Validation of an approach to measure high levels of neutralizing antibodies against AAV in human serum samples

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Introduction

A cell-based transduction inhibition assay was developed to measure neutralizing antibodies to AAV5 in human serum using a reporter vector encoding luciferase. The assay, which reports neutralizing antibodies (NAb) as semi-quantitative titer values, determined titers by calculating the mid-point (IC50) of a 7-point titration curve using 4-parameter logistic regression. Although overall variability of the assay was in line with expectations for a cell-based assay, a higher reporting range was desired in order to further develop the assay as a potential diagnostic. Because the curve fit algorithm for this method calculates titers as the mid-point of the titration curve, increased variability can occur when titration curves do not have clearly defined asymptotes (example in **Figure 1**) as the curve-fitting software extrapolates to determine asymptotes. This primarily affects samples with higher titers as such samples may not have enough points to the right of the IC50 to define a lower asymptote; this consequently impacts the upper reporting range of the assay. Therefore, an effort was undertaken to increase the reporting range of the assay by improving precision of the assay for higher titer samples.

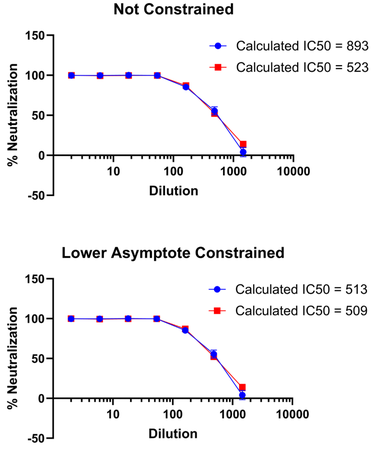


Figure 1. Calculated IC50 Variability. Two independent replicates of the same sample (each replicate consisting of 7 dilutions with triplicate wells per dilution) had different calculated IC50 values (52% relative difference) despite nearly overlapping neutralization curves (left panel). When the lower asymptotes of the same curves were fixed to zero (right panel), the IC50 values were comparable (1% relative difference), indicating the impact of asymptote extrapolation on calculation of IC50 values.

Results

Multiple validation data sets were re-evaluated by constraining the lower asymptote to zero. This approach improved precision of the assay (for example, for one higher titer sample without clearly defined lower asymptotes, precision improved after constraining the lower asymptote (**Figure 2**). Further, applying a curve constraint to multiple samples in a linearity study not only reduced %CV but also extended the linear range of the assay from a titer of 470 to a titer of 684 (**Figure 3**). One data point in the linearity study (the result shown above in Figure 1) was responsible for linearity limitations in this study. Although this approach had clear benefits, there were also drawbacks. First, if constraining one of the asymptotes, the 4-parameter logistic regression no longer truly has 4 parameters, calling into question the validity of IC50 calculations by this approach. Second, this approach would represent a fundamental change in the potential diagnostic assay compared to the original clinical trial assay. Therefore, an alternative approach was considered. The alternative approach, adding two points to the titration curve (meaning a 9-point titration curve rather than a 7-point titration curve) without changing the dilution factor or the method of IC50 calculation, was evaluated as a way to improve precision of the assay and extend the linear range while remaining consistent with the original assay (comparison of dilutions in **Figure 4**). Coupled with this change was a rule that there must be 3 points on the titration curve to the right of the calculated IC50 for the result to be valid; this rule was implemented to ensure that the lower asymptote would be clearly defined. Because the assay uses a three-fold dilution series, incorporation of two additional points extends titration curves 9-fold. The combination of these changes extended the linear range of the assay to a titer of 1100 (**Figure 5**) while improving precision: with 9-point titration curves, intra-assay precision (n=20 replicates per sample) ranged from 11% to 15% CV for four samples with titers ranging from 200 to 1100. Total variability (between operators, between runs, and between reagent lots) ranged from 18% to 25% CV for the same samples (n=at least 110 replicates per sample).

Additionally, a study was performed to compare titers of healthy donor serum samples (n=30 samples) tested with the original and modified methods. Linear regression of results between the two methods showed a strong correlation (R-squared 0.963) (**Figure 6**); therefore, results between the two methods differ in a predictable manner that can be described by a mathematical equation, thus facilitating comparisons between the two methods. Moreover, an additional optional reflex step (pre-dilution so that titration curves start at 1:18 dilution rather than 1:2 dilution) was investigated to further increase the reporting limit by retesting samples, if needed, at a higher starting dilution. With the reflex step, the assay was linear for titer values up to 4400 (**Figure 7**). Between-operator precision was also evaluated for two samples that required reflex testing (titer values of 1879 and 3747) and precision (n=45 replicates per sample) ranged from 13% to 15% CV.

Figure 2. Impact of Lower Asymptote Extrapolation on Two Samples. Two samples, with different titer values, were each tested in multiple replicates (each replicate consisting of 7 dilutions with triplicate wells per dilution to calculate IC50 values, represented by symbols on each graph). Each data set was evaluated with and without constraint of the lower asymptote to zero. For the higher titer sample (sample 1, left panel), constraining the lower asymptote improved variability, reducing the CV from 27% to 14%. By comparison, a lower titer sample (sample 2, right panel), which had more points to the right of the IC50 and therefore more clearly defined lower asymptote, had lower variability without constraining the lower asymptote (9% CV), though the CV was further reduced after constraining the lower asymptote.

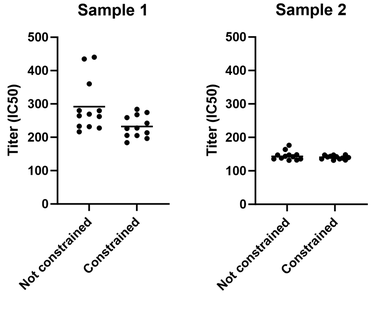
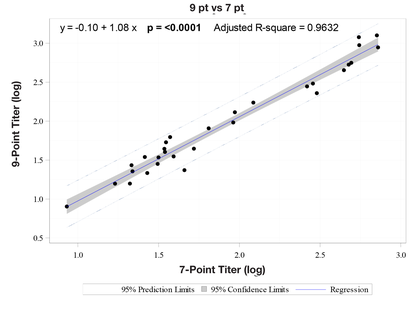


Figure 4. Comparison of Dilutions with 7-Point and 9-Point Titration Curves.

Dil 1	2
Dil 2	6
Dil 3	18
Dil 4	54
Dil 5	162
Dil 6	486
Dil 7	1458

Dil 1	2
Dil 2	6
Dil 3	18
Dil 4	54
Dil 5	162
Dil 6	486
Dil 7	1458
Dil 8	4374
Dil 9	13122

Figure 6. Correlation Between Results Obtained from 7-Point and 9-Point Titrations Serum samples from 30 healthy human donors were tested with 7-point titrations (no asymptote constraint) and 9-point titrations. Titers of samples are log-normal distributed; therefore, values were log10-transformed prior to regression analysis.



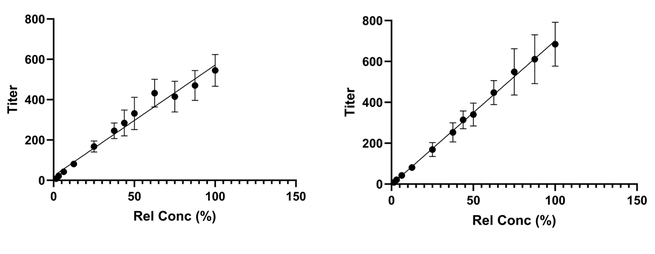


Figure 3. Impact of Lower Asymptote on Assay Linearity. One high titer sample was diluted in negative serum at several relative concentrations to generate a total of 12 linearity samples. Each of the samples was then titrated (consisting of 7 dilutions with triplicate wells per dilution to calculate IC50 values, represented by symbols on each graph). Each data set was evaluated without (left panel) and with (right panel) constraint of the lower asymptote to zero, and linear regression of the resulting IC50 values was performed to evaluate the linear range of the assay. Without constraint, the assay demonstrated linearity up to a titer value of 470, but increased to 684 following constraint of the lower asymptote to zero.

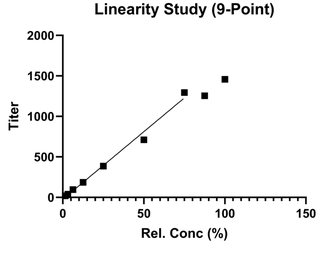


Figure 5. Impact of Increased Points on Curve on Assay Linearity. One high titer sample was diluted in negative serum at several relative concentrations to generate a total of 9 linearity samples. Each of the samples was then titrated (consisting of 9 dilutions with triplicate wells per dilution to calculate IC50 values, represented by symbols on each graph). Linear regression of the resulting IC50 values was performed to evaluate the linear range of the assay. With a 9-point titration, the linear range of the assay was extended to 1100 (compared to 684 using 7-point titration and constraining the lower asymptote to zero as shown in Figure 3).

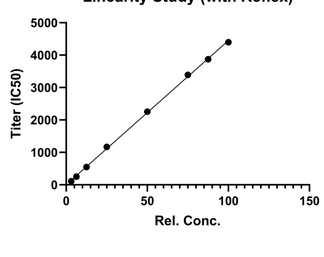


Figure 7. Linearity of 9-Point Titration Assay with Reflex. One high titer sample was diluted in negative serum at several relative concentrations to generate a total of 8 linearity samples. Each of the samples was then titrated (consisting of 9 dilutions with triplicate wells per dilution to calculate IC50 values, represented by symbols on each graph). Linear regression of the resulting IC50 values was performed to evaluate the linear range of the assay. The linear range of the assay with reflex was 109 to 4400.

Conclusions

The modified assay had an increased upper reporting limit allowing comprehensive measurement of high titers of pre-existing NAb while maintaining consistency with the predecessor assay. With an optional reflex step, the upper reporting limit was increased further. The modified assay had acceptable precision and results had a strong correlation to the original method. This approach can be applied to other projects or methodologies where it is important to extend the reporting range of a method without losing continuity with the original method.

Method

Two different approaches were considered to increase the upper limit of the assay: (1) changing the algorithm used to calculate titer values from titration curves, specifically to constrain the lower asymptote to zero; (2) addition of points to the titration curve, specifically increasing from 7 to 9 points. A retrospective evaluation of validation data was performed to test the first approach (changing the algorithm) whereas new data were generated to evaluate the second approach (addition of points to the titration curve). Subsequently, the modified assay was validated using native human serum samples at titer values spanning the range of the assay.

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