# Multiplexed Assays for Detection of IgG Antibodies against AAV Serotypes

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## PURPOSE

We present a quantitative, multiplexed serology assay that can be used to measure circulating antibodies against Adeno-Associated Virus (AAV) serotypes.

## OBJECTIVES

Our main goals were to assess the assay using pooled serum samples, monoclonal and polyclonal antibodies to demonstrate reproducible and specific antibody detection and to generate representative data with individual human serum samples. We also established a pooled serum calibration standard and demonstrated its effectiveness to quantify samples.

## METHODS

The assay utilizes 10-spot, 96-well assay plates directly coated with optimized concentrations of empty-capsid AAVs along with an electrochemiluminescent (ECL) detection system to simultaneously detect IgG antibodies to antigens in Table 1.

### MSD® Technology

MSD's electrochemiluminescence detection technology uses SULFO-TAG™ labels that emit light upon electrochemical stimulation initiated at the electrode surfaces of MULTI-ARRAY® and MULTI-SPOT® microplates.

| Spot # | Antigens |
|---|---|
| Spot 1 | AAV 1 |
| Spot 2 | AAV 2 |
| Spot 3 | AAV 5 |
| Spot 4 | AAV PHP.eB |
| Spot 5 | - |
| Spot 6 | AAV.rh10 |
| Spot 7 | AAV DJ |
| Spot 8 | AAV 6 |
| Spot 9 | AAV 8 |
| Spot 10 | AAV 9 |

*Table 1. AAV antigens used in study.*

### Electrochemiluminescence Technology

- Minimal non-specific background and strong responses to analyte yield high signal-to-background ratios.
- The stimulation mechanism (electricity) is decoupled from the response (light signal), minimizing matrix interference.
- Only labels bound near the electrode surface are excited, enabling non-washed assays.
- Labels are stable, non-radioactive, and directly conjugated to biological molecules.
- Emission at ~620 nm eliminates problems with color quenching.
- Multiple rounds of label excitation and emission enhance light levels and improve sensitivity.
- Carbon electrode surface has 10X greater binding capacity than polystyrene wells.
- Surface coatings can be customized.


*Figure 1. Schematic of MSD ECL technology.*

A prototype multiplexed AAV serology panel (AAV Panel 1) was developed to detect antibodies against AAV capsid antigens. The 96-well plate contains arrays of AAV antigens from different serotypes within each well. Antibodies in the sample bind to the antigens on the spots and an anti-human antibody (IgG) conjugated with MSD® SULFO-TAG is used for detection. The plate is read on an MSD instrument, which measures the light emitted from the MSD SULFO-TAG. The plate is used in combination with a calibrator for quantitation, controls, and a detection antibody (anti-human IgG).

## RESULTS

### Signal Reproducibility

AAV Serology plates were tested for signal reproducibility across wells and plates. All nine multiplexed AAV assays passed the intraplate reproducibility Coefficient of Variation (CV) specifications as designated in Table 2 below.

| | | Analyte and Signal Reproducibility | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **Metric** | **Spec** | **AAV 1** | **AAV 2** | **AAV 5** | **AAV PHP.eB** | **AAV.rh10** | **AAV DJ** | **AAV 6** | **AAV 8** | **AAV 9** |
| CV of Intraplate Averages | ≤ 18% | 9.9% | 6.2% | 9.1% | 10.4% | 7.4% | 8.6% | 12.8% | 15.4% | 4.8% |
| Average Intra-plate CV | ≤ 10% | 8.7% | 5.3% | 4.4% | 7.6% | 5.8% | 6.7% | 7.2% | 7.0% | 5.6% |
| Max Intra-plate CV | ≤ 13% | 11.3% | 7.8% | 4.8% | 8.6% | 6.7% | 6.8% | 10.1% | 10.2% | 7.0% |
| Average Signal by Batch (ECL) | 1,500-1,000,000 | 249,424 | 208,583 | 50,272 | 67,277 | 94,221 | 85,712 | 233,966 | 93,743 | 105,312 |
| **Result** | | PASS | PASS | PASS | PASS | PASS | PASS | PASS | PASS | PASS |

*Table 2. Representative data demonstrated consistent performance using a pooled serum based calibration standard, diluted to a level within the linear range of the assay, and tested using the standard assay protocol.*

### Specific Binding of Anti-AAV Monoclonal and Polyclonal Antibodies

Screening was performed to identify antibodies capable of distinguishing between AAV serotypes or with broad reactivity across serotypes. Selected antibodies can be used as QC reagents to confirm correct coating of antigen arrays. Testing of commercially-sourced normal human adult serum samples showed antibody activity was present against all the printed AAV serotypes.

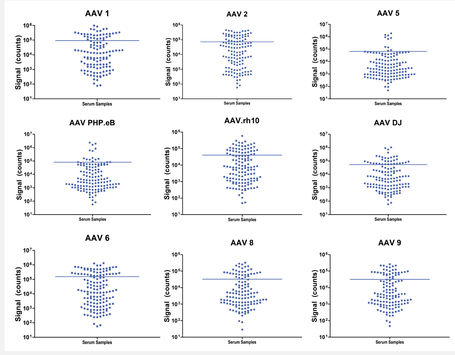
| | Antibody Target | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **Antibodies** | **AAV1** | **AAV2** | **AAV5** | **AAV PHP.eB** | **AAV.rh10** | **AAV DJ** | **AAV6** | **AAV8** | **AAV9** |
| **AAV 2 rAb** | 0.02% | 100% | 0.00% | 0.02% | 0.00% | 1.27% | 0.01% | 0.03% | 0.00% |
| **AAV 5 rAb** | 0.02% | 0.02% | 100% | 0.02% | 0.01% | 0.14% | 0.03% | 0.03% | 0.03% |
| **AAV 8 hAb** | 0.06% | 0.00% | 0.00% | 0.00% | 32.3% | 0.00% | 0.00% | 100% | 0.02% |
| **AAV 9 mAb** | 0.00% | 0.00% | 0.01% | 0.01% | 0.00% | 0.41% | 0.00% | 0.00% | 100% |
| **AAV X rAb** | 57.4% | 10.0% | 55.5% | 100% | 33.6% | 39.8% | 55.8% | 54.1% | 69.2% |
| **Serum-01** | 100% | 33.2% | 8.34% | 14.6% | 16.0% | 13.7% | 59.6% | 32.9% | 21.0% |
| **Serum-02** | 100% | 45.1% | 5.06% | 10.4% | 20.2% | 15.4% | 77.7% | 19.3% | 16% |

*Table 3. Representative percent differences in cross-reactivity between antigens and their respective antibodies. Data is normalized to the highest signal for each antibody/serum sample.*

### Sample Testing

A set of >100 commercially-sourced normal human adult serum samples were screened for the presence of anti-AAV antibodies. The horizontal line within each plot indicates mean ECL signal for each AAV serotype.

*Figure 2. Human serum sample screening for each AAV serotype.*




### Representative Data and Dynamic Range

Selected samples of the screened serum were used to build AAV Serology Calibrator 1 and AAV Serology Controls 1 and 2. The calibrator allows for quantification of serum antibody concentrations.
Using MSD assigned concentrations for the AAV Serology Calibrator 1, control concentrations as well as the Upper Limits of Quantification (ULOQ) and Lower Limits of Quantification (LLOQ) were assigned for each assay.

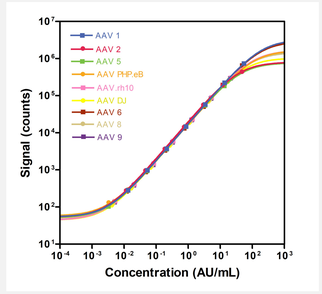



*Figure 3. Representative calibration curves demonstrate 3-4 logs of dynamic range for all assays.*

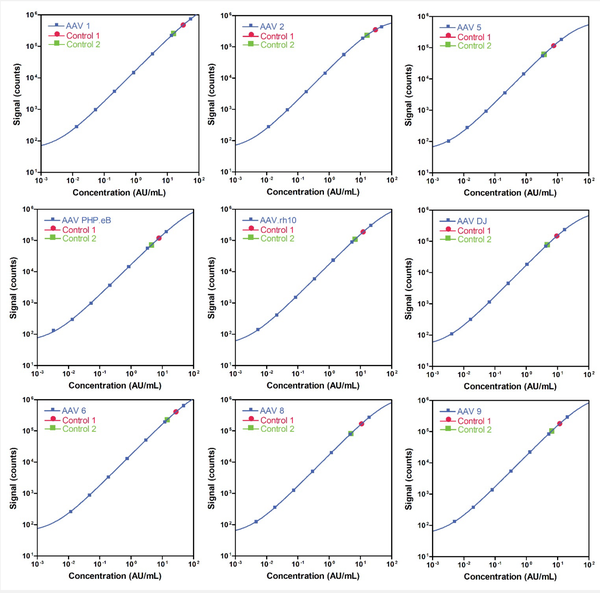



*Figure 4. Typical calibrator curves for all nine AAV assays using AAV Serology Calibrator 1. Calibrator curves use 7 points (1:4 serial dilutions) plus a blank (not shown). AAV Serology Control 1 and Control 2 are also shown.*

A typical IgG functional assay test consists of calibrator curves, controls, and samples representing ULOQ and LLOQ. Twelve commercially-sourced normal human serum samples were chosen for potential product release testing. Each sample was tested in 4 replicates. Precision was evaluated by calculating the CV for these 4 measurements.

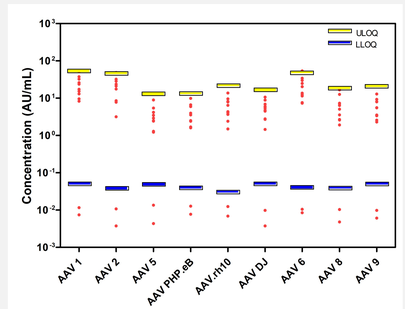



*Figure 5. IgG functional testing using a selected set of 12 purchased normal human adult serum samples demonstrated precision that met specifications (≤ 20% CV) for 10 positive samples. Two negative samples with concentrations below LLOQ are included.*

## CONCLUSIONS

The multiplex AAV ECL serology assay provides sensitive, high throughput, and simultaneous measurement of anti-AAV antibody (IgG) levels across multiple AAV serotypes, supporting its use in AAV-based gene therapy applications. Signal reproducibility, specificity, and sensitivity were assessed for individual serotypes using reference serum and monoclonal and/or polyclonal antibodies.

AAV vectors are commonly used in gene therapy but pre-existing anti-AAV antibodies pose a concern that may impact treatment efficacy. Serological analysis, such as is possible with this multiplex assay, is crucial for understanding these interactions. While virus neutralization assays are considered the gold standard for specificity, they are time-consuming and labor-intensive, highlighting the need for more efficient workflows in drug development.

We demonstrated that this prototype multiplex serology assay precisely quantifies IgG antibodies binding to multiple AAV serotypes (AAV1, AAV2, AAV5, AAV6, AAV8, AAV9, AAV DJ, AAV.rh10 and AAV PHP.eB) and offers a simpler, scalable alternative to traditional virus neutralization assays.

References: MSD-based assays facilitate a rapid and quantitative serostatus profiling for the presence of anti-AAV antibodies. 2022. Haar, J, et al. Mol Ther Methods Clin Dev:25:360-369. https://pubmed.ncbi.nlm.nih.gov/35573045/

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