Immunoassay automation is driving advancements in cell and gene therapies

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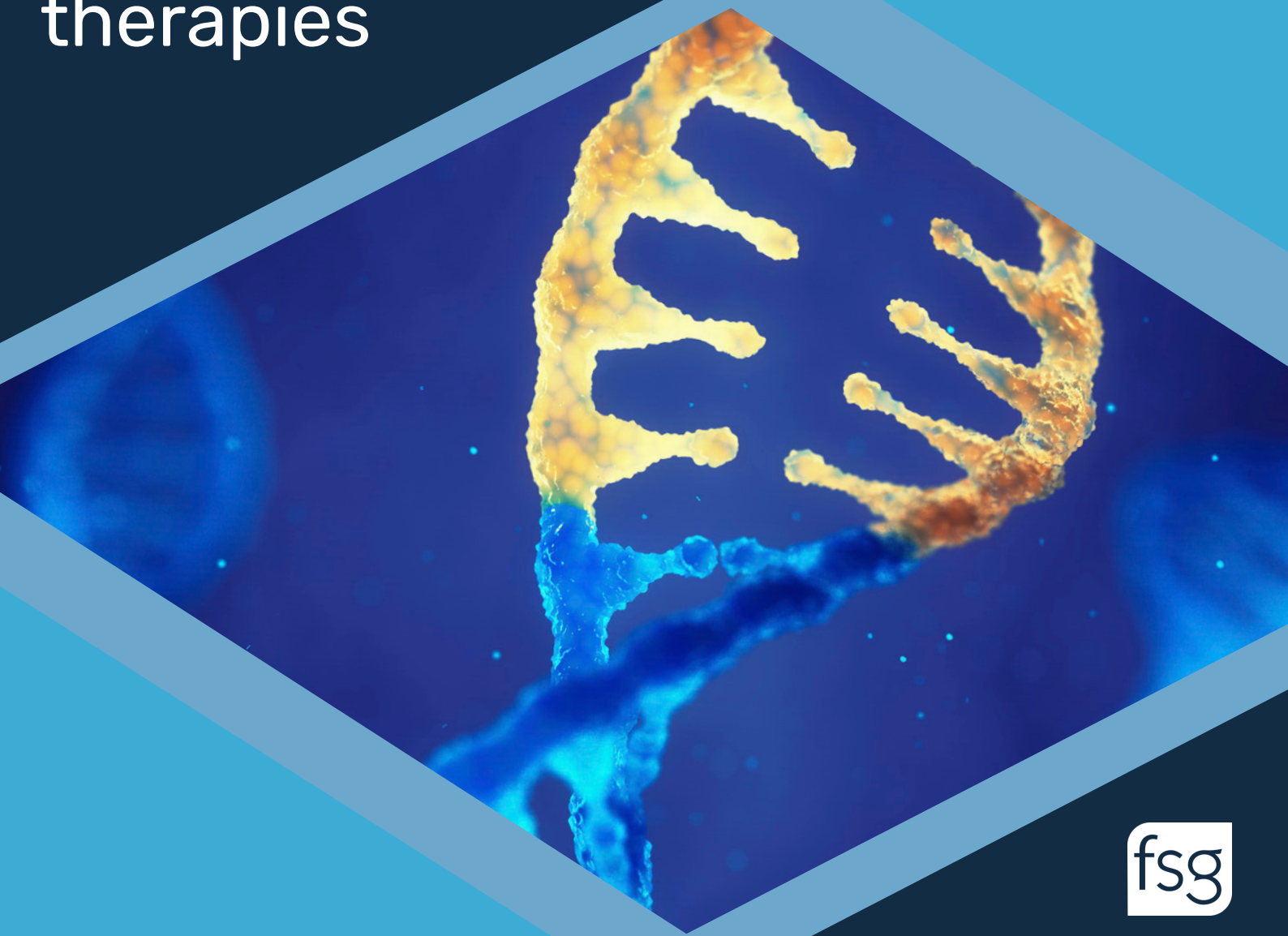

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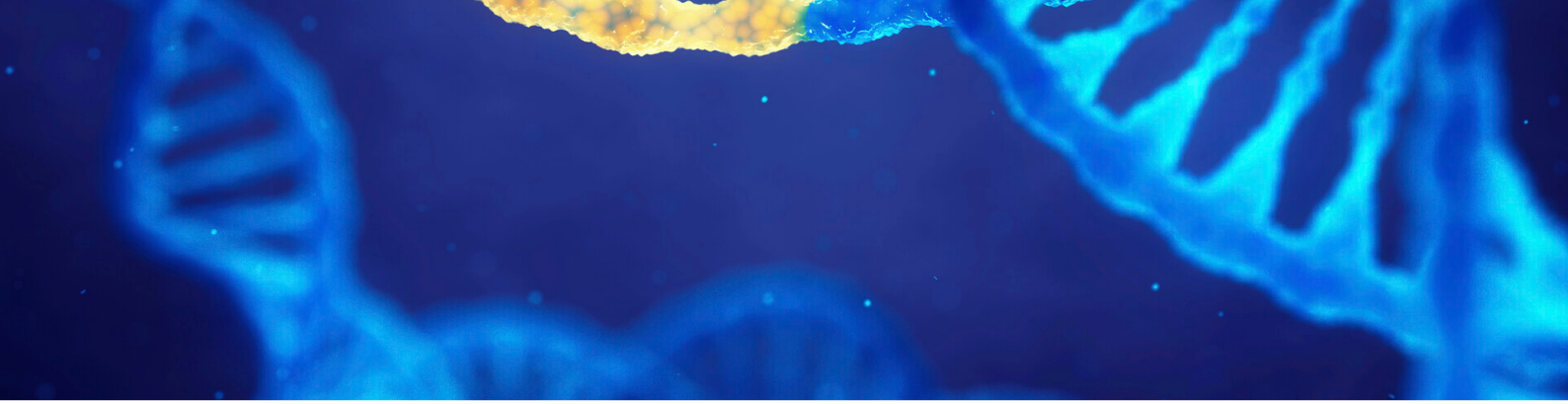

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Technology Digest: immunoassay automation is driving advancements in cell and gene therapies

by Naamah Maundrell
(Editor-in-Chief, Bioanalysis Zone)

Meeting the demands of personalized medicine

Cell and gene therapies (CGT) have ushered in a new era of personalized medicine and are a promising, growing area of research [1]. Personalized medicine is an approach that considers how individuals respond differently to medicines and treatment. By tailoring a treatment to an individual's specific disease state and unique physiology, it sidesteps the generic one-size-fits-all approach [2].

Since the first successful gene therapy study for humans was conducted back in 1989, thousands of CGT-related clinical trials have been completed. Gene therapy is the process that delivers therapeutic nucleic acids into a patient's cells to modify gene expression, and ultimately treats or prevents disease [3]. Cell therapies, by contrast, leverage a host's own cells to combat disease and may involve transgene expression to direct cells against specific targets. Disease treatment strategies that have benefited from CGT products include types of cancer, hematological, immunological, neurodegenerative, and metabolic disorders. Viral and nonviral vectors are used in cell and gene therapies to deliver the DNA or RNA into the host cells. Both come with inherent advantages and disadvantages, which is why it is important to choose the appropriate vector when developing the therapy. A common gene therapy vector is adeno-associated virus (AAV) as it is non-pathogenic, limiting the immune response. Different AAV serotypes infect distinct types of tissues, which creates more specific tissue targeting compared to other viral vectors [1].

Over the last few years, ground-breaking CGT drugs have been developed, but despite remarkable progress and an increased growth and investment within the field, CGT is still in its infancy. Despite many ongoing clinical trials, the number of approved therapies is still small and address a limited number of diseases. Developers in today's CGT market seek not only to target new diseases, but also to mitigate side effects and control costs. As such, researchers are intensifying their efforts to improve CGT drugs, along with the methods and platforms used to study them [2].

To ensure consistent results, effective analytical methods and platforms are vital from discovery to the manufacturing of CGT products [1]. Researchers rely on different analytical techniques to assess critical quality attributes as part of the viral quantification process. Immunoassays are a key bioanalytical tool used throughout CGT development. These assays are used to evaluate immunogenicity responses to gene therapy treatment as well as for quantifying viral vector titer.

Immunoassays are also used for characterization of cultured cells during expansion and post gene transduction within the cell therapy space. Traditional plate-based assays, such as ELISAs, can often be time consuming with lengthy assay development and analysis time [4]. To meet the growing need for fast and reproducible immunoassay data more automated analytical solutions have been developed, delivering more consistent results, and increasing analytical capacity and throughput [5].

Reaping the benefits of immunoassay automation

The advancement of CGT is driven by process improvements and technological innovations resulting in faster R&D, improved manufacturability, and more rigorous quality control. New automated platforms, such as the Ella™ platform, provide improved assay sensitivity and reproducibility while addressing issues of workflow and ease of use. CGT developers are leveraging these new automated immunoassay solutions to quickly and reliably quantitate viral vectors, characterize cultured immune cells, and investigate immune response [1].

Immunoassay platforms are valuable as they generate high quality data for chemistry, manufacturing, and control (CMC) and final product manufacturing. Key aspects to consider when choosing a platform include automation, scalability, method transferability and sample volume [1]. To deliver reliable results, it is important to use a high-quality platform. Automated immunoassay platforms from ProteinSimple, such as the Ella™ platform, can eliminate the hands-on steps that come with traditional immunoassays. By this, it reduces human error and increases result collection time [1,4].

Raising the bar in cell and gene therapy development

Offering a viable alternative to traditional plate-based ELISAs, fully automated immunoassays can provide a high level of throughput, reproducibility, and ease of assay transfer. One example being Simple Plex™ assay run on the Ella™ platform. These platforms enable multianalyte analysis from the same sample, providing researchers with a highly effective tool for cytokine profiling; measuring viral titer and process impurities; and characterizing cell expansion and functionality [1]. Additionally, the automated assay analysis software is 21-CFR Part 11 compliant [4].

> *"Process automation provides the dual benefits of time savings and reduction in operator-dependent variability. Simple Plex assays on Ella™ deliver these advantages along with unmatched ease of use to boost productivity and accelerate process development," stated Nathan Steere, Commercial Product Manager at Bio-Techne (MN, USA).*
>
> *Rajiv Pande, Director at Bio-Techne (MN, USA), added: "Furthermore, as a CGT drug advances to clinical studies and trials, Ella™ can be utilized as the platform of choice for real-time immune profiling."*

Automated immunoassays can aid in accelerating process development for different CGT solutions, enabling the characterization of bioprocesses, the analysis of biomarkers and the rapid monitoring of immune profiles [1]. Below are some common use case scenarios for automated immunoassays in CGT research.

Viral vector characterization

These platforms can be used to automate viral vector physical titer quantitation [6]. For example, the Simple Plex AAV2 assay, utilizes AAV2 antibodies from industry leader PROGEN to quantitate viral vectors. The assay offers a broad dynamic range and proven specificity, helping to ensure fast and reproducible viral titration across process matrices [4,7].

Lentiviral vectors (LVV) are an important tool for vaccine development and are one of the fastest growing vectors utilized within the cell and gene therapy industry. However, inefficiencies in LVV manufacturing have led to poor upstream yields. Using the Simple Plex HIV and p24 Lentiviral Titer Assay, researchers are able to compliment infectious titer data with physical titer data. To optimize their workflow, they are able to use the ratio of infectious titer to physical titer to calculate the specific lentiviral infectivity [8].

Characterization of cultured immune cells prior to adoptive cell transfer

To ensure drug safety and stability product characterization is essential. CAR-T cell therapies involve isolating an individual's T cells and then genetically modifying them to express a CAR on their surface, which is capable of recognizing tumor-associated antigens. Through using immunoassays, the engineered cells can be characterized, and an appropriate dose established, prior to adoptive cell transfer [1].

Immune response research

For cell therapies identification and monitoring of biomarkers related to T-cell activation and associated cytokine release syndrome (CRS) is required to fully understand the host response. Such biomarkers can be used to guide development of candidate therapies and help monitor toxicity by providing an evaluation of a patient's response. Automated immunoassay platforms, such as Ella™ with Simple Plex multianalyte assays, can measure a broad panel of analytes enabling fast and accurate quantitation [1].

Summary

Immunoassays provide researchers with an important bioanalytical tool that can be used throughout CGT development, helping to generate high quality data for CMC and final product manufacturing [1]. Automated immunoassay platforms, such as Ella™, can eliminate the hands-on steps that come with traditional immunoassays and therefore reduce human error, increase throughput, and accelerate results [4].

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Review

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Bioanalysis

Challenges and opportunities in bioanalytical support for gene therapy medicinal product development

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Gene and nucleic acid therapies have demonstrated patient benefits to address unmet medical needs. Beside considerations regarding the biological nature of the gene therapy, the quality of bioanalytical methods plays an important role in ensuring the success of these novel therapies. Inconsistent approaches among bioanalytical labs during preclinical and clinical phases have been observed. There are many underlying reasons for this inconsistency. Various platforms and reagents used in quantitative methods, lacking of detailed regulatory guidance on method validation and uncertainty of immunogenicity strategy in supporting gene therapy may all be influential. This review summarizes recent practices and considerations in bioanalytical support of pharmacokinetics/pharmacodynamics and immunogenicity evaluations in gene therapy development with insight into method design, development and validations.

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The first successful gene therapy study for humans was conducted in May 1989 [1]. Since then, over 2300 gene therapy related clinical trials have been conducted. In recent years, an increasing number of gene- and nucleic acid-based products have been advanced in late clinical development phases [2]. Gene therapy is an experimental technique that delivers therapeutic nucleic acid polymers into patient's cells to modify gene expression at DNA or RNA level to treat or prevent disease. Because of its unique ability to target 'undruggable' targets, gene therapy has long formed the third major drug platform in addition to traditional small- and large-molecule therapeutics. Many different terms have been used across industry, including gene therapy, nucleic acid-based therapy, oligonucleotide therapy and DNA/mRNA therapy. The EMA guidelines published in 2015 provide a more comprehensive definition as: "Gene therapy medicinal products (GTMPs) generally consist of a vector or delivery formulation/system containing a genetic construct engineered to express a specific therapeutic sequence or protein responsible for the regulation, repair, addition or deletion of a genetic sequence" [3]. To keep consistency, we use GTMP in this manuscript to describe all products that modify gene expression on DNA or RNA level in order to achieve therapeutic effects.

GTMPs have demonstrated patient benefits in specific areas of therapeutics to address unmet medical needs. Table 1 presents examples of current approved GTMPs and GTMP candidates in clinical trials [4–10]. Unlike other conventional therapeutic molecules, a GTMP contains both vector and transgene. Vectors used in GTMP can be designed and modified to target specific tissues or cells to avoid off-target effects or nonspecific toxic-

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Table 1. Examples of currently approved gene therapy medicinal product and gene therapy medicinal product candidates in clinical trails.

Company	Drug	Mechanism	Target	Status
Shenzhen SiBiono GeneTech	Gendicine	Oncolytic virus	Squamous cell carcinoma	SFDA approved
uniQure	Glybera	Gene therapy	Lipoprotein lipase deficiency	EMA approved
GlaxoSmithKline	Strimvelis	Gene therapy	Severe combined immunodeficiency due to adenosine deaminase deficiency	EMA approved
Biogen/Ionis	SPINRAZA	ASO	Spinal muscular atrophy	FDA approved
Sarepta Therapeutics	EXONDYS	ASO	Duchenne muscular dystrophy	FDA approved
Novartis	CTL019 (tisagenlecleucel)	Gene therapy	Relapsed or refractory pediatric and young adult patients with B-cell acute lymphoblastic leukemia	FDA review
Ionis	Volanesorsen	ASO	Familial chylomicronia syndrome	Phase III
Ionis	Inotersen (Ionis-TTRrx)	ASO	Familial amyloid polyneuropathy	Phase III
Alnylam Pharmaceuticals	Patisiran	siRNA	Hereditary ATTR amyloidosis	Phase III
Exicure	AST-005	SNA	Psoriasis	Phase II
eTheRNA immunotherapeutics	Unknown	mRNA	Melanoma	Phase II
CureVac	CV9104	mRNA	Prostate cancer	Phase II

ASO: Antisense oligonucleotides; SFDA: State FDA (Chinese FDA); SNA™: Spherical nucleic acid

ity. The modifications include deletion of genes associated with virulence, pathogenicity or replication-competence [11]. Two delivery systems are commonly used: viral- and nonviral-based delivery vectors [12–16]. Viral-based transgene delivery system demonstrated high transfection efficiencies and was used in the first generation of GTMPs. However, potential mutations, post-treatment recombination, potential oncogenic effect and high cost have been the concerns in employment of viral system to deliver GTMP to patients [17]. In contrast, nonviral vectors such as lipid nanoparticles (LNP) have shown less safety concerns due to their relative simplicity, though nonspecific cytotoxicity associated with cationic liposomes has been observed. In addition to delivery systems, the expressed gene moieties also present unique challenges in pharmacokinetics (PK) assessment and bioanalytical method development.

Informed by cumulative scientific data over several decades, the conventional wisdom regards the systemic drug concentrations as the driver for the pharmacological effects [18]. This general principle has to be altered as tissue concentration is the driver for the pharmacological effects for the majority of available gene therapies. Consequently, evaluating the PK of GTMP is an essential task in the pharmaceutical discovery research as well as during preclinical and clinical development. To GTMP, a prominent example of the utility of tissue exposure is the determination of exposure margin to facilitate the selection of starting dose for the first-in-human study. Furthermore, due to the dependency of pharmacological response (or pharmacodynamics, PD) on tissue drug concentration, a good understanding of the PK–PD relationship can be very useful in projecting the exposure and/or dose that would achieve the desirable response. The importance of the bioanalytical methods that measure drug concentrations to enable the PK characterizations, therefore, cannot be overemphasized.

In order to substantiate the reliability and validity of the PK data, regulatory agencies expect using validated methods to measure drug concentrations, provided their expectations in regulatory guidance/guidelines [19,20]. However, these guidance/guideline documents do not clearly articulate the regulatory expectations with respect to the assays used for support of GTMP preclinical and clinical development, such as PCR, hybridization methods and immunogenicity assays. Following the unique requirements of the tissue drug concentration being a driver for the pharmacological effects, it is generally recognized that drug concentrations representing analytes relevant to the pharmacological effects in both blood and tissue samples should be measured [21–24].

Bioanalytical methods for quantification of DNA or RNA transgene, the vector and the expressed gene

moieties and assessment of their immunogenicity are essential for understanding PK, PD and safety. Unlike most biotherapeutics, GTMPs may have specific tissue/cell tropism and can enter cells. Many products are administrated directly into the disease sites. For virus-based gene therapies, pre-existing antibodies to the viral capsid due to prior infection/vaccination with related viruses may affect the safety and efficacy of the GTMP. All of those add complexity of the bioanalytical support for GTMP development. The following sections summarize the current practices and trends in bioanalytical industry to illustrate the considerations and practices used in design, development, qualification/validation and implementation of bioanalytical methods as well as immunogenicity assessment for GTMP programs.

Pharmacokinetics method

PK assessment employs several analytical techniques to quantify GTMP based on the requirement of assay sensitivity, assay specificity, analyte property and technical expertise in each bioanalytical lab. Hybridization-ELISA, quantitative PCR (qPCR) and MS have been the method of choice for past years.

Hybridization-ELISA, HPLC and capillary gel electrophoresis–UV/fluorescence methods have been the traditional methods to quantitatively determine the analyte of interest for support of pharmacokinetics and toxicokinetics (PK/TK) evaluation of GTMP drugs [25–27]. Recently, more sensitive and more specific qPCR- and MS-based methods including LC–MS/MS, LC–high-resolution accurate mass and hybridization-based LC–fluorescence are added to the toolbox [28–30].

Comparing with PCR and MS methods, the traditional methods still offer the adequate sensitivity and operational convenience. The hybridization ELISA or the branched DNA (bDNA) assay employs traditional instrumentation, minimal sample processing and performing on the 96-wells platform that enables high-throughput and easy assay transfer to established bioanalytical laboratories. The bDNA assay, also known as Quantigene assay, is commonly used for mRNA quantification in various types of samples. It is a single step, probe-based-ELISA that works through cooperative hybridization of probes to the target mRNA. These probes range from 20 to 40 oligonucleotides in length and specifically bind to the target mRNA regions. The probe set consists of capture extenders to anchor the mRNA to the plate, label extenders that amplify the signal of the target mRNA and blocking probes to block the regions of mRNA that are not bound by probes within the region of probe design. Lysed study samples are added to the 96-well assay plates in duplicates and the ELISA-like assay protocol is followed. The luminescence signal obtained from each well is proportional to the RNA concentration in samples, which is back calculated against a calibration curve prepared in respective matrices. These methods can be validated following existing regulatory guidance for ligand binding assays. With the advanced amplification system of luminescence signal, the sensitivity of the hybridization-ELISA is comparable to the qPCR assays [27].

The qPCR assays have been recognized based on their high sensitivity, accuracy and practical ease, while spectrometry assays have been considered to have the best specificity. Real-time PCR or/and qPCR is the current gold standard for quantifying gene expression, detection of viral shedding and determination of virus copy number as analyte of interest [29,30]. However, it has been a task to design a suitable PCR assay for quantitative determination of GTMP with sequences of short length. For example, the average length of a miRNA product is around 20 nucleotides. In addition, assay contamination and cross reaction with other sources still remain one of the main challenges.

For viral-based gene therapies, US FDA has specific requirements to PK assays in regards of the sample collection and method sensitivity. For example, FDA 2006 guidelines state that "Use a quantitative, sensitive PCR assay to analyze the samples for vector sequences. You should submit data to your Investigational New Drug (IND) to demonstrate that your assay methodology is capable of specifically detecting vector sequence in both animal and human tissues." And "the assay should have a demonstrated limit of quantitation of <50 copies of vector/1 µg genomic DNA, so that your assay can detect this limit with 95% CI" [31]. The specific requirement on sensitivity poses the challenges in PCR method qualification and validation. Challenges in outsourcing lab work have to be considered in selecting PCR-based assays for regulated study support since qPCR is not a traditional bioanalytical assay platform. Bioanalytical CRO labs do not typically maintain instrumentation and expertise to validate qPCR methods and test samples.

LC–MS/MS-based methods have seen enormous growth in the last years. This type of assay provides higher analytical specificity than conventional HPLC and higher throughput than GC–MS. Utilization of LC–MS/MS for GTMP development is at the vanguard of preclinical candidate screening and nonviral-based vector quantification. For LNP quantification, multiple LC–MS methods can be developed and validated to ensure that the intact LNP can be quantitatively determined from various matrices. However, the absolute sensitivity and specificity is highly dependent

on the specific assay technique, assay reagents and the structure of the target analyte. Several limitations of LC–MS/MS such as concerns of sensitivity, throughput and transferability have been revealed [28]. Table 2 provides high levels of comparison among these methods.

For gene therapy entering clinical trials, their efficacy, safety, tissue distribution and immunogenicity must be assessed in preclinical animal models. Evaluation of biodistribution of gene therapy products focuses on localization of both the transgene and delivery vector, as well as the expressed protein. Since tissue exposure shall be the driver of clinical dose selection, the potential deleterious effects of the gene therapy vector or protein product on normal healthy tissues shall be evaluated in preclinical toxicology studies. It also requires an understanding of the probability of either entity reaching off target organs.

However, deciphering tissue exposure in clinical settings is very challenging since collection of human specimen is difficult and/or very limited. In particular, the consistent ratio of plasma/tissue from different animal species should be established to bridge the preclinical and clinical dose selections. The correlation between systemic and tissue exposure shall be established in the preclinical phases. To obtain a consistent ratio of plasma/tissue is very critical for the clinical dose selection to support clinical studies. In addition, multiple matrices shall be used for exposure characterization with sufficient analytical sensitivity. Therefore, multiple methods are often required to accomplish full PK characterization [32].

Immunogenicity assessment

The immunogenicity strategy in support of GTMP development shall consider three potential challenges: the immunogenicity against DNA/RNA transgene, the immunogenicity to the delivery vectors/vehicles, for example, viral capsid, liposome, and DNA/RNA encoded proteins as effective molecules for mRNA-based enzyme replacement therapy [33,34].

Risk-based approaches shall be used to assess potential immunogenicity of GTMP. Specifically, bioanalytical assays for determination of anti-DNA/RNA, antidelivery vector/vehicles, for example, antilipid component of LNP, and antiprotein antibodies shall be separately developed and validated. Considering the likelihood, clinical transformability and potential impact, the immunogenicity assessment will not apply to the samples from the toxicity/TK studies unless it is deemed to be necessary. However, it must be noted that the FDA guidelines on preclinical assessment of investigational cellular and gene therapy products indicate that the likelihood of immunoresponses to viral vector is high and need to be monitored [34]. In the case of GTMP using viral vector, pre-existing antibody to the viral capsid can significantly hamper effectiveness of the GTMP administration, and thus shall be tested for screening subjects or investigating the impact on PK/PD. For clinical studies, the immunogenicity strategy shall be implemented for all types of GTMPs. However, the utilization of differnt immunogenicity tests shall depend on development phases and the properties of each vector type. If immunogenicity is a concern (e.g., with viral capsids or allogeneic cellular products), then each subject's immune response to the product should be evaluated. This evaluation may include monitoring for evidence of both cellular and humoral immune responses [35]. In addition, other approaches to monitor acute immune-response shall be considered in the overall immunogenicity assessment.

Using mRNA therapies as example to illustrate the consideration and strategy, the anti-mRNA immunogenicity assays seem less needed since the current data do not indicate that there is significant induction of immunogenicity against mRNA itself unless in the autoimmune diseases. Therefore, the anti-mRNA antibody is considered as low risk and may not be necessary to be monitored routinely. Antiprotein antibodies can develop against proteins expressed from any mRNA, in particular, if repeat administration regimens are pursued. Anti-mRNA encoded protein antibody shall be screened for in clinical studies of mRNA-mediated protein replacement. Anti-LNP antibodies can be considered as low incident and may be determined to be of low impact on safety and efficacy.

Method validation

The analytical methods have to be qualified or validated to enable assessment. However, to qualify or validate the matrix specific assay individually is often operationally difficult. Therefore, fit for purpose approaches have been applied to address the challenges, including only validating the method using one type of tissue and qualify the method for other tissues samples via method selectivity test.

For the past years, many regulatory documents have touched upon the use of nontransitional bioanalytical methods to assess safety or clinical end points. These guidances include 'Guidance for Industry – Gene Therapy Clinical Trials – Observing Subjects for Delayed Adverse Events' (FDA 2005); 'ICH Considerations – General Principles to Address Virus and Vector Shedding' (EMEA, 2009); 'Pathogen Safety Data Sheets and Risk Assessment' (Public Health Agency of Canada, 2011); 'FDA Guidance for Industry – Clinical Considerations for Therapeutic Cancer Vaccines' (FDA, 2011); 'FDA Guidance for Industry – Design

and Analysis of Shedding Studies for Virus or Bacteria-Based Gene Therapy and Oncolytic Products' (FDA, 2015); and recent EMA draft guideline on the quality, nonclinical and clinical aspects of gene therapy medicinal products (EMEA, 2015) [36–41]. However, those regulatory guidelines still lack details on how assay validations should be designed and performed. Therefore, many bioanalytical labs follow the general method validation guidance for bioanalytical assays for ligand binding or chromatography assays [21].

For support of regulated studies, bioanalytical scientists have designed and conducted fit-for-purpose method validations to ensure suitability and data integrity of each method. The accuracy and precision, sensitivity, specificity and analyte stability in whole blood, various swab samples, tissue samples and urine specimens are tested to meet the requirements in the clinical setting. Of significance, the stability data shall be used to guide sample collection, shipping and storage. Having validated stability in multiple storage temperatures and conditions enables the extension of the study design for collection of more valuable data in both institutional and home settings. Very often the method shall be fully validated and implemented in a bioanalytical CRO, as in-house source can be a constraint for routine sample analysis.

Conclusion

Since GTMP has demonstrated clinical benefits serval disease, including inherited disorders, and cancers, it has formed the third major drug platform in addition to traditional small and large molecules. The pharmacokinetic behaviors of transgene, their vector and expressed gene moieties are still under study to be sure about GTMP's safety and effectiveness. To enable accurate and efficient PK/PD evaluation and immunogenicity assessment, a thoughtful bioanalytical strategy has to be established and implemented prior to supporting preclinical and clinical studies.

As more genetic therapeutics are advancing to late stage development, bioanalytical scientists are facing increased scientific and technical challenges and regulatory rigor in method design, optimization and validation. Fit-for-purpose approaches can be applied in order to demonstrate the analytical specificity, linearity and dynamic range, limit of detection and qualitative cut off, lower limit of quantification, diagnostic accuracy, intra-assay and interassay precision, and analyte stability in various samples. In addition, novel technologies in this area, for example, digital PCR, offer technical advantages but also make it more difficult to validate these assays to meet regulatory requirements.

Future perspective

Since 1989, because of their ability to target 'undruggable' targets, GTMPs have formed the third major drug platform in addition to small and large molecules. Despite several GTMPs being approved, many are still in the clinical investigation. Safety and sustainable efficiency are still the main concerns to drug develop-

Table 2. Comparison of conventional hybridization assays, PCR-based assays and MS-based assays for RNA/DNA quantification.

Methods	Hybridization assays	PCR-based assays	LC–MS
Sample preparation	miRNA, RNA and DNA isolation may not be needed	miRNA, RNA and DNA isolation is needed	Need sample extraction
Instruments	Traditional instrumentation, for example, plate washer, plate reader and incubators	PCR machine	LC–MS
Throughput	Easier to automate (possibility to expand to 384 well format)	Medium/high-throughput: reverse transcription and PCR reactions are time consuming	Medium-throughput
Sensitivity	High sensitivity	Highest sensitivity	Potentially acceptable
Interference/specificity	Need to distinguish exogenous (therapeutic) sequence from the endogenous sequence (cross-reactivity)	Endogenous RNA interference during isolation and prone to variations and contamination	High specificity
Reagent availability	Custom probe sets need to be designed for specific sequences	Combination of custom probes and commercially available reagents	Common reagents, no need of a specific probe
Method validation	Ligand-binding assay method validation guidance	No current guidance	LC–MS validation guidance

ers. Therefore, the pharmacokinetic behaviors and the immunogenicity of both GTMPs and the expressed gene moieties are essential for understanding pharmacology and safety. To enable accurate and efficient PK/PD evaluation and immunogenicity assessment, a thoughtful bioanalytical strategy has to be established and implemented prior to supporting preclinical and clinical studies. With more technical advancement, bioanalytical scientists have more tools to produce highly accurate and sensitive data. However, inconsistent bioanalytical approaches have been applied to PK and immunogenicity method design, development and validation. There are many underlying reasons for this inconsistency. Various platforms and reagents used in quantitative methods, lack of detailed regulatory guidance on method validation in gene therapy and uncertainty of immunogenicity impact in supporting gene therapy may all be influential. Bioanalytical labs often select one platform over others based on their own expertise and technical familiarity. For example, bDNA method offers adequate sensitivity and robustness with its signal amplification system. The method also can be validated following current method validation guidelines for large-molecule drugs. However, many labs have chosen qPCR as the default method to support GTMP development. How to adequately validate the qPCR assay remains a question for bioanalytical industry and regulatory agency to address.

Another example is the strategy of immunogenicity assessment for GTMPs. Since immunogenicity assessment can be stage specific, for example, preclinical versus clinical, some labs plan it based on the risk and impact of immunogenicity to safety and efficacy. Considering the likelihood, clinical transformability and potential impact, the immunogenicity assessment may not apply to the samples from the toxicity/TK studies. The anti-mRNA immunogenicity assays may not be necessary in clinical safety assessment since the current data do not indicate that there is significant induction of immunogenicity against mRNA itself unless in the autoimmune diseases. Contrarily, tests of the immune responses to the viral vehicles can be essential in case of virus-based gene therapy since pre-existing antibodies to the viral capsid due to prior infection/vaccination with related viruses may affect the safety and efficacy. Industry is still debating on the 'risk-based approaches' and looking for applicable perspectives from regulatory agencies.

Due to the mechanism of the actions of GTMP, the therapeutics may have specific tissue/cell tropism and can enter cells. To fully understand the bio-distribution of GTMP, a consistent ratio between systemic and tissue exposure has to be established in the preclinical studies. This has been extremely challenging for nonantisense-based molecules, for example, mRNA programs. Therefore, bioanalytical methods have to be suitable for quantification of analyte of interest in multiple matrices to provide evidences to bridge systemic and tissue exposure in preclinical PK and bio-distribution studies.

For the past years, many regulatory documents have touched upon the use of nontransitional bioanalytical methods to assess safety or clinical end points. However, those regulatory guidelines still lack details on

Executive summary

- Recently, an increasing number of gene- and nucleic acid-based products have been advanced in clinical development phases. Unlike traditional small- and large-molecule drugs, a gene therapy medicinal product (GTMP) contains both vector and transgene, and its therapeutic effect is mostly reached through altered protein expression. Therefore, the pharmacokinetic behaviors of both GTMPs and the expressed gene moieties are essential for understanding pharmacology and safety. To enable accurate and efficient pharmacokinetics/pharmacodynamics evaluation and immunogenicity assessment for GTMP, a thoughtful bioanalytical strategy has to be established and implemented prior to supporting preclinical and clinical studies. However, inconsistent bioanalytical approaches have been applied to pharmacokinetics and immunogenicity method design, development and validation.
- For pharmacokinetic assessment, bioanalytical methods shall provide evidences to bridge systemic and tissue exposure prior to the clinical study phases. Currently, hybridization-ELISA, quantitative PCR and MS have been the method of choice.
- The immunogenicity strategy in support of GTMP development shall consider three potential challenges: the immunogenicity against DNA/RNA transgene, the immunogenicity to the delivery vectors/vehicles, for example, viral capsid, liposome, and the anti-DNA/RNA encoded protein antibodies in the cases where the proteins are expressed in patients as effective molecules for enzyme replacement therapy.
- Current regulatory guidelines still lack details on how the assay validations for GTMP should be designed and performed. Therefore, many bioanalytical labs follow the general method validation guidance for bioanalytical assays for ligand binding or chromatography assays. It may create substantial divergence between the bioanalytical data and data interpretation. Further discussions and input from the industry regulatory agencies will be valuable for providing the best practices for managing this type of method validation.

how the assay validations should be designed and performed. Therefore, many bioanalytical labs follow the general method validation guidance for bioanalytical assays for ligand binding or chromatography assays. It may create substantial divergence between the bioanalytical data and data interpretation.

All of those add to the complexity of the bioanalytical support for GTMP development. Looking forward, as more genetic therapeutics are advancing to late stage development, it opens opportunities for bioanalytical industry to work with technology providers and regulatory agencies. Through the close collaboration among pharmaceutical companies, bioanalytical CROs with expertise and regulatory agencies, we optimistically look forward to having a well-established set of industry practices and regulatory guidance in the future.

Financial & competing interests disclosure

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

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Automated immunoassays are fast tracking cell and gene therapy workflows

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Dr Dana Holzinger studied Biology at the Ruprecht Karls University in Heidelberg, Germany. Her dissertation at the German Cancer Research Center and the University Hospital in Heidelberg focused on the identification of human papillomavirus-associated head and neck squamous cell carcinomas. Afterwards, Dana worked as a postdoctoral researcher leading several molecular epidemiological studies on the identification of biomarkers for different HPV-associated pathologies. In 2019, she joined PROGEN as Product Manager and became Head of Product Management in 2020.

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Questions

What is viral titration and why is it important?

The term titration originates from chemistry, representing a method for quantitative chemical analysis. The method is based on a standard solution (titrant), which reacts with an analyte to determine its concentration. However, it has been used by biologists in a slightly modified form describing the determination of concentrations based on a titration curve. In regard to our AAV ELISAs, it means to determine the concentration of the viral particles in an unknown AAV sample based on a specific standard with a known concentration that is provided with our ELISA kits.

The determination of the viral concentration is a very important step during analytical characterization of AAV-based gene therapies. A comprehensive characterization and quality control is mandatory for the production of safe gene therapy products, which is substantiated by the guidelines from the regulatory authorities. Therefore, the decision of which methods to use for characterization is a very crucial factor.

It is beneficial to choose methods, which are robust and reproducible, especially methods showing low variabilities between labs or even between people from the same lab meet this requirement.

To make sure our AAV ELISAs generate accurate data, PROGEN established internal gold standards for the calibration of the ELISA kits. While there are commercially available Reference Standard Materials (RSM) available for the serotypes AAV2 and AAV8, the AAV-based gene therapy field lacks corresponding RSMs for the other AAV serotypes. However, standard materials for calibration are indispensable tools for the quality control of our AAV ELISAs but also for the development of new AAV ELISA kits. For this reason, our team established a process for comprehensive characterization of our internal gold standards inspired by the process published for the AAV2 and AAV8 RSM characterization. Each ELISA lot is carefully calibrated based on the corresponding internal gold standard to make sure our ELISA kits not only measure accurate capsid titers but also have low inter-lot variabilities to assure consistency for our customers. The precise characterization and high-level quality control of PROGEN´s AAV ELISAs enable a standardized workflow and accurate data for AAV capsid titers.

There seems to be a widespread need for frequent, robust and rapid viral titer measurements in drug discovery and development – why is that?

The gene therapy market is growing rapidly which clearly determines the demand. More and more companies enter the field of gene therapy, which increases the exertion of pressure, specifically time pressure on the companies and the scientists. The healthcare industry has always been a highly competitive field, and the promising results from previous and current clinical studies using gene therapies and in particular AAV-based gene therapies contribute to this development.

The transfer from preclinical studies to clinical trials also increased the pressure from regulatory authorities asking for robust, reliable and reproducible data to ensure the safety of the gene therapy products. It is mandatory to have the product fully characterized to be transferred into clinical trials, which includes data that can be reproduced, and show a consistency throughout the process.

However, characterization of viral-based products is not trivial since there are several components that can influence efficiency and safety. The term viral titer as such is not a very precise description since there are different viral titers to be measured for a comprehensive characterization. The field discriminates between capsid titers, genome titers and infectious titers. While the capsid titer which you can determine using PROGEN´s AAV ELISAs describes the amount of fully assembled viral capsids including full and empty particles, the genome titer refers to the number of genomes carrying the transgene that have been packed into the viral capsids.

There can be significant differences between these two titers since not each viral particle necessarily contains a transgene. The packaging efficiency depends on different factors including production processes as well as the specific transgene that has been integrated into the genome sequence.

Though it sometimes is underestimated, the capsid titer provides very important information. First of all, it gives information about the packaging efficiency and puts the genome titer in relation to the total number of viral particles. It is known that empty particles can induce an unwanted immune response when administered to patients. These particles lack the transgene so they do not have a therapeutic effect. Consequently, empty particles might interfere with the efficient delivery of the transgene by inducing an immune response against the AAV vector and additionally do not contribute to the therapy. This obviously needs to be prevented, especially considering the single vector application to each patient. Since gene therapies have been designed to restore dysfunctional genes permanently, a single application is supposed to be sufficient to achieve a lifelong cure. This ambitious objective clearly emphasizes why an efficient initial treatment is so important.

What are the benefits of measuring viral titers with an immunoassay approach (vs a PCR-based approach)?

As described previously, there are different viral titers to be determined. In this context, it is important to say that the AAV ELISA, which is an immunoassay approach, measures the AAV capsid titer while the PCR-based methods are used to determine the genome titer. Since both titers are indispensable for the comprehensive characterization of an AAV-based gene therapy product, there is no either or with ELISA and PCR.

However, in general, a PCR-based approach shows much more variabilities than the AAV ELISA, which is due to the method itself. In a PCR approach, small amounts of DNA are amplified for detection and quantification. This makes the method highly sensitive but also prone for high variabilities of the final data since everything gets amplified during this process. Thus, small differences such as pipetting errors occurring by pipetting small volumes, which by the way all pipettes carry when pipetting amounts lower than 10µl, will be amplified as well and might contribute to the high variabilities.

Since the PROGEN AAV ELISAs are not based on amplification processes, naturally occurring small differences only have a minor effect on the final result compared to PCR-based approaches. In 2010 and 2014 when the AAV2 and AAV8 Reference Standard Materials (RSM) [1,2] were characterized, the inter-lab variances of qPCR and the PROGEN ELISAs were analyzed in contributing labs around the world. The results clearly demonstrate that the PROGEN ELISAs showing CVs of 34% [1] (rAAV2) and 40% [2] (rAAV8) were superior compared to qPCR (rAAV2: 78% [1] and rAAV8: 113% [2]) in terms of inter-lab variances.

Another critical factor of the PCR-based approaches is the selection of specific primers. While the PCR-based methods, depending on the primers used, might not be able to discriminate between the amplified full-length DNA product and amplified DNA fragments of a certain size, the PROGEN ELISA only measures fully assembled capsids due to the unique antibodies used for the detection, which exclusively bind conformational epitopes present on fully assembled AAV particles.

However, it is very important to have data for all of the different viral titers including the genomic titer provided by PCR as well as the capsid titer provided by ELISA, thus making a comparison of PCR-based methods and our ELISA very difficult. We strongly recommend determining the different titers ideally with several orthogonal methods to gain a comprehensive understanding of the gene therapy product and ensure the highest possible level of safety for the patients.

The move towards near complete automation of life science tools is becoming prevalent across large pharma and biotech. What could be the benefits of determining viral titers with an Ella like platform?

We would like to hand this question over to our partners at proteinsimple since we are convinced that proteinsimple will give you a more sophisticated answer on the advantages of the Ella platform. However, as final remark, we really enjoy working with proteinsimple and join forces by combining PROGEN´s expertise in the field of AAV gene therapy with the expertise on automated systems of proteinsimple. In addition, working with proteinsimple has been a great experience on a personal level, which is a major factor for the successful cooperations.

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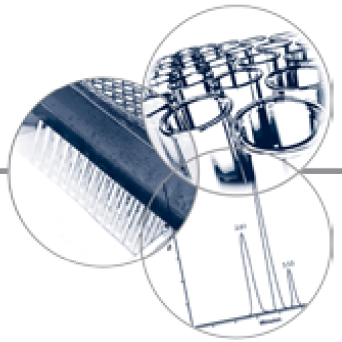

Automated immunoassay equipment platforms for analytical support of pharmaceutical and biopharmaceutical development

Laboratory automation is not new, but few scientists have been exposed to the wide range of analytical equipment platforms, which have been available from diagnostic and research companies, with many workers focusing on one or the other disciplines throughout their career. However, many such instrument platforms play an important role in drug-development in laboratories around the world. This review covers some of the experiences I have had in what is nearly 40 years in laboratory analysis – the last 18 years being in CROs supporting pharmaceutical development. There are many platforms that I have used, which are not included here since the focus of the article is on immunoassay techniques. I think it is worthy to note that many of the capabilities within modern platforms, from a wide range of manufacturers, would appear to me to have a 'genetic' link back to the first automated analyzers launched over 50 years ago. It has been interesting to take a walk down the development road of these platforms over that timeframe and, no doubt, will continue to be at least equally of interest in the future.

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Analytical support in drug-development today covers a wide range of techniques and equipment platforms. Whilst areas in analytical sciences such as diagnostics have continually made progress and developed an increasing number of automatic utilities into a wide range of platforms, instrumentation used in research laboratories, it could be argued, by comparison, has developed little over the last 15–20 years.

Furthermore, many laboratories involved in bioanalysis in drug-development (whether pharmaceutical companies, biotechnology companies or CROs) often do not get exposed to instrumentation used in diagnostics and so do not learn of the capabilities already present in these platforms that can enhance their use today. Consequently, feedback to manufacturers of **robotics** used in research seems to have been spasmodic at best and thus the manufacturers would appear to have had little impetus to introduce new developments and enhancements to the capabilities of their systems. Notable efforts have been made such as the discussion groups within the American Association of Pharmaceutical Scientists; for example, 'The Twenty-First Century Laboratory', where a 'wish-list' of equipment capabilities was proposed. Having attended at least one of those meetings, the point made above was very relevant, since virtually everything that was on that list was already available on a number of diagnostic platforms, and had been for some time (see list of capabilities later). A very good chapter called 'The Application of Automation in Ligand-Binding Assays' that addresses research platforms can be found in the excellent book edited by Khan and Findlay [1].

Today, we see 'new' models of many xyz robotics that still lack some of the enhancements – particularly in user-interface programming – that have been present in some open diagnostic platforms for over 15 years.

The purpose of this article is to look at how we can use **automation** in bioanalytical immunoassay services today to improve and enhance both the quality of the science as well as assist in the management of the laboratory – including increasing analytical capacity and throughput.

The history of automation

Automation in laboratories is by no means a new concept. It has – not surprisingly – been lead by clinical diagnostic services, owing to the very large workload they endure and also seeking quicker and quicker turnaround times to better serve patient healthcare due to the critical clinical situations that often present themselves to hospital emergency rooms.

Nearly 40 years ago, some of the methods used for the measurement of certain analytes in blood, for which results were needed 'urgently,' could take from 30 min to several hours. They were done separately using different instruments

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Key Terms

Robotics: The application of automated machinery to tasks traditionally done by hand.

Automation: The techniques and equipment used to achieve automatic operation.

and turnaround time did not meet clinical demand requirements. Therefore, many systems have been developed over the years to improve upon this situation. These started in the areas that had the largest routine workload – mainly 'wet' chemistry methods, and saw the first fully automated analyzers launched in 1957 (Figure 1) – some 54 years ago. These were 'continuous flow' systems – basically a single stream of air-segmented fluid continually added to or modified – that allowed the reactions of the assays to take place, have the final spectrophotometric reading using a flow cell and then discard to waste. A test-tube assay automated start to finish. It revolutionized clinical laboratories worldwide.

These early systems – and their more computerized second and third generations – were seen to be unnecessarily wasteful of reagents and samples and R&D groups with manufacturers looked for ways to improve this. What developed were known as 'random access' analyzers. Here, the continuous flow system was replaced by discrete reaction vessels reducing waste almost to zero, but also allowing a specific selection of tests rather than having to conduct a panel of tests on all samples. Since this time, other areas of analysis have attracted attention for automation and immunoassay is one of those key areas – translating technology developed in standard 'wet' chemistry to the requirements of immunoassay.

Automation development in immunoassay

Immunoassay originated as radioisotopic methods – good old RIA (radio-immunoassay). However, these methods left little room for automation of the tube techniques and most development focused on improving throughput on the radioactive gamma counters used – moving from single- to multi-well detectors. With the development of 96-well microplate technology, the first 'xyz' robotics were seen and used in the early 1980s (e.g., Tecan [101], Hamilton [102]). At this stage, they were simply programmable on a protocol-by-protocol basis and were unsophisticated in being unable to organize batches of different assays simultaneously in an automated and efficient way.

Here, we saw the same systems entering both research and diagnostics laboratories but whereas little really developed to dramatically change the field of robotics in research, much was happening in the diagnostic industry.

In 1979, Abbott diagnostics introduced the Quantum II – an automated immunoassay system for enzyme immunoassays. In 1981, Abbott again launched the TDx system, which incorporated the first commercial application of fluorescence polarization, and followed it in 1988 with the IMx system. The TDx and IMx were widely used in many labs. These were systems designed to conduct one batch of a specific method at a time – typically 20–30 samples. What followed these 'first-generation' platforms were systems that would be able to have the same random-access capabilities from a large assay repertoire, which was a feature of the earlier wet chemistry analyzers.

The early 1990s saw a number of these launched including the Immulite (1993) [103] by DPC (now Siemens) and the Axsym (1994) by Abbott (Figure 2) [104].

These latest systems saw some additional capabilities that enhanced laboratory management and sample processing of immunoassays. Again, many of these had been developed on previous clinical chemistry systems and were simply incorporated into these multi-analyte capability systems. This included:

- Barcode reading of primary sample tubes;
- Liquid-level sensing of both samples and reagents;
- Clot detection;
- Volume checks of reagents on board;
- Checks on authenticity, placement and expiry dates of reagents via barcodes;
- Calibration protocols;
- On-board QC programs;
- Data reduction and direct output of final concentration results;
- Uni- and bi-directional interfaces with laboratory information management systems.

Figure 1. The Technician AutoAnalyzer 1. From right to left: reagents, autosampler (rear), peristaltic pump (fluidics), dialyser (protein removal), incubator (rear), double-beam spectrophotometer (front), chart record.
Images courtesy of manufacturers.

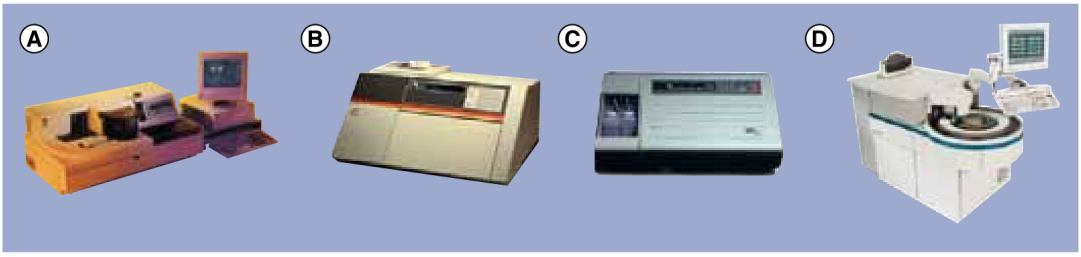

Figure 2. Automated immunoassay platforms. (A) Immulite 1000 (now Siemens). **(B)** Abbott TDx. **(C)** Abbot IMx. **(D)** Abbott Axsym.
Images courtesy of manufacturers.

The drawback of these systems versus the xyz robotics and 96-well microplate assays were two-fold. Firstly, they were (and still are) 'closed' systems, meaning that you could only use reagents from the same manufacturer as the platform itself and existing methods could not be reoptimized on these systems. Secondly, new assays for different analytes to those on the equipment's repertoire could not be developed, so that they had little place to play in large parts of the drug-development pathway – especially for pharmacokinetic (PK) and immunogenicity assays.

During the same period, whilst advances in xyz robotics were being made, and some of the components above added to their capabilities, what was largely lacking was a user-friendly interface to program the instruments for new assay protocols. Most systems today still suffer from this problem with new protocols often taking hours or even days to program. Thereafter, some require significant checking to ensure that the platform actually executes the program as expected and that there are no conflicting instructions to the robot that could cause problems.

Some manufacturers of 96-well microplate reagent kits in diagnostics looked upon this as an opportunity and sought to develop xyz robotics that could automate a microplate assay – including in-house ones – but also overcome the programming issue for new protocols. Others looked to developing everything, but the robotics, coming up with what was basically a specific plate washer and reader for their assays (e.g., Amersham Amerlite). Moreover, there have been a number of platforms that have developed to run a specific part of an assay protocol such as SPE or liquid extraction. Some of these moved quickly into 96-pipette head processing, allowing simultaneous processing of 96 samples, which dramatically improved throughput and capacity over manual methods. The most common of these that I have come across are those manufactured by Tomtec (Figure 3) [105]. It should be noted that more recent models discussed later also offer this as part of a larger platform that can process the other parts of the analytical method in addition to the extraction.

From my experience, the first truly 'open' system that I used that could claim full automation from start to finish for these methods and also demonstrate user-friendly software as its front-end interface with the user was launched in the UK in 1998 by Grifols – the Triturus (Figure 3) [106]. Typically, we trained

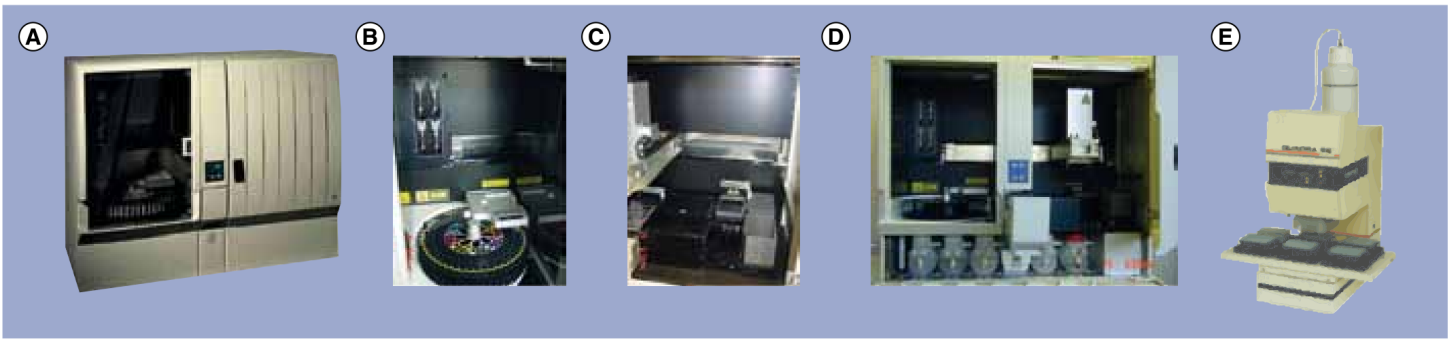

Figure 3. The Grifols Triturus. (A) Triturus. **(B)** Sample compartment with carousel for samples, QCs and calibrators. **(C)** Reagent, shaker/incubator, washer and reader compartment. **(D)** Front of instrument showing all compartments including shared fluidics and waste collection/disposal. **(E)** TomtecQuadra 96 – one of the earliest 96-pipette head processing robots.
Images courtesy of manufacturers.

Key Term

Multiplex: Multiple analytical methods that are run simultaneously within the same reaction vessel.

new operators on the instrument in less than half a day and, thereafter, new assay protocols would take about 30 min or so to program. The system has multiple self checks in the software that assist the operator and numerous automatic checks that are performed when the 'go' button is pressed. These typically ensure that all the samples and reagents are in the right positions and that there are adequate volumes on board for the programmed batches.

For those of us that really evolved immunoassay services for our laboratories throughout this period, it is interesting to look back upon how it has changed in its application from the period immediately prior to development of these platforms. Whilst outside the scope of this article, for those with an interest to review the history of immunoassays more fully, I recommend 'Immunoassays for the 80s' by Voller, Bartlett and Bidwell [2].

Throughout this period of development, it was clear that as well as looking at automation of manual processes, manufacturers were also investigating the use of new techniques to improve the performance of the analytical methods. For example, improving analyte specificity was (and still is) a driving force for some analytes and different detection and separation technologies became common in some platforms. These included fluorescence (e.g., delayed enhanced lanthanide fluorescence immunoassay [DELFIA] and time-resolved fluorescence), chemiluminescence (e.g., Amerlite) and magnetic bead separation. Additionally we saw the introduction of electrochemiluminescence to the assay repertoire.

Multiplex & other specialist platforms

Not long after the Triturus was launched, we also saw the arrival of new immunoassays called '**multiplex**' methods. This seemed, and was, a big technological step forward in the science itself. Luminex was the first in the field, introducing its xMap technology in 1999 [107]. We were fortunate to buy one of the first instruments in the UK and have gained some really interesting knowledge and experiences using it and the many methods that have now been developed for it. One of the benefits of this is the fact that it is really a flow cytometer and reagents and commercial methods using the technology have

Figure 4. XYZ Robots with integrated specialist detectors. (A) Automated Multiplex Platforms (AIMS). **(B)** AIMS incorporating a Luminex xMAP platform (AtheNA Multi-Lyte® version). Images courtesy of manufacturers.

been developed by a wide range of manufacturers – so it is an 'open' system. That is, we are not restricted to the manufacturer of the instrument also being the sole supplier of reagents.

xMap technology platforms are not automated systems but really are the detectors for the end point of the methods. However, I have included them here since recently some progress has been made with robotics that allows instruments, such as the Luminex, to be incorporated into the robotic system itself and supply a fully automated 'hands-off' approach from start to finish of the assay method. One such platform is the Automated Immunoassay Multiplex System (AIMS®) from ZEUS Scientific (FIGURE 4) [108].

Whilst AIMS was one of the earliest to integrate these machines, other manufacturers now have platforms that can integrate a fairly wide variety of analytical equipment – not only detectors but instruments such as centrifuges, decappers, dryers, thermocyclers, cryostats and others. Hamilton and TECAN, for example, are two companies that claim this type of capability.

One of the benefits that we have seen with xMAP technology is that it has been outlicensed to multiple companies (e.g., Bio-Rad [Bioplex], ZEUS Scientific [AtheNA Multi-Lyte], Millipore [Milliplex] and so on), and all of these companies have developed a range of assays that are commercially available. Some companies have made physical hardware changes to the instrument or developed their own software (e.g., Bio-Rad). This gives a tremendous advantage in terms of variety of possible uses and availability of assay methods that are not restricted to just the instrument manufacturer itself – a potential problem with other platforms.

In addition to having less flexibility if a platform is restricted to a single provider of kits/reagents, there is also a potentially higher business risk to companies using such platforms depending upon the manufacturer's size and financial stability. Many organizations are very risk-averse in this particular area following the issues raised by the Bioveris demise a few years ago.

As well as xMAP technology, other examples of multiplex systems include the Mesoscale Discovery [109] and Aushon Searchlight systems [110]. xMAP platforms use microspheres as the solid-phase upon which to build an immunoassay. Spheres can be specifically labelled with dye combinations and antibodies to allow analyte coding and detection. The end point is measured using a flow cytometer that both identifies the sphere by the dye (and hence identifies the analyte) and also the end point of the immunoassay by the intensity of the final signal. Mesoscale Discovery uses electrochemiluminescence with proprietary microplates that have multiple detection electrodes located in the bottom of each well of the plate – each with a specific label for a particular analyte. The Aushen Searchlight uses chemiluminescence and also has multiple analytes per well by utilizing antibody array spotting. Here, the brightness of each luminescent end point is captured using a charge-coupled device camera in a standalone detector.

However, innovation continues to thrive in analytical sciences and manufacturers continue to invest in R&D of equipment platforms – some of these making radical moves in terms of technology. For many years we have seen that the most widespread technique has been the microplate – for various functions – not only the standard 96-well plate we see in many immunoassays, but also deep-well plates used for sample storage and sampling or extraction processes. Other microplates with larger numbers of smaller wells have been used with automated platforms, however most of these techniques have been in the discovery and high-throughput screening arena and rarely do these assays make their way through to 'production' assays that can be used in many laboratories or a more routine environment.

Randox evidence

In 2004, I presented at the Bioval Conference in London, UK, on the topic of 'Biomarkers in Drug Development,' where I covered a range of analytical platforms available. In that presentation, I put a slide about the Randox Evidence – the world's first protein Biochip Array Technology system.

My slide questioned whether this system may be the future of biomarker analysis, since some of the metrics being claimed were highly impressive:

- >1500 tests/h;
- 25 markers per chip;
- As little as 7 µl of sample required;
- Multiple matrices;
- Other automated capabilities as discussed above for diagnostic systems.

I believe that Randox concentrated in the early days on routine diagnostic assay arrays (cardiac, endocrine, fertility, metabolic, thyroid and tumor markers). In addition, they developed

Key Term

Microfluidics: The behavior, precise control and manipulation of fluids that are geometrically constrained to a small, typically submillimeter scale.

a number of separate immunoassays useful in diagnostics and therapeutic drug monitoring; molecular arrays related to microbiological diseases, toxicology arrays for drugs of abuse and arrays for drug residues have now been developed on this analyzer. Furthermore, and of more interest to this article, is their range of research arrays – comparable to many of the multiplex arrays available from manufacturers of assays for xMAP, MSD and Aushon technology.

Performance claims are impressive and there are a number of articles in the public domain illustrating its performance over long periods of time in independent laboratories [3].

Interestingly, they have developed different size models to fit different lab requirements – from a small bench-top detector/reader, where the chip assays are processed manually, through full automation in both random-access and batch-based models.

Randox appears to be one of the few major diagnostic companies to get heavily involved in the development of research assays for use on their accredited diagnostic platforms. Their longevity and experience over their 30 years existence of manufacturing research and diagnostic products for use over a wide range of analytical platforms gives a lot of confidence for future support and financial stability. Moreover, their attitude to developing and manufacturing research assays in exactly the same way as they do their diagnostic products gives confidence in the quality of their products. The range of platforms can be seen below in Figure 5.

Gyros gyrolab

Early in 2000, we started hearing about a new nanotechnology workstation, manufactured by Gyros of Sweden – the Gyrolab [111]. This was a true innovation whereby immunoassays were developed in a microvessel within what appears to be very similar to a compact disc – indeed most users today call them CDs.

The Gyros evolved from early microfluidic system research at Pharmacia Biotech (which later became Amersham Biosciences) in Uppsala, Sweden, which began in 1989 and ended with the forming of Gyros AB as an independent company in 2000. At this stage, Gyros owned an extensive portfolio of over 40 patents related to **microfluidics**, CD manufacture, system components, surface chemistry and specific application areas.

Gyros had a wide range of challenges to overcome in entering the microfludic world, which are beyond the scope of this article, but one very interesting point is that when working at the nanoliter scale, scaling laws become very significant, in that surface tension becomes a more dominant force than gravity [4].

Unlike microplate technology where both manual and automated methods rely on very precise volume control pipettes or fluidics, respectively, here all the volume control was contained within the prefabricated CD. Everything is overfilled slightly with excess removed by gentle centrifugation and critical volumes maintained within the microvessel by hydrophobic barriers. Once the excess is spun to waste in this way, more rigorous centrifugation forces the accurate volume of remaining fluids through the hydrophobic barriers and over the solid-phase contained in the bottom of the vessel (Figure 6).

The Gyrolab brings a number of significant benefits to the laboratory with regard to immunoassay methods in biological fluids that can be challenging:

- Reagents: the nanotechnology significantly reduces the volume of reagents used and this means that assays developed to support large projects (e.g., a PK assay in a Phase III study) or large numbers of samples, will require a much

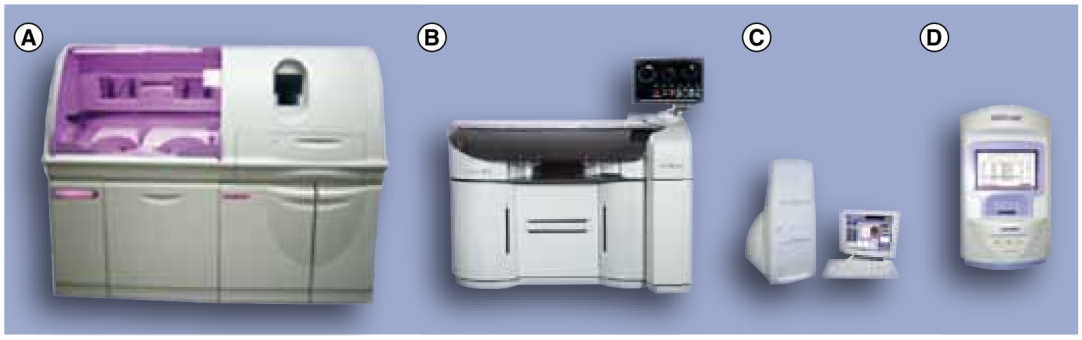

Figure 5. Range of Randox Evidence Platform. (A) Evidence, **(B)** Evidence Evolution **(C)** Evidence Investigator and **(D)** Evidence Multistat.
Images courtesy of manufacturers.

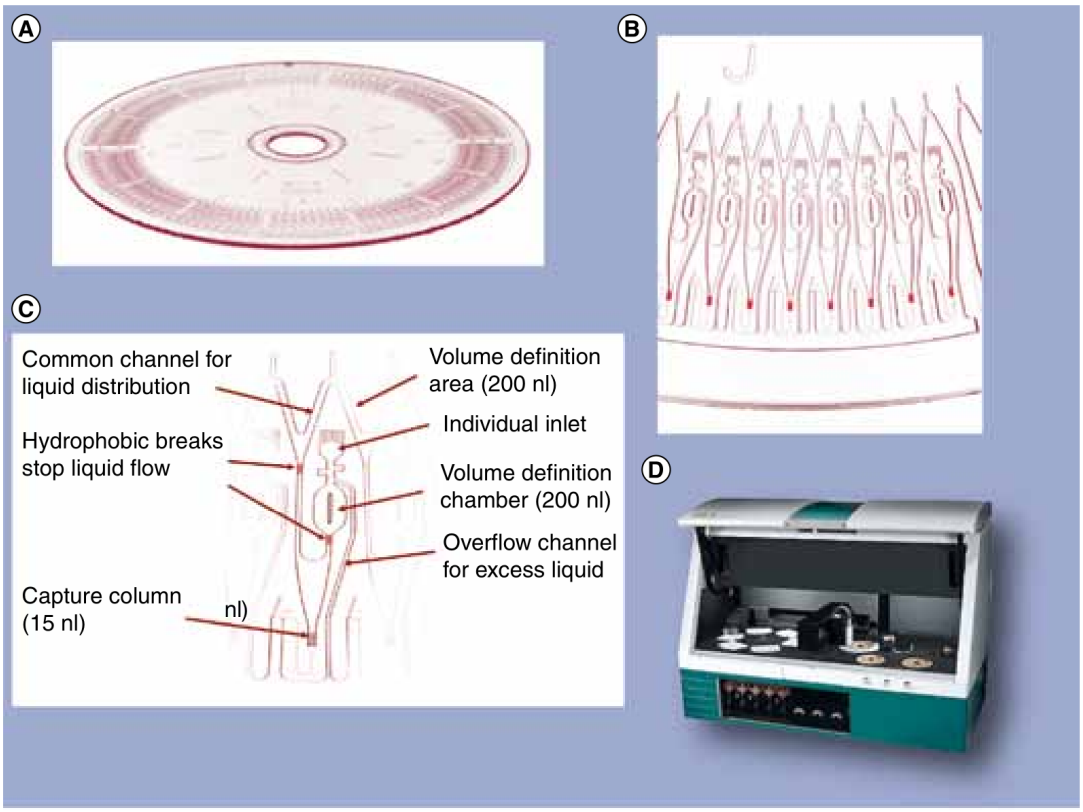

Figure 6. The Gyros Gyrolab. (A) CD. **(B)** Eight microvessel section of CD. **(C)** Microvessel with description of components. **(D)** Gyrolab instrument.
Images courtesy of manufacturers.

smaller amount of antibody to support the continued use of the method. Antibody production is not an insignificant cost and so this has considerable cost-saving implications over the course of the study or drug-development program;

- The reaction times of the antigen–antibody interactions are vastly reduced – it takes 5–7 s for the sample to flow through the 15 nl column, thereby minimizing incubation and contact time. This has three major effects. It reduces the overall time of the analytical method (each method takes around 1 h to complete). Due to this, new methods can be developed much more quickly, since multiple runs can be conducted in a single day (there is also a very good method development software package onboard that further assists this). The shortness of the antigen–antibody interaction times means that there is very little chance for nonspecific binding and so potential matrix effects seen in some methods can be eliminated in many cases; and is also why the system can tolerate 50% matrix. A further effect of this, of course, is that antibodies typically need to be have a reasonable degree of, or be high affinity;
- Samples: as with reagent volumes, the same is true for sample volume. Even given minimum dead volume requirements, the volume of sample required equates to around 5–10 µl. Since most biological fluids are diluted a minimum of 1:2; this volume allows for multiple sampling, which makes it a very useful tool where biological fluid matrix is rare – whether due to the patient (e.g., pediatrics) or the matrix (e.g., cerebrospinal fluid or tears).

Miniaturize and integrate sandwich immunoassays

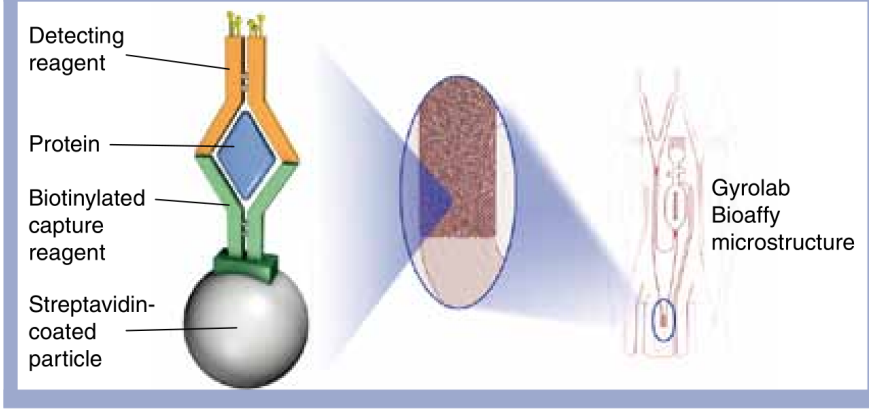

Figure 7. Illustration of sandwich immunoassay.
Image courtesy of manufacturers.

The immunoassay reactions take place using this solid-phase and the final signal is detected using laser-induced fluorescence (Figure 7).

Personally, I am very interested in the potential future developments of this platform – and understand that fully automated antidrug antibody (ADA) assays, which require an acid dissociation step, may be available soon.

Practical use of instrument platforms in drug-development

Clearly, these different instrument platforms have many utilities to help laboratories support the analytical requirements of drug-development. Depending upon assay methods used, they can have considerable impact upon processing samples for various requirements.

In small-molecule development, immunoassays have largely been limited to analysis of biomarkers for various purposes. Here, as demonstrated above, we have a potentially wide selection of platforms from both research and diagnostics to assist us in the support of this work. However, in the larger-molecule development of biological therapies, the scope for immunoassay widens into both PK and ADA/immunogenicity assays, in addition to biomarker requirements.

In certain circumstances, where trial design and analyte choice has allowed, we have found it possible to use a fully automated platform to measure multiple analytes (in separate methods – i.e., not multiplexed) to produce PK and biomarker pharmacodynamic (PD) data simultaneously on the same sample aliquot. Obviously, when such opportunities arise, there are tremendous efficiencies and other benefits to the management and conduct of the study. Typically, smaller volumes of biological fluid and fewer number of aliquots are required to be produced. When you consider the organization of producing these, labeling appropriately, the cost of consumables, storage post collection at investigator sites and laboratories and the logistics costs of fewer shipments, financial savings can be considerable and the practical aspects of conducting the collection, storage and so on is much simpler.

Automated versus manual methods

It is relatively easy to see how automation can improve performance characteristics of methods when compared with the same technique conducted manually. If we take a look at a 'standard' 96-well microplate ELISA method, it can be broken down into several different steps:

- Dilution of samples;
- Pipetting calibrators, QCs and samples into the plate;
- Pipetting reagents into the plate;
- Incubation(s) (with or without shaking) – possibly at different temperatures;
- Washing the plate;
- Reading the end point of the reaction.

All these steps have intrinsic errors to them and some of those errors can be reduced by automation, notably:

- Accurate and precise fluidics that usually have better precision than manual pipetting. This actually has a cumulative reduction in the overall error of the method, since there are always several pipetting steps in each assay – each one with a potential improvement. So, even if the difference in the precision of the automated versus manual pipetting is only marginally improved, the cumulative effect has a real impact on the overall system performance;
- Incubation times that are always accurately controlled. How many times does a manual assay overrun its incubation due to the operator being unavailable to conduct the next step at the exact time required? Whilst this will not necessarily affect the results of the individual plate significantly, it can contribute to increased variability on an interbatch basis, since prolonged incubation will have an impact upon final raw data response;
- Temperature control for incubation – in our experience, fewer problems occur within automated systems than moving plates manually between separate instruments for each of the steps in an assay. Platform-to-platform variation is also overcome;
- Plate washing – some robotics allow for different and specific wash programs for different types of plates (e.g., flat, rounded or conical-bottomed). This degree of sophistication may not be available in some standalone plate washers. Problems with plate washing often causes problems in immunoassays and having this process standardized can improve a method's performance when viewed over multiple batches dramatically;
- Reading the final end point of the method – the real advantage of automation here is that the time of the reading is always the same.

Once again, the major impact here is across multiple batches when looking at assay performance.

The overall impact of all of these together is seen in the full system response. Firstly, the raw data tends to be more reproducible from batch-to-batch. FIGURE 8 shows comparison of the raw data responses of several batches for three assays for the same biomarker (Amyloid AB 1-42 in cerebrospinal fluid) being conducted in three different ways – two manual assays (a Luminex and DELFIA method) with different end points and a sandwich ELISA, which was fully automated.

What is clear from these charts is the improved precision of the overall system response on the automated platform. This will nearly always translate into better interassay precision and accuracy.

One interesting point – though not really the subject of this article – which I feel it is worth mentioning here, is that many workers routinely tabulate and evaluate calibration data

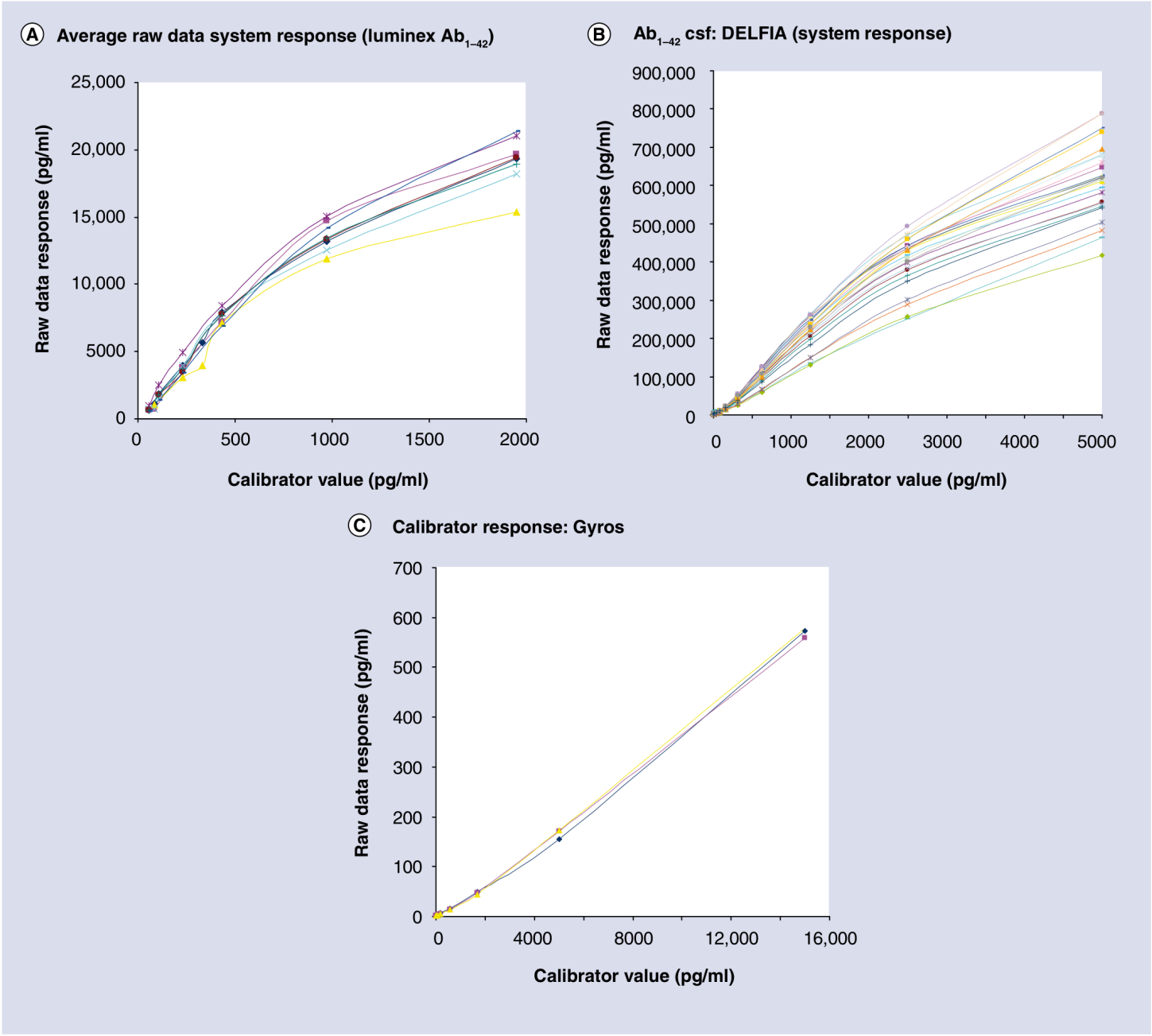

Figure 8. Comparison of raw data responses from manual and automated systems. (A) Luminex xMAP method. **(B)** DELFIA method with PE Victor reader. **(C)** Fully automated – Gyros system.
Images courtesy of manufacturers.

when validating assays – often including those tables in reports. However, in most instances that I have seen, these tables have included back-calculated results as opposed to raw data. This will often not demonstrate what is happening in the system responses that are shown in Figure 8. Indeed, if we graphically represent the same data as back-calculated results, we see the graphs shown in Figure 9.

Since these assays are nonlinear and use algorithms, such as four- and five-parameter logistic fits, it is not surprising that the observed versus expected results always show relatively good data. The actual overall method performance is better evaluated by looking at raw data as shown in Figure 8 and demonstrates the different performance of the automated versus manual techniques.

As a further example of how automation may improve overall performance, Figure 10 represents the interassay precision (CV %) of a PK immunoassay method for a biological molecule.

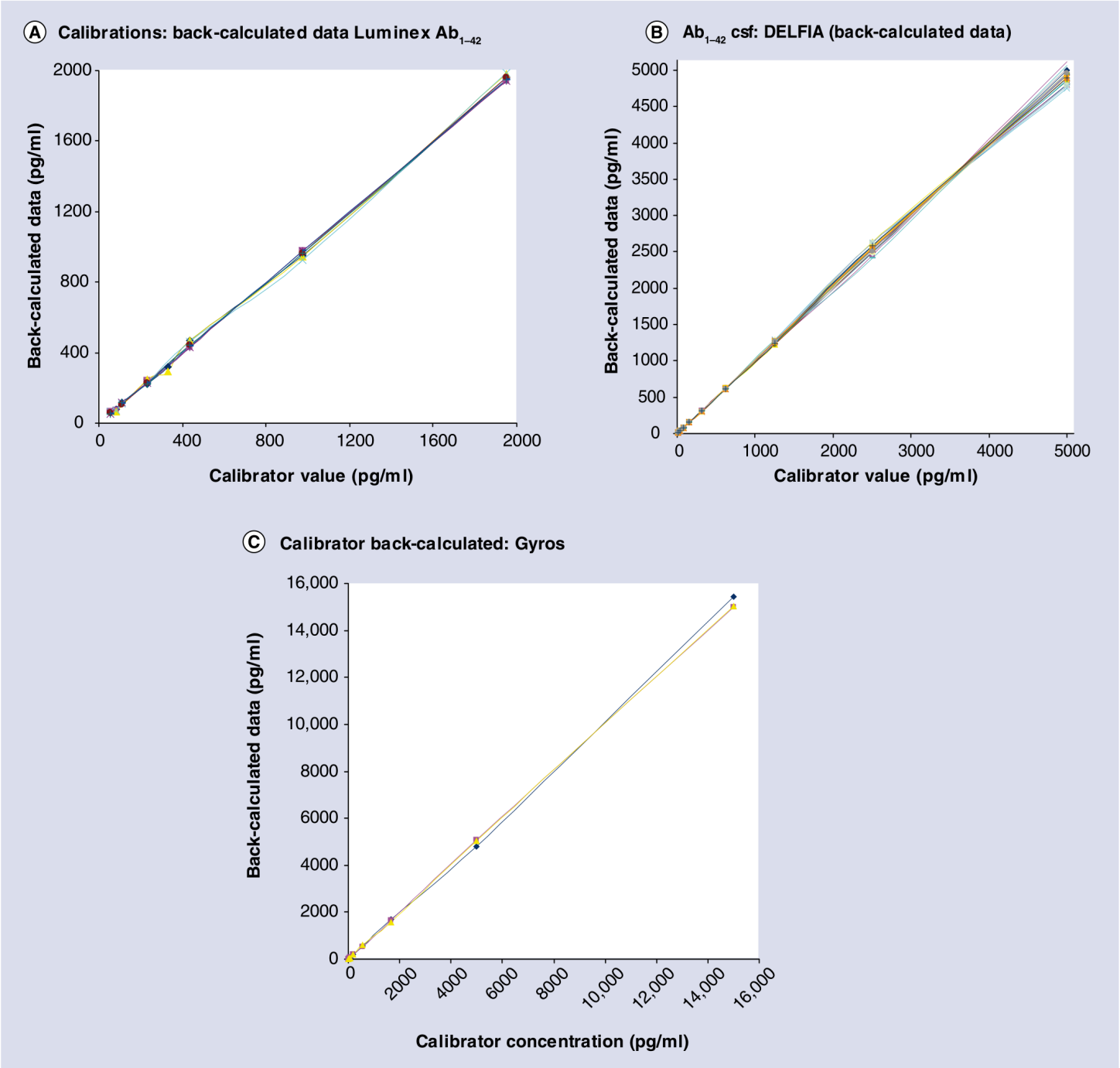

Figure 9. Back-calculated results of the data represented in Figure 8.
Images courtesy of manufacturers.

The manual method results in this chart, generated in our laboratory matched closely to the data from the original published method for this molecule. Transferring the exact method to an automated platform, we saw two major improvements. Firstly, the performance in terms of precision improved dramatically (as did accuracy [relative error] and total error) and, secondly, we were able to extend the analytical range of the method.

It is often surprising – to me at least – to hear that immunoassay methods can never be as precise as other methods, such as LC–MS/MS, due to the inherent variability that is generally expected within immunoassays. However, when we interrogate performance data of many immunoassay methods available on these platforms, we often find that this is not true at all.

As an example, Figure 11 shows Levey-Jennings QC charts from a project our laboratory was once involved with about 14 years ago. This is a peptide hormone assay, analyzed on a fully automated platform and used for PD analysis (it could equally be used for PK analysis if this peptide hormone was developed as a drug). The validation of the assay showed that the method had interassay precision (CV) of 4% across the whole analytical range.

Now, to use this as a PK assay under latest guidelines would require QC results in sample batches to pass '4–6–20' rules (4 out of 6 QCs within ±20% of its target value [25% at the LLOQ] with at least 50% of QCs passing at each level). It is clear that this method is far better than the criteria demand, and indeed accepting results up to five-times CV of the method we would argue is inappropriate as, statistically speaking, results with that level of performance would actually demonstrate that the method is 'out-of-control' according to its performance criteria at validation. However, that is probably another argument for another article.

Using it as a PD assay, we would more appropriately use acceptance limits linked to the method performance itself. These could be 95% confidence limits (±2 SD) as 'warning' limits on method problems, and ±3 SD as batch failure limits. When we look at the validation performance, this would translate to failing a batch if even a single QC result in a sample batch was outside ±12% of its target value.

Hence, looking back on immunoassay data, the expectations of them always being poorer than other method types is clearly not correct. In fact, we have a number of immunoassays with CVs similar to the peptide hormone assay quoted above.

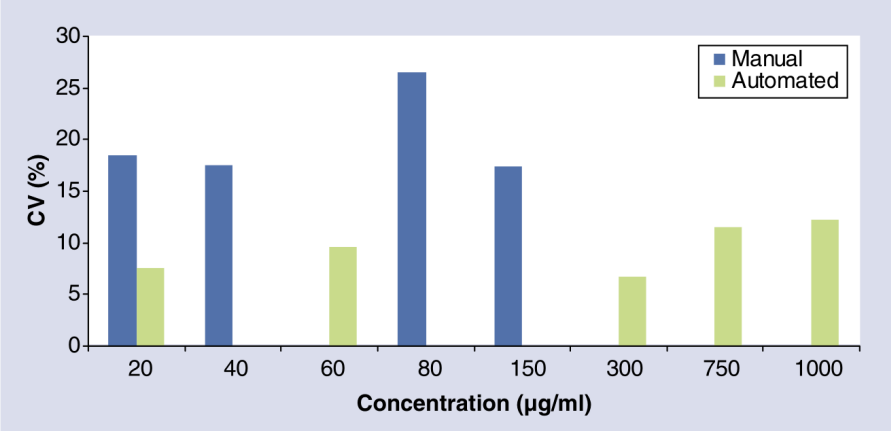

Figure 10. Method performance of a manual immunoassay compared with the fully automated version of the same method.
Image courtesy of manufacturers.

PK assays

Virtually all immunoassay methods for PK analysis can be automated. The degree of automation

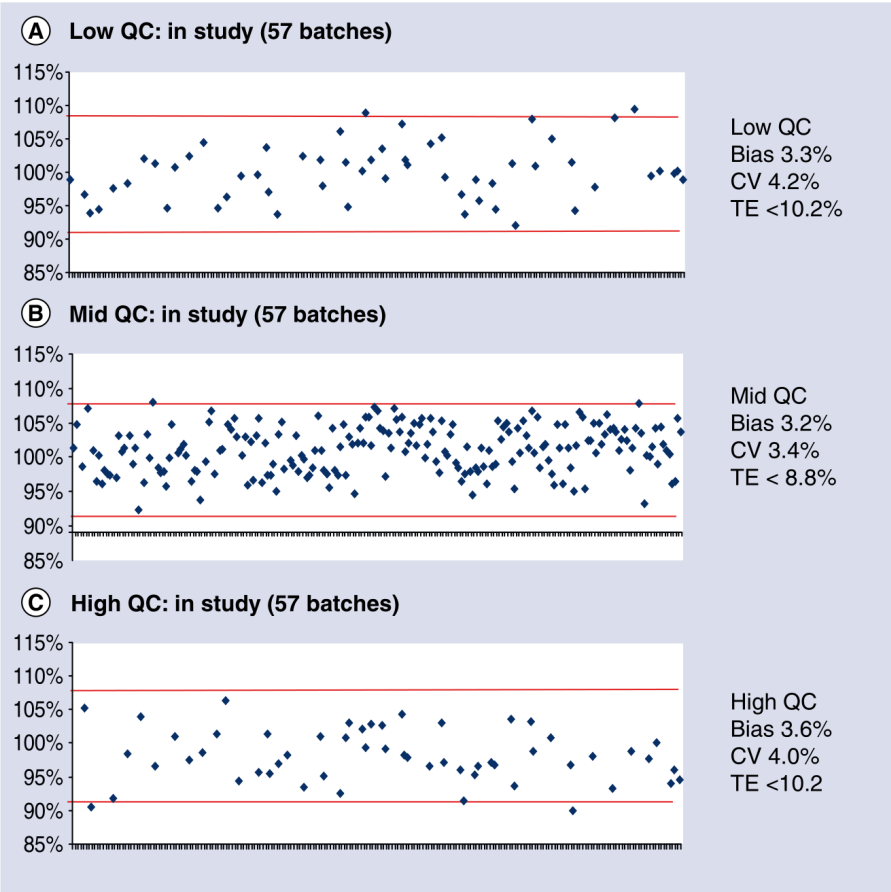

Figure 11. Levey–Jennings charts of low, medium and high quality controls over study timeline with performance metrics of the method over 57 analytical sample analysis batches covering several months.
Images courtesy of manufacturers.

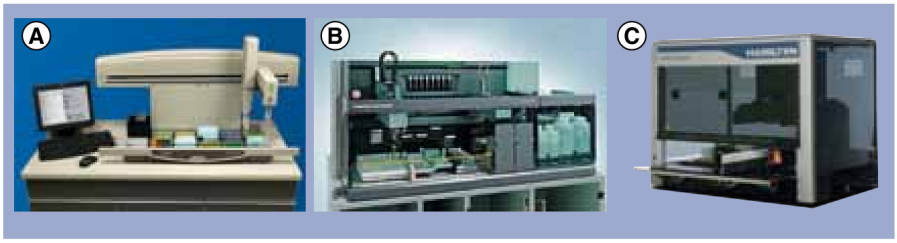

Figure 12. Some current xyz robotics platforms. (A) Beckman-Coulter's Biomek 3000 [112]. **(B)** Tecan's Freedom Evolyzer. **(C)** Hamiltons STARLET. Images courtesy of manufacturers.

depends on individual laboratories, although the benefit of automation for immunoassays has been demonstrated repeatedly in the diagnostics industry, and, certainly in our experience, the argument for automation is very strong. Clearly, there are many ways to organize an analytical laboratory and many factors come into play when choosing how to conduct each assay. Busy laboratories are regularly conducting a large number of different assay methods every day and if the batch numbers of each assay are relatively small in this scenario, full automation becomes more difficult – especially on platforms that have user interfaces, which are not that efficient. As mentioned previously, platforms like the Grifols Triturus have major benefits over many others due to the ease of programming new method protocols into the instrument. Where fully automated systems really come into their own is on projects that have large numbers of samples that require analysis for either one method or a small number of methods.

Immunogenicity assays

Whilst overall assay formats may differ in some ways for immunogenicity assays, the same basic premise exists as for other immunoassays – many will be capable of automating. There will almost certainly be some issues to overcome with integrating some platforms that are really end point detectors, which will be more difficult for some and may make that step inefficient or too costly, whilst other fully automated systems are already being used in this field. Moreover, a number of these platform manufacturers are already investing in development of their systems to enable their use in this field, where previously such platforms may not have been considered. I am certain this is an area that we will hear much more of in the future.

Figure 13. Bioscale's VIBE Workstation. Described as a 'A New Generation of Protein Analysis'.
Image courtesy of manufacturer.

Use of accredited biomarker assays on automated platforms for PK & PD analysis

One of the real benefits that the diagnostic industry can bring to drug-development is that when biological drugs are being evaluated and PK assessment is required, it is often possible to use accredited diagnostic kits with only very small modifications. Here, we can get the benefit of very robust methods – sometimes having been in use in thousands of laboratories around the world for many years. In addition, where they have been developed on fully automated platforms, such as some of those mentioned previously, we also get all of the benefits that automation brings to the method, as discussed above.

Examples of some of the drugs where we may take advantage of these methods would be molecules such as growth hormones, insulin and vitamins (e.g., D and B12), which all exist as endogenous molecules and have very good robust methods available on a wide range of platforms (e.g., Abbott Axsym/Architect, Siemens Immulite, Roche Elecsys, Beckman-Coulter Access and others).

Future perspective

It is clear that much research and development has been invested into laboratory equipment platforms used for immunoassays over many years now. In the xyz robotics field, we are seeing more instruments increasing their repertoire of capabilities. Some of those platforms, currently available, are shown below in Figure 12 [112]. Whilst this is not an exhaustive list, it will be interesting to see how these capabilities develop and how well received the platforms are – only time will tell.

Additionally, I expect that there will be other platforms that come to the forefront, which may be more critically developed around more specific methodologies (as opposed to the typically totally open and flexible approach seen in the

Figure 14. Point-of-care immunoassay platforms. (A) Alere Triage system. **(B)** LifeAssays system. **(C)** Avantra Q400 Multiplex biomarker system.
Images courtesy of manufacturers.

xyz robots discussed in this article). One such instrument is Bioscale's VIBE™ (Figure 13) [113] – others I am sure will follow – all aimed at simplifying analytical techniques in the laboratory, whilst trying to improve upon method performance criteria.

I also expect there to be more moves towards companion diagnostic assays, which developers may wish to get closer to the doctor's office or even bedside via point-of-care (POC) testing equipment. Some appear to be already there, for example, Alere's Triage system (formerly Biosite [114]), and Radiometer's AQT90 FLEX. LifeAssays also have a system based on a sandwich immunometric immunoassay principle, where the test system uses magnetic nanoparticles (Figure 14) [115].

It is also interesting that some manufacturers share this vision. I recently learned that Bioscale (VIBE, above) have already started miniaturizing the proprietary nonoptical detection capabilities of its assay into a compact device for diagnostic and POC applications. I look forward to seeing the outcome.

Moreover, having recently viewed Courtagen Life Sciences, Inc.'s Avantra Q400 Multiplex biomarker system [116], I was impressed at the capability of this POC equipment – based on a fully enclosed cartridge system using microfluidics and quoting very respectable performance criteria for a number of popular research assays in plasma/serum. It is the most advanced POC immunoassay system that I have seen to date and I am looking forward to evaluating it soon.

Many assays are already available on such platforms and I believe we will see others come along – perhaps on new platforms, such as those Bioscale have in mind – in the future. Here, microfluidics and perhaps nanotechnology will also play a part in their development, as has been witnessed with some of the platforms already discussed. Whilst these platforms are obviously directed towards and used in diagnostics, they do potentially have a major part to play in drug-development in the future. With the advent of using (or wishing to use) new biomarker assays for stratifying patient populations for study

Executive summary

- Laboratory automation is not new, but few scientists have been exposed to the wide range of analytical equipment platforms, which have been available from diagnostic and research companies, with many workers focusing on one or the other disciplines throughout their career.
- Many such instrument platforms play an important role in drug-development in laboratories around the world.
- Analytical support in drug-development today covers a wide range of techniques and equipment platforms.
- Today, we see 'new' models of many xyz Robotics that still lack some of the enhancements – particularly in user-interface programming – that has been present in some open diagnostic platforms for over 15 years.
- Throughout this period of development, it was clear that as well as looking at automation of manual processes, manufacturers were also investigating the use of new techniques to improve the performance of the analytical methods.
- However, innovation continues to thrive in analytical sciences and manufacturers continue to invest in R&D of equipment platforms – some of these making radical moves in terms of technology.
- One very interesting point is that when working at the nanoliter scale, scaling laws become very significant, in that surface tension becomes a more dominant force than gravity.
- Virtually all immunoassay methods for pharmacokinetic analysis can be automated.
- It is clear that much research and development has been invested into laboratory equipment platforms used for immunoassays over many years now.

inclusion and 'companion diagnostics', it is clear that as analytical methods for new biomarkers are developed, the opportunity to perform technology transfers to POC platforms such as those discussed here has very attractive advantages for global clinical trials in the future.

One thing is certain – this field of analytical science is not standing still. I, for one, look forward to seeing whatever new developments come our way.

Acknowledgements

All images are used at the courtesy of the companies listed in the references. The instruments are used as examples in this article and are not meant to be an exhaustive list of those available; they simply represent those that the author has either used or has learned of during the course of his career.

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Perspective
SPECIAL FOCUS ISSUE: The Exciting World of Oligonucleotides

Bioanalysis

Bioanalysis of adeno-associated virus gene therapy therapeutics: regulatory expectations

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The number of gene therapy (GTx) modality therapies in development has grown significantly in the last few years. Adeno-associated virus (AAV)-based delivery approach has become most prevalent among other virus-based GTx vectors. Several regulatory guidelines provide the industry with general considerations related to AAV GTx development including discussion and recommendations related to highly diverse bioanalytical support of the AAV-based therapeutics. This includes assessment of pre- and post-treatment immunity, evaluation of post-treatment viral shedding and infectivity, as well as detection of transgene protein expression. An overview of the current regulatory recommendations as found in currently active and published draft US FDA and EMA guidance or guideline documents is presented herein.

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Keywords: gene therapy • genome integration • immune response • infectivity • neutralizing antibody • total antibody • transgene protein • viral vector shedding

In recent years a variety of gene therapy (GTx) approaches using viral vectors to treat a variety of conditions have been quickly gaining momentum. Many of these gene therapies are aimed at treating rare genetic conditions by introducing a functional copy of a defective gene. Application of these approaches presents an opportunity for a transformative, potentially curative treatment able to correct the root condition caused by a genetic deletion or mutation in a critical gene. In addition to this increase in development of GTx for the treatment of various rare disease conditions, other applications have been increasingly explored, including treatment of cardiovascular diseases [1], Alzheimer, Parkinson's and other chronic life threatening conditions [2,3].

Multiple viral vectors have been investigated for GTx delivery, including adenovirus, lentivirus and many serotypes of adeno-associated virus (AAV) [4]. Due to multiple factors AAV-based delivery has taken the leading position in the field, with more than 130 individual clinical trials currently registered on the ClinicalTrial.gov. AAVs are nonenveloped, single stranded DNA viruses that belong to the parvovirus family. Serotypes of AAV viruses package a relatively small single stranded DNA genome ranging from 4.7 to 5 kb [5] with a relatively simple genome that encodes three capsid proteins: VP1, VP2 and VP3. Wild-type AAV is nonpathogenic and requires a helper virus, for example, adenovirus, for efficient replication to occur. Without a helper virus, AAV vectors will infect a cell, causing a latent state infection with an episomally maintained genome, but viral replication will not occur. Many natural serotypes of AAV have been identified including human and nonhuman primate as well as avian species specific [6]. Several subtypes have been particularly attractive for GTx development, including AAV1 through 9 and other *in vitro* genetically modified serotypes. AAV serotypes can infect dividing as well as quiescent postmitotic cells, which presents an opportunity for long-term expression of transgenes encoded in the viral DNA, without the requirement for target cells to be actively dividing. Currently, two GTx-based therapeutics have been approved in the EU: an AAV-based treatment of lipoprotein lipase deficiency (UniQure: Glybera, taken off market in 2016) [7] and lentiviral based *ex vivo* GTx for treatment of adenosine deaminase severe combined immunodeficiency (ADA-SCID, GlaxoSmithKline, Strimvelis) [8]. In addition, LUXTURNA®, an AAV-2 vector-

newlands press

based therapy was approved in the USA for treatment of vision loss due to balletic RPE65 mutation-associated retinal dystrophy [9]. In the coming years, there is every likelihood that multiple other AAV-based treatments will file for approvals.

Regulatory guidelines have been provided to the industry by both US FDA and EMA to describe general principles of GTx development, including questions related to nonclinical and clinical evaluation, long-term monitoring for serious adverse events, follow-up studies and product quality-related topics [10–15]. Additionally, several indication specific guidelines have been published by the FDA, including GTx development for treatment of retinal [16], hemophilia [17], and rare diseases [18]. However, many questions related to the bioanalytical assessments conducted during development of viral delivery of GTx modalities remain. These and related questions pertinent to the development of AAV-based GTx are reviewed herein with the focus on presenting the current regulatory recommendations based on information available from active or draft FDA and EMA guidance or guideline documents. As many regulatory guidance documents exist to describe development of genetically modified cells and other than AAV viral vector-based treatments, these will remain outside of the scope for the present review manuscript.

Detection of anti-AAV therapeutic immunity

Immunogenicity responses to the components of AAV therapeutic

A significant body of information has been accumulated regarding immunogenicity risks associated with viral vector-based GTx modalities. Immunogenicity concerns include those related to the anticapsid protein and antitransgene protein responses. Both may play a significant role in the success or failure of a GTx-based treatment. For both antivector and antitransgene protein immune responses a potential induction of humoral or cellular-based responses are possible. Relatively high prevalence of antivector antibodies has been described in general population, including healthy individuals. The prevalence of these antibodies, both neutralizing and non-neutralizing, has been reported to be as high as 50% or more in the individual donors tested [19,20]. Antibodies with no clear ability to inhibit viral transduction are typically referred as total antibodies (TAb, also referred to as binding antibodies) while inhibiting antibodies are described as neutralizing (NAb). In addition to neutralizing antibodies, existence of other matrix factors able to negatively impact cellular uptake and/or inhibit transgene protein expression have been proposed [21]. Up until now, limited correlation between TAb and NAb titers have been reported in the literature, although it is reasonable to expect that a higher TAb titer sample should contain some degree of neutralizing activity against viral transduction [22]. Recently, it has been shown that antibodies that bind AAV capsid, but do not have neutralizing activity may enhance transduction of the liver cells [23]. The study highlights the need for a greater understanding of the type of pre-existing anti-AAV capsid antibodies and their impact on the treatment and importance in selecting patients.

The degree of reported pre-existing TAb and NAb immunoglobulins in human subjects prior to GTx treatment depends on several parameters, including the AAV serotype in question, populations tested and specifications of the assay used to detect antibody presence. A variety of reasons for the diversity of anti-AAV immune response have been proposed, including living conditions, population density, hygienic conditions and quality of the health care. A highly diverse geographical distribution of the prevalence of anti-AAV immunity, including neutralizing antibodies, has been presented and discussed [19,20,24,25]. The viral serotype-based variation in pre-existing response has been reviewed elsewhere and is often used as the basis for GTx treatment serotype selection [19,25,26]. Whether measured as TAb or NAb, pre-existing antibody-based immunity against the administered GTx viral serotype may have a negative impact on treatment efficacy [13–15,17,18,27–29]. Regulatory guidelines state that the presence of GTx AAV serotype specific antibodies may prevent delivery of the transgene into the target cells (e.g., liver) therefore limiting treatment efficiency and efficacy [17]. As a consequence, regulatory guidelines recommend that a consideration should be given to the possible impact in subjects that are found to be positive for the pre-existing antibodies to the serotype of AAV used for the GTx [13]. An impact on both safety and efficacy may be anticipated and is recommended to be evaluated and discussed with the regulatory agencies. Importantly, it is recommended that patients that have pre-existing antibody titers above a predetermined and potentially GTx therapeutic specific value may need to be excluded from the treatment [17,18]. Specific recommendations on pre-existing titer values that should be used as a patient exclusion criterion and how they may be defined are not discussed in the currently available regulatory guidelines which may be a reflection of highly diverse nature of diseases intended to be treated by the GTx modality as well as the diversity of the assay types applied to detect NAb and TAb activities.

Therefore, a specific maximum NAb titer value may be assigned as a patient inclusion criterion. The specific cut-off may depend on the NAb assay characteristics, including type of cells used in the assay, multiplicity of infection value or use of a helper virus to improve assay sensitivity. It may be challenging to assign one universal NAb assay-based cut-point value without appropriate degree assay harmonization or standardization. Currently, no nonclinical model exists that would allow for an accurate prediction of pre-existing NAb or TAb impact in human subjects and direct translation of immunogenicity information from animal models to human subjects may not be linear [13]. It may still be possible to apply conclusions derived during preclinical development of the GTx therapeutic, particularly when the same or highly similar assays are used to evaluate presence of NAb or TAb activity in samples collected prior to the GTx application in animals, for example in nonhuman primate (NHP) studies. In the absence of other information, it might be possible to propose how low level NAb or TAb may be expected to inhibit or completely block virus transduction, particularly when applying NHP study data. For example, a broadly referenced NAb titer value of 1:5 was substantiated in the report by Wang *et al.* where a significant suppression of viral transduction and subsequent reduction in the production of the transgene protein (Factor IX) was described in cynomolgus monkeys with higher than the 1:5 threshold NAb titers [27]. Significantly higher NAb threshold values of 1:500 were reported elsewhere demonstrating high dependency of this critical parameter on the assay characteristics [30,31]. The importance of evaluating of anti-AAV NAb activity and its impact on the AAV treatment in large animals other than monkey models had been specifically noted [32].

In addition to pre-existing antibodies, the possible presence of pre-existing cellular immunity against the viral serotype of the GTx vector has been reported [33–35]. Although the exact degree of impact of pre-existing antiviral cellular immunity is not entirely clear at this point and requires further investigation, regulatory guidelines note a potential connection with the selection of GTx treatment route of administration and dosing regimen [28,29].

The host immune system is expected to produce a robust immune response to the nonhuman protein components of the AAV GTx vector. It has been broadly demonstrated that innate and adaptive immunity against GTx modality components, including antivector and antitransgene protein responses, may present a significant challenge for successful development of an AAV-based treatment [34,36]. Regulatory guidelines list both antivector and antitransgene protein responses as potential risk factors impacting GTx treatment safety and efficacy outcome [18]. Generally, early access to methods designed to measure immune response against GTx components early in the GTx development life cycle is recommended [18]. If assays are not available or fully validated at the start of study sample collection, the pretreatment (baseline) and post-treatment material should be stored at appropriate conditions, for example, frozen, before testing [12]. As the assays become available, a fit-for-purpose subject monitoring for the presence of neutralizing and non-neutralizing anti-GTx component antibodies throughout the course of a trial is suggested [18].

A prolonged exposure to transgene protein is expected postadministration of a GTx therapeutic. This is particularly important for patients lacking the endogenous protein (CRIM negative) as their immune system may recognize the transgene protein as foreign. It has been shown that immune responses in CRIM negative patients treated with a protein-based biotherapeutic can present a significant risk and needs to be strongly considered [37]. An immune response against transgene proteins may result in a highly undesirable autoimmune phenomenon and; therefore, a consideration for an assessment of antitransgene protein immune response is requested by the regulators [14]. The exposure to the transgene protein may continue for several years or potentially for the life of the patient. As a result, testing for antitransgene protein immune response may become an important post-treatment monitoring tool [38,39]. Both neutralizing and non-neutralizing antitransgene protein antibodies may impact the efficacy of the treatment either by blocking the specific protein activity or impacting circulating concentration of the protein by accelerating its clearance from the blood compartment. Regulatory guidelines highlight the need to understand both neutralizing and non-neutralizing antitransgene protein immunity as an important element when determining potential impact on treatment safety and efficacy [18]. At the same time, it has also been proposed that testing may be avoided when it is demonstrated that the antitransgene protein immune response is non-neutralizing, not targeting epitopes linked to the specific protein activity and; therefore, may not be impacting product efficacy [11]. Guidelines propose that, where available, neutralizing antibody response to transgene protein should be assessed based on the specific transgene protein activity evaluation [17]. For example, coagulation Factor VIII and IX activity assays can be used to determine impact of antitransgene protein antibody response in the case of GTx therapeutics design to treat hemophilia A and B conditions [40].

In contrast to the transgene protein, circulation of the free viral vector is not expected to exceed days or weeks although viral genetic material can be detected for up to several months postadministration [41]. Because a robust

postdose immunity against viral vector should be expected the value of detection of both TAb and/or NAb response may be questioned. The antiviral response information may still be valuable to understand potential hypersensitivity reactions observed immediately after the treatment or during later observation period and is indirectly proposed by the regulators [18]. As the current GTx treatment paradigm is commonly based on a single administration of the therapeutic, a possibility of repeat dosing remains a highly attractive option and it has been already applied to treat biallelic RPE65 mutation-associated retinal dystrophy [42]. Bilateral subretinal injections of AAV2-hRPE65v2 (LUXTURNA), an AAV2 vector-based therapy carrying gene of retinal pigment epithelial 65 kDa protein, were given to patients during two sequential separate procedures separated by 6–18 days [43]. As such an assessment of post-treatment immune response to both viral vector and transgene protein can be a significant element of the repeat treatment decision process as was pointed out in the Retinal Disorders Indication specific regulatory guidance [16].

Understanding of the potential of cellular antivector and antitransgene protein immune response to impact safety and efficacy of GTx treatment is viewed by the Regulators as a critical element of postdose immunity assessment of GTx products [13]. AAV viral protein processing by the transduced cells followed by presentation within the major histocompatibility class I complex was proposed as a mechanism of induction of a $CD8^+$ cytotoxic T-cell response which has a potential to eliminate virus carrying cells and result in a decline in the transgene protein expression [33–35]. It is recognized that the exact degree of impact of anti-GTx immune response on the safety or efficacy of the GTx treatment may vary from a transient response without any clinical significance to a severe life-threatening condition [11]. Therefore, periodic monitoring for cellular immune response is recommended with the frequency determination based on the anticipated impact and observed clinical signal [17].

Due to the potential of the anti-GTx immune response to impact both treatment safety and efficacy, regulatory guidelines recommend an extended follow-up and monitoring as part of the prolonged observation period that is sufficient to ensure that appropriate clinical signals are detected [11]. The monitoring may include evaluation of both antibody and cellular immunity against the transgene protein, particularly when clinical relevance between specific response type and clinical impact has to be established [11,12]. The exact duration of follow-up monitoring may greatly depend on the serotype of the virus vector, anticipated safety risks and treatment indication. In the example of GTx products designed to treat hemophilia conditions, a short-term monitoring of up to 2 years following GTx product administration is proposed to include antibody and cellular-based immunity to the vector, as well for the presence of neutralizing antitransgene protein specific antibodies (inhibitors). A long-term monitoring scheme (>2 years) should include presence of antitransgene protein neutralizing antibodies [17].

Looking beyond the clinical development phase, a post-BLA approval availability of an appropriate and specific companion diagnostic (CDx) assay to detect antivector immunity in potential human subjects is requested in the regulatory guideline documents [17,18,44]. Development of a therapeutic specific CDx should be conducted in parallel with the pre-approval clinical investigation of the GTx therapeutic allowing for a coordinated submission to regulatory agencies [17]. Industry continues to debate the appropriateness of selecting TAb versus a NAb method as part of the patient inclusion criteria. For both method types, an alignment is still needed to determine whether the regulatory guidelines describing development and validation of assays for detection of immunogenicity against therapeutic protein products apply when working on GTx supporting methods [45].

Detection of anti-AAV therapeutic immune response

The pharmaceutical industry has significant experience developing fit-for-purpose assays designed to detect unwanted immune responses to protein-based biotherapeutic compounds. Information can be found in several regulatory guidelines and industry White Papers [45–48]. Industry and regulatory agencies are generally aligned on the expectations for the requested analytical specifications, including assay sensitivity, methods used to calculate assay cut-point parameter, assay minimal dilution – among others [49]. Typically, a tier-based approach to detect antibody-based immune response is applied [45]. Using this approach, initial screening if conducted for putative total binding antibody is followed by antibody drug-binding specificity test. If the presence of therapeutic specific antibodies is detected and specificity of binding is confirmed, additional characterization of antibody specificity, including analysis for neutralizing antibody activity, follows. Specific details regarding validation of assays designed to detect antibody-based immune responses against biotherapeutics have been discussed in the industry White Papers as well as regulatory guidance documents [45,46,48].

Current GTx-focused guideline documents provide a limited level of detail regarding modality specific TAb and NAb assays while referencing protein biotherapeutics immunogenicity guidance documents mentioned above [18].

Development of appropriate assays designed to detect antivector as well as antitransgene protein antibody response may not require additional regulatory clarification although several assay specific questions remain, mainly whether the methodologies applied to assess unwanted immunogenicity against protein-based biologics are applicable in full when developing similar assays for a GTx therapeutic. For example, sponsors will need to determine whether a tier-based approach to assess immune response is appropriate for a GTx therapeutic, including assessment of antivector and antitransgene protein immune response or should the evaluation be focused on the detection of neutralizing activity only. Sponsors will also need to determine whether a statistically based assay cut-point calculation, which is required for protein biotherapeutics is applicable for a GTx modality, including anti-GTx NAb assay protocols. Other details to be discussed and agreed on include cell lines to be used in anti-GTx NAb protocols, acceptable NAb and TAb assay sensitivity and the nature of positive and negative suitability controls used in the assays. Overall, additional effort will be needed to standardize protocols applied to detect anti-GTx specific immune responses.

Current regulatory guidelines do not provide an in-depth comprehensive discussion of methods designed to evaluate innate immune responses. The innate pathway plays a critical role in the early non-specific response to a viral infection. Various cell types and cellular receptors, such as Toll-like receptors, may recognize and respond to the presence of highly structured viral particles [50,51]. The AAV type viruses have limited potential to interact with toll-like receptors although other mechanisms of innate anti-AAV virus response have been described and reviewed [52].

The Enzyme-Linked ImmunoSpot (ELISPOT) analytical platform was proposed for the purposes of screening peripheral blood mononuclear cells ability to produce INF-γ in the presence of viral and/or transgene protein generated peptides [17]. The ELISPOT methodology has been referenced as the main approach to evaluate cellular immunity against GTx components. Although currently, there are no regulatory guidance documents that provide agencies position on the ELISPOT method development and validation, industry guidelines are available [53,54]. Flow cytometric protocols designed to detect antigen specific T cells have also been developed and may be applied as an alternative approach to the ELISPOT platform [55,56].

Detection of AAV genome material

Exposure & biodistribution in clinical subjects

Biodistribution studies are often a requirement for preclinical studies in AAV GTx development; however, translating preclinical data into the clinic are recognized as being challenging [13]. Regulators require that sponsors of GTx trials understand the kinetics and load of AAV transgene particles shed from patients after dosing. These shedding assay requirements are discussed below. In comparison, a true biodistribution study is not commonly asked for or possible in clinical studies but is discussed in guidance documents [10,13,15]. Standard absorption, distribution and metabolism studies are not directly relevant for AAV GTx; however, either blood or other matrix samples can be used to assess the systemic persistence of the vector, along with potential environmental exposure to caregivers and the general population to the AAV transgene construct [10].

Assessment of shedding in clinical studies

In comparison to typical small and large molecule treatment modalities, traditional pharmacokinetic (PK) measurements using MS approaches are not appropriate for measuring viral GTx kinetics in patients. Instead a quantitative polymerase chain reaction (qPCR)-based assay needs to be developed to measure AAV GTx in patient blood samples. In addition, regulatory agencies require measurement of shed AAV particles from patients in a variety of matrix types, including whole blood, or plasma/PBMCs, saliva, urine, semen and stool depending on route of delivery. These are often required for part of an environmental risk assessment of the AAV GTx. In addition to providing information on the rate of detectable shed AAV, the data from these assays are informative on the kinetics of AAV clearance from these biological matrices. Whole blood samples, or blood components such as plasma and PBMCs, should be assessed to build a profile of clearance from circulation for each AAV GTx in lieu of a more traditional PK measurement. In several countries and regions AAV GTx fall under genetically modified organism regulations and as such the environmental risk of exposure to both caregivers, healthcare workers and the general population will need to be defined through an understanding of the levels of shed viral particles from study participants.

There are limited published guidance's specifically addressing regulatory agency expectations for shedding assays. In general, the validation of these assays should be carried out as per the general biomarker and analytical assay guidance's that are available for the regulatory agencies that the sponsor proposes to seek approval from [57,58]. The serious long-term event monitoring guidance specifies a detection limit of 50 copies per microgram of genomic

DNA [14]. While that number can be calculated for tissue and cells such as PBMCs, it becomes a more difficult number to quantify for whole blood or urine samples. The sponsor is responsible for determining the lower limit of quantification (LLOQ) for the qPCR assays in each matrix to be assessed. Given the high sensitivity of qPCR assays, coupled with the increased specificity of a probe-based assay, it is reasonable to expect that most assays will approach or surpass this recommendation. Care should be taken during the development of the assay(s) to ensure that the extraction efficiency of the AAV GTx in each matrix is reasonable and well understood by the testing laboratory. Additionally, the assessment of PCR inhibitory effects of the matrix, buffers, and storage kit(s) should be investigated as part of assay development and be part of the validation package. A primer target that covers at least a portion of the inserted transgene should be used, in order to minimize potential false-positive numbers from wild-type AAV infections. While AAVs have been shown to be stable [59], short and long-term stability assessments in each matrix, as well as the impact on freeze–thaws should be established. It is reasonable to expect freeze–thaw assessments to be done early in assay development, whereas longer-term stability assessments can be carried out in parallel to assay development. Given the prevalence of AAVs in the general population, special care should be taken during assay development to build in multiple levels of negative controls throughout the extraction and qPCR assay. To avoid differences in manufacturing of preclinical versus clinical material, the clinical lots of AAV material should be used as the standards for the assay, as well as for the initial development and validation of the qPCR assay.

Given that a qPCR assay has the potential to detect fragments of DNA as well as the full AAV/transgene complex, most regulatory bodies will additionally expect a DNAse treatment of each sample type. This will allow for the differentiation between AAV protected transgene DNA versus fragmented single strand DNA in samples which could artificially increase AAV/transgene quantification.

Assessment of infectivity in clinical studies

As a component of the environmental risk assessment, regulatory agencies may request further characterization of the shed material for infectivity and growth. The EMA guidance [58] specifically requests that sponsors develop an infectivity type assay but indicate that if the LLOQ of that assay once fully characterized is found to be significantly higher than results found from shedding assays, that a sponsor may not need to run the infectivity assay. Since the assay may utilize the same end point qPCR as the shedding assays, it therefore may be recommended to develop both assays in parallel. Currently, no regulatory guidance exists requiring these cell-based infectivity assays to be validated to a specific standard. There are clear expectations for infectivity cell-based assays for other delivery modalities based on replication competent viral vectors [57].

The development of an infectivity assay is difficult, as AAV alone does not induce cytopathic effect in cell culture and while AAV may be internalized in cells, the virus may not be infectious. To assess infectivity, a susceptible cell line is treated with the shed material, in the presence or absence of a helper virus, incubated and then assessed for viral internalization using a qPCR-based assay, ideally using the same assay used to assess shedding. During the development of the infectivity assay, the cell line of choice, seeding density, and incubation time must be optimized, a limit of detection (LOD) and LLOQ established, the reproducibility of these results determined, and the stability of the infectious material assessed. Using a qPCR-based readout, PCR inhibitors should also be assessed and minimized.

Assessment of genome integration in clinical studies

The genomic payload of recombinant AAV vectors does not need to integrate into the host genome in order to be biologically active and the DNA remains primarily episomal. AAV are considered to be nonintegrating vectors in regulatory guidelines [10,13,15]. However, random integration events can be observed in AAV with a low frequency of between 0.1 and 1% of transduction events [60]. There are some studies that have shown increased liver tumors when neonatal mice were injected with AAV in association with integration into specific sites in the mouse genome that do not have a human homolog [61]. However, others have not found tumor induction when adult mice are treated with AAV [62–64]. Long-term follow-up of 135 hemophiliac dogs for greater than 10 years [65–67], and NHP for greater than 5 years [68] that were treated with AAV vectors replacing factor IX have not found any evidence of tumor induction. Additionally, there have been no reported incidences of AAV vector-induced neoplasia in humans in over two decades of AAV use in clinical trials. However, developers of AAV gene therapeutics should plan to discuss AAV genomic integration with regulatory agencies early in the development process to ensure there is no concern regarding the specific AAV construct or intended disease population that the vector will be used in.

Regulatory expectations for qPCR-based assays

To accurately quantify the amount of shed and/or infectious material as required by multiple regulatory guidance documents, a validated, quantitative qPCR assay is necessary. The Minimum Information for the Publication of Quantitative Real-Time PCR Experiments (MIQE) document provides methodology considerations for the design of qPCR assays and experiments [69,70]. When designing qPCR-based assays, nonspecific dyes or sequence-specific probes are available as reporters. Nonspecific dyes (such as SYBR Green, SYTO-13 and SYTO-82, etc.) intercalate into dsDNA, but could detect nonspecific amplification leading to concentration overestimation or false-positive signals. The use of fluorogenic probes leads to specific binding of the probe to the targeted sequence, providing greater specificity in qPCR amplification [71]. When designing qPCR assays, special consideration in primer and probe design should be given with regards to amplicon size, GC content and location [72]. When considering GTx constructs, the qPCR amplicon should include a portion of the transgene to verify the delivery of the genetic payload and avoid an over estimation based on the potential presence of empty capsids. After initial screening of potential qPCR assays, primer and probe concentrations should be optimized. Several approaches are available for optimization of qPCR assays, one of which is the use of full factorial design. This approach estimates the effect of each PCR factor on assay performance and may detect interactions between PCR factors while determining optimal reagent concentrations [73]. The optimal assay should be selected based on a combination of amplification efficiency, highest fluorescence values and lowest cycle threshold (Ct) counts. Amplification efficiency is determined based on the slope of the standard curve using the formula $E = 100 \times (10\text{-}1/\text{slope} - 1)$ [74]. The ideal slope for 100% amplification efficiency is approximately -3.3, but the acceptable range for a quantitative PCR assay is between -3.1 and -3.6 (90–110%) [70,74]. Sensitivity is critical to the quantification of shed material. Sensitivity should be assessed across a linear dynamic range ($R^2 > 0.98$) of ideally 5–6 $\log_{10}$ and determine the LOD and LLOQ and to verify the assay is sensitive enough to reach the 50 copies per µg of genomic DNA, as referenced above [69,75]. Assessing the linear range of the assay requires the development and characterization of an appropriate standard curve.

Developing a standard curve for GTx constructs, with respects to shed material, requires optimizing the standard curve in all sample type matrices. This will ensure accurate quantification in each sample type and help identify any matrix-induced effects during DNA extraction [76]. For initial primer/probe evaluation, linearized plasmid can be used; however, the use of plasmid for quantification of AAV material can lead to an overestimation of the titer [77]. For AAV constructs, standard curve development and final assay validation should be performed using encapsulated DNA, preferably using the same serotype capsid used in clinical dosing. For quantification of shed material, internal spike-in controls should be used to characterize extraction efficiency and evaluate PCR amplification quality and inhibition [71]. Multiplexing assays in a single well should be considered to evaluate the target amplicon as well as the internal control as this provides greater power to the qPCR analysis [69]. Validating a multiplexed assay requires evidence demonstrating that the presence and amplification of multiple targets in a single well is not impaired by the other assays and that the efficiency and LOD of the assays are the same as when the assays are performed on their own [69].

Specificity, reproducibility and robustness must be determined in order to validate qPCR assays. Specificity should be assessed using tools such as NCBI BLAST or equivalent technologies and should be assessed during optimization by testing target sequence in the presence of human genomic DNA [69,78]. Reproducibility experiments should be performed to verify the precision of results for the same method using the same samples performed by different operators and multiple instruments. If the assay is to be performed at multiple laboratories, reproducibility should be evaluated across all locations. A well-designed qPCR assay should demonstrate a percent CV less than 25% for these reproducibility experiments [74]. As a component of validation, robustness must also be assessed by testing different master mixes, different qPCR instruments, accounting for variation in assay run parameters (i.e., annealing/extension temperatures and times, etc.) and performing guard-banding experiments [79].

An alternate approach to standard qPCR assays is digital droplet PCR, which has become a more established and mature technology over the past several years. During a droplet digital PCR (ddPCR) assay, DNA and the target assay are dispersed into thousands of individual oil droplets where individual PCR reactions can occur [70,80]. This reduces the amount of background DNA in each reaction enabling greater detection of low copy amplicons. ddPCR has advantages over qPCR in that the technology provides an absolute quantification of copies/ml without the use of a standard curve, is less affected by sample inhibitors, and is considered more precise [81]. However, ddPCR is generally more expensive, has a smaller dynamic range, and has limited multiplexing capacity [81]. As

ddPCR is a relatively new technology, most of regulatory guidance's reference the use of qPCR for quantification of genomic material. We anticipate that as the use of ddPCR becomes more prevalent that this will change. However, if ddPCR is selected as the end point assay of choice we would recommend that, bridging studies be carried out to verify the assay of choice performs as well, if not better, when using ddPCR as compared with conventional qPCR.

Detection of transgene protein

As a growing number of GTx products enter clinical development, particularly in rare diseases [82], the need for transgene protein detection has become increasingly pressing as an important contribution to GTx development. While some regulatory guidance documents specifically mention the use of the transgene protein measurement in the context of preclinical toxicology or preclinical and clinical pharmacodynamics [10,15]; detailed guidance does not yet exist for clinical samples. Depending on the disease pathophysiology, some GTx program may use suitable functional end points or efficacy biomarkers instead of, or in addition to, transgene protein expression. Examples of such biomarkers are factor activity as primary end point in hemophilia GTx clinical trials [17], or serum phenylalanine levels in a mouse model of human phenylketonuria [83]. However, a more detailed discussion of considerations for biomarkers in GTx is beyond the scope of this article.

The objective of most AAV GTx approaches is gene replacement to achieve long-term stable transgene protein expression at levels that are therapeutic [39]. While scientific advancements are expected to be made also in diseases with multigene defects, current GTx approaches focus mainly on treating monogenic diseases, in other words, with a single-gene defect. Therefore, the aim is to achieve transgene expression of a single protein at an expression level resulting in meaningful clinical benefit.

Measuring the transgene protein can be a critical aspect of GTx development, both preclinically and clinically. Preclinically, this assessment contributes to the selection of drug constructs for further development, is recommended to help with setting a suitable dose for preclinical studies and to determine an initial clinical dose in a subsequent first in-patient trial [10]. Assessment of preclinical transgene protein expression profile is also recommended to identify the potential for induced toxicity if expression is too high or if aberrantly expressed in nontarget tissues [10]. In early clinical studies, understanding of the transgene protein expression contributes to dose selection and importantly enables correlation with effectiveness of the treatment and clinical outcome, as shown in GTx clinical trials for hemophilia B [84]. In a rare disease guidance document, the FDA lays out issues for evaluation and validation of surrogate biomarkers, including transgene protein [85]. In some GTx strategies, transgene protein expression may be pursued as a surrogate end point and if it is considered reasonable likely to predict clinical benefit, it may be used as a basis for accelerated approval.

Across this evolving field of GTx, there is a broad range of classes of transgene proteins requiring their detection including soluble proteins, enzymes, structural and membrane proteins as well as intracellular proteins located in specific subcellular compartments. Many of these proteins or even protein classes have not been encountered in bioanalytical laboratories. Furthermore, significant technical challenges can exist when the transgene protein and the endogenous counterpart are not fully identical but need to be analytically differentiated. Reasons for this difference could be mutations or other modifications in the endogenous protein leading to its absence or reduction in function. In addition, in some preclinical investigation, the human transgene protein derived from the clinical drug product needs to be differentiated from the respective endogenous protein in a preclinical species [86]. Furthermore, AAV packing size limitations of the plasmid-encoded vectors may result in the design of shortened, truncated transgene proteins compared with the endogenous form [87]. At last, depending on the etiology of the disease, these proteins need to be measured in a range of tissues and biofluids. For tissue-based transgene protein assays, like for any other tissue protein assays, optimized extraction conditions need to enable high and reproducible recovery [88], while being compatible with the downstream analytical method. A variety of technologies are required for transgene protein detection accommodating the above considerations. These range from ligand binding assays, western blots, tissue staining techniques, to more recently protein MS. The latter technique is particularly suitable for the quantification of tissue proteins [89,90]. For example, it has been shown to be able to quantifiably measure dystrophin protein in skeletal muscle tissue where previously only western blot methods were available [91,92]. However, it has become clear that to address the emerging, significant bioanalytical needs for transgene protein expression and associated regulatory expectations, improved and advanced bioanalytical methods are needed.

The expression of transgene protein in patients can be compared with a control, normal population and depending on the disease and sample availability also to the patient's own baseline. Furthermore, depending on what is known about the course a disease takes in the absence of intervention (natural history) and the associated

Table 1. Analytical tests requested by the current guidance documents in support of GTx modality therapeutics.

Phase of GTx treatment or therapeutic development	Test type	Additional details	Request level	Ref.
Pre-administration	Antivector immunity	Total antibody response	Recommended	[13–15,17,18,27–29]
		Neutralizing antibody response	Recommended	
		Cellular response	Discussed	
Post-administration	Antivector	Total antibody response	Discussed	[12,18]
		Neutralizing antibody response	Discussed	
		Cellular response	Discussed, potentially critical	
	Antitransgene protein	Total antibody response	Discussed	[11,13,14,17,18]
		Neutralizing antibody response	Discussed, potentially critical	
		Cellular response	Discussed, potentially critical	
	Vector shedding	Vector shedding	Required until subject negative	[57,58]
	Infectivity	Infectivity	Dependent on shedding data	
	Transgene protein	Transgene protein	Discussed, possible biomarker	[10,15]
Follow-up	Antitransgene protein	Total antibody response	Discussed	[11,12,17]
		Neutralizing antibody response	Discussed, proposed	
		Cellular response	Discussed, proposed	
	Vector shedding	Vector shedding	Required if subject not negative during trial	[57,58]
	Infectivity	Infectivity	Dependent on shedding data	
Post-approval	Antivector CDx	Total antibody response or neutralizing antibody response	Required	[17,18,44]

CDx: Companion diagnostic assay; GTx: Gene therapy.

expression of the endogenous protein that is replaced by the GTx, a prospective natural history study can be conducted [18,93]. As outlined in the regulatory guidance documents, natural history studies can help design and conduct clinical trials. Target protein expression can be monitored through different phases or clinical stages and considers demographic, genetic, environmental and other variables that correlate with disease and outcomes in the absence of GTx treatment [93]. Target protein data can then contribute to building a correlation between endogenous protein expression and function that will help with establishing a correlation of transgene protein expression and outcome in GTx clinical trials.

Regulatory guidance documents dedicated solely to the bioanalysis of transgene protein expression do not exist. The FDA document provides some guidance for analytical validation of surrogate biomarkers that include the transgene protein [85]. It states that analytical validation should be confirmed before starting the clinical trial and should evaluate several factors including assay sensitivity, specificity, range of results that can be measured, standardized methods of sample collection, shipment and preparation as well as reproducibility of the results. However, for more specific analytical guidance the FDA bioanalytical method validation and EMA bioanalytical guidance documents are applied where possible and appropriate to the protein class, detection method and importantly the use of the data (Table 1) [94,95].

Conclusion

Immune response potential against viral vector-based GTx modality therapeutics is highly unique and impactful. While pretreatment immunity against protein-based biotherapeutics is not commonly expected, it has been broadly shown that antiviral antibody and cellular immunity is highly prevalent and potentially detrimental for the GTx treatment. It is therefore imperative to understand and assess potential impact and ability to measure antivector immune response before treatment is initiated. Although antibody-based pretreatment immunity has been viewed as the main risk element, cellular immunity can also be considered during patient assessment prior to the treatment. Development of specific companion diagnostic tests, hence becomes an expectation for a successful approval and launch of a GTx treatment.

Due to highly foreign nature of the virus and potentially transgene product protein as well, post-treatment immunity can be expected. Hence, subject monitoring becomes an important element of the treatment protocol. Regulatory guidelines describe monitoring for the specific immune response to the virus and transgene protein.

Guideline documents and industry publications also focus on the need to assess potential cellular response and potential patient treatment implications.

Guidelines list immune response assessment is a part of the follow-up monitoring, particularly when there is evidence of clinical relevance between antiviral or antitransgene protein immunity and clinical impact.

Multiple countries and regions classify GTx therapies under genetically modified organism regulations. As such understanding the potential environmental risk of exposure to caregivers and to the general population by an AAV GTx is a requirement. Additionally, understanding the PK of systemically delivered AAV GTx requires additional whole blood, or blood component, assay development and modeling.

At last, transgene protein expression can be an important measurement for some GTx programs, both preclinically and clinically. Guideline documents and literature focus primarily on its use as a biomarker.

Future perspective

With an increasing number of GTx therapeutics in development, we believe that further alignment around regulatory expectations and requirements will be needed in the future. Questions related to the relevance of certain tests, the degree of analytical method fit-for-purpose validation, analytical data relevance to the patient selection and requirements for post-treatment monitoring will likely to be further discussed and refined. Although several GTx-related guidance documents are already available, additional GTx bioanalytical guidance documents may be needed.

Executive summary

- Pretreatment immunity to gene therapy (GTx) therapeutic can be expected and should be evaluated due to high potential to impact treatment outcome.
- Antibody based and cellular anti-GTx immunity have been detected pre- and post-treatment.
- Post-treatment immune responses to viral vector proteins as well as to the transgene protein can be expected.
- Shedding assays are required by health agencies for multiple matrices, as well as whole blood or blood components to assess environmental risk and pharmacokinetics.
- Detailed analysis of post-treatment antitransgene protein immune response can be critical to understand GTx treatment impact.
- Companion diagnostic assay-based assessment of pretreatment antiviral vector immunity is likely to be required after the regulatory approval.
- Transgene protein expression can be an important biomarker measurement for some GTx programs.

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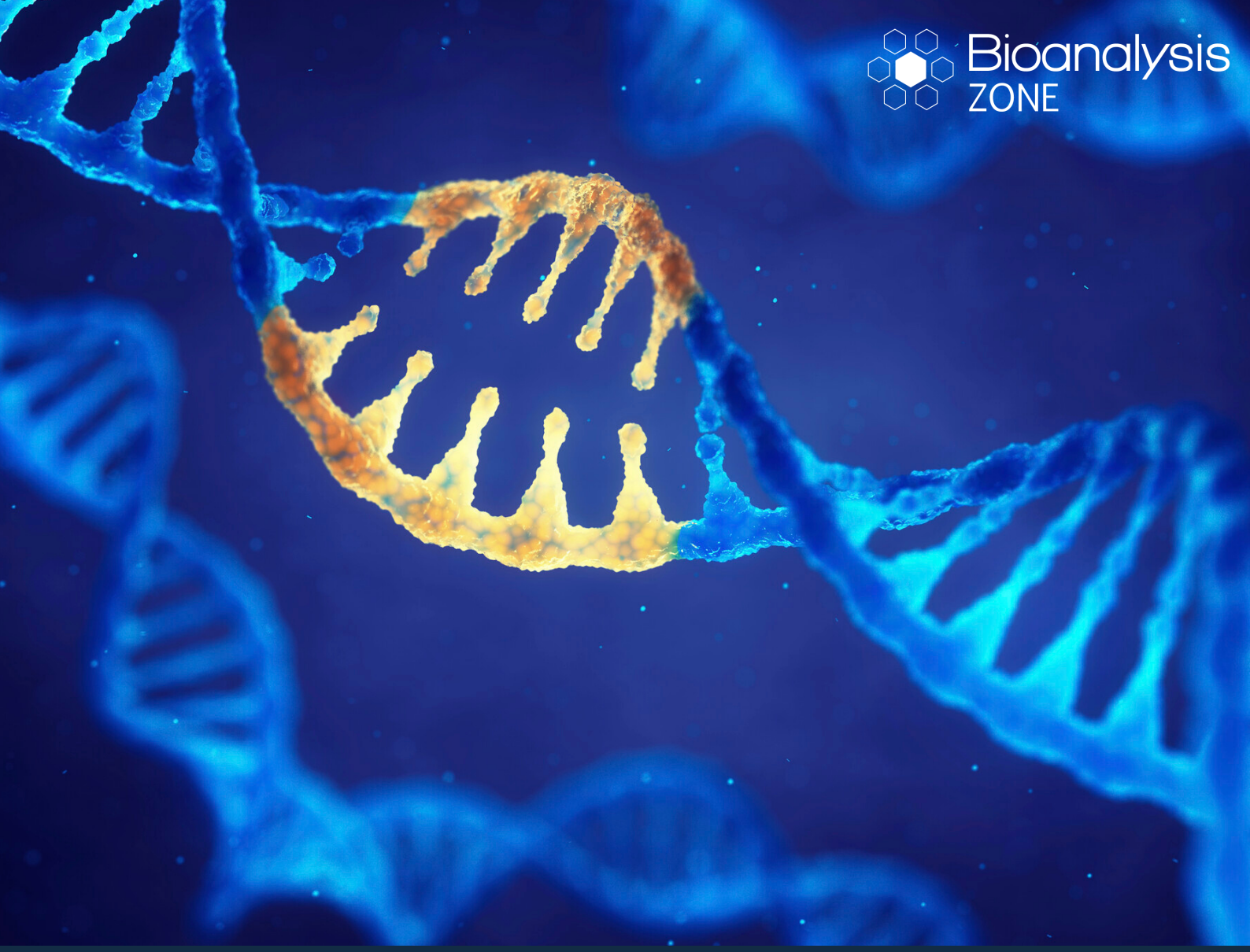

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