OPTIMIZING AAV-MEDIATED NEURAL GENE THERAPY: A NEXT-GENERATION NEUTRALIZATION ASSAY FOR PATIENT STRATIFICATION

PhD thesis book

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1 Introduction

Modern neuroscience increasingly depends on genetic tools to map, monitor, and manipulate defined neuronal populations in vivo. Among available platforms, adeno-associated viruses (AAVs) are widely used, as they can be tailored for brain delivery, achieve sustained expression, and are compatible with diverse experimental approaches across a wide range of model species, from rodents to non-human primates.

In clinical contexts, AAV-based therapies have already gained regulatory approval for spinal muscular atrophy and inherited retinal dystrophies, with many more in active clinical development across neurology, ophthalmology, and systemic indications.

However, a major obstacle to the broader application of AAV-based therapies is the presence of neutralizing antibodies (NAbs) targeting the viral capsid. These antibodies compromise both safety and efficacy. NAbs typically arise following natural infection with wild-type AAVs.

Pre-existing immunity to AAV is widespread across populations and varies by serotype and geography; serological surveys report NAbs against AAV1, AAV5, and AAV9 in 74.9%, 63.9%, and 57.8% of adults, respectively.

NAbs are typically measured using functional assays that quantify the ability of blood serum to inhibit AAV transduction in vitro. Currently, however, assays are developed independently by individual laboratories and companies, leading to differences in design, sensitivity, threshold definitions, and data interpretation.

This lack of harmonization highlights the need for a unified method that combines an optimized experimental design with a rigorous analytical strategy. In response, I developed the core Transduction Inhibition Assay (coreTIA), a framework that integrates an optimized constant serum concentration assay format with a Bayesian statistical analysis pipeline.

2 Objectives

The primary aim of my thesis was to establish a harmonized method for quantifying AAV neutralizing antibodies, integrating experimental optimization with robust data analysis to enhance patient stratification and translational research. To achieve this, I defined three main objectives:

1. **Establishment of an optimized experimental protocol:** To systematically evaluate assay variables and develop a core Transduction Inhibition Assay (coreTIA) that eliminates matrix-induced artifacts and maximizes sensitivity.

2. **Implementation of a statistical framework:** To develop a robust computational workflow that resolves the limitations of conventional data analysis methods by providing reliable ND50 values with quantifiable credible intervals, even from incomplete dilution series.

3. **Comparative validation across species:** To compare the performance of the newly developed core assay against standard methodologies using human, feline, and macaque serum samples, including longitudinal seroreversion models.

3 Methods

3.1 Experimental Procedures

Bioluminescent assay reporters

Plasmids encoding CAG-NLuc-3xFLAG-10His-WPRE-SV40 and CAG-FLuciferase-WPRE-SV40 were used to generate reporter vectors.

AAV production.

HEK293T cells were co-transfected with pAAV vector, pHelper, and pRC plasmids using PEI. AAV particles were purified by iodixanol gradient ultracentrifugation and titered by qPCR.

Variable Serum Concentration (VSC) Assay

HEK293T cells (1×10^5/well) seeded in DMEM + 1.25% FBS, incubated 2h at 37°C/5% CO_2. Test serum was 2-fold serially diluted in DMEM, pre-incubated with AAV (1×10^7 vg/well, MOI 100), and added to cells. Luminescence was measured after 24-48h using Nano-Glo assay reagent.

coreTIA: The Constant Serum Concentration (CSC) Assay

Test serum was diluted in FBS to maintain constant total serum concentration (10%); cells were seeded in FBS-free DMEM. All other steps were identical to the VSC assay.

Serum samples

Sera were obtained from human samples (Semmelweis University), C57Bl/6 mice (AAV9-hsyn injected, HUN-REN RCNS), domestic short-hair cats (naïve/CRP-vaccinated/CAP-B22 injected, HUN-REN RCNS), and rhesus macaques (naïve, KU Leuven/University of Pécs).

3.2 Statistical and Computational Framework

ND50 definition

Neutralizing Dose 50: serum dilution (or antibody concentration) required to reduce transduction by 50% relative to the antibody-free control.

Non-statistical 50% inhibition estimation

ND50 was estimated as the first dilution where the mean response fell below 50% inhibition.

Linear-bootstrap 50% inhibition estimation

Interpolation was performed between bracketing data points (one above and one below 50%), using all combinations of technical replicates to generate a bootstrap distribution of ND50 estimates and credible intervals.

Hill-MCMC 50% inhibition estimation

A Bayesian Markov Chain Monte Carlo (MCMC) approach fitted a 4-parameter Hill curve to dose-response data (2000 posterior draws, truncated normal priors on slope/ND50), modeling log-normal noise for robust ND50 estimation with credible intervals.

Data Analysis

Data representation and documentation is built upon the Wellmap Python package. Raw luminescence data were converted to percentage inhibition relative to the serotype-matched antibody-free control. Effect sizes between conditions (e.g., VSC vs. CSC) were quantified using non-parametric Cliff's delta. A practical equivalence threshold of 0.3 log2 units was established to distinguish biological differences from technical noise.

4 Results

4.1 Identification of key assay parameters and establishment of an optimized protocol.

Conventional cell-based AAV neutralization assays typically employ a variable serum concentration (VSC) protocol, in which test sera are serially diluted in cell culture medium. However, when applied to complex biological samples, this format introduces significant matrix effects. Instead of the expected sigmoidal curve, human serum samples unexpectedly showed increasing transduction levels at higher serum concentrations, which masked true neutralizing activity and prevented reliable ND50 calculation (Figure 1/A-C dashed lines).

To overcome this major obstacle, I developed the Constant Serum Concentration (CSC) assay format. By diluting test sera in fetal bovine serum (FBS) instead of culture medium, the total serum concentration remains constant across all dilutions (Figure 1/D). This modification successfully stabilizes the baseline transduction, eliminates matrix-induced artifacts, and restores well-behaved sigmoidal dose-response curves, enabling robust quantification of neutralizing antibodies (Figure 1/A-C solid lines).

To maximize the overall sensitivity of the assay, I implemented a high-sensitivity NanoLuc (NLuc) reporter system. The NLuc reporter provided an approximately 1000-fold increase in bioluminescence compared to the conventional Firefly luciferase (FLuc).

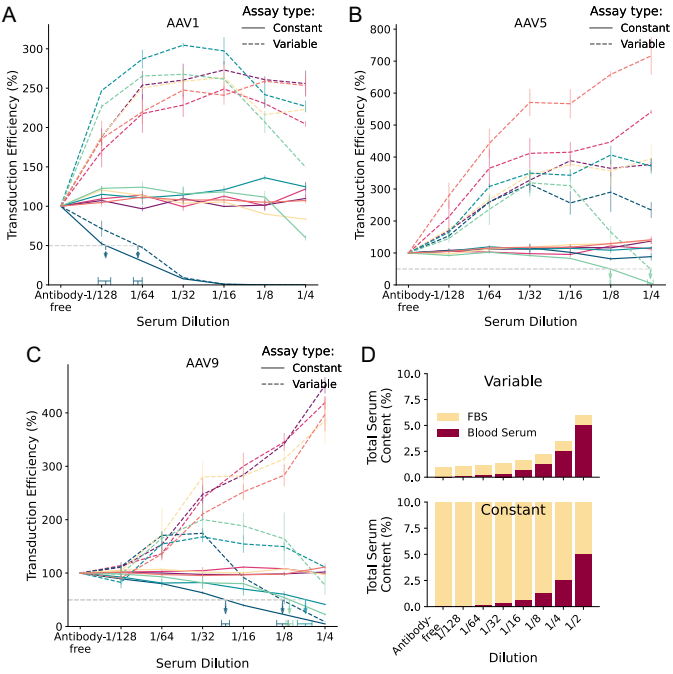

Figure 1. Optimized pre-incubation conditions and constant serum concentration yield an improved AAV neutralization assay across different serotypes.

(A-C) *Transduction curves for seven human sera tested against AAV9, AAV1, and AAV5. The Variable format (dashed lines) suffers from baseline inflation exceeding 100%, whereas the optimized Constant format (solid lines) stabilizes baseline transduction and accurately reveals neutralization activity.* ***(D)*** *Schematic illustration of design differences between Variable Serum versus Constant Serum Concentration assay formats.*

Furthermore, I systematically evaluated key procedural variables to maximize analytical sensitivity and practical utility. I established that a viral input of MOI 100 provides the optimal balance for robust

neutralization readouts across multiple AAV capsids. I also demonstrated that omitting serum heat-inactivation preserves optimal analytical sensitivity. Finally, temporal evaluations showed that antibody-virus binding reaches equilibrium rapidly (within 15 minutes) and that a 24-hour post-transduction readout performs equivalently to 48 hours. These temporal optimizations shorten the experimental timeline and provide the flexibility needed for high-throughput processing.

Collectively, these methodological optimizations establish the experimental foundation of the coreTIA (core Transduction Inhibition Assay). By eliminating matrix artifacts and maximizing sensitivity, this protocol provides a highly robust platform for neutralization quantification.

4.2 Implementation of a statistical framework for quantifying neutralizing activity.

Conventionally, neutralizing antibody levels are determined using a simple threshold-based approach that identifies the highest serum dilution inhibiting transduction by more than 50%. While straightforward, this non-statistical method yields only a single point estimate, providing no information about measurement confidence or precision.

To address this fundamental limitation, I integrated the coreTIA framework with a Bayesian statistical pipeline that estimates neutralizing potency using two complementary methods. The first is a Linear-bootstrap method, which performs a local linear interpolation between the two adjacent data points that bracket the 50% threshold,

coupled with resampling to generate uncertainty metrics. The second, more advanced approach is the Hill-MCMC method, which employs non-linear curve fitting to model the entire sigmoidal dose-response dynamic based on the Hill equation.

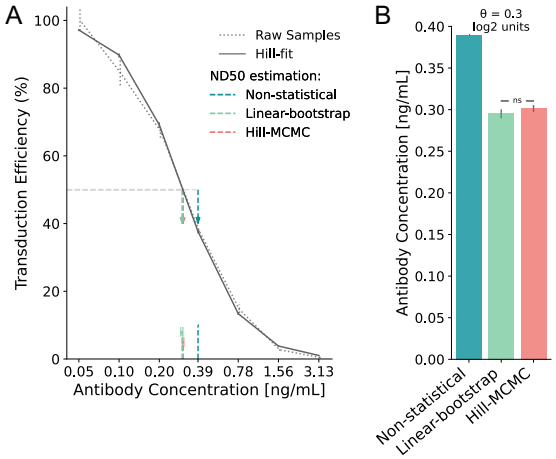

Figure 2. Statistical framework for estimating 50% inhibition.

(A) *Neutralization curve obtained with an ADK9 antibody dose series. The dotted grey curve shows raw sample means (with 95% CI); the solid grey curve represents the Hill-fit model (with 95% credible intervals). Dashed vertical arrows denote ND50 estimates, with horizontal bars indicating uncertainty for the Linear-bootstrap and Hill-MCMC methods.* ***(B)*** *Application of the practical equivalence threshold (θ = 0.3 log2 units). Vertical error bars represent credible intervals. The Linear-bootstrap and Hill-MCMC estimates differ by less than the threshold (marked "ns"), indicating practical equivalence.*

Unlike the conventional method, both statistical approaches generate credible intervals, explicitly quantifying the uncertainty of each

measurement. Using both synthetic and experimental data, I demonstrated that these statistical models produce significantly more accurate ND50 estimates than the conventional approach (Figure 2/A). Furthermore, by analyzing the distribution of credible interval widths, I established a data-driven practical equivalence threshold (0.3 log2 units) to reliably distinguish meaningful biological differences in ND50 from mere technical variability (Figure 2/B).

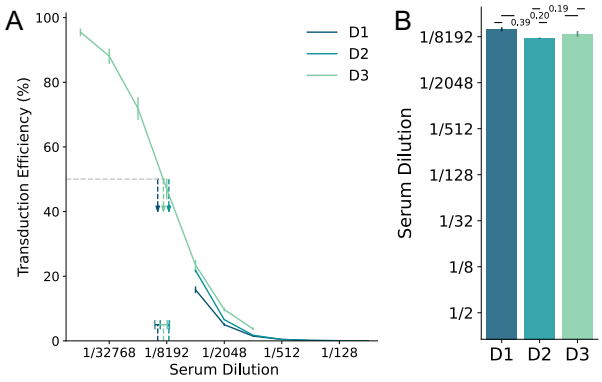

Figure 3. Robust ND50 estimation from incomplete dilution series using the Hill-MCMC model.

(A) *Hill-MCMC extrapolation applied to the same mouse serum sample tested on three different days using varying dilution ranges. On Days 1 and 2 (D1, D2), the tested ranges did not cross the 50% inhibition threshold, whereas on Day 3 (D3) an adjusted range successfully bracketed the 50% point. Solid lines show Hill-MCMC fits, and vertical dashed arrows indicate the posterior mean ND50 estimates with corresponding 95% credible intervals (horizontal bars).* ***(B)*** *Comparison of posterior ND50 estimates across days. Bars represent the posterior mean ND50 estimates derived via Hill-MCMC for D1, D2, and D3, with error bars indicating 95% credible intervals.*

Patient samples frequently produce incomplete dose-response curves, where the tested dilution series fails to cross the 50% transduction threshold. Under these conditions, conventional non-statistical and Linear-bootstrap methods fail to calculate ND50.

To resolve this, I demonstrated that the Bayesian Hill-MCMC model can be effectively applied. By fitting the overall dynamics of the available data, the Hill-MCMC approach can reliably extrapolate the neutralizing potency even from partial curves (Figure 3).

Together with the optimized experimental protocol, this statistical framework ensures robust and precise NAb quantification across a wide range of biological samples.

4.3 Comparative validation of the optimized core assay across human and animal models.

The enhanced sensitivity and reliability of the coreTIA platform have direct implications for patient safety and clinical eligibility. Misclassification of serostatus is a major risk in gene therapy, as even low neutralizing antibody levels can compromise transduction efficacy. I evaluated the clinical utility of the Constant Serum Concentration (CSC) strategy using a human serum cohort, comparing it directly against the conventional Variable Serum Concentration (VSC) format.

First, I aimed to identify truly seronegative sera suitable for use as an antibody-free control or background matrix. For this purpose, I applied a stringent threshold of >90% transduction at a 1/4 serum dilution (Figure 4A-C). The results revealed a striking difference in classification sensitivity. The CSC assay identified significantly more

samples as "ineligible" than the VSC format across all tested capsids. These findings indicate that the conventional VSC assay frequently fails to detect low-level neutralizing activity, potentially compromising the quality of reference materials used for assay validation and, ultimately, patient eligibility determinations.

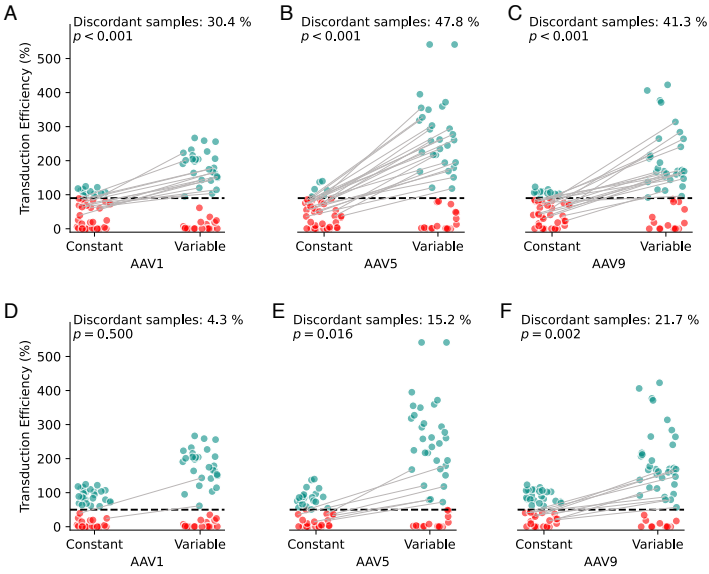

Figure 4. Constant serum concentration (CSC) assay provides more consistent subject stratification than the variable serum concentration (VSC) assay format.

Identification of seronegative (teal) vs. ineligible/neutralizing (red) samples from a cohort of 46 sera using CSC and VSC assays at a 1/4 dilution. (A-C) Classification using a stringent 90% transduction threshold (dashed line). (D-F) Classification using a standard 50% transduction threshold. Discordant samples are connected by gray lines, with p-values indicating significant classification differences across AAV serotypes.

Next, I re-evaluated the same cohort using the standard clinical criterion: 50% transduction at a 1/4 dilution to distinguish neutralizing from non-neutralizing samples (Figure 4D-F). Under this less stringent threshold, the CSC format still reclassified a substantial proportion of samples that were deemed non-neutralizing by the VSC assay. The discordance was serotype-dependent; this variation likely reflects the differential magnitude of serum-induced transduction enhancement observed across serotypes.

Beyond human clinical applications, establishing a consistent NAb methodology across species is essential for reliable translational comparisons. To validate whether the CSC format's baseline stabilization extends to preclinical species, I analyzed sera from domestic cats and rhesus macaques. Consistent with the human data, animal sera analyzed with the conventional VSC protocol showed artifactual baseline transduction levels above 100% across multiple AAV capsids. In contrast, the optimized CSC format effectively eliminated this matrix-induced signal boost in both species, restoring well-behaved sigmoidal curves and enabling accurate ND50 quantification. This confirms that the coreTIA framework generalizes across species, offering a standardized tool for translational neuroscience.

Patients often undergo seroreversion, transitioning from a seropositive to a seronegative state. Determining this status accurately is critical for establishing safe eligibility windows for AAV re-administration. To evaluate how assay sensitivity influences the detection of long-term antibody persistence, I tested two preclinical feline models

representing distinct immune scenarios: vaccination-induced cross-reactivity and direct AAV vector administration (Figure 5).

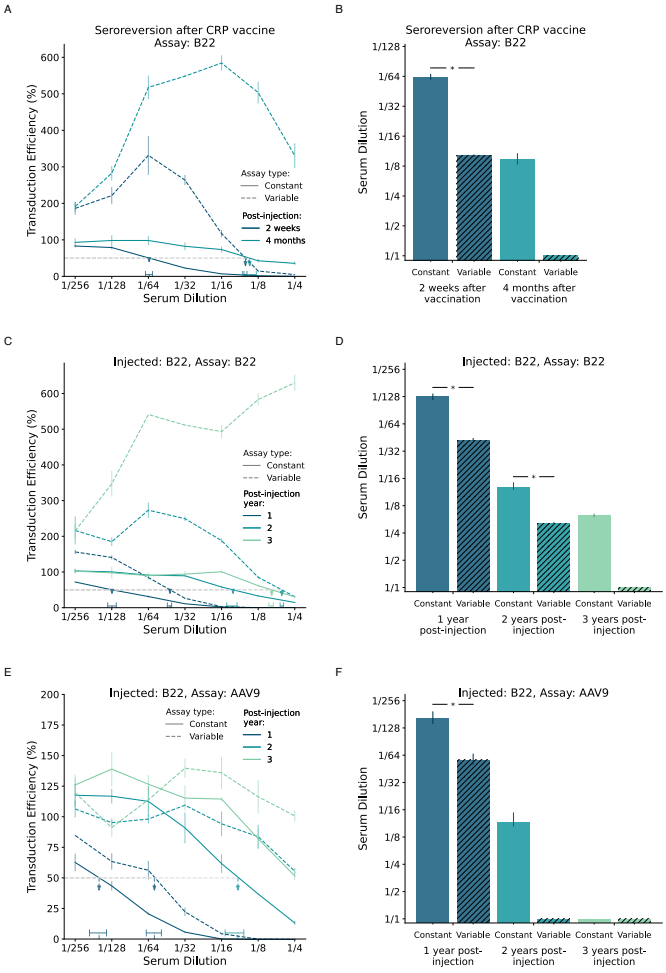

Figure 5. Performance of CSC assay in preclinical seroreversion models ***(A, C, E)*** *Transduction curves for serum samples collected from feline subjects at various time points. Vertical error bars indicate standard*

deviations from the mean. Colored vertical arrows indicate ND50 dilutions, pointing to horizontal bars that denote 95% credible intervals. ***(B, D, F)*** *corresponding ND50 estimates derived from the curves for both Constant (filled bars) and Variable (striped bars) assays. Error bars represent 95% credible intervals, and asterisks denote significant differences. For samples with minimal (ND50 < 1/4) or no neutralization, a 1/1 dilution is used as a placeholder.* ***(A–B)*** *Serum samples collected from one cat two weeks and four months after vaccination with a feline CRP vaccine.* ***(C–D)*** *Serum samples collected from one cat one, two, and three years after injection with CAP-B22 capsid.* ***(E–F)*** *Same serum samples as in (C-D) tested against the parental capsid of CAP-B22, AAV9.*

In both models, the conventional VSC assay demonstrated severe matrix-driven masking effects. Crucially, while the VSC assay suggested a near-complete loss of neutralizing activity after four months (vaccination model) or three years (injection model), the high-sensitivity CSC format continued to detect functionally significant neutralization. These longitudinal observations confirm that neutralizing activity persists significantly longer than suggested by conventional methods. By preventing the premature declaration of seronegativity, the coreTIA framework supports safer decision-making for AAV re-dosing strategies.

5 Conclusions

AAV vectors have become indispensable tools in basic neuroscience, enabling precise circuit mapping, optogenetic manipulation, and the treatment of neurological disorders. However, the successful application of these tools is often hindered by pre-existing immunity.

The primary aim of my work was to establish a harmonized method for quantifying AAV neutralizing antibodies to improve the applicability of these interventions. The major scientific contributions and new results of my thesis are summarized below:

Thesis I: Elimination of matrix effects via the Constant Serum Concentration (CSC) design

- I demonstrated that conventional AAV neutralization assays suffer from dilution-dependent baseline inflation caused by variable serum content.
- I showed that maintaining a constant serum concentration stabilizes assay baselines and unmasks neutralizing activity that is otherwise obscured by matrix effects.
- I showed that this optimized experimental design increases sensitivity without compromising procedural simplicity.

Thesis II: Establishment of a Bayesian statistical framework

- I integrated the Linear-bootstrap and Bayesian Hill-MCMC approaches into the coreTIA framework to quantify the uncertainty of every ND50 estimate.
- I demonstrated that the Hill-MCMC model accurately estimates ND50 values even from incomplete dilution series where the 50% inhibition point lies outside the measured range.
- I established a data-driven "practical equivalence" threshold (0.3 log2 units) to distinguish meaningful biological differences from technical variability.

Thesis III: Enhanced patient stratification and long-term immune monitoring

- I validated the clinical and translational utility of the coreTIA framework across human and animal cohorts including feline and rhesus macaque subjects.
- I showed that coreTIA reclassifies a significant proportion of false-negative human samples as seropositive, improving the safety of patient stratification.
- Using longitudinal feline models, I proved that functionally relevant neutralizing antibodies persist significantly longer (up to 3 years) than detected by conventional assays, providing critical data for determining AAV re-administration windows.

My thesis resolves a methodological bottleneck in neurological gene therapy. By enabling reliable quantification of immunity against both standard serotypes and novel synthetic capsids, coreTIA supports the planning of translational studies and improves the safety assessment of gene therapies for CNS disorders.

6 Bibliography of the candidate's publications

Publications related to the thesis

Kovács B, Somogyi F, Szabó V, Nagy ZZ, Hernádi I, Mátyás F, Vanduffel W, Szemlaky Z, Rózsa B, Ulbert I, Hillier D. CoreTIA: a modular, statistically robust transduction inhibition assay for AAV neutralization. Front Immunol. 2025;16:1623848. doi: 10.3389/fimmu.2025.1623848.

Kovács B, Szabó V, Horváth D, Dobos AB, Nagy ZZ, Vanduffel W, Szemlaky Z, Szepesi Á, Ulbert I, Rózsa B, Hillier D. Overcoming matrix effects in AAV neutralization assays with a constant serum concentration approach. Gene Ther. 2025. doi: 10.1038/s41434-025-00567-0.

Publications independent of the thesis

Domahidy F, **Kovács B**, Cseri L, Katona G, Rózsa B, Mucsi Z, Kovács E. Comprehensive study of thiazole-orange-based DNA dyes. ChemPhotoChem.2024;8:e202400080. doi: 10.1002/cptc.202400080.

Varga N, Pomozi V, Kozák E, Erdei Z, Tóth S, Kutás A, Mucsi Z, Rapi Z, **Kovács B,** Matuscsák A, Bánhídi O, Váradi C, Rácz T, Német K, Viskolcz B, Szepesi Á. Development and validation of combined in vitro and in vivo assays for evaluating the efficacy of strontium-chelating compounds. Sci Rep. 2025;15:43006. doi: 10.1038/s41598-025-06618-1.