinylated Antibody**	**SA-Donor beads**	**Plate recommendation**
AL3181HV	50	100 µL	20 µL	20 µL	10 µL	50 µL	White OptiPlate-96 (cat # 6005290)
	100	50 µL	10 µL	10 µL	5 µL	25 µL	½ Area AlphaPlate-96 (cat # 6002350) White OptiPlate-384 (cat # 6007290) Light gray AlphaPlate™-384 (cat # 6005350)
AL3181C	250	100 µL	20 µL	20 µL	10 µL	50 µL	White OptiPlate-96 (cat # 6005290)
	500	50 µL	10 µL	10 µL	5 µL	25 µL	½ Area AlphaPlate-96 (cat # 6002350) White OptiPlate-384 (cat # 6007290) Light gray AlphaPlate™-384 (cat # 6005350)
	1 250	20 µL	4 µL	4 µL	2 µL	10 µL	Light gray AlphaPlate-384 (cat # 6005350) ProxiPlate™-384 Plus (cat # 6008280) White OptiPlate-384 (cat # 6007290)
	2 500	10 µL	2 µL	2 µL	1 µL	5 µL	Light gray AlphaPlate-1536 (cat # 6004350)
AL3181F	5 000	50 µL	10 µL	10 µL	5 µL	25 µL	½ Area AlphaPlate-96 (cat # 6002350) White OptiPlate-384 (cat # 6007290) Light gray AlphaPlate-384 (cat # 6005350)
	12 500	20 µL	4 µL	4 µL	2 µL	10 µL	Light gray AlphaPlate-384 (cat # 6005350) ProxiPlate-384 Plus (cat # 6008280) White OptiPlate-384 (cat # 6007290)
	25 000	10 µL	2 µL	2 µL	1 µL	5 µL	Light gray AlphaPlate-1536 (cat # 6004350)

Common Steps for Preparing Reagents (Manuals 1 & 2)

The 3-Steps Manual described below is for 500 assay points including one standard curve (48 wells) and samples (452 wells). *If different amount of samples are tested, the volumes of all reagents have to be adjusted accordingly.*

1) Preparation of AlphaLISA Lysis Buffer 1X (if needed to lyse samples):
Add 1 mL of 5X AlphaLISA Lysis buffer to 4 mL of Milli-Q® grade H_2O.

2) Preparation of 1X AlphaLISA HiBlock Buffer:
Add 5 mL of 10X AlphaLISA HiBlock Buffer to 45 mL Milli-Q® grade H_2O.

3) Preparation of AAV1 capsid analyte standard dilutions:

a. Reconstitute lyophilized AAV1 (1E+11 VP) in 100 µL Milli-Q® grade H2O. Stock AAV1 particles solution concentration is 1E+12 VP/mL. The remaining reconstituted analyte should be aliquoted immediately and stored at -80 ºC for future assays (see page 4 for more details).

b. Prepare standard dilutions as follows in 1X AlphaLISA HiBlock Buffer (change tip between each standard dilution):

Tube	Vol. of AAV1 (µL)	Vol. of diluent (µL) *	[AAV1] in standard curve (VP/mL)
A	10 µL of reconstituted AAV1	90	1.00E+11*
B	60 µL of tube A	140	3.00E+10
C	60 µL of tube B	120	1.00E+10
D	60 µL of tube C	140	3.00E+9
E	60 µL of tube D	120	1.00E+9
F	60 µL of tube E	140	3.00E+8
G	60 µL of tube F	120	1.00E+8
H	60 µL of tube G	140	3.00E+7
I	60 µL of tube H	120	1.00E+7
J	60 µL of tube I	140	3.00E+6
K	60 µL of tube J	120	1.00E+6
L	60 µL of tube K	140	3.00E+5
M ** (background)	0	100	0
N ** (background)	0	100	0
O ** (background)	0	100	0
P ** (background)	0	100	0

* Dilute standards in diluent (e.g. 1X AlphaLISA HiBlock Buffer or 1X AlphaLISA Lysis buffer).
At low concentrations of analyte, a significant amount of analyte can bind to the vial. Therefore, load the analyte standard dilutions in the assay microplate within 60 minutes of preparation.

** Four background points in triplicate (12 wells) are used when LDL is calculated. If LDL does not need to be calculated, one background point in triplicate can be used (3 wells).

Manual 1: Default Manual (3 incubation Steps) with 10 µL of sample volume: Use of this manual results in higher assay sensitivity

4) Preparation of 5X Mix AlphaLISA Anti-HRP Acceptor beads (25 µg/mL) + HRP Anti-AAV1 (0.625 nM):

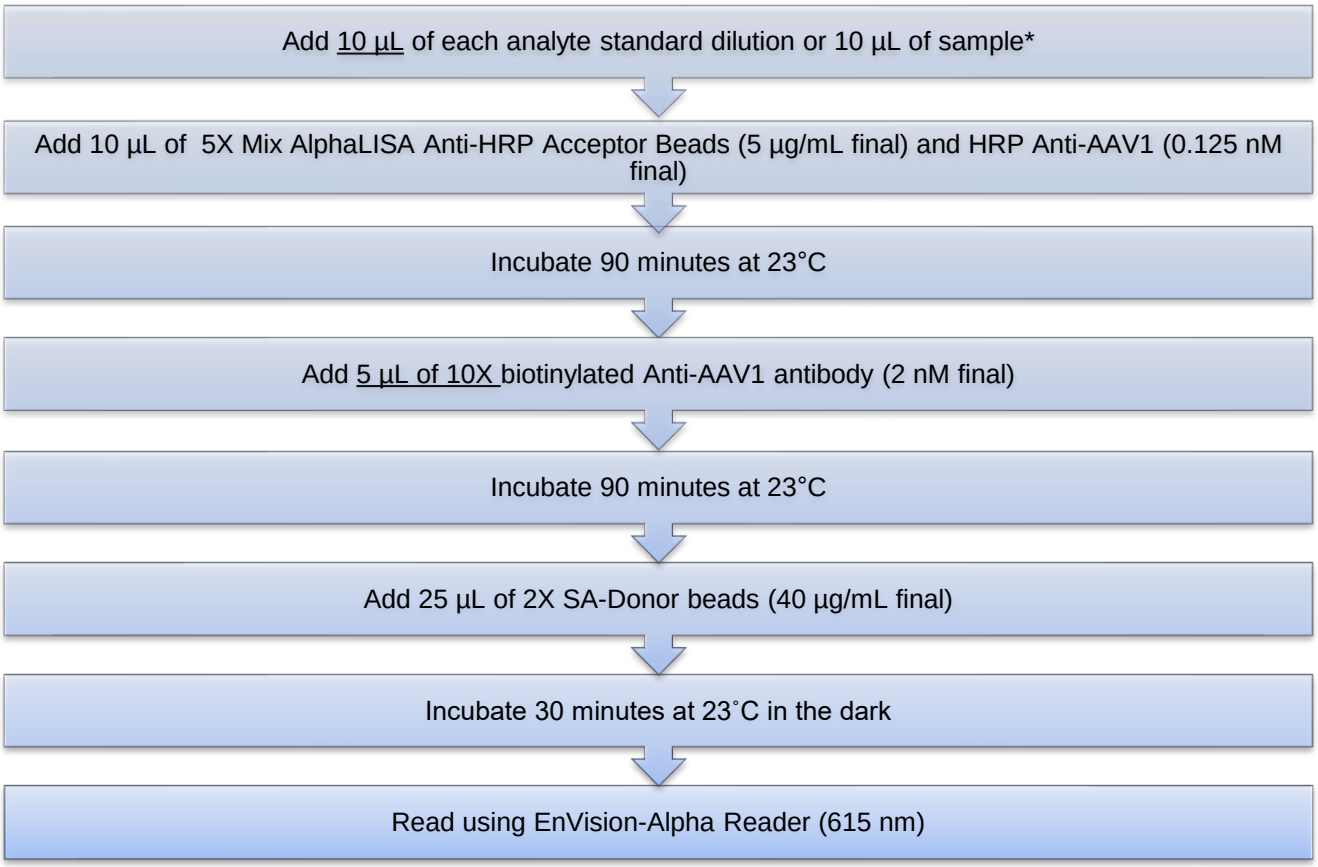
a. Prepare just before use.

b. Add 25 µL of 5 mg/mL AlphaLISA Anti-HRP Antibody Acceptor Bead and 6.25 µL of 500 nM HRP Anti-AAV1 to 4968.75 µL of 1X AlphaLISA HiBlock Buffer.

5) Preparation of **10X** biotinylated anti-AAV1 antibody (20 nM)
   a. Prepare just before use
   b. Add 100 µL of 500 nM biotinylated antibody to 2400 µL of 1X AlphaLISA HiBlock Buffer.

6) Preparation of 2X Streptavidin (SA) Donor beads (80 µg/mL):
   a. Prepare just before use.
   b. Keep the beads under subdued laboratory lighting.
   c. Add 200 µL of 5 mg/mL SA-Donor beads to 12300 µL of 1X AlphaLISA HiBlock Buffer.

7) In a Light Gray AlphaPlate (384 wells):



* CAUTION: The AAV product line will use a sample input volume of 10 µL instead of the usual 5 µL. Pay attention to use 10 µL sample when doing the manual.

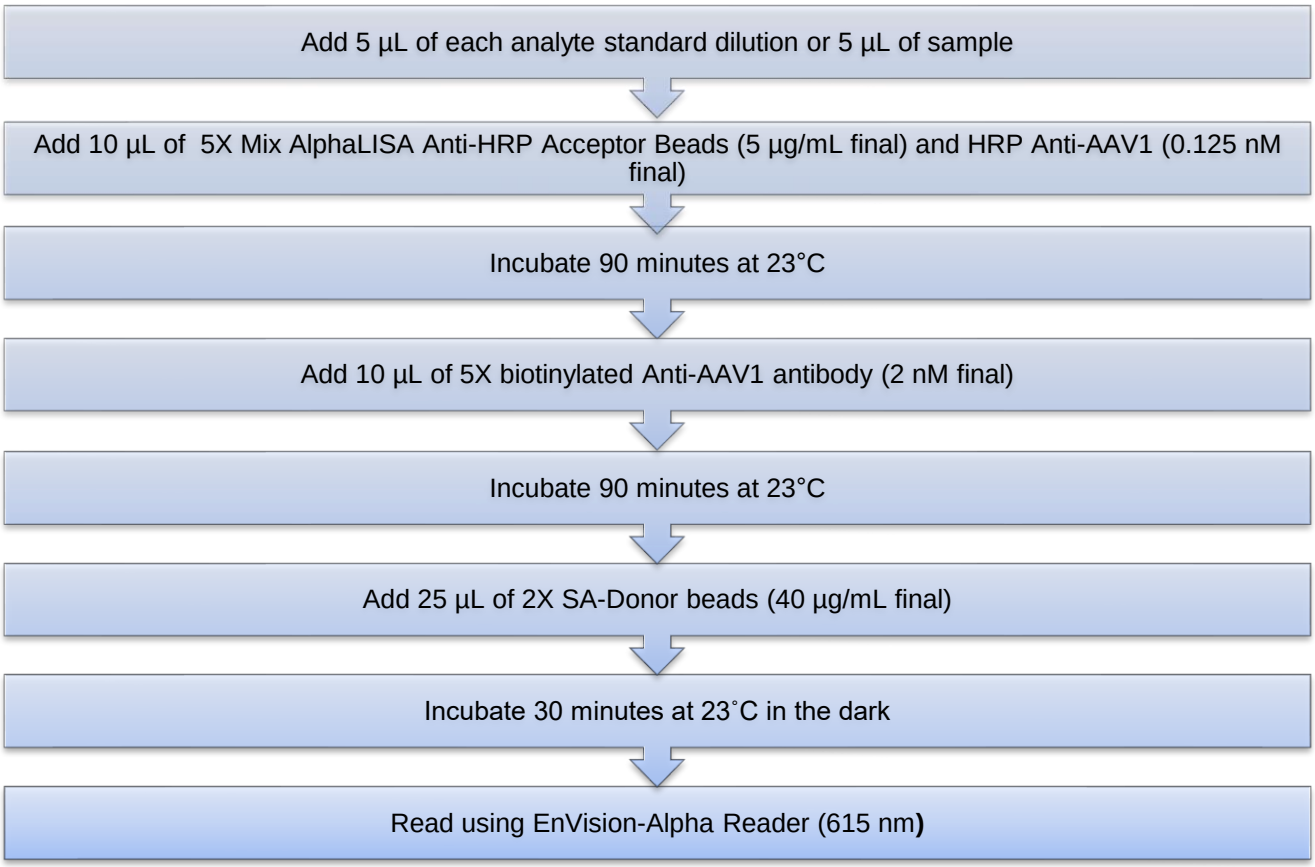
Manual 2: Alternative Manual (3 incubation Steps) with 5 µL of sample volume: recommended when using culture media containing free biotin

8) Preparation of 5X Mix AlphaLISA Anti-HRP Acceptor beads (25 µg/mL) + HRP Anti-AAV1 (0.625 nM):
   a. Prepare just before use.
   b. Add 25 µL of 5 mg/mL AlphaLISA Anti-HRP Antibody Acceptor Bead and 6.25 µL of 500 nM HRP Anti-AAV1 to 4968.75 µL of 1X AlphaLISA HiBlock Buffer.

3) Preparation of 5X biotinylated anti-AAV1 antibody (10 nM)
   a. Prepare just before use
   b. Add 100 µL of 500 nM biotinylated antibody to 4900 µL of AlphaLISA HiBlock Buffer.

4) Preparation of 2X Streptavidin (SA) Donor beads (80 µg/mL):
    a. Prepare just before use.
    b. Keep the beads under subdued laboratory lighting.
    c. Add 200 µL of 5 mg/mL SA-Donor beads to 12300 µL of 1X AlphaLISA HiBlock Buffer.

5) In a Light Gray AlphaPlate (384 wells):



Data Analysis

- Calculate the average count value for the background wells.
- Generate a standard curve by plotting the AlphaLISA counts versus the concentration of analyte. A log scale can be used for either or both axes. No additional data transformation is required.
- Analyze data according to a nonlinear regression using the 4-parameter logistic equation (sigmoidal dose-response curve with variable slope) and a $1/Y^2$ data weighting (the values at maximal concentrations of analyte after the hook point should be removed for correct analysis).
- The LDL is calculated by interpolating the average background counts (12 wells without analyte) + 3 x standard deviation value (average background counts + (3xSD)) on the standard curve.
- The LLOQ as measured here is calculated by interpolating the average background counts (12 wells without analyte) + 10 x standard deviation value (average background counts + (10xSD)) on the standard curve. Alternatively, the true LLOQ can be determined by spiking known concentrations of analyte in the matrix and measuring the percent recovery, and then determining the minimal amount of spiked analyte that can be quantified within a given limit (usually +/- 20% or 30% of the real concentration).
- Read from the standard curve the concentration of analyte contained in the samples.
- If samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

Assay Performance Characteristics

AlphaLISA assay performance described below was determined using the default manual (10 µL of sample volume and 3-steps manual) using AlphaLISA HiBlock Buffer as assay buffer. The analytes (standards) were prepared in HiBlock Buffer, DMEM, AlphaLISA Lysis Buffer and RPMI. All other components were prepared in HiBlock Buffer.

- Assay Sensitivity:

The LDL was calculated as described above. The values correspond to the lowest concentration of analyte that can be detected in a volume of 10 µL sample using the recommended assay conditions.

LDL (VP/mL)	(Analyte diluent)	# of experiments
7.61E+06	AlphaLISA HiBlock Buffer	38
6.19E+06	AlphaLISA Lysis Buffer	6
1.95E+07	DMEM	9
2.12E+08	RPMI (containing free biotin)	7

- Assay Precision:

The following assay precision data were calculated from the three independent assays using two different kit lots. In each lot, the analytes were prepared in AlphaLISA HiBlock Buffer, AlphaLISA Lysis Buffer, DMEM or RPMI. All other components were prepared in HiBlock Buffer. Each assay consisted of one standard curve comprising 12 data points (each in triplicate) and 12 background wells (no analytes). The assays were performed in 384-well plate format.

  - Intra-assay precision:

The intra-assay precision was determined using a total of at least 12 determinations that were interpolated in concentration, data of variability are shown in CV%.

[AAV1] VP/mL		AlphaLISA HiBlock Buffer	AlphaLISA Lysis Buffer	DMEM	RPMI
CV (%) Intra	**2.00E+10**	10%	8%	7%	13%
	2.00E+09	5%	3%	7%	16%

  - Inter-assay precision:

The inter-assay precision was determined using a total of 3 independent determinations in triplicate that were interpolated in concentration, data of variability are shown in CV%.

[AAV1] VP/mL		AlphaLISA HiBlock Buffer	AlphaLISA Lysis Buffer	DMEM	RPMI
CV (%) Inter	**2.00E+10**	4%	2%	4%	6%
	2.00E+09	7%	3%	5%	6%
	8.00E+07	11%	4%	N/A	N/A

- Spike Recovery:
    - Buffer and cell culture media

Five known concentrations of analyte were spiked into AlphaLISA HiBlock Buffer, AlphaLISA Lysis Buffer, DMEM or RPMI. All samples, including non-spiked diluents were measured in the assay. Note that the analytes for the respective standard curves were prepared in AlphaLISA HiBlock Buffer, AlphaLISA Lysis Buffer, DMEM or RPMI. All other assay components were diluted in HiBlock Buffer.

Spiked AAV1 capsid (VP/mL)	Antigen Spike Recovery (%)			
	AlphaLISA HiBlock Buffer	AlphaLISA Lysis Buffer	DMEM	RPMI
1.00E+10	100%	100%	100%	100%
4.00E+09	95%	97%	96%	90%
1.60E+09	94%	94%	94%	85%
2.56E+08	94%	82%	99%	90%
1.02E+08	94%	103%	91%	N/A

    - Cell lysates

AAV1 capsid at 1.0E+10 VP/mL was spiked in several dilutions of control SF9 or HEK293 cell lysates not expressing AAV capsids (corresponding to different amount of total protein concentration). All samples, including non-spiked diluents were measured in the assay. Note that the analytes and cell lysates were prepared in AlphaLISA Lysis Buffer (1X). All other assay components were diluted in HiBlock Buffer.

AAV1 (VP/mL)	spiked in SF9 cell lysate (mg/mL of total protein)	% Recovery
1.00E+10	2	72%
	1.5	78%
	1	83%
	0.5	95%
	0.25	100%

AAV1 (VP/mL)	spiked in HEK293 cell lysate (mg/mL of total protein)	% Recovery
1.00E+10	2	66%
	1.5	75%
	1	88%
	0.5	97%
	0.25	102%

As expected, no AAV1 was detected in non-spiked SF9 or HEK293 control cell lysates. Antigen spiking recovery data underlined unbiased AAV1 concentration measurement in cell lysates when total protein concentration was below or equal to 0.5 mg/mL for SF9 and 1 mg/ml for HEK293. When analyzing AAV1 content in cell lysates, it is important to ensure that total protein concentration is below 0.5 or 1 mg/mL depending on cells by using dilution with the AlphaLISA Lysis Buffer. At higher cell lysate total protein concentration, there is an interference that could bias the AAV1 read concentration.

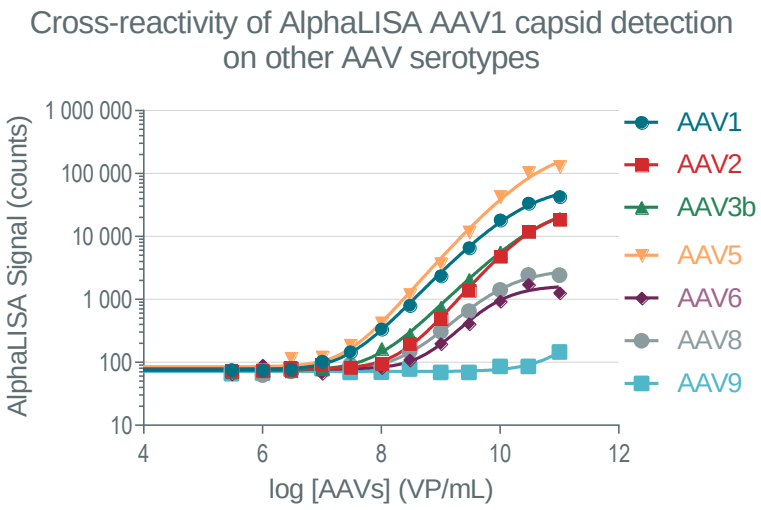
Three known concentrations of analyte were spiked into SF9 or HEK293 cell lysates that are representative of AAVs manufacturing processes at 0.5 mg/ml of total proteins. All samples, including non-spiked diluents were measured in the assay. Note that the analytes for the respective standard curves and samples were prepared in AlphaLISA Lysis Buffer (1X). All other assay components were diluted in HiBlock Buffer.

AAV1 capsid (VP/mL)	Antigen Spike Recovery (%)	
	SF9 cell lysate (0.5 mg/ml)	HEK293 cell lysate (0.5 mg/ml)
1.00E+10	92%	93%
1.00E+09	93%	87%
1.00E+08	101%	86%

- Specificity:

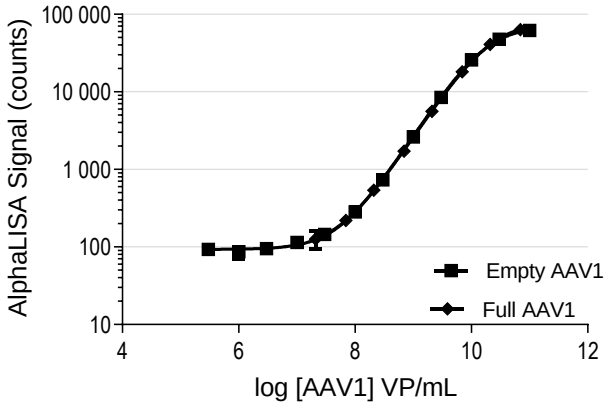
Cross-reactivity of the AAV1 capsid AlphaLISA Detection Kit was tested using different AAVs capsids in HiBlock Buffer.
The cross reactivities were calculated using concentrations ranging from 1.00E+8 to 3.0E+10 VP/mL.

Empty AAVs Particles	Cross Reactivity (%)					
	AAV2	AAV3B	AAV5	AAV6	AAV8	AAV9
1.00E+08	9%	37%	140%	4%	6%	0%
3.00E+08	17%	27%	150%	5%	10%	1%
1.00E+09	17%	27%	153%	5%	9%	0%
3.00E+09	18%	27%	195%	4%	8%	0%
1.00E+10	21%	24%	771%	3%	5%	0%
3.00E+10	20%	20%	N/A	2%	3%	0%



The assay shows differential affinities depending on the serotype but does not recognize AAV9.

To demonstrate the detection of both full and empty AAV1 capsids, recognition of full AAV1-CMV-eGFP and empty AAV1 capsids was analyzed in the assay. A large range of AAV1-CMV-eGFP concentration (GC/mL) was convert in VP/mL.

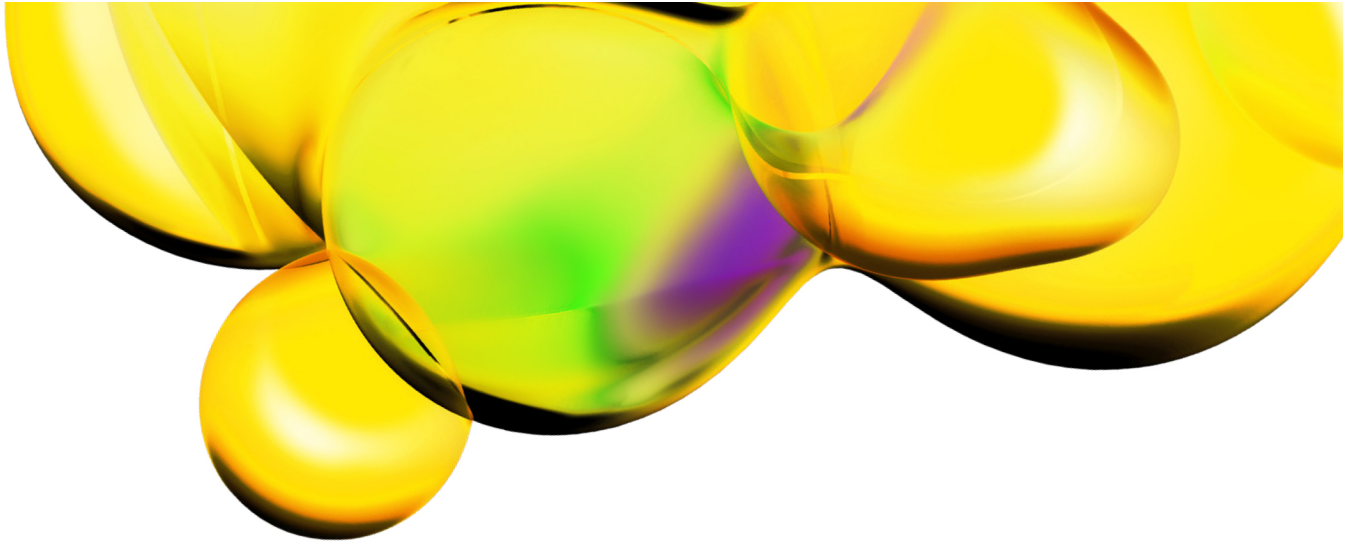


The AlphaLISA AAV1 capsid detection assay can detect in the same way both full and empty AAV1 capsids within the linear detection range of the assay.

Troubleshooting Guide

You will find detailed recommendations for common situations you might encounter with your AlphaLISA Assay kit at: www.revvity.com

FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.



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