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Molecular Therapy
Advances
Review

AAV serotype IVDs for patient selection in gene therapy development: Technical and regulatory considerations by ARDAT consortium

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Currently, most clinical trials for adeno-associated virus (AAV)-based gene therapy (GT) exclude patients with pre-existing anti-AAV antibodies (AAV Abs) due to potential impact on treatment efficacy and safety. Diagnostic regulations require sponsors to define pre-existing AAV Ab level cutoffs and validate patient selection assays before beginning clinical studies. In the absence of robust clinical outcome data early in development, sponsors often rely on thresholds derived from literature or preclinical studies, or default to assay detection limits. The transferability of these thresholds to actual clinical outcomes remains uncertain, leading sponsors to use conservative cutoffs to avoid costly revalidations and development delays. This may prevent some patients from accessing potentially beneficial treatments. We advocate for more flexible validation approaches based on current understanding of antibody characteristics and the commonly used assay formats. We propose an AAV serotype-specific *in vitro* diagnostic (IVD) assay, an "AAV serotype IVD," with pragmatic analytical validations for single AAV serotypes. This approach would allow sponsors to establish clinically meaningful cutoffs through adaptive clinical trials that progressively include patients with escalating pre-existing AAV Ab levels by implementing multiple cutoffs during clinical development. These measures could expedite AAV GT development while expanding access for patients with elevated pre-existing AAV Ab levels.

INTRODUCTION

Anti-adeno-associated virus antibodies (AAV Abs) present a significant challenge for AAV-based gene therapies (AAV GTs) as they could trigger or amplify adverse immune reactions[1] that may harm a patient or interfere with the efficacy[2–4] of an AAV GT treatment. Due to these risks, patients with pre-existing AAV Abs are often excluded from clinical trials evaluating AAV GTs.[5] As AAV Abs are predictive biomarkers that are associated with the safety and efficacy of the AAV GT and not with a particular disease, the clinically relevant cutoff for patient exclusion can only be established during interventional studies. However, strict US and EU regulations[6,7] require that the assays used for patient selection are fully validated at the patient selection cutoff value before a trial begins. This creates a dilemma particularly in early-phase trials where the optimal cutoff is unknown.

This paper reviews the current state of companion diagnostic (CDx) development for AAV GTs in the EU and USA, highlighting regulatory differences and their impact on patient selection. We outline how validation effort could be reduced without compromising assay quality, drawing on scientific insights from well-characterized bridging electrochemiluminescence immunoassays (ECLIAs)[8,9] and the properties of antibodies as analytes. We propose a quantitative, serotype-specific *in vitro* diagnostic (IVD) approach, an "AAV serotype IVD," to enable flexible cutoffs in early studies, aiming to standardize testing, reduce validation burden, and accelerate patient access to AAV GTs.

PATIENT SELECTION FOR AAV GT TREATMENT

Risk-benefit considerations

Patient selection for AAV GT treatment is influenced by various factors[5] like disease severity, co-morbidities, and existing standard of care. Biodistribution to target and non-target tissues, including transduction efficiency and speed, dosage, and route of administration (ROA), influences vector clearance from circulation and thus the potential for immune-related adverse events. In addition, the seroprevalence and distribution of AAV Ab levels are critical factors to assess, particularly within the intended patient population. These

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factors should be considered when evaluating the overall risk-benefit profile of AAV GT in patients with pre-existing AAV Abs. If patient selection based on pre-existing AAV Abs is deemed necessary for ensuring safe and effective treatment with an AAV GT, all seropositive patients or those exceeding a specified pre-existing AAV Ab level should be excluded. In such instances, a CDx is required and the labeling for AAV GT and its corresponding CDx will include cross-references to ensure alignment of their intended uses and associated instructions. If the expected benefit outweighs the risk for the patient, a stepwise study design that includes patients with progressively higher AAV antibody levels, accompanied by careful monitoring, to expand access to AAV GT for seropositive individuals may be considered.

While seropositive patients are in most cases permitted to participate in trials for locally administered AAV GTs (e.g., ocular or central nervous system delivery), they are typically excluded from systemic treatments. To date, only two authorized intravenous AAV GT trials have included patients with higher AAV Ab levels with mixed results.

AAV Ab cutoff evaluation: Clinical outcomes and preclinical data translatability

Roctavian (valoctocogene roxaparvovec) was evaluated in a phase 1/2 trial (NCT03520712) in hemophilia A patients with pre-existing AAV5 Abs. According to ClinicalTrials.gov, the study was terminated early after two of three patients with pre-existing total antibody (TAb) titers $\leq$ 500 showed insufficient factor VIII (FVIII) activity ($\sim$5 IU/dL).

For Hemgenix (etranacogene dezaparvovec), initial exclusion of hemophilia B patients with neutralizing antibodies (NAbs) was reconsidered after retrospective analysis of clinical data indicated efficacy at AAV5 NAb titers $\leq$ 340.[10] An additional non-human primate study supported efficacy up to titers of $\sim$1,030,[10] and seroprevalence analyses suggested that up to 84% or 95% of patients could benefit, depending on the cutoff.[11] In the HOPE-B trial (NCT03569891), patients with NAb titers $\leq$350 (n = 47) achieved higher factor IX (FIX) activity (42% ± 22%) compared to those with higher titers.[4] Although patients with titers up to 700 (n = 3) still benefited (27% ± 17%),[4] very high titers (3212; n = 1) abolished FIX expression.[12]

Overall, the Hemgenix case shows that pre-existing AAV Abs can be tolerated to a certain degree. However, defining clinically relevant AAV Ab cutoffs remains challenging, and current preclinical approaches require further validation in human studies.

Meaningful clinical cutoff selection

Establishing meaningful AAV Ab cutoffs requires "all-comer" studies with comprehensive risk-benefit assessments that account for seroprevalence, AAV Ab levels, disease severity, standard of care, ROA, dose, and safety and efficacy endpoints. However, in high-dose systemic GT—where serious adverse events (SAEs) have been reported[13]—all-comer study designs may expose patients with high antibody levels to risk without providing meaningful benefit, potentially diminishing therapeutic value and adversely affecting study efficacy outcomes. An alternative is adaptive trial design with staggered inclusion of patients across increasing AAV Ab cutoffs. However, implementing multiple cutoffs is impractical due to current regulatory and assay validation constraints.

Current cutoff strategies and their long-term impact on patient inclusion

Consequently, sponsors typically use assay detection limits or predefined AAV Ab cutoffs based on literature or preclinical data, which may be overly conservative and exclude potentially benefiting patients. Changing cutoffs requires full assay revalidation and clinical verification, causing high costs and significantly delaying development and access.

Unlike conventional biomarkers, AAV antibodies are therapy-specific and require interventional studies to define meaningful cutoffs. In contrast to some disease biomarkers where post-approval cutoff adjustments have occasionally been implemented,[14,15] such adjustments are unlikely due to treatment irreversibility, safety risks, and cost constraints. As a result, initial cutoffs are rarely revised and may permanently limit patient access to potentially life-saving therapies.

APPROVED AAV GTs AND CDx

By September 2026, only three of the ten USA and/or EU approved AAV GTs utilize a CDx (Table 1). The Roctavian[16] label in the USA advises using the approved AAV5 DetectCDx[24] to prevent treatment of seropositive patients. Conversely, the European label simply requires demonstrating the absence of AAV5 Abs via an "appropriately validated assay"[17] as Roctavian was approved prior to the CDx. In the USA, Beqvez (fidanacogene elaparvovec-dzkt)[18] is restricted to patients who test seronegative using the NAbCyte anti-AAVRh74var HB-FE assay.[25] In the EU, the label allows the use of an alternative validated test if no CE-marked IVD is available, reflecting that the CDx had already been approved in the USA at the time of Beqvez authorization in Europe. The prescribing information recommends administering Elevidys (delandistrogene moxeparvovec-rokl)[20] only to patients with AAVrh74 Ab titers < 400, as measured by the AAVrh74 antibody ELISA CDx.[26] Most recently, Genglycos (pariglasgene brecaparvovec-opnr),[27] approved in USA under accelerated approval for a pediatric population with high unmet medical need, requires selection of patients without detectable anti-AAV8 Abs.[28] Although no FDA-authorized CDx is currently available, the FDA mandated analytical and clinical validation studies to support implementation of an authorized CDx and corresponding label updates. Completion of these studies is expected by February 2027, with submission of a final report by May 2027.[29] The currently low adoption of CDx can be attributed to various clinical and regulatory factors. Therapies like Luxturna (voretigene neparvovec), Upstaza/Kebilidi (eladocagene exuparvovec), Itvisma (onasemnogene abeparvovec), and Otarmeni (lunsotogene parvec-cwha) are delivered locally to body areas that are separated from blood via a barrier.[30] Meanwhile, Zolgensma (onasemnogene abeparvovec) was approved during the initial wave of AAV GTs for highly critical, life-threatening

Table 1. Overview of approved AAV GTs and their corresponding CDx

AAV GT	Roctavian®[16,17]	Beqvez®[18,19]	Elevidys®[20]
Disease	hemophilia A	hemophilia B	Duchenne's muscular dystrophy
AAV serotype	AAV5	AAVrh74var	AAVrh74
Transgene	human FVIII	human FIX (Padua)	micro-dystrophin transgene
EU approval	August 24, 2022 (voluntarily withdrawn: February 2026)[21]	July 24, 2024 (withdrawn: May 15, 2025)[19]	not approved
USA approval	June 29, 2023 (voluntarily withdrawn: February 2026)[21]	April 26, 2024 (discontinued; February 2025)[22]	June 22, 2023
CDx	**AAV5 DetectCDx**	**NAbCyte Anti-AAVRh74var HB-FE**	**AAVrh74 Antibody ELISA CDx**
Assay format	bridging assay	cell-based TI assay	indirect assay
AAV Abs detected	AAV5 TAbs	AAVRh74var NAbs	AAVrh74 TAbs (IgG)
Readout	ECL	luminescence	HRP/TMB
Selection cutoff	TAb negative	NAb negative	TAb titer <400
Establishing laboratory	ARUP Laboratories	Labcorp Drug Development	Quest Diagnostics
CE mark (EU)	November 2023[23]	no CE marked test	no CE marked test
PMA approval (USA)	P190033[24] (June 29, 2023)	H230005[25] (April 25, 2024)	P250002[26] (July 24, 2025)

CE, Conformité Européenne; CDx, companion diagnostic; ECL, electrochemiluminescence; HRP, horseradish peroxidase; NAbs, neutralizing antibodies; TAbs, total antibodies; TI, transduction inhibition; TMB, 3,3′,5,5′-tetramethylbenzidine.

conditions,[31] and Genglycos was granted accelerated approval for a pediatric population with high unmet medical need, while analytical and clinical validation studies required to support an FDA-authorized CDx remain ongoing. Hemgenix bypassed the need for a CDx because its pivotal HOPE-B trial demonstrated safety and efficacy in most seropositive patients. However, regulators noted lower FIX activity in patients with higher pre-existing AAV5 NAb levels and required the Hemgenix label to reference an ongoing Anti-AAV5 Antibody Study, encouraging patient participation to assess the impact of these antibodies on bleeding risk.

ANTI-AAV ANTIBODY ASSAYS—CURRENT STATUS

Ongoing delays in clinical studies are often caused by revalidation of patient selection assays that were not initially developed under diagnostic standards. This highlights the need to distinguish between bioanalytical and diagnostic assays. Although drawing on experience from testing for anti-drug antibodies (ADAs) developed against biologic drugs, this work focuses exclusively on diagnostic assays for pre-existing AAV Abs and excludes post-treatment immunogenicity assessments.

Table 2. Comparative analysis of bioanalytic and diagnostic methods: Key characteristics and applications

Method	Bioanalytic	Diagnostic
Measure	analyte (substance) in preclinical/clinical study samples	biomarker (measurand incl. analytes) before or after treatment with a therapy
Examples of analytes/ biomarkers	small molecule or biologic drug, components or metabolites of a drug or GT, antibodies raised against a drug, PD and safety biomarker	measurands like heart rate, blood pressure, analytes like endogenous molecules (e.g., glucose, hormones, peptides, proteins, and antibodies)
Use of data	evaluate PK/PD, biodistribution, persistence, metabolism, excretion, shedding of an administered drug or immune response raised against the drug; support evaluation of safety and effectiveness of drugs and biological products	make a treatment decision, diagnose a disease, assess a prognosis
Method validation	according to its intended use; following ICH M10, Immunogenicity guidelines,[32,33] BMV for biomarkers[34]	according to its context of use; following national diagnostic guidance and standards like IVDR[7] (EU) or CLSI standards[35] (USA)
Performing laboratories	bioanalytical laboratories	diagnostic laboratories, in-house clinical laboratories, commercial provider, IVD manufacturer
Laboratory standards	GLP,[36] GCP[37]	*EU:* IVDR (IVD manufacturers and health institutions using qualifying in-house IVDs),[7] EN ISO13485:2016/ A11:2021 (IVD manufacturers),[38] ISO15189:2022[39]; *USA:* CAP/CLIA, 21 CFR part 820/FDA QMSR (IVD manufacturers)

This table was adapted based on Braun et al.[5] BMV, bioanalytical method validation; CAP, College of American Pathologists; CLIA, clinical laboratory improvement amendments; GLP, good laboratory practice; CLSI, Clinical & Laboratory Standards Institute; HDE, humanitarian device exemption; ICH, International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use; ISO, International Organization for Standardization; IVDR, In Vitro Diagnostic Regulation; PD, pharmacodynamic; PK, pharmacokinetic; PMA, premarket application.

Bioanalytical versus diagnostic testing for AAV antibodies

Bioanalytical assays typically measure drugs and metabolites, while diagnostic assays target endogenous analytes (Table 2). This distinction blurs in immune response testing, as both assess endogenous antibodies. For ADA testing, pre-dose samples are used only for analytical purposes and do not affect treatment decisions. In

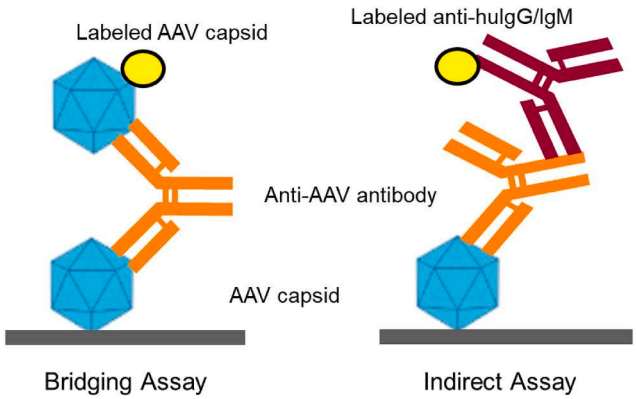


Figure 1. TAb assay

TAb detection assay formats: bridging and indirect ELISAs or ECLIAs. In the bridging format, the AAV Ab builds a bridge between the AAV capsid that is immobilized on a surface and the AAV capsid labeled with a molecule (e.g., Sulfo-Tag) that is able to generate a detectable signal (e.g., electrochemiluminescence). Alternatively, a biotinylated AAV capsid is used for detection. This allows streptavidin-conjugated HRP to bind and generate a substrate-dependent chromogenic or luminescent signal. The indirect assay format identifies antibodies that bind to the immobilized AAV capsids using a labeled secondary antibody (e.g., Sulfo-Tag, HRP), which preferentially detects IgG and IgM, if not all isotypes. Assay samples are AAV Ab-positive when their S/N ratio or relative concentration exceeds the technical cutoff—a predetermined threshold specific to each assay. The technical cutoff value (upper reference limit) of a qualitative assay is determined by assaying a statistically sufficient number of samples known to be AAV Ab-negative. In a (semi-)quantitative assay the LoD represents the technical cutoff.

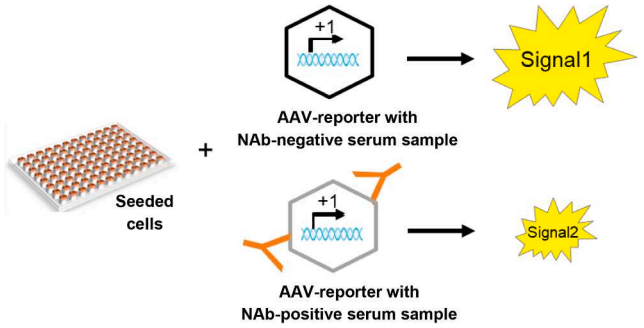


Figure 2. Functional cell-based TI assay for NAb detection

Functional cell-based TI assays are used for NAb detection. An AAV reporter vector (e.g., expressing luciferase) is preincubated with an AAV Ab-negative control serum expected to generate 100% transduction (signal S1) when the mixture is added to human cells like HEK293T. In parallel, a test serum sample is preincubated with the AAV reporter vector generating signal S2 when transducing the cells. The normalized response (NR = S2/S1) and % inhibition ((1 − NR) × 100) are calculated. Sponsors define an assay sample as NAb-positive when either >50% inhibition is achieved or when % inhibition exceeds a specific assay cutoff. The technical cutoff value (upper reference limit) of a qualitative assay is determined by assaying a statistically sufficient number of samples known to be AAV Ab-negative. In a (semi-)quantitative assay, the LoD represents the technical cutoff.

contrast, for AAV GT, pre-existing AAV Abs directly guide patient selection, requiring diagnostic-grade assays and accredited laboratories. Therefore, it is crucial to consider a method's context of use and align assay validation with the intended clinical application.

Common anti-AAV antibody assay formats

While TAb assays (Figure 1) measure both non-neutralizing and neutralizing antibodies (NAbs), offering insights into the overall presence of AAV Abs, cell-based transduction inhibition (TI) assays (also called NAb assays) (Figure 2) assess the functional ability of these antibodies to inhibit viral transduction.

Overall, TAb assays are known for their high reproducibility and fast turnaround time. These are simpler to perform and involve less complex development and validation processes than TI assays, as they do not require live cells. Consequently, TAb assays are increasingly preferred over TI assays. Nonetheless, both types of assays are employed for patient eligibility testing before AAV GT treatments and have been used to develop CDx approved in the EU[23] or USA[24–26] (Table 1).

Regulatory pathways for AAV GT CDx: classification, evidence, and co-approval

Assay classification

CDx are defined by regulatory authorities from multiple regions[7,40–42] as IVDs that are essential for the safe and effective use of their associated therapeutic products. AAV Ab assays, used to identify patients who may benefit from AAV GTs qualify as CDx. Such IVDs are most often classified as high risk, class III in the USA or class C under (EU) in vitro diagnostic regulation (IVDR),[43] and require referencing of the corresponding therapeutic in their labels. Notably, the Food and Drug Administration (FDA) has recently proposed reclassifying certain post-amendments class III nucleic acid-based test systems—including companion diagnostics—into class II with special controls, shifting them from premarket approval (PMA) requirements to 510(k) premarket notification pathway. In the USA and Japan, concurrent approval of the CDx and the corresponding therapeutic product is generally expected, whereas in Europe co-development of the CDx and the therapeutic product is recommended, if the CDx has not yet been Conformité Européenne (CE)-marked or submitted for conformity assessment at the time of market authorization, the dossier should describe the candidate CDx and provide anticipated timelines for its conformity assessment, although that assessment is not a legal prerequisite for approval of the therapeutic product.[44]

Co-development and evidence requirements for novel AAV GT CDx

While a follow-on CDx could be developed outside a therapeutic product trial by showing comparability to an approved CDx, development of a novel CDx (without a predicate) typically requires clinical trial data. Therefore, adherence to the requirements for the co-development of biomarker assays during clinical trials is essential, as it impacts clinical development strategies, which will be discussed in the following paragraphs. Regulatory approval for a novel CDx typically follows two pathways: (1) using prospective data from the

CDx prototype assay (investigational use only—IUO, under USA regulations) to determine patient eligibility in pivotal trials, or (2) conducting retrospective bridging studies comparing the CDx prototype with the assays which determined the eligibility of the patient in the trial, i.e., local laboratory tests (LLTs) or clinical trial assays (CTAs).

Biomarker assay use in clinical trials in the EU

In the EU, a biomarker test used to select patients for a clinical trial must be either CE-marked for the intended purpose in the trial or approved for use under a performance-study application (PSA)[45] before the trial starts. Under Article 5(5) of the (EU) IVDR, devices developed and used in-house by a health institution, including for use in clinical trials, are exempt from CE marking, and a PSA is generally not required.

Before placing a CDx on the EU market, the manufacturer must submit technical documentation to a notified body for conformity assessment prior to CE marking. A notified body is an independent commercial organization designated by an EU Member State to assess the conformity of medical devices and IVDs with applicable regulatory requirements under the IVDR. Some of the key parameters described in the documentation are scientific validity, analytical performance, and clinical performance. Scientific validity confirms the scientific rationale behind a biomarker and refers to the association between an analyte and a clinical condition or physiological state. Analytical performance is the ability of the device to accurately detect or measure a particular analyte. Clinical performance is the ability of the device to produce results that correlate with a particular clinical condition or physiological/pathological process or state in the target population.

For centralized authorized therapeutic products such as AAV GTs, notified bodies must request a scientific opinion from the European Medicines Agency (EMA) on suitability of the CDx for the therapeutic product.

USA requirements for investigational and marketed CDx

In the USA, IUO assays intended for patient selection, that are not exempt from investigational device exemption (IDE) requirements must undergo a study risk determination (SRD) before the start of the clinical trial. If the SRD concludes that the investigation is "non-significant risk" the study is subject to the abbreviated IDE requirements under 21 CFR 812.2(b)[46] and a formal IDE submission to FDA is not required. If the assay's use in the clinical trial is classified as a "significant risk" device study, an IDE application must be submitted to Center for Devices and Radiological Health (CDRH) at the FDA for approval of the assay before use as a CTA. Ideally, an IUO assay is used and clinically validated in the pivotal trial before a PMA application is submitted to FDA in parallel with the therapeutic product (e.g., AAV GT) Biologic License Application (BLA). The FDA CDRH will review the CDx PMA, whereas the Center for Biologics Evaluation and Research (CBER) will be responsible for the review and approval of the therapeutic product. The FDA may approve a therapeutic product without prior approval or clearance of a CDx when the therapeutic's benefits clearly outweigh the risks posed by the absence of an approved or cleared CDx. In such cases, the agency generally expects subsequent CDx approval or clearance,[40,47] typically outlined as a post-marketing commitment in the FDA approval letter. In June 2023, the FDA launched a voluntary oncology pilot program to define performance expectations for diagnostic tests used for patient selection, acknowledging that lab developed tests may be used for treatment decisions in cases where an approved CDx is not yet available.

In Table 3, we provide an overview of processes and documentation requirements for clinical development of IVDs under EU and USA regulations.

Global validation standards and remaining gaps

Unlike the USA, EU lacks specific guidelines providing detailed instructions for conducting required analytical validation of IVDs. Consequently, sponsors and laboratories outside the USA frequently refer to American guidance documents, particularly the Clinical and Laboratory Standards Institute (CLSI) standards (Table 4). This is especially common when seeking global approval for an assay.

Current CDx validation requirements: Implication on cutoff selection and patient's access to AAV GT

Existing regulatory frameworks mandate that analytical validation of assays for patient selection for investigational therapies must be performed in suitable laboratory settings prior to clinical implementation. Although validation requirements share common elements between EU and USA, the EU IVDR and Medical Device Coordination Group (MDCG) guidance establish performance-evaluation principles and are generally less prescriptive about step-by-step experimental protocols or standardized study templates than CLSI standards. In the USA, sponsors can obtain in-depth alignment on analytical and clinical validation for CDx through FDA QSubmission Program (PreSubmissions/Pre-subs) consultations.[61] In contrast, in the EU, due to key European Database on Medical Devices (EUDAMED) modules in the EU being still offline and formal routes to consult notified bodies on study design being limited, manufacturers often lack reliable access to predicate technical documentation or meaningful summaries from CE-marked AAV CDx and therefore adopt conservative validation approaches.

In the EU, the possibility of worst-case validation allows scientists to validate assays by testing at the minimum and maximum boundaries of an assay's linear concentration range, thereby establishing validity across the entire spectrum between these points. However, researchers must provide robust scientific rationale for the validation approach that adequately supports a specific cutoff value across this concentration range. In the context of AAV GT, this approach means that any patient selection threshold falling within the validated AAV Ab concentration range could be considered validated for use in clinical studies.

Table 3. IVDR performance study application Dossier requirements vs. investigational device exemption requirements under 21 CFR Part 812[46]

Aspect	IVDR—Performance study application (EU)	IDE—Investigational device exemption (USA)
Regulatory basis	regulation (EU) 2017/746 (IVDR),[7] MDCG guidance (e.g., MDCG 2022–19,[48] MDCG 2025–5)[45]	21 CFR Part 812,[46] FDA guidance on IDE responsibilities and risk determination[49]
Purpose	authorize clinical IVD studies intended to demonstrate safety and performance, including interventional or high-risk procedures for patients	allow investigational use of a device in clinical studies
Prerequisites	applies, when patients are enrolled or testing occurs in any IVDR jurisdiction, including the EU and EEA	IDE applies when patients are enrolled or testing occurs in the USA; IRB reviews and documents NSR/SR classification; NSR studies follow abbreviated IDE requirements, FDA IDE approval is not required; SR studies require FDA IDE approval before study initiation
Key deliverables	*PSA dossier including:*	*IDE application including:*
	Application form, including list of sites, PIs and IRBs	list of sites, PIs and IRBs
	PEP & PSP	investigational plan
	device label	device label
	device description (included in IB)	device description
	scientific and analytical performance documentation (included in IB)	report of prior investigations (including report of all prior clinical, animal, and laboratory testing)
	risk management documentation (included in IB)	risk analysis (included in investigational plan)
	list of general safety and performance requirements (included in IB)	
	declaration of conformity and general data protection procedures	
	instruction for use (if applicable)	instruction for use
	informed consent documents (if applicable)	informed consent documents
	financial disclosure statements (if applicable)	financial disclosure statements
	proof of insurance, suitability of site, and investigator (if applicable)	signed investigator agreements/certification
Timelines	NCA review 55 or 75 days (including validation of submission)	FDA review 30 days
Regulatory oversight	NCA	CDRH (FDA)

CDRH, Center for Devices and Radiological Health; CFR, Code of Federal Regulations; EEA, European Economic Area; EU, European Union; FDA, Food and Drug Administration; IB, investigator brochure; IDE, investigational device exemption; IRB, Institutional Review Board; IVD, in vitro diagnostic; IVDR, In Vitro Diagnostic Regulation; MDCG, Medical Device Coordination Group; NCA, National Competent Authority; NSR, non-significant risk; PEP, performance evaluation plan; PI, principal investigator; PSA, performance study application; PSP, performance study plan, SR, significant risk.

In the USA, CLSI standards can create a substantial validation burden because assay performance must be demonstrated at clinically relevant decision limits, which may complicate worst-case assay validation approaches.[54] Following CLSI EP12,[54] a clinically relevant patient-selection cutoff should be established and its performance characterized before study initiation, creating challenges during early clinical development when the optimal cutoff may not yet be known. Based on author's experiences, regulatory authorities and diagnostic partners frequently expect performance to be evaluated within a region around the clinical decision limit, often approximately ±20% of the selected cutoff, although CLSI EP12 does not prescribe a universal ±20% acceptance interval.

Notably, the EU and the FDA do not differentiate between early phase clinical trials and pivotal studies, imposing additional burden during an early phase when assay cutoff values may still be premature for their clinical relevance. Therefore, sponsors often use conservative cutoffs precluding some patients with life-threatening diseases from accessing potentially life-saving therapies.

ANTI-AAV ANTIBODY ASSAYS—FUTURE PERSPECTIVE

More flexibility in assay validation is required and alternative assay designs are necessary to establish clinically meaningful cutoffs for patient screening. Embracing these changes would accelerate patient access to novel AAV GTs that utilize the same AAV serotype, while

Table 4. CLSI standards relevant for investigational and market authorizations

CLSI standard	Title
EP05[50]	Evaluation of Precision of Quantitative Measurement Procedures
EP06[51]	Evaluation of Linearity of Quantitative Measurement Procedures
EP07,[52] EP37[53]	Interference Testing in Clinical Chemistry, Supplemental Tables for Interference Testing in Clinical Chemistry
EP12[54]	Evaluation of Qualitative, Binary Output Examination Performance
EP15-A3[55]	User Verification of Precision and Estimation of Bias
EP17-A2[56]	Evaluation of Detection Capability for Clinical Laboratory Measurement Procedures
EP24-A2[57]	Assessment of the Diagnostic Accuracy of Laboratory Tests Using Receiver Operating Characteristic Curves
EP25[58]	Evaluation of Stability of In Vitro Medical Laboratory Test Reagents
EP28-A3c[59]	Defining, Establishing, and Verifying Reference Intervals in the Clinical Laboratory
EP34[60]	Establishing and Verifying an Extended Measuring Interval Through Specimen Dilution and Spiking

also expanding treatment opportunities for individuals with high pre-existing AAV Ab levels.

While it is understood that broad diagnostic guidelines must mandate the validation of numerous parameters to cover a wide array of assay types and analytes, the relevance of each validation parameter for an individual assay system strongly depends on the analyte and assay format used. In AAV Ab assays, the target analytes—antibodies—are already highly characterized. Furthermore, bridging ECLIAs have been extensively studied within bioanalytical laboratories.[8,9] Their strengths and limitations are well understood. Given this extensive scientific experience regarding both the antibody analytes and the ECLIA methodology, the regulatory validation burden could be safely streamlined. Reducing these requirements would not compromise the accuracy, quality, or reliability of the diagnostic results.

In the following sections, we propose an AAV serotype IVD assay for clinical development, outlining key validation and potential revalidation considerations for CDx use, including worst-case validation where appropriate. Our recommendations focus on TAb assays, because of the broad scientific experience and the industry preference for these assays due to their relative simplicity and robustness versus cell-based TI assays.

AAV serotype IVD per AAV serotype as game changer

Implementing AAV serotype IVDs that are validated across a wide linear AAV Ab concentration range for screening trial participants could overcome current clinical challenges. This approach could enable stepwise identification of the most clinically relevant cutoff in early-phase studies employing multiple cutoffs. Consequently, this data-driven strategy would expand treatment eligibility for patients with higher baseline AAV Ab levels and accelerate patient access to emerging AAV GTs that utilize the same AAV serotype. Additionally, this strategy supports the creation of multiple CDx from a single foundational AAV serotype IVD. It would also standardize AAV Ab testing, enhance comparability of data across different studies, and lower overall development expenses. Ultimately, this more flexible framework could prevent costly delays in launching both first-in-human (FiH) and later-stage clinical trials, particularly when clinical data dictate cutoff adjustments.

AAV serotype IVD design and test result reporting

We recommend establishing bridging ECLIAs or, alternatively, bridging enzyme-linked immunosorbent assays (ELISAs) employing horseradish peroxidase (HRP) with a chemiluminescent substrate. Comparative data will be published separately by ARDAT. In addition, we recommend validation of a broad linear range for the determination of AAV Ab concentrations using a reference antibody.

Employing a monoclonal reference antibody for relative AAV Ab concentration determination would facilitate assay transfer between laboratories and companies. It is crucial for developing assay kits to support global testing and allows for long-term consistency, standardization, and comparison across different methods and laboratories. Executing field studies (e.g., conducted for FVIII products)[62] would be achievable if a centralized organization, like the World Health Organization (WHO), were to maintain and distribute standardized reference antibodies. Furthermore, including a broad predefined AAV Ab quantification range in the initial submission could significantly reduce the need for supplemental IDEs or substantial PSA amendments, as it would allow evaluation of multiple cutoffs within the approved investigational framework during clinical development.

Instead of calculating a statistical technical cutoff based on seronegative samples, we recommend using bridging TAb assays for AAV antibody assessment and applying the limit of quantitation (LoQ) for sensitivity assessment and the limit of detection (LoD) as the technical cutoff (Figure 3), as described in CLSI EP17-A.[56] Although using the LoD as the technical cutoff may slightly reduce assay sensitivity, we expect the impact to be minimal given the inherently high sensitivity and low background of bridging TAb assays compared with indirect TAb and NAb assays. Additional supporting data generated by ARDAT will be published separately. Pooled matrix (e.g., serum and plasma) from multiple seronegative individuals can be used as negative control to monitor assay performance. Importantly, in accordance with CLSI standards, AAV Ab-positive validation samples must consist of authentic human specimens. Different AAV Ab levels can be generated by diluting high-level positive samples with seronegative human matrix. In contrast, seronegative human samples spiked with polyclonal or monoclonal AAV Ab

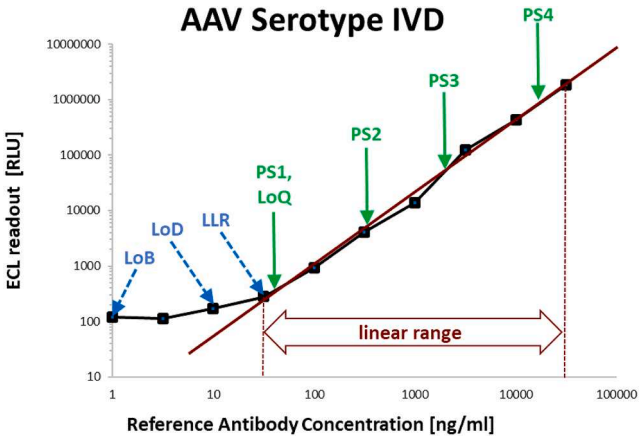


Figure 3. AAV serotype IVD

This figure is a schematic representation intended to illustrate the concept of an AAV Serotype IVD and is not based on experimental data. Potential patient selection cutoffs (PS1-PS4) can be defined as relative AAV Ab concentration within the validated linear range of the assay. The limit of blank (LoB) is the highest value in a series of results on a sample that contains no analyte. The LoB refers to an observed test result, while all of the other limits refer to actual concentrations of the analyte. The LoQ is the lowest actual concentration at which the analyte is reliably detected and at which the uncertainty of the observed test result is less than or equal to the goal set by the laboratory, or by the manufacturer of the method. The LoD is the actual concentration at which an observed test result is very likely to exceed the LoB and may therefore be declared as "detected." The lower limit of linear range (LLR) is the lowest concentration at which the method response has a linear relationship with the true concentration. It will always be that LoB < LoD ≤ LoQ. The other limits may not have a consistent relationship to each other and some of them may be the same. ECL, electrochemiluminescence; RLU, relative light units.

preparations are not acceptable for validation purposes. Such spiked samples may, however, be used as system suitability controls during patient sample analysis.

Because antibodies vary widely, biologic treatment-induced ADAs are usually assessed by titer rather than concentration, as equal concentrations can differ in binding or neutralizing capacity and thus affect pharmacokinetics, efficacy, and safety differently. For AAV GT patient selection, however, the key question is only whether antibody levels exceed a defined cutoff. Since titers, signal-to-noise (S/N) ratios, and relative concentrations should correlate linearly within the assay range,[63] any consistent metric should be suitable in the absence of a hook effect. We therefore recommend using relative AAV Ab concentrations based on a monoclonal reference antibody to define both the cutoff and patient sample results.

We recommend avoiding titer validation for patient selection assays due to added complexity, time, cost, and error risk. Relative concentration testing is simpler (requiring fewer wells and dilutions), while titration involves multiple dilution steps and error propagation. For example, reaching a titer of 1,280 requires six 1:1-dilution steps beyond a minimum required dilution (MRD) of 1:20. In contrast, measuring relative concentration only requires diluting the sample to the MRD and classifying it as above or below the cutoff. We recommend testing at three dilutions in case a hook effect is observed during assay validation. We recommend conducting precise quantification separately as exploratory analysis, if needed.

Discussion of relevant validation parameters

Limited reagent stability or cross-reactivity with non-target antibodies reduces the availability of active assay reagents. These limitations primarily impact the upper and lower extremes of an assay's linear range. Therefore, validating these factors using high- and low-AAV Ab concentration samples at the assay's boundary limits should be sufficient, provided stability is demonstrated.[64,65]

Substances that interfere by binding to the analyte (such as an endogenous interferent or exogenous concomitant medication) disproportionately impact low-concentration samples. As the analyte concentration increases, it overwhelms the fixed amount of interferent. Because the most severe interference occurs at the lower end of the linear range, testing for interference at this lower boundary is generally adequate.

Optical crosstalk and physical cross-contamination (e.g., through pipette tip contamination) are most problematic when a high-signal sample is adjacent to a low-signal sample. For example, a 0.1% signal spillover from a 100,000 RLU sample adds 100 RLU to neighboring wells. This causes a massive 100% increase for a baseline signal of 100 RLU, but only a 1% increase for a 10,000 RLU signal. To mitigate these risks, strategic plate layouts (e.g., placing buffer in adjacent wells) can be used for patient sample testing. Furthermore, ECL systems only require a single crosstalk evaluation per reader, assuming plate type and signal range remain consistent. These findings can be scientifically extended to all assays whose boundary values and the expected values of the test samples (e.g., pre-existing AAV Ab concentration range in human samples) remain within the assayed parameters. Chromogenic assays experience negligible crosstalk due to build-in optical isolation in most plate readers.

Analyte stability testing for AAV Abs in subject samples is still performed by diagnostic partners although research has consistently shown that antibodies targeting various proteins or viruses demonstrate remarkable stability in human serum across different conditions and concentration ranges. Antibodies remained stable for 72 h at 22°C, 2 weeks at 4°C, and 6.3 years at −20°C,[66,67] also applying to common transport conditions using dry ice. Further studies confirmed antibody stability after multiple freeze-thaw cycles (ranging from 3 to 176).[66,67] The FDA recognizes that establishing analyte stability for ADAs in subject samples is generally not feasible and therefore no longer requires formal subject-sample stability assessments. Instead, samples should be stored under conditions that preserve antibody reactivity and freeze-thaw cycles should be minimized through appropriate aliquoting.[32] From a scientific standpoint, it is challenging to justify why FDA/CDRH or notified bodies would still mandate such studies. We suggest engaging with relevant regulatory authorities to discuss the necessity of AAV Ab stability

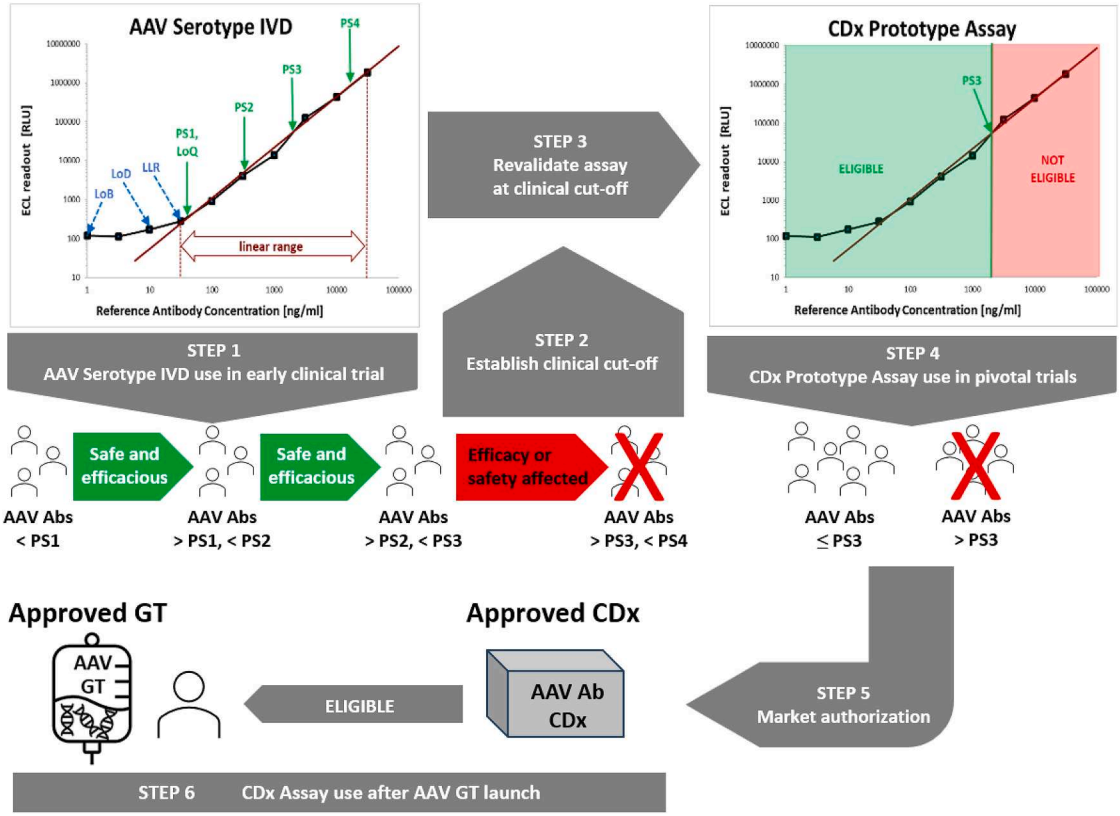


Figure 4. Assay journey from AAV serotype IVD to an approved CDx

Step 1: use the AAV serotype IVD for patient selection in early clinical trials. Sequentially increase the AAV Ab patient inclusion cutoff (PS1, PS2, PS3, etc.) as long as all enrolled patients continue to demonstrate acceptable safety and efficacy. Step 2: identify the highest antibody cutoff that does not adversely affect safety or efficacy and establish it as the clinically relevant patient selection cutoff. Step 3: revalidate the relevant assay performance characteristics at the established clinical cutoff. Step 4: freeze the assay design and use the CDx Prototype assay for patient selection in pivotal clinical trials. Step 5: submit the assay for market authorization after clinically verifying both the assay performance and the selected patient selection cutoff. Step 6: use the approved CDx assay for routine patient selection following commercial launch of the AAV GT. ECL, electrochemiluminescence; LLR, lower limit of linear range; LoB, limit of blank; LoD, limit of detection; LoQ, limit of quantitation; PS, potential patient selection cutoff; RLU, relative light units.

assessment in subject samples, using the cited research and FDA guidance as supporting evidence for reconsideration.

Short-term, freeze-thaw, refrigerated, and room temperature stability may still be relevant for positive-control AAV Abs used as reference standards or system-suitability controls. Reagent stability should be addressed separately from subject-sample stability.[32] Assay precision represents a critical parameter in diagnostic methodology and should be evaluated across the complete measurement range. Within the linear range, precision should maintain consistency without significant variations. CLSI EP05[50] provides guidance on estimating assay precision. For clinical decision limits, acceptance criteria of ±20% around the target concentration are frequently applied; however, such thresholds are assay- and application-specific and are not prescribed by CLSI EP05.

Overall, these validation considerations support a streamlined, science-based validation strategy for AAV Ab assays. Focusing validation on boundary conditions could simplify assay development, validation, and revalidation without compromising assay reliability.

Application of the AAV serotype IVD in clinical development

A single AAV serotype IVD assay could be used for studies involving different AAV GTs that use the same serotype but require different AAV Ab cutoff levels due to factors such as dosing differences and ROA. It could also be used when a single AAV GT is administered to different patient populations requiring different cutoffs based on risk-benefit considerations. AAV serotype IVDs would be particularly valuable when they could be used in early-phase clinical trials to determine the relevant clinical cutoff as illustrated in Figure 4. Following this approach, seronegative patients would be treated first and individuals with higher AAV Ab levels would progressively be included thereafter by incrementally increasing the selection cutoff within the AAV serotype IVDs linear range. When a correlation of a specific AAV Ab level with either AAV Ab-related SAEs or reduced treatment efficacy is observed, enrollment of patients at or above this

antibody range would be stopped. A clinically meaningful cutoff could subsequently be established based on these observations. This strategy would improve patient risk-benefit profiles and enhance study outcomes while expanding access to potentially life-saving therapies for all suitable candidates. However, in clinical trials for (ultra-)rare diseases with very limited patient numbers, establishing a correlation between pre-existing AAV Abs and efficacy or safety outcomes may be challenging.

Use of a non-approved AAV serotype IVD or an approved CDx in new clinical studies (e.g., different AAV GT or indication) would require an IDE or PSA submission, as the intended use is not covered. However, this approach would avoid *de novo* assay development and shorten validation timelines, enabling faster study initiation. This would be primarily applicable to programs using the same serotype within a single company, or where companies funding the AAV serotype IVD or CDx validation through their diagnostic partner out-license the assay to other companies.

An AAV serotype IVD could receive independent approval as an IVD—for example, for determining AAV5 antibody concentrations. In this case, it may be worthwhile to explore with the respective EU authorities whether the approved test could be used for patient selection in clinical studies without PSA. In the USA, it may be useful to engage with the FDA/CDRH to assess whether such an assay could be considered a "non-significant risk" device, thus qualifying for an abbreviated IDE pathway (21 CFR 812.2(b)).[35] A specific CDx would be required at the time of market authorization of the GT.

Before an AAV serotype IVD is used for a slightly modified capsid, a scientific justification should demonstrate its suitability for detecting antibodies against the modified variant. For example, substituting the original capsid with the modified one in the assay and testing a representative set of human samples could confirm comparable seroprevalence and concentration profiles. This approach should be aligned with relevant regulatory authorities.

Revalidation of the AAV serotype IVD for use as a prototype CDx in pivotal studies

After establishing a clinically relevant cutoff during early studies, regulatory authorities may require revalidation of the AAV serotype IVD specifically at this threshold for its use in pivotal studies (Figure 4). We recommend, at a minimum, revalidating precision for each new cutoff, as this represents a critical diagnostic parameter, though it typically remains stable within the linear range. We also suggest reassessing interference from potential endogenous interferents, common medications in patient population, and disease-specific alternative treatments at the new cutoff if not previously validated sufficiently for the AAV serotype IVD.

Regulatory bodies may additionally request revalidation of analyte and reagent stability, cross-reactivity, interference, cross-talk, and cross-contamination directly at the selected cutoff. We suggest engaging with the regulatory bodies to discuss the need for these additional revalidation experiments considering the scientific aspects presented in this paper. If regulatory authorities follow the scientific rationale presented here, substantial delays in clinical study initiation could be avoided, thereby accelerating patient access to GT. Based on the authors' collective experience from supporting CDx and patient-selection assay development programs, the process of establishing a technical cutoff, completing the required validation activities, and implementing the assay for clinical use can delay first-in-human studies by approximately 1.5 years or longer.

Stepwise validation approach for "single use" patient selection assays

If an AAV capsid is intended to be used for a single AAV GT application only, the validation effort for an AAV serotype IVD may exceed the effort required for a one-time validation of the CDx. In such cases, we suggest engaging with the regulatory bodies to discuss the possibility of a stepwise validation approach. This would allow for the initiation of the study without delays caused by assay validation.

Before using the assay for patient selection in the FiH study, the minimum required parameters and their validation scope should be defined in alignment with the relevant regulatory authorities. Based on the authors' experience, we recommend completing at least partial precision validation, sensitivity, reactivity/true positivity, hook effect assessment, and endogenous (e.g., triglycerides and hemoglobin) and exogenous interference (common medications and disease-specific therapies) testing before FiH use.

Full precision, expected-value studies, population characteristics, and ruggedness/robustness should be completed before pivotal-study initiation. Crosstalk and cross-contamination assessments, if necessary, should also be completed before pivotal studies are started, provided a plate layout is chosen that limits both phenomena.

From a scientific perspective, repeated antibody-stability assessment in subject samples should not be required if samples are stored and handled under conditions that preserve antibody reactivity; however, any residual stability expectations should be aligned with regulators. If subject-sample stability assessment is still deemed necessary, we suggest discussing the extent of the stability study with the regulatory bodies based on scientific rationale. Limited short-term, transport, and room temperature handling assessments should generally be sufficient before FiH when single-use aliquots are used. Reagent stability may be minimized by using single-use aliquots and fresh reagents within their specified shelf life, with further evaluation deferred if necessary.

It is critically important to ensure that the assay is established in a laboratory suitable for patient selection assays and that real human samples, ideally those of the intention-to-treat patient population, are used as validation samples.

Lab-developed tests

In the USA, beyond previously discussed IDE considerations,[6] lab-developed tests may be used as clinical trial assays instead of kitted IVDs when conducted in College of American Pathologists (CAP)-accredited/Clinical Laboratory Improvement Amendments (CLIA)-certified laboratories; however, these assays cannot support market authorization.

Establish in-house assay in a health institution

In the EU, the IVDR article 5(5) exemption[7] applies to EU-based health institutions operating on a non-industrial scale without transferring devices to other legal entities; since May 2024, in-house IVDs must meet Annex I[7] safety and performance requirements, implement a quality management system (ISO 15189,[39] with ISO 13485[42] principles where relevant), and maintain appropriate documentation. Proposed amendments may further increase flexibility, including for central laboratories conducting clinical trials to fall under the in-house exemption.[68]

In the UK, investigational IVDs fall under UK MDR 2002 No. 618[69] and require the Medicines and Healthcare products Regulatory Agency review of technical documentation (e.g., Study Checklist or a Tabular Summary) before study initiation.

SUMMARY AND CONCLUSIONS

The development of appropriate patient selection methods for AAV GT requires a critical balance between ensuring treatment efficacy and safety and maximizing patient access to potentially transformative therapies. Current regulatory approaches, while intended to protect patients, may inadvertently create barriers that delay clinical development and limit access for some in principle eligible patients.

Therefore, it would be beneficial to enhance consistency across regulatory frameworks and revise validation criteria specifically for AAV Ab analytics and the CDx assay formats commonly employed in AAV GT based on scientific evidence. Antibodies are well-characterized analytes with extraordinarily high stability. Additionally, bridging ECLIA formats have been extensively characterized during method validation for ADA determination in biologics projects. The insights gained from these studies should enable adjustment of validation requirements without compromising assay quality. Based on these insights, we suggest the implementation of AAV Serotype IVDs for use in early clinical studies along with limited revalidation at the established clinical cutoff before its use as prototype CDx in pivotal trials.

The benefits of the AAV Serotype IVD approach are numerous. First, this approach would allow for the gradual inclusion of patients with increasing AAV Ab levels into clinical trials by utilizing adaptive study designs. This would enable the determination of optimal cutoffs based on actual clinical outcomes without increasing risks for patients or inefficiently treating some. Second, this approach would enable the use of the same base assay for multiple products with the same AAV serotype, even if the products rely on different clinical cutoffs for patient selection. Furthermore, the proposed AAV serotype IVD assays would be comparable due to their concentration-based methodology employing monoclonal reference AAV Abs for calibration. Conducting field studies would become feasible if a central institution, such as the WHO, stores and provides appropriate reference antibodies for comparative testing in various laboratories. Finally, the cost and time savings in analytical and partially in clinical validation could make the use of the AAV Serotype IVD approach attractive and contribute to assay harmonization. This approach has the potential to lower the barrier for initiating FiH studies, ultimately leading to faster market access for AAV GTs and earlier availability for patients. In some cases, the cost savings could make an AAV GT economically viable, especially for smaller biotech companies or when targeting ultra-rare diseases.

We would like to encourage researchers, scientific consortia, working groups, AAV GT sponsors, and regulatory authorities to actively participate in reviewing the ARDAT initiative's proposals and to explore their implementation through a dedicated guideline for AAV Ab assays in GT. This effort aims to expedite therapeutic access for a greater number of patients.

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AUTHOR CONTRIBUTIONS

M.B. drafted and finalized the manuscript; all authors have contributed to the contents, review, and references of the article.

DECLARATION OF INTERESTS

The authors are listed on the title page of the manuscript. The authors are employed by, are shareholders of, and receive compensation from companies that are involved in development of GT modality therapeutics. M.A. is co-founder and shareholder of Blackfin Bio and Crucible Therapeutics. The authors have no other relevant affiliations or financial involvements with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed. No writing assistance was utilized in the production of this manuscript. The views and conclusions presented in this paper are those of the authors and do not necessarily reflect the representative affiliation or company's position on the subject.

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