Application Note

AAV9-induced Complement Activation ELISA

Enzyme immunoassay for the evaluation
of complement activation driven by AAV9 capsids

Doc. no. LABEL-DOC-0750 1.0
Effective Date: 21-Sep-2026

For research use only.

Not for use in diagnostic procedures.

This application note contains a suggested protocol
and pertaining performance data.

Each individual laboratory must set up their own method
and perform relevant validations.

REF AAV9COMPLRUO

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1 PURPOSE

The AAV9-Induced Complement Activation ELISA is a Research Use Only (RUO) immunoassay developed to evaluate and characterize complement activation induced by adeno-associated virus 9 (AAV9) vectors *in vitro*.

Example research applications include:

- Functional evaluation and characterization of complement activation associated with AAV9 *in vitro*, including both antibody-mediated and antibody-independent mechanisms.
- Characterization of complement activation and generation of supporting data for AAV9 gene therapy vector research and development.
- Assessment of variability in complement responses across samples.
- Evaluation of complement activation independent of total antibody (TAb) levels.
- Complement-mediated functional properties of Tabs.
- Characterization of sample-to-sample variation in complement responses to AAV9 exposure.

2 BACKGROUND

AAV9 vectors are widely used in gene therapy, but their interaction with the immune system can lead to unintended complement activation (1). Excessive complement activation has been associated with adverse events in clinical studies, highlighting the need for tools that can evaluate how individual samples respond to AAV9 exposure (2). The AAV9 COMPL Assay provides a plate-based method to measure AAV9-induced complement activation.

3 GENERAL ASSAY INFORMATION

The key characteristics and technical specifications of the assay are summarized in **Table 1**.

Table 1: Overview of assay characteristics.

Parameter	Description
Technology	ELISA
Format	Solid-phase functional ELISA
Size	96 well
Plate coating	AAV9 capsids
Target	Terminal Complement Complex (TCC, C5b-9)
Detection system	HRP/TMB, absorbance read at 450 nm
Analytical method	Semi-quantitative
Sample Type	Serum
Reactivity	Human
Assay time	~4.5 hours
Assay temperature	37°C and room temperature (20-25°C)
Working volume	100µL per well
Sample volume	~55 µL (no dead volume; sufficient to prepare required dilutions)
Sample dilution	1st Step: 1:4 (1:4 – 1:16), 2nd Step: 1:75 (recommended, adjustable)
Throughput	45 samples per plate measured in duplicate

4 PRINCIPLE OF THE AAV9-INDUCED COMPLEMENT ACTIVATION ELISA

This is a solid-phase enzyme immunoassay for evaluating complement activation triggered by AAV9. AAV9 particles are immobilized on a microtiter plate, enabling comprehensive capture of the entire process of AAV9-mediated complement activation.

This includes interactions between AAV9 and anti-AAV9 reactive components, as well as the complement system, all within a single well from initiation to readout.

The assay principle and step-wise workflow are summarized in **Figure 1**.

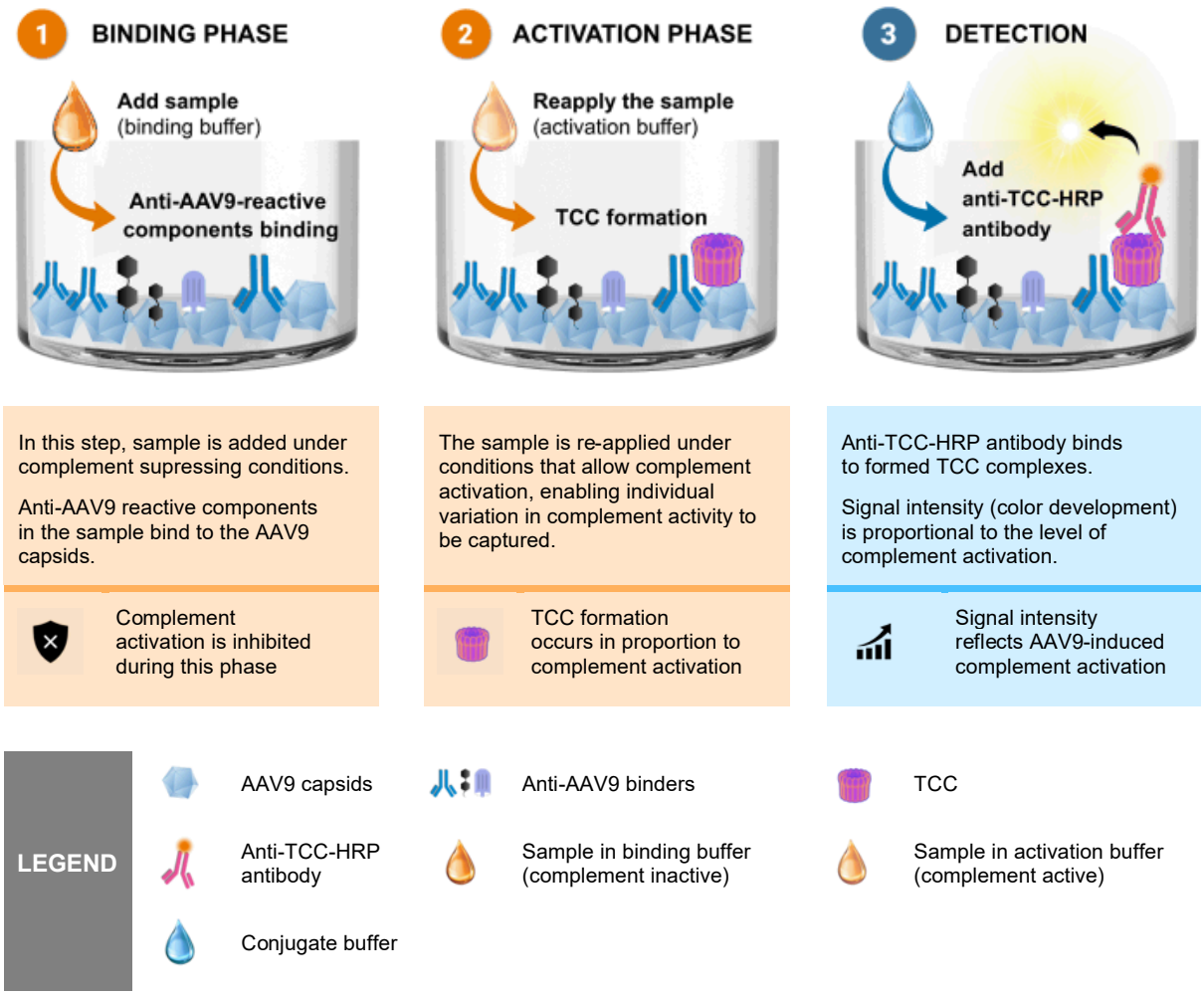


Figure 1: Step-by-step progression of the assay (for consistency throughout this document, the terms BINDING step and ACTIVATION step are used to describe the two main phases of the assay procedure).

5 WARNINGS AND PRECAUTIONS

- **FOR RESEARCH USE ONLY – NOT FOR DIAGNOSTIC USE.**
- The human serum components used in the preparation of the lyophilized serum have been tested using FDA CBER-licensed donor-screening tests under 21 CFR 610.40 and found negative for antibodies to human immunodeficiency virus types 1 and 2 (HIV-1 and HIV-2), hepatitis C virus (HCV), and hepatitis B surface antigen. However, because no test method can provide complete assurance that HIV, HCV, hepatitis B virus, or other infectious agents are absent, this reagent should be handled as potentially infectious. In accordance with the recommendations of the Centers for Disease Control and Prevention (CDC) and the National Institutes of Health (NIH), the material should be handled at Biosafety Level 2.
- The lyophilized serum (SERUM LYO) is packaged and shipped separately from the kit.
- All kit components are classified according to Regulation (EC) No 1272/2008 (CLP). Relevant hazard information, including hazard classifications, hazard statements (H), precautionary statements (P), and supplemental information (EUH), is summarized in **Table 2**.
- All reagents should be handled in accordance with standard laboratory safety practices. Wear appropriate personal protective equipment (PPE) and avoid ingestion, inhalation, or contact with skin, eyes, and mucous membranes. Certain reagents (see **Table 2**) contain isothiazolinone preservatives (CMI/MI mixture) and may cause irritation or allergic reactions. Do not pipette by mouth. For detailed information on safe handling and first-aid measures, refer to the Safety Data Sheet (SDS).
- Do not use components past their expiration date.
- Do not mix kit components or reagents from different lot numbers.
- Any serious incident during the use of this product should be reported to Svar Life Science AB.

Material SDS for all components contained in this kit are available via the Svar Life Science AB website (www.svarlifescience.com) or upon request.

Table 2: Safety and labelling information for kit components.

Component	Pictogram	Hazard status	Contains	Statement
Wash buffer, conjugate	 WARNING	Hazardous	CMI/MI mixture (CAS 55965-84-9), <0.015% (Reaction mass of 5-chloro-2-methyl-4-isothiazolin-3-one and 2-methyl-2H-isothiazol-3-one)	Hazard statements: H317 - may cause an allergic skin reaction. H412 - harmful to aquatic life with long lasting effects. Precautionary statements: P261 - avoid breathing spray. P273 - avoid release to the environment. P280 - wear protective gloves. P333+P313 - if skin irritation or rash occurs: Get medical advice/attention.
Diluents, positive and negative controls	 WARNING	Not classified	CMI/MI mixture (CAS 55965-84-9), <0.0015%	EUH208 – Contains CMI/MI. May produce an allergic reaction. EUH210 - SDS available on request
Stop Solution	NA	Not classified	0.5 M sulfuric acid (H_2SO_4)	EUH 210 - SDS available on request
Serum, lyophilized		Not classified (chemical); Biological Agent Group 2	Human-derived lyophilized serum	Handle as potentially infectious. Tested negative for HBV, HCV, HIV.
AAV9-coated 96-well plate	NA	NA	NA	No declarable substances/mixtures, exempted from remainder of SDS

6 KIT CONTENT

All components provided in the kit, including their descriptions, formats, and preparation requirements are listed in **Table 3.**

Table 3: Kit content.

Component	Description	Volume / Format	Ready to Use	Solution / container identification
Ag	Antigen (AAV9)-coated 96-well plate, sealed	1 plate	Yes	-
CONTROL/CTRL +	Positive Control; Anti-AAV9 antibody	1.2 mL	Yes	Red solution, black cap
CONTROL/CTRL -	Negative Control; No anti-AAV9 antibody	1.2 mL	Yes	Red solution, white cap
SERUM LYO	Human serum, lyophilized	0.2 mL	No, reconstitute and dilute before use (see Section 10.1)	Clear bottle, white cap
DIL	Dilution buffer: Assay binding diluent	32 mL	Yes	Red solution, clear bottle
AAV COMPL DIL	Dilution buffer; Complement activation diluent	35 mL	Yes	Colorless solution, clear bottle
CONJ	Conjugate; HRP-conjugated anti-human TCC antibodies	15 mL	Yes	Blue solution, amber bottle
SUBS TMB	Substrate; TMB solution	15 mL	Yes	Colorless solution, amber bottle
H_2SO_4	Stop Solution; Sulfuric acid stop solution	15 mL	Yes	Colorless solution, clear bottle
BUF WASH	Wash buffer; 30× concentrated wash buffer	30 mL	No, dilute before use (see Section 10.1)	Colorless solution, clear bottle
-	Sealing films; Adhesive plate seals	2 pieces	Yes	-

7 MATERIALS OR EQUIPMENT NEEDED BUT NOT PROVIDED

- Microplate reader with filter 450 nm and 620 nm.
- Incubator capable of maintaining +37°C. If a CO_2 cabinet is used, make sure that the CO_2 supply is disconnected/off.
- Precision pipettes with disposable tips.
- Automatic microtiter plate washer, multichannel pipette, or similar for washing.
- Absorbent tissue.
- Vessels for sample and reagent dilution.
- Ice.
- Timer.
- Deionized water.
- Optional: 96-well deep round-bottom plates (see Section 10.1.3, *Dilution of Samples*, for details).

8 STABILITY AND STORAGE

All reagents, except SERUM LYO, should be stored at 2–8°C, and are stable under these conditions until the kit's expiration date. This also applies to the diluted wash solution.
The SERUM LYO should be stored at ≤ -18°C or below. After reconstitution, the serum should be frozen at ≤ -70°C and may be thawed once.

9 SAMPLE REQUIRMENT AND HANDLING

9.1 Sample types

Serum is the recommended sample type for this assay. Other matrices may be suitable following laboratory-specific assessment.

9.2 Required sample volume

Approximately 55 µL serum is required for analysis of one sample in duplicate. Additional volume may be required to accommodate dead volume and potential repeat testing.

9.3 Sample conditions and limitations

Only properly collected and handled samples should be used.

Samples exhibiting visible hemolysis, lipemia, microbial contamination, or improper handling, including pre-activated complement, may affect assay performance.

Samples with inherent or acquired complement deficiencies or dysfunctions, including those resulting from ongoing complement activation or the presence of inhibitors or complement-modulating agents, may yield altered results.

9.4 Specimen collection, storge and thaw limits

Collection

- Collect whole blood using an aseptic venipuncture technique.
- Allow blood to clot and process according to standard laboratory procedures to obtain serum.

Processing

- Centrifuge the blood sample and carefully transfer the cell-free serum into a clean tube.
- Store serum at 2–8°C for immediate analysis.
- If analysis is delayed, freeze serum at ≤ -70°C.

Thawing of frozen specimens

Partially thaw frozen sera by briefly placing the tubes in a 37 °C water bath with gentle mixing. Immediately transfer the tubes to ice and allow them to thaw completely. Mix briefly using a vortex mixer.

Freeze-thaw

Avoid repeated freeze–thaw cycles, as repeated freezing may affect performance.
Each laboratory should verify acceptable storage conditions for test samples, as pre-analytical factors may influence stability.

10 PROCEDURE

10.1 Preparation and handling of reagents

10.1.1 Wash buffer

Mix 30 mL of 30× wash buffer with 870 mL of deionized water.
If salt crystals are observed in the vial containing the concentrated wash buffer, place the vial in a 37 °C water bath until the crystals have fully dissolved before dilution.

10.1.2 Reconstitution of SERUM LYO

Reconstitute SERUM LYO according to the following procedure:

- Gently tap the lyophilized material to the bottom of the vial and remove the cap.
- Add the 200 µL of deionized water directly to the lyophilized material.
- Replace the cap.
- Allow the vial to stand on ice for 5 minutes, then gently shake or vortex occasionally until completely dissolved.
- Dilute the reconstituted serum in the same way as a sample (see 10.3 Assay Procedure).

The reconstituted SERUM LYO can be stored for up to 4 hours prior to use if kept at +2-8°C or on ice. It should be stored at ≤ -70°C and may be thawed once.

10.1.3 Dilution of samples

Sample dilution may be defined by the user. As guidance, a suggested dilution for the BINDING step is 1:4 in DIL buffer, and for the ACTIVATION step is 1:75 in AAV COMPL DIL buffer. **Samples must be applied to the same well positions in both steps, performed sequentially.**

To minimize handling errors and reduce the risk of sample misplacement, the use of 96-well deep round-bottom plates is recommended for sample dilution and transfer.

10.2 Plate Layout

An example plate layout is shown in **Figure 2**. The layout may be adjusted as needed; however, the well positions used during the ACTIVATION step must correspond to those used during the BINDING step. The well positions of SERUM LYO in the ACTIVATION step must correspond to the well positions of the associated controls in the BINDING step, while test samples must occupy the same well positions in both steps.

BINDING

	1	2
A	Ctrl Pos	Ctrl Pos
B	Ctrl Neg	Ctrl Neg
C	Blank - DIL	Blank - DIL
D	S1	S1
E	S2	S2
F	S3	S3
G	S4	S4
H	etc	etc

ACTIVATION

1	2
Serum Lyo	
Blank – AAV COMPL DIL	
S1	S1
S2	S2
S3	S3
S4	S4
etc	etc

Figure 2: Example plate layout for the AAV9-induced complement Activation ELISA. Ctrl Pos – positive control; Ctrl Neg – negative control; Blank-DIL – Blank-assay binding diluent; Serum Lyo – lyophilized serum; AAV COMPL DIL – complement activation diluent; S1-S4 – test samples.

10.3 Assay protocol

Washing steps: Wash the plate 3 times with 300 µL of wash buffer per well. After the third wash, tap the plate on tissue to remove excess liquid.
Use adhesive plastic film for each step performed in 37°C. Please note that no incubation should be performed under a CO_2 atmosphere. If a CO_2 incubator is used, ensure that the CO_2 supply is turned off.

Bring plate to room temperature.

1. BINDING
Keep controls, samples, and DIL buffer (red) on ice while working.

- Apply controls, blank and samples:

Positive and negative controls – add 100 µL/well of ready-to-use controls in duplicate.
Blank – add 100 µL/well of DIL buffer (red) in duplicate.
Samples – dilute samples 1:4 in cold DIL buffer - load 100 µL/well in duplicate.

- Incubate the plate for 1 hour at 37°C, covered with a plate sealing film.
- Prepare components for step 2, while incubating the plate:

Keep SERUM LYO, original samples, and AAV COMPL DIL on ice during preparation and until application in Step 2.

SERUM LYO: reconstitute lyophilized serum according to section 9.1 *"Preparation and handling of reagents, Reconstitution of SERUM LYO".* After reconstitution, dilute serum 1:75 in AAV COMPL DIL for use in Step 2.
Samples: prepare a separate 1:75 dilution of each original sample during the time of incubation: prepare the samples by diluting them 1:75 in AAV COMPL DIL.

- After incubation at 37°C is complete, wash the plate according to instruction above.

2. ACTIVATION
Keep SERUM LYO, samples and COMPL DIL on ice while working.

- Apply diluted serum and samples:

Positive and negative controls – add 100 µL/well of SERUM LYO diluted 1:75 in AAV COMPL DIL to the corresponding wells from Step 1.
Blank – add 100 µL/well of AAV COMPL DIL.
Samples – add 100 µL/well of the 1:75 diluted sample to the same sample positions used in Step 1.

- Incubate the plate for 2 hours at 37°C, covered with a plate sealing film.
- Bring detection reagents to room temperature, protected from light.
- After incubation at 37°C is complete, wash the plate according to instruction above.

3. DETECTION

- Add 100 µL/well of HRP-conjugated anti-TCC Antibody (CONJ).
- Incubate for 1 hour at 37°C, covered with a sealing film.
- Wash the plate according to instruction above.
- Add 100 µL/well of TMB Substrate (SUBS).
- Incubate for 30 minutes at room temperature, protected from light.
- Stop Reaction – add 100 µL/well of Stop Solution (H_2SO_4). Shake the plate before reading. Read at 450 nm (620 nm reference) within 5 minutes.

10.4 Assay protocol overview

All incubations at 37 °C should be performed with the plate sealed using a sealing film. Do not incubate under a CO_2 atmosphere - if a CO_2 incubator is used, ensure the CO_2 supply is turned off. Wash 3× with 300 µL wash buffer per well between assay steps unless otherwise stated. Bring plate to room temperature before use.

1. Binding: 1:4 sample dilution
Keep controls, samples, and DIL buffer (red) on ice while working; plate at room temperature.

- Add 100 µL/well of positive & negative controls, blank (DIL buffer), and samples diluted 1:4 in DIL buffer.
- Incubate 1 hour at 37°C → wash 3x.
- During incubation, reconstitute SERUM LYO with 200 µL deionized H_2O and dilute 1:75 in AAV COMPL DIL. Prepare a separate 1:75 dilution of each original sample in AAV COMPL DIL for Step 2. Keep the prepared materials on ice until application in step 2.

2. Activation: separate 1:75 sample dilution
Keep SERUM LYO, samples and COMPL DIL on ice while working.

- Add 100 µL/well of 1:75 diluted SERUM LYO to control wells, AAV COMPL DIL (blank), and samples diluted 1:75 to the same well positions used in the Binding step.
- Incubate 2 hours at 37°C → wash 3x.
- During incubation, bring reagents to room temperature for subsequent steps.

3. Detection and Readout

- Add HRP-conjugated anti-TCC antibody (100 µL/well); incubate 1 hour at 37 C → wash 3x.
- Add TMB substrate; incubate 30 minutes at room temperature, protect from light.
- Add stop solution, shake the plate shortly and read at 450 nm with 620 nm reference within 5 minutes.

10.5 Disposal of kit contents

Kit materials and waste generated during the analysis must be disposed of as hazardous waste. The disposal of such waste is governed by national and regional regulations.
For guidance on proper disposal procedures, contact your local authorities or licensed waste-management companies, who can advise you on the appropriate handling and disposal of hazardous materials.

11 EVALUATION OF RESULTS

Calculation of Optical Density (OD)

For each sample, calculate OD by subtracting A620 from A450 for the corresponding well, followed by subtraction of the mean OD Blank (typically two wells):

$$OD = (A450 - A620) - OD\ Blank\ (mean)$$

Calculation of Percent of Complement Activity

Calculate percent complement activity using the following formula:

$$Complement\ activity\ (\%) = \frac{OD\ Sample}{OD\ Positive\ Control} \times 100$$

12 QUALITY CONTROL

Specifications for the positive and negative controls are provided in the Certificate of Analysis (CoA), which is lot-specific and included with the kit. The results indicated in the CoA are intended as a guideline only; results obtained by individual laboratories may differ. The OD values of the positive and negative controls are used to monitor assay and reagent performance. If the measured OD for either control falls outside its specified acceptance range, the assay is considered invalid and must be repeated in its entirety.

13 PERFORMANCE CHARACTERISTICS

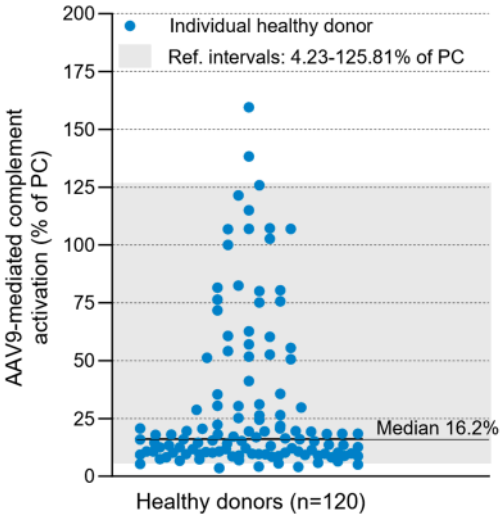
A summary of the assay performance of the product is given below.

13.1 Reference range

The reference interval was established using 120 serum specimens from healthy individuals, with CLSI guideline EP28 used as guidance. The 2.5th and 97.5th percentiles were 4% and 126% of the positive control, with 90% confidence intervals of 3.6–5.8% and 107–160%, respectively.

Individual results showed substantial variability, with relatively high complement activation in some healthy individuals (**Figure 3**). As pre-existing anti-AAV9 antibodies may occur naturally and mediate activation in this assay, the reference interval should not be interpreted as a cutoff for complement activation.

Figure 3: Distribution of AAV9-mediated complement activation in healthy individuals.



13.2 Precision

Assay precision (**Table 4**) was evaluated using 6 samples spanning low to high OD levels. Samples were tested at 1:4 and 1:75 dilutions following the AAV9-induced Complement Activation ELISA procedure. Results are reported as mean OD and relative response (% of positive control).

Intra-assay variability was assessed using 8 replicates within a single run.
Inter-assay variability was assessed using 8 replicates across three independent runs.

Table 4: Assay precision summary.

	Intra-Assay				Inter-Assay			
Sample Description	Mean OD	CV OD (%)	Mean Rel. Response (% Pos Ctrl)	CV Rel. Resp. (%)	Mean OD	CV OD (%)	Mean Rel. Response (% Pos Ctrl)	CV Rel. Resp. (%)
Low-Level 1	0.3	10	11	11	0.3	11	12	11
Low-Level 2	0.4	13	17	14	0.4	13	18	14
Mid-Level 1	0.7	12	31	12	0.8	13	37	13
Mid-Level 2	1.2	12	54	12	1.2	10	57	9
High-Level 1	1.6	7	69	7	1.6	6	76	7
High-Level 2	2.3	7	98	7	2.3	7	107	9
Average:		10		11		10		11

The overall intra-assay and inter-assay variability for relative response were both 11%, calculated as the mean CV% across all samples.

13.3 Lot-to-lot variability

Lot-to-lot reproducibility was assessed using two fully independent kit lots manufactured at different time points, incorporating independently produced component and raw-material lots, providing a robust assessment of assay reproducibility. Sample classifications were maintained across all QC levels. For samples within the measurable response range, inter-lot variability was low to moderate (CV 4.9–28.7%). One negative sample showed a higher relative CV (50.3%) due to very low signal levels (4.5–9.4% of PC), without affecting its classification.

14 LIMITATIONS

Results are semi-quantitative and reported relative to the positive control provided; absolute complement activity is not determined. Complement activation is measured under *in vitro* conditions and may not fully reflect in vivo activity.

Assay performance depends on strict adherence to the protocol described in this Application Note. Use of non-specified reagents or buffers, or variability associated with manual pipetting, may impact assay precision. Use of multichannel or automated pipetting may help reduce variability.

15 LITERATURE REFERENCE

1. *Kropf, E., Markusic, D. M., Majowicz, A., Mingozzi, F. & Kuranda, K. Complement System Response to Adeno-Associated Virus Vector Gene Therapy. Hum. Gene Ther. (2024) doi:10.1089/hum.2023.194*
2. *Salabarria, S. M. et al. Thrombotic microangiopathy following systemic AAV administration is dependent on anti-capsid antibodies. J. Clin. Investig. (2023) doi:10.1172/jci173510*

16 TROUBLESHOOTING

Table 5: Summary of common assay problems, possible causes, and suggested corrective actions.

Problem	Possible Cause	Solution
Low signal	Reagent degradation or improper storage	Verify storage conditions, expiry dates, and avoid contamination
	Insufficient incubation or incorrect timing	Ensure correct incubation temperature and duration
	Procedure errors or incorrect plate handling	Follow protocol with a checklist; verify wash buffer and washing steps
	Instrument or reader issues	Verify plate reader settings (wavelength, filters, shaking)
	Lack of complement activity in sera	Avoid freeze–thaw cycles; store samples properly
Low signal in samples only	Complement inhibitors in matrix	Use inhibitor-free serum
	Complement degradation	Work with thawed samples on ice; verify complement integrity; Standardize sample processing workflow
Low signal in positive control only	Possible control-related issues	Verify correct reconstitution, storage conditions, expiry, and avoid freeze–thaw cycles
High background	Reagent contamination	Verify storage, expiry and contamination status
	Procedure errors (insufficient washing, over-incubation)	Follow protocol with checklist; verify washing efficiency
High background in samples	Sample too concentrated	Increase sample dilution
	Nonspecific complement activation	Standardize sample handling
	Interference from heterophilic antibodies	Evaluate potential interfering substances
High variability	Pipetting variability	Use multichannel or automated pipetting
	Inconsistent washing	Ensure uniform washing and aspiration across wells
	Reader issues (bubbles, dirty plate)	Clean plate bottom; remove bubbles
	Slow reagent addition	Use consistent timing; use multi-tip or auto-dispenser
Edge effects	Evaporation or temperature gradients	Seal plates; avoid outer wells; maintain stable incubation
Saturated signal	Over-incubation or high HRP activity	Reduce TMB incubation; dilute detection reagent or samples
Poor reproducibility between plates	Protocol deviations or lot differences	Standardize workflow; use same lots; check calibration

17 DESCRIPTION OF SYMBOLS

	Batch code
	Catalog number.
	Use-by-date.
	Temperature limit.
	Biological risk.
	Consult instructions for use.
	Manufacturer.
	Contains sufficient for 45 tests.
	Warning

Ag	Antigen (coated plate).
CONTROL +	Positive control.
CONTROL -	Negative control.
SERUM LYO	Human serum, lyophilized
DIL	Assay binding diluent.
AAV COMPL DIL	Complement activation diluent
CONJ	Conjugate.
SUBS TMB	Substrate TMB.
H_2SO_4 0.5M	Stop solution: sulfuric acid, 0.5 molar
BUF WASH 30X	Wash buffer, 30x concentrate.

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