Mini Review
Volume 14:10, 2022

Journal of Bioanalysis & Biomedicine

ISSN: 1948-593X

Open Access

Bioanalytical Assays of AAV Therapeutics: Recent Advances and Considerations

Xiaolong Tom Zhang[1]*, Niancao Chen[2] and Mike Mencer[1]

[1]*Worldwide Clinical Trials, Texas, USA*
[2]*Fate Therapeutics, California, USA*

Abstract

Over the last decade, the advance of manufacture, delivery and immunity modulation, gene therapeutics (GTs) has resurged as most popular modalities. Due to its special nature in delivery and biodistribution as well as the unique mechanism of actions, conventional bioanalytical approaches have limitations in supporting the development of GTs. This review summaries the challenges and considerations in designing GTs related bioassays.

Keywords: AAV • Bio analysis • Bio distribution • Shedding • Immunogenicity • Potency

Introduction

GTs that use genes to treat or prevent disease have gained significant attention to address various unmet medical needs [1]. Though the concept of GTs was proposed in the early 1970s, the discovery and development of GTs experienced interruption until the 2010s with an exciting resurgence and significant improvements in safety and efficacy [2]. In the past thirty years, more than 2,500 clinical programs have been performed for a broad range of applications from a variety of cancers to neurodegenerative disorders and infectious diseases. Several gene therapy products have been approved by different regulatory agencies (Table 1). Based on the assessment of the current pipelines and clinical success rate, the U.S. Food and Drug Administration (FDA) predicts the approval of 10-20 gene and cell therapies every year from 2025 onward, announcing it a turning point in the development of these technologies and their applications to human health [3]. During the development of GTs products, assessment of bio distribution, shedding, immunogenicity and potency are four critical integral parts for measuring safety and efficacy across various development phases. This section reviews recent practices, challenges and considerations in designing bioassays to support the development of adeno-associated virus (AAV)-based GTs (Figure 1).

Description

Bio distribution

The evaluation of vector distribution profile following gene therapeutic administration is one of the key components to preclinical and clinical programs [3]. The biodistribution data serves to investigate the presence of vectors in both desired and undesired body fluids, tissues and organs. Q-PCR/dd-PCR is the popular approaches for characterizing the viral vector, including the presence, persistence, and clearance [4]. During the development of qPCR based bioanalytical assays, scientists must optimize the extraction, amplification and detection of the nucleic acid analyte. Method validation is expected in supporting GxP level studies, and the validation should cover assay accuracy, assay precision, limit of quantification (LOQ), assay range, linearity and robustness [5]. As there is no bioanalytical guidance from regulatory agencies, the assay criteria are often defined according to the purpose of the studies. Despite the prevalence of qPCR in bioanalysis of gene therapeutics, it has several limitations. During the qPCR analysis, a large number of cells are required for extracting sufficient amount of DNA. During the extraction of nucleic acid, the cross-contamination across the experimental procedures limits the application of qPCR in some laboratories [5].

Liquid chromatography mass spectrometry (LC-MS): is one of the most popular platforms for the qualitative and quantitative bioanalysis of various therapeutics in laboratories [6]. Due to the advance of ion-paring reversed phase, LC-MS is also favoured in analyzing nucleic acids. The sample preparation is critical during the analysis and Liquid-liquid extraction (LLE) or/and anion exchange solid phase extraction (SPE) are recommended in this step [6]. Beyond GTs, LC-MS is used to measure the change of proteins regulated by gene therapeutics because it offers attractive sensitivity, broad range and high specificity [7].

Ligand binding assay: Such as 'cutting ELISA' (oligonucleotide probe hybridization/S1 nuclease protection) and fluorescent oligo probe hybridization is another approach that can be used to support the bio analysis of gene therapeutics. Hybridization ELISA often offers improved assay sensitivity in comparison with LC-MS. There are FDA and ICH guidance with LBAs that support the assay development and validation. In addition, LBA is comparable

Table 1. Approved gene therapy products.

Approval Year	Approving Agency	Tradename	Manufacturer	Indication
2020	FDA	Tecartus	Kite/Gilead	Mantle cell lymphoma (MCL)
2019	FDA	Zolgensma	AveXis/ Novartis	Spinal muscular atrophy in children
2017	FDA	Kymriah	Novartis	Acute lymphoblastic leukaemia
2017	FDA	Yescarta	Kite Pharma	Non-Hodgkin lymphoma
2017	FDA	Luxturna	Spark Therapeutics	Inherited retinal dystrophy
2016	EMA	Strimvelis	GlaxoSmithKline	Adenosine deaminase deficiency
2015	FDA/EMA	Imlygic	Amgen	Metastatic melanoma
2012	EMA	Glybera	uniQure	Lipoprotein lipase deficiency
2003	NMPA	Gendicine	Shenzhen SiBiono GeneTech	Head and neck squamous cell carcinoma

***Address for Correspondence:** Xiaolong Tom Zhang, Worldwide Clinical Trials, Texas, USA, E-mail: tom.zhang@worldwide.com*

Date of Submission: 02 October 2022, Manuscript No. jbabm-22-75677; **Editor assigned:** 04 October 2022, PreQC No. P-75677; **Reviewed:** 16 October 2022, QC No. Q-75677; **Revised:** 21 October 2022, Manuscript No. R-75677; **Published:** 28 October 2022, DOI: 10.37421/1948-593X.2022.14.349

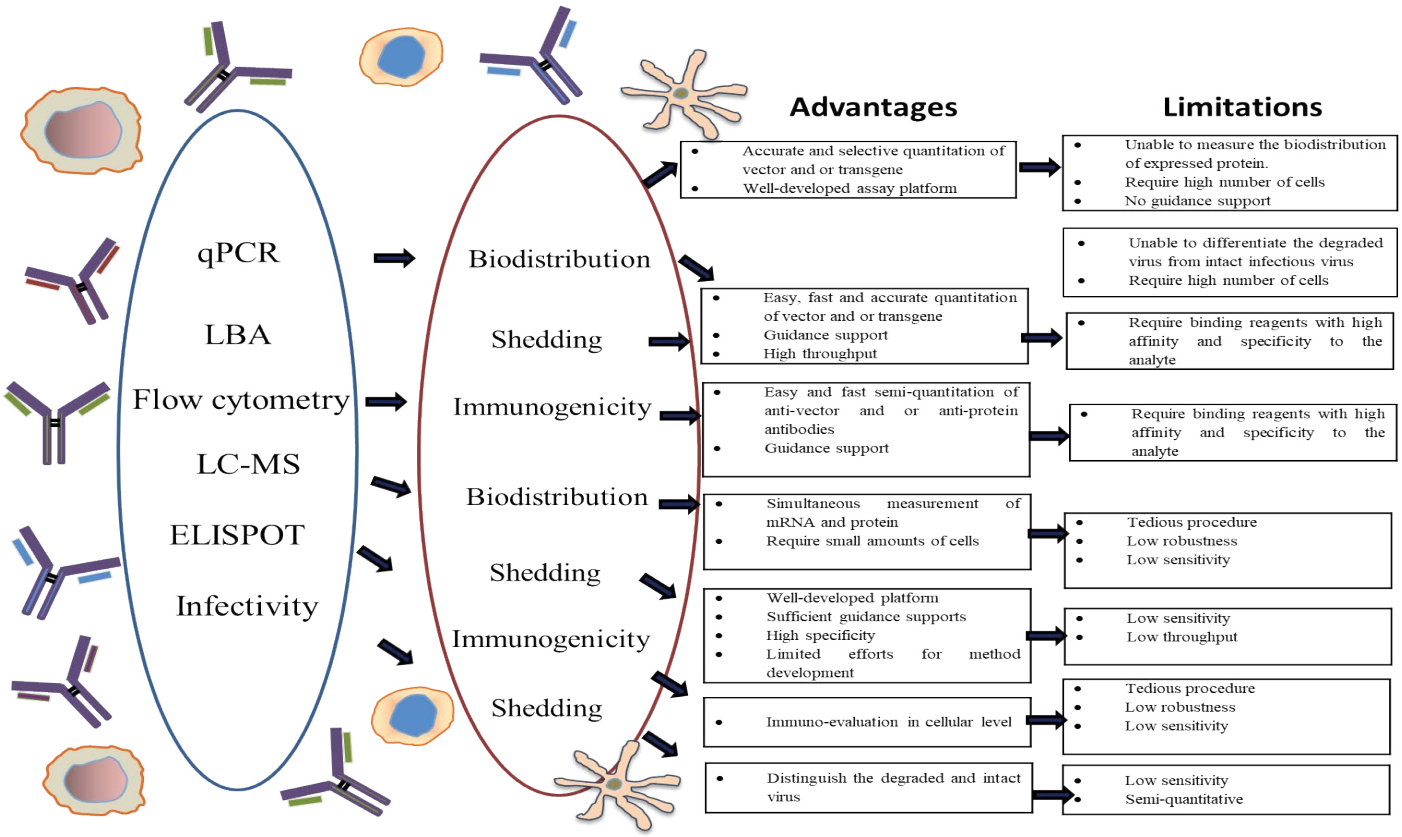

Figure 1. Bioanalytical comparison of qPCR, LBA, Flow Cytometry, LC-MS, ELISPOT and infectivity assays in supporting the biodistribution, shedding and immunogenicity of gene therapeutics.

with bio analytical automation system that provides high data quality and sample analysis efficiency. One limitation is the assay specificity challenge. Probes may often interact with matrix factors, which trigger non-specific assay signals.

Shedding: According to The International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use (ICH), shedding is the release of the virus-based gene therapeutics through secreta, excreta or skin of the patient. Shedding is different from bio distribution, which refers to the spread of viral gene therapeutics within the body system and its localization in tissues and organs. Instead, shedding assesses the potential risk to the environment and the impact to untreated humans and other species [7]. The U.S. FDA published guidance "Determining the Need for and Content of Environmental Assessments for Gene Therapies, Vectored Vaccines, and Related Recombinant Viral or Microbial Products" in 2015. The guidance explains that the U.S. FDA expects applications of gene therapeutics (e.g., INDs, BLAs and supplements to BLAs) to be accompanied by an Environmental Assessment (EA). Failure to include an EA within the application may result in the filing suspension unless a claim of categorical exclusion is granted under 21 CFR 25.15(a) [8].

In general, AAV is replication-incompetent, exists for a short duration and displays a different shedding profile compared to the oncolytic or other replication-competent viruses. However, manufacturing gene therapeutics is a complex procedure. The investigation of any potential replication-competent recombinants is recommended during the manufacturing of replication-incompetent vector/gene therapeutics. During the shedding assay design, the analyse of interest can be either nucleic acids or infectious viruses. The selection of a suitable bioassay is critical in generating meaningful and high-quality shedding data (i.e., data that accurately presents the shedding profile of the gene therapeutics and can be successfully applied to assess the risk of potential transmission to untreated individuals).

PCR and hybridization ELISA are common methods to assess shedding because of they are well developed assay platforms in high throughput formats and have the ability to procure high assay sensitivity, and fast turnarounds [9]. Scientists can use both assays to quantitatively report the number of genome copies for shedding evaluation by detecting the nucleic acids. One limitation of nucleic acid-based assays is that it fails to differentiate the intact infectious virus from degraded non-infectious virus As a result, the nucleic acid assay itself may not be sufficient in evaluating the viral shedding. Infectivity assay, an approach of *in vitro* titrating viral material into cell for reaching 50% infective dose, can be used measure the amount of infectious virus from the clinical samples. However, infectivity assay can only quantify the infectious virus that is mostly intact another limitation is that the sensitivity of infectivity assay is not comparable with qPCR or hybridizing LBA assays. Due to the reasons above, it is recommended to combine nucleic acid-based assays with infectivity assays in analyzing shedding samples. For example, suppose a PCR or hybridization ELISA method is used as a quantitative approach specific for nucleic acids of a virus as tier 1 level of sample analysis. According to the level of detected nucleic acids (i.e., the presence of high PCR signal above baseline) the corresponding study samples are further analyzed by infectivity assay in tier 2 level. However, the recommended analysis approach can be limited when the PCR assay has lower sensitivity than infectivity assay or the cell culture of infectivity is unsuitable due to the interference of special study samples (i.e., excreta).

Immunogenicity: Among the developed GTs, AAV is favoured resulting from the extensive transduction capability across the different tissue types and non-integrative property, promoting efficient and consistent transgene expressions. However, clinical trials reveal the humoral and cellular immunity on AAV and transgene that limit gene therapeutics development [9]. Pre-existing immunity against AAV has been widely reported in humans. As it is common to be infected with a broad spectrum of AAVs, anti-AAV antibodies have been reported to be prevalent in 30-60% of the population. Thus, developers should assess pre-existing humoral immunity against AAV vectors Scientists often use ligand binding assays for the assessment of humoral immunity. During the assay development, reagent labelling and PC selection are critical. Compared with the other biologics, the AAV vector has limited amino groups for the chemical conjugation of the biotin or sulfo-tag groups. The undesirable labelling ratio can limit the assay sensitivity. Though AAV-

based gene therapeutics are different from regular biologics, the general clinical ADA guidance can serve as the method development and validation for the clinical studies. Among the anti-AAV antibodies, the frequencies of AAV1 and AAV2 antibodies in humans were close to 70%, followed by AAV6, AAV9 and AAV8 [10]. The statistical investigation indicates that the seroprevalence geographically varies. For example, Sweden reported 48% of AAV1 neutralizing antibodies in the population while Poland and Hungary reported 79%, illustrating the geographical discrepancies [10]. This is also prevalent in U.S. states, where the state of Wisconsin reports anti-AAV1 antibodies at 32%, and the number increases to 67% in South Carolina. This fact requires the scientific rationale in selecting volunteers in studying the pre-existing immunity for AAV vectors.

It is better to have assays to verify the titer correlation between anti-drug antibodies and neutralizing antibodies in trials. Despite a few cases of enhanced gene delivery from volunteers with anti-AAV antibodies, many trials have shown the limited efficacy of AAV mediated delivery into these patients These patients tend to receive less benefits or even experience side effects after the administration of AAV gene therapeutics. The further characterization reveals the inhibition results from multiple steps, including receptor recognition, AAV entry, intracellular trafficking, nuclear transport and the synthesis of second-strand viral DNA. The characterization of anti-AAV IgG antibodies demonstrates that IgG1 is the predominant immunoglobulin subclass followed by IgG2 and IgG3. The high titer of IgG1 is often observed in the development of AAV neutralization. Studies indicate that IgG2 may be related to the route of administration. Alternatively, IgG3 may be associated with T cell reactions to AAV. Thus antibody subtype characterization assay is expected for this investigation. In addition to humoral immunity, pre-existing cellular immunity may be against AAV and limit the applications of AAV-based gene therapeutics. Scientists can detect cellular immunity by performing a few cell-based functional assays, including flow cytometry and Enzyme-Linked Immunospot (ELISPOT). The detection of T cells secreting IFN-gamma in the presence of the AAV antigen is a popular approach in assessing cellular immunity. However, the correlation of T cells secreting IFN-gamma with the presence of anti-AAV antibodies is not always observed in the trials.

Recent studies indicate that IFN-gamma is not the only signature cytokine for AAV mediated cellular immunity (2013). A few studies have reported two activation mechanisms of cellular immunity. The presence of AAV antigen induces transient activation of NK cells, which secret IFN-gamma and TNF-alpha in the seronegative population. Alternatively, AAV activates memory T cells that secrets TNF-alpha, IFN-gamma, IL-2 and cytolytic granules such as granzyme B and CD107a. Compared with the ligand binding assays, these cell-based assays have relatively low sensitivity. When designing these assays, it's critical for scientists to consider PBMC preparation and the selection of controls. During the assay development, scientists need to optimize parameters such as cell density, antigen concentration, detection concentration and image capture. When the assay supports regulated studies, assay sensitivity, cut point, precision, specificity and linearity laboratories must test under GXP guidelines. Due to the lack of guidance, there are no strict assay acceptance criteria based on the fit-for-purpose principle.

Potency: Potency is defined as "the specific ability or capacity of the product, as indicated by appropriate laboratory tests or by adequately controlled clinical data obtained through the administration of the product in the manner intended, to effect a given result." Potency assays measure the biological activity of a viral vector (i.e., AAV), which should be relevant to its therapeutic effects. Regulatory agencies such as the U.S. FDA recommends acquiring an appropriate understanding of the biological properties by exploring different characterization aspects (e.g., physical, chemical, biochemical, biological attributes) to develop a meaningful and relevant potency assay. Ideally, the potency assay will reflect, or to some extent, mimic the GT product's mechanism of action (MOA). However, GT products are complex and come with many challenges (Figure 2). For example, many AAV products rely on more than one biological activity to perform their therapeutic effects, and the MOA is not fully understood by the time potency assay is developed, making it challenging to have one assay fully representing the relevant therapeutic effects. Therefore, scientists are encouraged to develop multiple complementary bioassays to characterize and measure the relevant biomarkers at different stages along the gene transduction course. For instance, scientists can develop two individual assays focusing on the following two steps using appropriate analytical instruments and methods respectively, i.e., transferring genetic materials to the target cells and expressing the gene of interest in the target cells to perform its biological effects.

In vivo potency assays using appropriate animal models (rodents or NHP) have been widely utilized to support both preclinical trials and product development. These assays can be used as the release testing for some indications because they have directly measurable biomarker endpoints expressed by the transgene or their biological effects (e.g., factor VIII/IX or bleeding rate in a hemophilia model In addition, animal models can better represent the physiological conditions and the target cell microenvironment where the surrounding immune cells, blood, and extracellular matrices (ECMs) might significantly impact the viral vectors' efficiencies. Moreover, AAVs with specific promoters might be efficient in transducing target cells *in vivo* while performing poorly in cell-based systems. Although *in vivo* platforms serve as an adequate potency platform for supporting GT product development and preclinical/clinical trials, cell-based *in vitro* potency assays (Figure 3). are still highly desirable and recommended by the U.S. FDA for the following reasons: 1) high throughput while low cost of materials; 2) less variation than *in vivo*

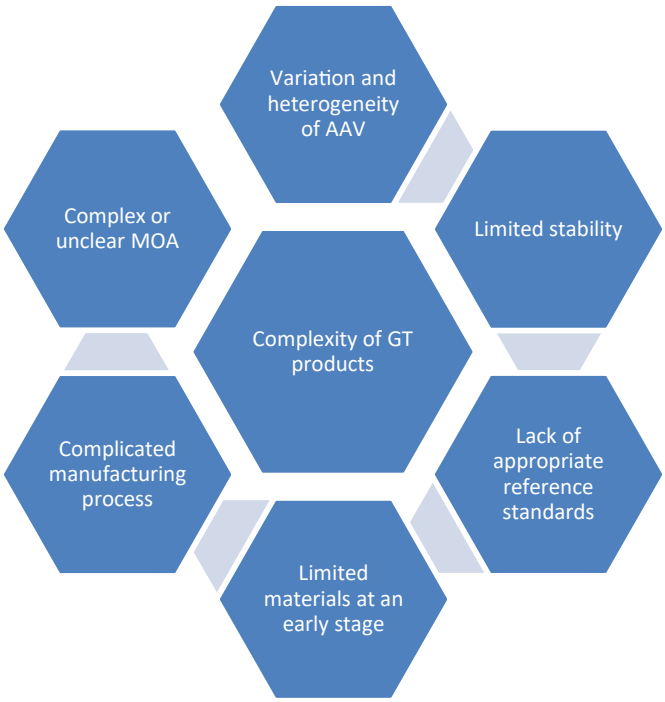

Figure 2. Complexity of GT products.

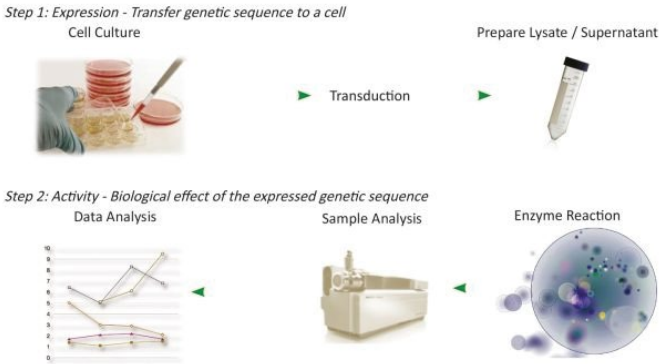

Figure 3. Stepwise in vitro potency assay. In step 1, tansgenes in cells are quantitated. In step 2, protein expression and/or its associated biological effects are measured. The scheme is adopted from a literature(Doucette and Shujath 2020).

assays; 3) suitable for validation and routine release testing and adaptable to automation; 4) reduce the use of animals according to 3 Rs. Because of the complexity of GT products, some unique challenges need to be considered and tackled to establish an optimal and reliable bioassay. To achieve this, a stable cell line, which serves as one of the most critical reagents, should be in place and it must be compatible with the vector capsid and promoter. In some cases, a commercially available cell line might serve as an appropriate platform for assay development. For instance, the HepG2 human liver cell line has been successfully adopted for AAV8 cell infectivity/transduction. In other cases, a customized cell line may be needed to achieve this goal.

Another challenge associated with *in vitro* assays is that AAV vectors generally have a relatively poor transduction efficiency in cells, which requires a high multiplicity of infection (MOI). These costs significant amount of materials for assay development and validation, and it may not be sustainably available especially during early development. To solve or alleviate this challenge, scientists have tried to streamline the efforts in optimization of assay and vector engineering for capsid/genome to augment the cell transduction efficiency. As mentioned earlier, a variety of analytical tools can be utilized to measure the transgene in cells or its protein expression. Scientists use quantitative PCR (qPCR) as the gold standard for quantitating transgene delivery inside cells. Recently, the reverse-transcription droplet digital PCR (RT-ddPCR) has also emerged as a more accurate, reproducible, and robust technique to quantitate AAV titers and potency in the industry. Scientists developed a one-step RT-ddPCR method to quantitate vector expression *in vitro* and in an NHP with good precision and linearity with a range of 0.05 to 25ng of RNA input. To quantitate the transgene expression (i.e., protein), scientists can utilize various immunological binding assays, such as western blot, flow cytometry and ELISA, to quantitate the target protein expression. Additionally, a scientists can develop a functional assay for the protein expression to quantify the vector transduction efficiency. For instance, a cell-based quantitative assay was developed and validated for potency assessment of a clinical-grade AAV vector to treat Crigler-Najjar syndrome. In this assay, both UGT1A1 transgene expression and its biological activity were quantified using flow cytometry and a bilirubin conjugation assay. Finally, to establish a validated bioassay, it is vital to understand and control the variations and include a reference standard material as the control. Therefore, assay parameters need to be optimized and controlled. These factors include cell seeding density, AAV incubation time, chemical enhancers for transduction, and transduction medium. Appropriate statistical analysis is also highly recommended to draw meaningful and reliable conclusions in assay development and correlating with *in vivo* potency data set.

Conclusion

With the recent progress on control of GTs manufacture, monitor of nucleic acid delivery and modulation of immune system, significant resources have been invested into GTs modality. At the same time, efforts are required in developing new bio analytical technologies and standards, which help to set up a solid foundation for the development research.

References

1. Allay, James A., Susan Sleep, Scott Long and David M. Tillman, et al. "Good manufacturing practice production of self-complementary serotype 8 adeno-associated viral vector for a *hemophilia* B clinical trial." *Hum gen ther* 22 (2011): 595-604.
2. Anguela, Xavier M., and Katherine A. High. "Entering the modern era of gene therapy." *Annu Rev Med* 70 (2019): 273-288.
3. Arnaboldi, Sara, Roberto Benevenia, Barbara Bertasi and Elisa Galuppini, et al. "Validation of a real-time PCR method on pta gene for *Clostridium tyrobutyricum* quantification in milk." *Food Control* 130 (2021): 108250.
4. Aronson, Sem J., Robert S. Bakker, Sascha Moenis and Remco van Dijk, et al. "A quantitative *in vitro* potency assay for adeno-associated virus vectors encoding for the UGT1A1 transgene." *Mol Ther Methods Clin Dev* 18 (2020): 250-258.
5. Atkinson, Barry and Eskild Petersen. "SARS-CoV-2 shedding and infectivity." *The Lancet* 395 (2020): 1339-1340.
6. Bartel, Melissa, David Schaffer and Hildegard Büning. "Enhancing the clinical potential of AAV vectors by capsid engineering to evade pre-existing immunity." *Front Microbiol* 2 (2011): 204.
7. Bartlett, Michael G., Jaeah Kim, Babak Basiri and Ning Li. "Sample preparation for LC-MS bioanalysis of oligonucleotides." Sample preparation in LC-MS bioanalysis (2019): 319-334.
8. Bartus, Raymond T., Marc S. Weinberg and R. Jude Samulski. "Parkinson's disease gene therapy: success by design meets failure by efficacy." *Mol Ther* 22 (2014): 487-497.
9. Basner-Tschakarjan, Etiena, Enoch Bijjiga and Ashley T. Martino. "Pre-clinical assessment of immune responses to adeno-associated virus (AAV) vectors." *Front immunol* 5 (2014): 28.
10. Broeders, Sylvia, Ingrid Huber, Lutz Grohmann and Gilbert Berben, et al. "Guidelines for validation of qualitative real-time PCR methods." *Trends Food Sci Technol* 37 (2014): 115-126.

How to cite this article: Zhang, Xiaolong Tom. Niancao Chen and Mike Mencer. "Bioanalytical Assays of AAV Therapeutics: Recent Advances and Considerations." *J Bioanal Biomed* 14 (2022): 349.