

AVIPure® AAV5 Affinity Ligand ELISA Kit

User Guide



ELISA AAV5 UG 9-EL-0020 - 2

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Contents

1. Introduction.....	5
2. About this Document.....	5
3. Product Description	6
3.1 Pre-assay Reagent Preparation.....	7
3.1.1 All Kit Components.....	7
3.1.2 1X Sample Diluent	7
3.1.3 PBS Solution	7
3.1.4 PBS-Tween 20 Wash Solution	7
3.1.5 TMB Substrate Solution	7
3.1.6 Test Samples	7
3.2 Sample Preparation Methods.....	7
3.2.1 Method A: Buffer Exchange	8
3.2.2 Method B: Dilute and Go	8
3.2.3 Method C: Boil and Boost	8
3.3 Test Sample Dilution Preparation.....	8
3.4 Standard Preparation.....	9
3.5 Plate Set-up.....	9
3.6 ELISA Procedure.....	10
3.7 Important Points Regarding Assay Sensitivity	11
4. Calculation of Results.....	11
5. Troubleshooting.....	12
6. References and Additional Resources.....	13
6.1 Journal Articles	13
6.2 Online Resources	13
7. Index	14

List of Tables

Table 1. Explanation of User Attention Phrases	5
Table 2. Safety Precautions.....	5
Table 3. Product Characteristics	6
Table 4. Preparation Method Overview (with Starting Concentrations; desired LOQ = 0.2 ng/mL)	7
Table 5. Method Attribute Table	8
Table 6. Concentrated Standard Solution Preparation	9
Table 7. Representative Plate Set-up for One Protein Sample	10

List of Figures

Figure 1. Representative AVIPure AAV5 Affinity Ligand Standard Curve by 4-Parameter Logistic Fitting	11
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Abbreviations

AAV5	adeno associated virus serotype 5
dH ₂ O	distilled water
ELISA	enzyme-linked immunosorbent assay
HPLC	high performance liquid chromatography
HRP	horseradish peroxidase
LOQ	limit of quantification
N	normality
ng/mg	nanogram per milligram
ng/mL	nanogram per milliliter
nm	nanometer
PBS	phosphate buffered saline
PPE	personal protective equipment
rpm	revolutions per minute
TMB	tetramethylbenzidine
μm	micrometer

1. Introduction

The AVIPure® AAV5 Affinity Ligand ELISA Kit (part number 9-EL-0020) from Repligen provides accurate and precise quantitation of residual AVIPure AAV5 Affinity Ligand in protein products purified with AVIPure AAV5 Affinity Resin.

Testing for residual AVIPure AAV5 Affinity Ligand occurs in several different phases of development and commercial manufacturing that may include:

- Process development: leaching characteristics of the resin under specific conditions
- Manufacturing: eluted samples taken throughout several points in the purification process
- Finished product release: document process containment levels and lot-to-lot consistency

This user guide provides general guidance for the use of AVIPure AAV5 Affinity Ligand ELISA Kit. For further optimization or troubleshooting support, please contact Customer Service team at Repligen (email: customerserviceUS@repligen.com, customerserviceeu@repligen.com; phone: +1 (781) 250-0111).

2. About this Document

Table 1. Explanation of User Attention Phrases

Phrase	Description
Note:	Points out useful information.
IMPORTANT	Indicates information necessary for proper instrument operation.
PRECAUTION	Cautions users of potential physical injury or equipment damage if the information is not heeded.
WARNING!	Warns users that serious physical injury can result if warning precautions are not heeded.

Table 2. Safety Precautions

Symbol	Description
WARNING 	Wear standard laboratory personal protective equipment (PPE), including lab coat, protective eye wear, and gloves.
WARNING 	This product is for laboratory and manufacturing production use only. Not for administration to humans.
WARNING 	Flammable liquid and vapor. <ul style="list-style-type: none"> • Keep away from heat/spark/open flame/hot surfaces. No smoking. • Keep container tightly closed. • Ground/bond container and receiving equipment. • Store in a well-ventilated place. Keep cool.
IMPORTANT 	Dispose of contents/container in accordance with local/regional/national/international regulations.

3. Product Description

The ELISA Kit is supplied with AVIPure AAV5 Affinity Ligand standard, which is used in affinity chromatography for the purification of AAV5.

The polystyrene microtiter plate provided in this kit is coated with an antibody with affinity to the AVIPure AAV5 Affinity Ligand. Standards and test samples are diluted with sample diluent (Reagent A) and incubated with the immobilized antibodies. Captured AVIPure AAV5 Affinity Ligand is then detected by the addition of a biotinylated antibody detection probe (Reagent C). The high substitution of the probe allows maximum binding of streptavidin peroxidase conjugate (Reagent D). The final detection step involves adding tetramethylbenzidine (TMB; Reagent E) to give a highly sensitive colorimetric reaction. The color intensity is proportional to the amount of AVIPure AAV5 Affinity Ligand present in the sample.

Table 3. Product Characteristics

Reagent	Description	Volume	Storage
Reagent A	Sample diluent (5X) concentrate	20 mL	2 - 8°C
Reagent B	AVIPure AAV5 Affinity Ligand standard solution, concentration of 1.0 mg/mL in purified water	200 µL	2 - 8°C
Reagent C	Rabbit anti-Protein A: Biotin probe, contains 0.02% sodium azide	200 µL	2 - 8°C
Reagent D	Streptavidin-HRP (horseradish peroxidase) conjugate	200 µL	2 - 8°C
Reagent E	TMB peroxidase substrate, contains 3, 3', 5, 5'-tetramethylbenzidine in buffer	20 mL	2 - 8°C
PBS packs	Final volume of each pack when reconstituted is 1 L	2 packs	Ambient
ELISA plate	96-well microtiter plate coated with anti-Protein A, packed with desiccants	Dried Plate	2 - 8°C

Note: Reagents are specific to the kit lot and should be discarded once all plate strips have been consumed.

The following reagents, supplies, and equipment are not provided with the kit:

- dH₂O or HPLC-grade water (preferred)
- 1 L graduated cylinder
- 1.5 mL Eppendorf® Tubes
- 15 mL and 50 mL plastic centrifuge tubes
- Tween 20
- Reagent reservoirs
- 5 mL and 10 mL serological pipettes
- Plate sealers
- Filter (0.22 µm) and 1 L bottle
- Phosphoric acid
- Micro-pipettors and 12-channel pipettor
- ELISA plate reader with wavelength capability at 450 nm
- Timer
- Vortex mixer
- Micro-centrifuge
- Water bath

3.1 Pre-assay Reagent Preparation

3.1.1 All Kit Components

Allow all kit components to equilibrate to room temperature [see note below].

3.1.2 1X Sample Diluent

Dilute 4.0 mL of Reagent A (5X sample diluent) in 16 mL of purified water in a 50 mL plastic centrifuge tube. Vortex for 5 - 20 seconds or invert 10 - 15 times for thorough mixing. The 1X sample diluent is stable for 2 weeks at room temperature [see note below].

3.1.3 PBS Solution

Dissolve the contents of one PBS pack in 800 mL of dH₂O. Once dissolved, bring to a final volume of 1000 mL. Mix well. Filter PBS solution through a 0.22 µm filter. Solution can be stored at room temperature for one week.

3.1.4 PBS-Tween 20 Wash Solution

Pour 700 mL of the PBS solution (prepared and filtered per instructions above) into a 1 L graduated cylinder. Add 700 µL of Tween 20. Mix well. Save the remaining 300 mL PBS solution for the final ELISA wash. Filter PBS-Tween solution through a 0.22 µm filter. Solution can be stored at room temperature for one week.

3.1.5 TMB Substrate Solution

For a full-plate assay, use the whole bottle of TMB. For a half-plate assay, aliquot 8 mL of TMB into a 15 mL conical centrifuge tube and cover with aluminum foil to protect from light. Ensure TMB is equilibrated to room temperature before use. Return unused bottle to a 2 - 8°C refrigerator.

3.1.6 Test Samples

Allow all test samples to equilibrate to room temperature [see note below].

Note: An ideal room temperature range of 65 - 77°F (18 - 25°C) is important for optimum assay performance.

3.2 Sample Preparation Methods

Sample preparation methods for the AVIPure AAV5 Affinity Ligand ELISA Kit have been optimized to allow end users to select the method most appropriate for their assay needs. A representative preparation method overview is shown in [Table 4](#).

Table 4. Preparation Method Overview (with Starting Concentrations; desired LOQ = 0.2 ng/mL)

Input Sample Concentration Constraint	Method	Description
N/A	A: Buffer Exchange	Samples are buffer-exchanged into PBS (by dialysis or spin column) then diluted to concentration below 3×10^{12} in PBS with 0.1% Tween 20 prior to test sample dilution prep (Section 3.2.1).
$\geq 3 \times 10^{12}$ vp/mL AAV5	B: Dilute and Go	Samples are diluted in PBS with 0.1% Tween 20 to concentration below 3×10^{12} vp/mL before performing test sample dilution prep (Section 3.2.2).
$< 1.0 \times 10^{13}$ vp/mL AAV5	C: Boil and Boost	Samples are neutralized and adjusted to final 0.1% Tween 20. Samples are boiled for 5 minutes and centrifuged prior to test sample dilution prep (Section 3.2.3)

Table 5. Method Attribute Table

Description	A: Buffer Exchange	B: Dilute and Go	C: Boil and Boost
High performance	X	X	X
Assay completion <3 hours	X	X	X
Reduced sample preparation steps		X	
Enhance Limit of Quantitation			X
High Starting Sample Concentration			X

3.2.1 Method A: Buffer Exchange

Prior to running the assay, samples must be buffer exchanged into PBS (0.01 M phosphate buffer, 0.15 M sodium chloride, 3 mM potassium chloride, pH 7.2 - 7.4) and diluted to an AAV5 concentration of $\leq 3 \times 10^{12}$ vp/mL. Dialysis or a desalting column may be used.

Note: The PBS packs provided in the kit are not intended for this buffer exchange. They are to be reconstituted and used as directed in the ELISA protocol.

3.2.2 Method B: Dilute and Go

This method is designed to dilute out any interfering substances. It can be performed with common process buffers such as 100 mM citrate, glycine, and acetic buffers neutralized with Tris base. Prior to running the assay, dilute AAV5 samples with starting concentrations greater than 3×10^{12} vp/mL directly into phosphate buffered saline (PBS) with 0.1% Tween 20 to reach a final concentration $\leq 3 \times 10^{12}$ vp/mL. For best performance, characterize the assay with process-specific buffers and proteins.

Note: No buffer exchange is required when the dilution step is performed. If sample concentration is less than 3×10^{12} vp/mL, the Dilute and Go method (Method B) is not recommended. Instead, the user should proceed with Buffer Exchange (Method A).

3.2.3 Method C: Boil and Boost

Boil and Boost is an alternative sample treatment method to minimize substance interference. It can be performed with common process buffers such as 100 mM citrate and acetic buffers neutralized with Tris base. The assay should be characterized using process-specific buffers and proteins.

Note: Recovery in glycine buffers or with >0.2% Polysorbate was observed to be significantly lower than other buffers when this method was used. It is recommended that samples containing glycine or high concentrations of surfactants be buffer exchanged into PBS prior to running this method.

Add at least 0.5 mL of each sample to 1.5 mL centrifuge tubes (the assay procedure requires 0.25 mL). Tween 20 should be added to each sample to a final concentration of 0.1%. Create a pin hole in the cap of each centrifuge tube and boil for 5 minutes in a water bath. After cooling the samples, centrifuge the tubes at 13,000 x g or 1,000 rpm for 5 minutes. Boiling causes disassociation from AAV5 ligand and precipitation of AAV5 viral vector. Transfer the supernatant to a new tube (optional). The supernatant will be used when preparing sample dilutions in the assay procedure.

3.3 Test Sample Dilution Preparation

1. After test samples have been prepared, label an Eppendorf® tube for each test sample. Add 200 μ L of 5X sample diluent (Reagent A) to each. Next add 550 μ L of dH₂O to each of these tubes. Vortex for 5 - 10 seconds to ensure thorough mixing. Then add 250 μ L of each test sample to the corresponding labeled tube (e.g., add 250 μ L of Test Sample 1 to the Eppendorf tube prepared for Test Sample 1 above). Vortex for 5 - 10 seconds to ensure thorough mixing. These are the 1:4 starting sample dilutions. Place these tubes off to the side.

3.4 Standard Preparation

1. When Reagent B reaches room temperature, mix by vortex. If reagent remains on the sides or cap of the tube, briefly spin in a micro-centrifuge.
2. Label three 1.5 mL Eppendorf tubes as Tube 1, Tube 2, and Tube 3. Prepare the standard solutions by diluting Reagent B with 1X sample diluent ([Table 6](#)). Vortex each tube thoroughly between dilutions. Low protein binding tubes should be used.
3. Place Tube 3 (5 ng/mL AVIPure AAV5 Affinity Ligand standard) aside.

Note: Standard curve points may be added at lower concentration to extend range

Table 6. Concentrated Standard Solution Preparation

Tube	Volume Reagent B (AVIPure AAV5 Affinity Ligand Standard)	Volume 1X Sample Diluent
1	10 µL of Reagent B	990 µL
2	10 µL of Tube 1	990 µL
3	50 µL of Tube 2	950 µL

3.5 Plate Set-up

These instructions describe preparation of a 7-point standard curve in triplicate. Two-fold dilution series of the standard curve and protein samples are used starting in wells H1 - H3 and D4 - D6, respectively.

Note: The following pipetting and suggested dilution instructions are specific to a single sample assay ([Table 7](#)). Analogous steps should be taken when performing the assay according to your personal design. Alternatively, users may choose to prepare standards and samples in a dilution plate and transfer to an assay plate. A low protein binding dilution plate is recommended.

1. Using a 12-channel pipettor, add 100 µL of 1X sample diluent into wells A4 - A6, B1 - B6, C1 - C6, D1 - D3, E1 - E3, F1 - F3, and G1 - G3.
2. Transfer 200 µL of 5 ng/mL AVIPure AAV5 Affinity Ligand standard solution (Tube 3) into wells H1 - H3.
3. Transfer 200 µL of 1:4 protein sample dilution into wells D4 - D6.
4. Make 2-fold serial dilutions of the AVIPure AAV5 Affinity Ligand standard and protein samples by transferring 100 µL from each set of triplicate wells into the wells directly above them. Mix thoroughly by pipetting 5 times.

Note: In a single sample assay format, the same tips can be used for each row.

5. After making the last AVIPure AAV5 Affinity Ligand standard serial dilution in wells C1 - C3, remove 100 µL and discard. Also discard 100 µL from the final protein sample dilution in wells A4 - A6.

Table 7. Representative Plate Set-up for One Protein Sample

	1	2	3	4	5	6	7	8	9	10	11	12
A	Plate blank			1:32								
B	0 ng/mL			1:16								
C	0.156 ng/mL			1:08								
D	0.313 ng/mL			Sample #1, 1:04								
E	0.625 ng/mL											
F	1.25 ng/mL											
G	2.5 ng/mL											
H	5 ng/mL											

3.6 ELISA Procedure

1. After the AVIPure AAV5 Affinity Ligand standards and sample dilutions have been prepared, cover the plate and incubate at room temperature for 60 minutes.
2. After incubation, remove all liquid from the wells. Using a wash bottle or automated plate-washing system, wash the plate with PBS-Tween 20 solution. Remove the liquid and dry thoroughly by inverting the plate on clean paper towels and tapping gently. Repeat the wash and dry cycle three (3) more times for a total of four (4) washes.
3. Prepare the rabbit anti-Protein A biotin probe solution:
 - a. Briefly vortex the Reagent C vial. If reagent material remains on the sides or cap of the tube, briefly spin in a micro-centrifuge.
 - b. For a full-plate assay, prepare 12 mL of rabbit anti-Protein A biotin probe solution by combining 70 µL of Reagent C with 12 mL of prepared PBS-Tween 20 in a 15 mL conical centrifuge tube. Mix solution thoroughly.
 - c. For a half-plate assay, prepare 6 mL of rabbit anti-Protein A biotin probe solution by combining 35 µL of Reagent C with 6 mL PBS-Tween 20 in a 15 mL conical centrifuge tube. Mix solution thoroughly.
4. Using a 12-channel pipettor, add 100 µL of the diluted Reagent C probe solution to each well containing a test sample or standard. Leave wells A1 - A3 (plate blanks) empty.
5. Cover the plate and incubate at room temperature for 30 minutes. After incubation, wash the wells four (4) times with PBS-Tween 20 and remove the liquid. Dry thoroughly by inverting the plate on clean paper towels and tapping gently.
6. Prepare the streptavidin horseradish peroxidase conjugate solution:
 - a. Briefly vortex the Reagent D vial. If reagent material remains on the sides or cap of the tube, briefly spin in a micro-centrifuge.
 - b. For a full-plate assay, prepare 12 mL of streptavidin horseradish peroxidase conjugate solution by combining 12 µL of Reagent D with 12 mL of prepared PBS-Tween 20 in a 15 mL conical centrifuge tube. Mix solution thoroughly.
 - c. For a half-plate assay, prepare 6 mL of streptavidin horseradish peroxidase conjugate solution by combining 6 µL of Reagent D with 6 mL PBS-Tween 20 in a 15 mL conical centrifuge tube. Mix solution thoroughly.
7. Add 100 µL of the diluted Reagent D conjugate solution to each well containing test sample or standard. Leave wells A1 - A3 (plate blanks) empty.
8. Cover the plate and incubate at room temperature for 30 minutes.
9. After incubation, discard the conjugate solution from the plate. Wash the wells twice with PBS-Tween 20. Wash twice more but with PBS only. After each wash, discard the liquid by inverting the plate on clean paper towels and tapping gently.

Note: Before proceeding with the next step, make sure the TMB solution is at room temperature (65 - 77°F (18 - 25°C). If the lab is too warm, move the assay to a cooler location for the development step.

10. Using a multi-channel pipettor, add 100 µL of the TMB substrate to each of the wells, including A1 - A3 (plate blanks).
11. Incubate plate for 10 minutes. Stop reaction by adding 100 µL of 1 N phosphoric acid to each well, including A1 - A3 (plate blanks), in the same order of pipetting used for the TMB substrate solution.

Note: Other strong acids typically used as stop solutions in ELISA may be substituted for 1 N phosphoric acid. If bubbles are present in the wells, agitate slightly before reading.

12. Read the plate at 450 nm.

3.7 Important Points Regarding Assay Sensitivity

1. Numerical results of this assay are expressed as nanograms per milliliter (ng/mL) of AVIPure AAV5 Affinity Ligand.
2. The sensitivity of the assay is typically 0.2 ng/mL.

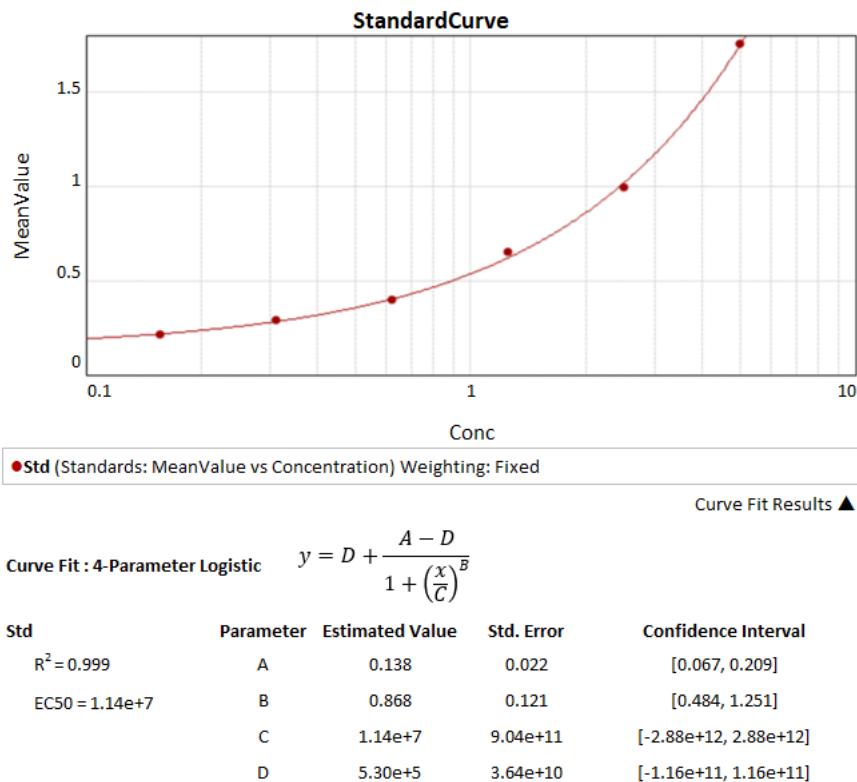
4. Calculation of Results

1. Calculate the mean absorbance value for the plate blank wells (A1 - A3) and subtract from all remaining wells on the plate (including the 0 ng/mL standard curve.) Determine the average absorbance value for each standard concentration and all test samples.

Note: Method of calculation for standard curve should be based on internal standards. Other curve fits may be used as deemed appropriate.

2. Calculate the standard curve:

Figure 1. Representative AVIPure AAV5 Affinity Ligand Standard Curve by 4-Parameter Logistic Fitting



The standard curve points can be fitted using curve-fitting software. Model fits such as quadratic or 4- parameter logistical equations are recommended. Such a fit is the acknowledged reference model for sigmoidal immunoassay data (1, 2).

The regression line can be used to determine the AVIPure AAV5 Affinity Ligand concentration for the samples.

$$\frac{ng}{mg} = ppm = \frac{\text{mean concentration } \left(\frac{ng}{mL} \right)}{\frac{mg}{mL} \text{ of sample concentration per well } \left(\text{e.g., } 0.125 \frac{mg}{mL} \right)}$$

Sample AAV5 Ligand Conc. (ng/ml) = AAV5 Conc. Calculated from standard curve \times Sample Dilution

5. Troubleshooting

Problem	Possible Cause	Remedy
Not enough of required reagent.	Splashing of reagent on sides or cap of reagent tube during mixing, shipping, or handling.	Briefly centrifuge tube.
Inconsistent results between sample dilutions.	Protein sample was not fully equilibrated in PBS, pH 7.0 – 7.4, before assay. The protein sample concentration in the undiluted sample was $>3 \times 10^{12}$ vp/mL.	Re-dialyze sample in PBS. Ensure pH is 7.0 – 7.4 and re-run assay. Ensure sample concentration is $\leq 3 \times 10^{12}$ vp/mL.
Outliers, where one replicate has an abnormally high or low absorbance value.	Small amount of peroxidase conjugate left on the plate before color development (i.e., wells were not thoroughly washed).	Discard outliers and average duplicates. Ensure thorough washing in any subsequent ELISA testing.
Color development time to reach 0.9 AU is >10 minutes.	TMB solution (Reagent E) was not at room temperature before adding to wells. Room temperature too low, or too cool.	Solution can be warmed before adding to wells. Use incubator set at 65 – 77°F (18 – 25°C) for all incubations or develop longer than 5 minutes. Note: Absorbance of <0.9 is acceptable if overall signal to noise ratio is ≥ 6 .
Background signal is >0.150 .	Color development for TMB substrate was >10 minutes. Temperature of TMB substrate $>77^{\circ}\text{F}$ (25°C). Insufficient plate washing.	Start timer immediately after adding TMB substrate to 5 ng/mL standard wells. Store TMB in a location that is between 65 – 77°F (18 – 25°C) until use. Ensure plate was washed 4 times.
O.D. values consistently high for all samples or low recovery of AVIPure AAV5 Affinity Ligand in samples.	Buffer component interference.	Buffer-exchange sample into neutral buffer or perform a greater fold dilution into neutral buffer Section 3.2.1 .

6. References and Additional Resources

6.1 Journal Articles

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6.2 Online Resources

User Guides and Safety Data Sheets are available on the Repligen website, repligen.com.

7. Index

Boil and Boost	7, 8	Reagent B.....	6, 9
Buffer exchange	7, 8	Reagent C.....	6, 10
Calculation of results	11	Reagent D	6, 10
Dilute and go.....	8, 11	Reagent E.....	6, 12
Important.....	5	Reagents	6
Note	5, 6, 7, 8, 9, 10, 11	Sample preparation	7
Plate set-up	9	Standard curve.....	9, 11
Precautions	5	Standard preparation	9
Reagent A.....	6, 7	Warning	5

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ELISA AAV5 UG 9-EL-0020 - 2