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QUANTITATIVE PCR (qPCR) AND DROPLET DIGITAL (ddPCR) – LEADING-EDGE ANALYSIS FOR YOUR GENE THERAPY PROGRAMS

Gene therapy continues to accelerate through preclinical and clinical research arenas. These programs are developed with targeted and personalized medicines in mind. The goal of gene therapy is to safely deliver and incorporate a genetic alteration to restore/repair the protein of a missing or faulty gene. Preclinical assessments of gene therapies consider the general absorption, distribution, metabolism, and excretion (ADME) of the drug, as well as data from tailored assessments to evaluate delivery and cell incorporation.

Gene therapies require, by design and definition, DNA and/or RNA delivery and analysis. While most ongoing research involves gene therapies being delivered *in vivo* via adeno-associated viral (AAV) vectors, new *in vivo* delivery mechanisms are on the rise, such as other types of delivery vectors including lipid nanoparticles.

In this issue, we will review the applications of PCR analysis for your gene therapy programs, and present two case studies.

INTRODUCTION TO qPCR, dPCR, AND RT-PCR

At the most basic level, PCR involves thermal cycling (repeated cycles of heating and cooling) of a reagent cocktail. Each reaction contains buffers to facilitate DNA polymerase enzymatic amplification of nucleic acids, and target-specific primers and probes. Primers and fluorescent probes are designed and optimized for each individual assay and platform. Determining the appropriate region(s) or target(s) of amplification and compatible fluorescent probe design is paramount for accurate quantification.

Quantitative PCR (qPCR), often referred to as real-time qPCR, allows for an absolute or relative quantification of a DNA target, and is a reliable and well-established method of quantification and amplification. A standard curve is utilized for each target, and it is easily multiplexed based on the scope of the study. Copy number assessment typically ranges from 5.00e+01 to 5.00e+07.

Digital PCR (dPCR) analysis, specifically with respect to the droplet digital PCR **(ddPCR)** platform, uses the same preparations as a qPCR assay, except that prior to amplification, the reaction is partitioned into nanoliter-sized droplets. ddPCR is advantageous on studies where target copy numbers are expected to be low, between 30 and 150,000 copies. Statistical analyses used in analysis improve the signal-to-noise ratio of inhibitors and an exact copy number is counted within the droplets generated. In ddPCR, there is no standard curve required for copy number quantification, and the assay can be multiplexed based on the scope of the study.

Reverse Transcription PCR (RT-PCR) uses a reverse transcriptase enzyme to transcribe a specific piece of RNA into complementary DNA (cDNA) to then be amplified by DNA polymerase. To quantify RNA transcripts within a biological sample, a reverse transcription step is required prior to PCR amplification, and this reaction can use target-specific priming or more promiscuous priming techniques, such as oligo (dT). RT-qPCR also requires appropriately designed primers and probes, and can be adapted for the ddPCR platform. The PCR-amplified DNA copies determine whether a specific RNA molecule is being expressed in an organ sample.

The most common tissues assessed for biodistribution and transcript copy number count are: the injection site(s), gonads, adrenal gland, brain (various sections), spinal cord (cervical, thoracic, and lumbar), liver (various lobes), kidney, lung, heart, spleen, and blood. This is not conclusive, and additional tissues may be selected based on the understanding of the gene therapy product, existing data, and the general scope of the toxicology study.

Regulations

In the regulated spaces, collaborative efforts are underway to establish harmonized practices for method development, qualification, validation, and sample analyses.

Overall, there is little regulatory guidance for validation of PCR assays in preclinical or clinical research. FDA Guidance for Industry S12 Nonclinical Biodistribution Considerations for Gene Therapy Products, released in May of 2023, aimed to harmonize the conduct for biodistribution studies with that of gene therapy products. Groups that validate PCR assays for GLP studies evaluate accuracy, precision, linearity, sensitivity, specificity, selectivity (or matrix effect), and stability, similar to other regulated bioanalysis platforms. The most notable parameter with regard to regulations in PCR is the detection of 50 copies of a gene target in a background of 1 µg of background nucleic acid (RNA or DNA).

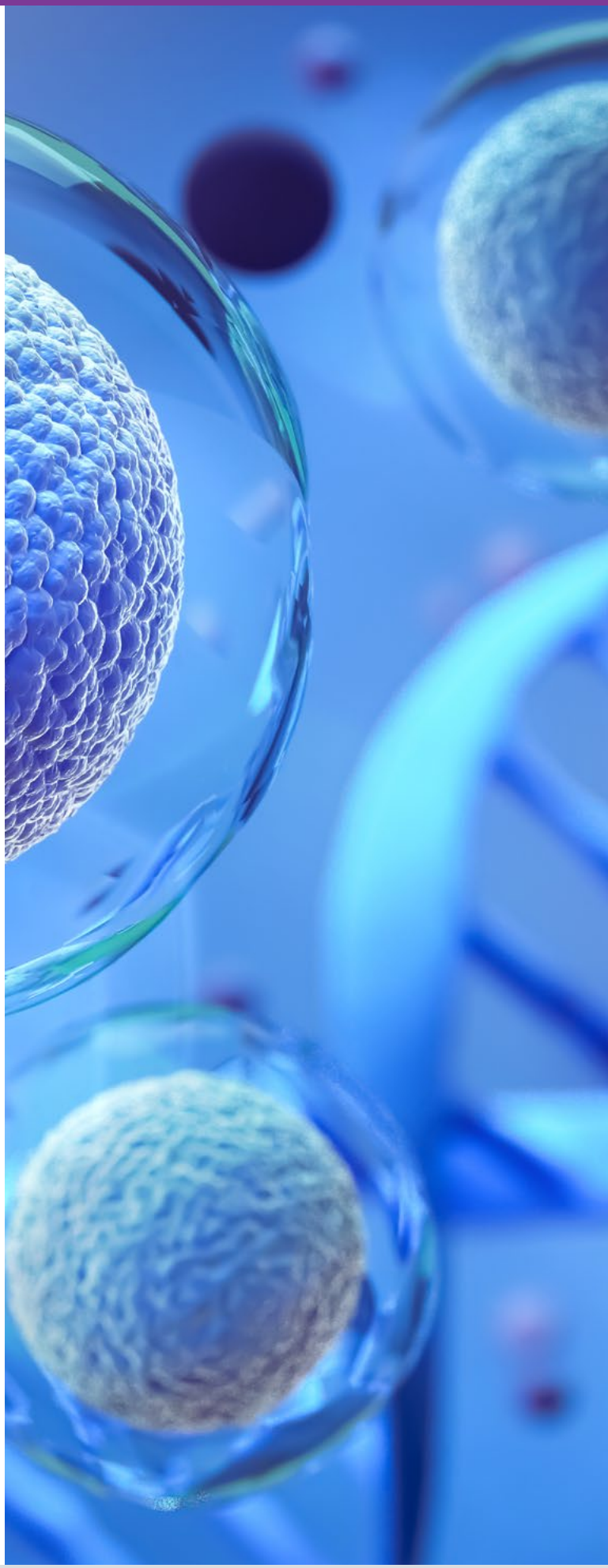
UTILITY OF qPCR, ddPCR, AND RT-PCR

Established methods used in gene therapy development and research comprise the various PCR methodologies. Traditionally, qPCR has been utilized to assess biodistribution of gene therapies in preclinical animal testing, with biodistribution assessment performed as a DNA assay. To analyze a gene therapy's ability to incorporate itself into a target genome and be expressed as an RNA transcript, reverse transcriptase qPCR (RT-qPCR) is also commonly utilized.

Leading-edge technology has vastly improved digital PCR methodology. Using statistical methods and impeccable assay design, digital PCR methods determine absolute copy number, or gene target counts in a given sample of DNA or RNA. Thus far, the ddPCR platform has been the most thoroughly investigated of the digital methodologies, and can successfully quantify gene target copy numbers in DNA and RNA samples in multiplex formats.

Depending on your needs, ddPCR is more sensitive than qPCR and is usually considered the superior choice. qPCR is suitable for applications that require a broad dynamic range, whereas ddPCR is for those applications that require higher precision analysis. In a gene therapy application, for example, ddPCR would be used for accurate viral vector quantification because ddPCR represents an absolute count of DNA target.

RT-PCR uses reverse transcription to produce a DNA template from an RNA source that can then be amplified. There are several applications using this technology. For instance, this platform has been used to detect SARS-CoV-2 RNA, and to diagnose COVID-19. RT- PCR is also widely used for mutation detection, providing excellent sensitivity and specificity. It has been the platform of choice to detect COVID-19 mutations, which led to the identification of subvariants such as Omicron. Viral load, a sign of potential active infection, is also assessed by RT-PCR.



PCR APPLICATIONS

PCR applications continue to expand in the nonclinical and clinical drug development space:

- Development and/or design of cell and gene therapies: viral vector delivery mechanisms, virus identification and analysis, oligonucleotide drug research, RNA and DNA plasmid customization, CAR-T cell applications, and stem cell applications.
- Gene target biodistribution, migration, and persistence analysis to support pharmacodynamic (PD) and toxicological studies.
- RNA transcript distribution and expression to determine if the gene/therapy is being detected in a particular organ or tissue and then is it being transcribed to RNA; all prior to any protein analysis being performed.
- Vaccine development: viral shedding and viral load determination are key assays performed in support of vaccine efficacy trials.
- Detection/confirmation of infections, as well as in the development of novel therapies against microbial infections.
- PCR for clinical diagnosis and treatment of cancer and inherited disease research.
- Providing support for gene expression and immunological assessments for efficacy and PD endpoints.
- Personalized medicine and therapy applications which include development areas of next-generation sequencing, genotyping, target gene knockout, and CRISPR/Cas9 technologies.

ADVANTAGES AND APPLICATIONS

With the wide variety of applications for PCR, it is important to understand the difference between qPCR and ddPCR uses. Their usage will depend on the scope of the drug study, the gene therapy PD, and the desired endpoints. **Each method has a distinct and appropriate range of detection which can be dependent on the target delivery mechanism and distribution.** All PCR methods require stable and optimized reagents, proper primer and probe design, and well-established cycling conditions, optimized for enzyme and reaction efficiency.

ddPCR is a digital technology that counts individual molecules with high precision and linearity over a five-log range. With ddPCR, a sample is partitioned into 20,000 droplets. After amplification, droplets containing target sequence are detected by fluorescence and scored as positive, while droplets without fluorescence are scored as negative. ddPCR outperforms qPCR by not only measuring the absolute copy number but also overcoming the limits of detection, i.e., detecting small fold-change differences expressed as 10% precision and mutation rates less than one percent.

During initial drug development, when undertaking dose-range finding or non-GLP exploratory studies, it may be appropriate to utilize both qPCR and ddPCR to assess expected distribution or delivery/uptake information. qPCR would be appropriate for quantification at higher copy numbers or ranges, and then ddPCR can be employed to support data generated from qPCR and explore low-copy number tissues or those samples with restrictive volume quantities.

An additional consideration during study design and endpoint discussion is whether RNA analysis is required in addition to DNA analysis. Both qPCR and ddPCR can be utilized for both DNA and RNA analysis, with the difference being a reverse transcription step when analyzing RNA study samples.

Quantitative PCR

qPCR can be employed to determine relative and absolute quantification of a gene therapy target by comparing the expression of a target gene to a reference gene (i.e., housekeeping gene), a control sample, or a target. An appropriately characterized reference standard for standard curve generation and positive controls is of paramount importance, and a reference standard for each gene therapy target is required.

When conducting regulated studies, qPCR parameters assessed during validation include:

- linearity (in conjunction with amplification efficiency)
- accuracy (QCs of nominal value)
- precision
- sensitivity (approximately 50 copies per 1 µg of nucleic acid)
- specificity (of all respective naïve tissue types)
- matrix effect (also known as selectivity)
- stability (benchtop, freeze/thaw, and storage)

In the biopharma industry, qPCR is most often used to assess biodistributionf gene therapy drug products. Expression and transcript analyses are often used to supplement the biodistribution data. qPCR is also commonly used in the development process for monoclonal antibodies, vaccines, novel cell and gene therapies, and biosimilars, as a tool for quality control and genetic analysis. PCR applications are rapidly expanding as scientific technology advances.

Droplet Digital PCR

ddPCR quantifies an exact target copy number in a given sample by partitioning a standard PCR reaction into approximately 20,000 nanoliter-sized droplets, and performs an individual PCR reaction on each droplet. Each droplet is then drawn through a fluorescent detector (the QX200 droplet reader) to count each droplet's reaction as containing, or not containing, the target. This droplet/target copy count is based on an established statistical manipulation and therefore, a standard curve for relative quantification is not required.

DNA and RNA applications are available in the ddPCR platform. Target copy numbers are absolute and not reliant on any standard for comparison. Digital PCR formats are also adept for low-copy number and low-sensitivity analyses, such as: 2.00e+01 to 1.60e+05. In addition to impeccable sensitivity, digital PCR methods offer high tolerance which increases performance by controlling for effects of inhibitors.

Table 1. Comparison of qPCR and ddPCR Characteristics

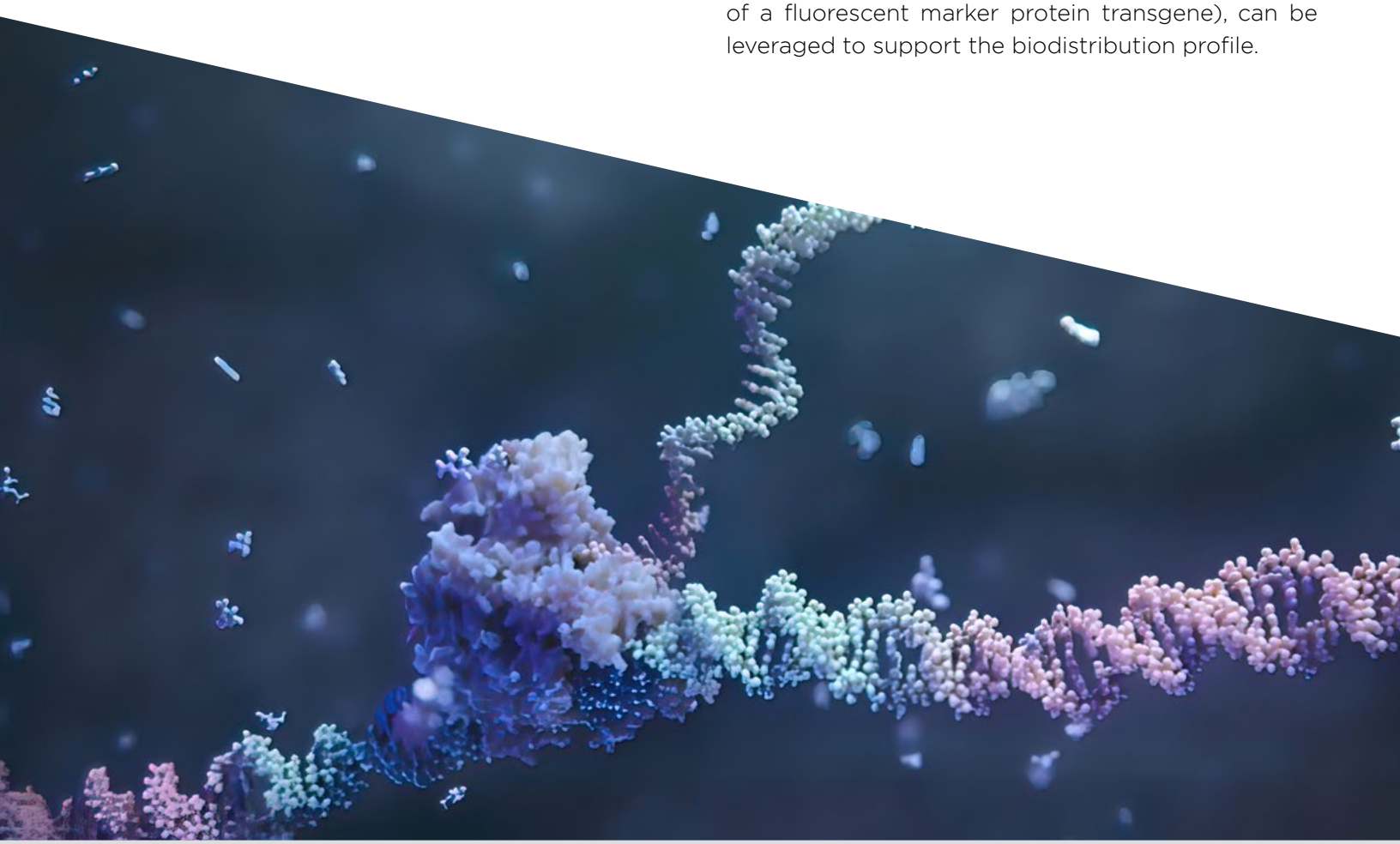
qPCR	ddPCR
Standard curve required for quantification; quantification based on a reliable standard.	No standard curve required for quantification; quantification is absolute count.
Lower limit of detection is often 50 copies of a target (generally accepted regulatory requirement).	Adept at measuring smaller quantitative differences and increased lower-level sensitivity, down to approximately 20 copies of a target.
Can detect between 5.00 x 10^1 and 5.00 x 10^7 copies of target.	Can detect between ~2.00 x 10^1 and 2.00 x 10^5 copies of target (narrow range).
Inexpensive, generally speaking, for standard to high-throughput analyses.	Overall, expensive reagents and consumables.
A reliable and trusted method of quantitation; relative expression can be adapted.	Precise, binary results. Low error rate, overcomes PCR inhibitors.
Great for a large majority of targets; reliable if there are some sample inconsistencies since the standard curve is required.	Overcomes any required normalization from housekeeping genes.

The increasing demand for preclinical and clinical safety assessments of cell and gene therapy test articles (TAs) have created the need for regulated biodistribution, vector shedding, gene expression, and/or pharmacokinetic bioanalysis studies. Currently, quantitation of vector genome and/or transgene DNA/RNA relative to input genomic DNA by established nucleic acid amplification methods (e.g., qPCR), dPCR) is considered the standard for detection of gene therapy products in tissues/biofluids over the course of time.

Although the FDA S12 guidance provides recommendations when conducting biodistribution and vector shedding PCR-based assays supporting nonclinical studies, only preclinical biodistribution assay sensitivity is specified in these documents. **While criteria such as accuracy, precision, and repeatability are not yet defined in the FDA S12 guidance, the EMA, a number of white papers, as well as discussions among field experts at workshops, have provided recommendations to help complement the regulatory requirements.**

FDA S12 guidance states that nonclinical studies for biodistribution assessment can be conducted as standalone or in conjunction with nonclinical pharmacology and toxicology studies. Biodistribution assessment should be conducted in a biologically relevant animal species or model that supports transfer and expression of the genetic material, using a route of administration (ROA) that reflects the intended clinical ROA as much as possible. The dose levels studied should provide sufficient characterization of the biodistribution profile.

The TA administered in nonclinical biodistribution studies should be representative of the intended clinical gene therapy product, considering the manufacturing process, important product characteristics such as titer, and the final clinical formulation. In some situations, nonclinical biodistribution data generated with a gene therapy product consisting of the same vector intended for clinical use, and a different therapeutic transgene or an expression marker gene, (e.g., AAV vector of the same serotype and promoter that directs expression of a fluorescent marker protein transgene), can be leveraged to support the biodistribution profile.



ALTASCIENCES' CASE STUDIES

Case Study I

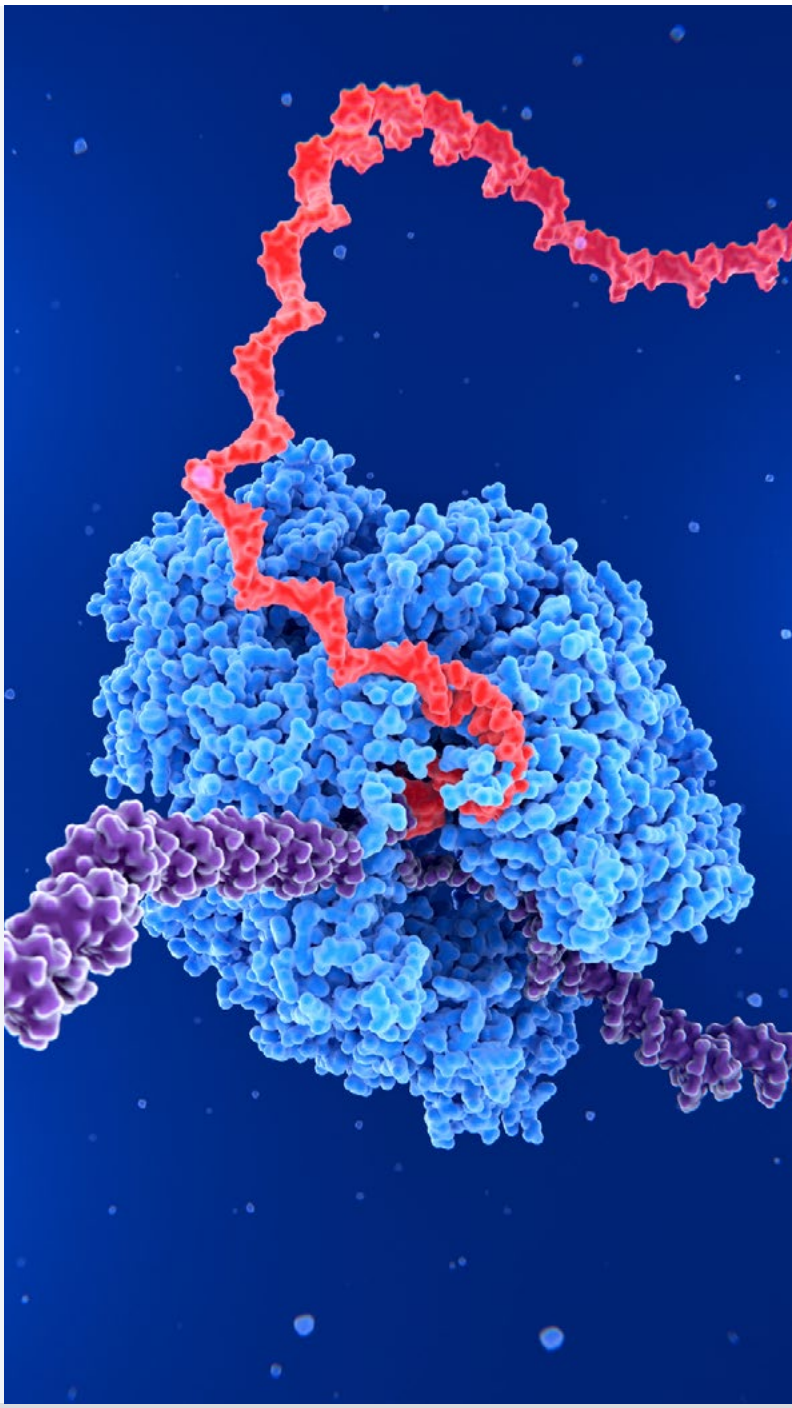
Biodistribution and Transcript Quantification in Mouse Tissues Using ddPCR

Summary: A preclinical study in mice was completed for an AAV vector-delivered drug compound with a fully validated method in dPCR and RT-ddPCR. ddPCR and RT-ddPCR were both utilized to assess vector copy number and vector-derived transcript copy number, respectively, in the following mouse tissues: blood, bone marrow, brain, heart, kidney, liver, lung, lymph node, spleen, and gonads.

Methods: The sponsor provided primers and probe sequences and linearized plasmid reference standards, and ddPCR and one-step RT-ddPCR assays were optimized for the Bio-Rad Droplet Digital PCR system. QCs, positive controls, and negative controls were prepared in a background of 1 µg genomic DNA using naïve tissues supplied by Altasciences. Both assays were validated to a sensitivity of 50 copies of AAV vector target.

All study samples were received from the sponsor's designated testing facility and processed on-site at Altasciences in Seattle according to site-specific SOPs. Samples were cut frozen to comply with isolation kit input and subsequently homogenized using a bead-beating procedure. After tissue DNA and RNA were isolated using Qiagen DNeasy® and RNeasy isolation kits, respectively, all samples were quantified using UV spectrophotometry and diluted to working concentrations for downstream ddPCR analyses. Blood for RNA analysis was collected in PAXgene reagent to preserve RNA sample; the coordinating PAXgene RNA isolation procedure was utilized to obtain RNA, which was then quantified and diluted as other sample isolations, prior to RT-ddPCR analysis.

Results: Validated range of detection was up to 180,000 copies of vector target. No vector copy number or vector-derived transcripts were quantified in vehicle control animals. Two exposure timepoints were assessed, and at both timepoints all tissue types reported quantifiable vector and vector-derived transcript copy numbers.



Case Study II:

Vector Shedding Analysis via qPCR From Nonhuman Primate Shedding Matrices

Summary: A preclinical qPCR study was conducted in NHPs to assess vector shedding of a gene target of a recombinant AAV drug compound. Shedding matrices included blood, urine, feces, saliva, nasal swabs, and tears. Primers and fluorescent probe specific to the gene target contained within the drug were designed by Altasciences and supplied by an outside vendor. The assay developed selectively quantified vector copy number of a specific DNA target-sequence after drug administration and the assay was validated for use on GLP studies.

Methods: The reference material was a linearized DNA plasmid containing the TA-specific target sequence. Standards, QCs, spike-positive controls, and no-template control (NTC) samples were prepared in a DNA background obtained from in-house naïve NHP tissues.

Shedding samples were aliquoted and DNA was isolated using Qiagen, column-based isolation kits. Fecal sample DNA isolation was performed using the automated Qiacube HT isolation system via the QIAamp® Fast DNA Stool Mini Kit. Blood, urine, tears, saliva, and nasal secretion isolations were performed using QIAamp® Mini DNA Kits. All sample DNA was quantified using UV spectrophotometry and diluted to working concentrations as appropriate for downstream qPCR analysis. Amplification and PCR fluorescence data acquisition was performed using a ThermoFisher QuantStudio™ 7 Pro qPCR system.

Results: The vector copy number means were less than LLOQ for all control and acclimation study samples. Vector copy number was quantifiable in all blood samples after dosing for treated groups. Vector copy number means were quantifiable in fecal and urine samples for a limited time interval.

HOW ALTASCIENCES CAN HELP

Nonclinical and clinical studies assessing pharmacokinetic (PK) and toxicological properties are essential to support claims of safety and efficacy for cell and gene therapy products. These include studies on gene vector and transgene biodistribution, protein expression, viral shedding, vector integration, and other areas.

Altasciences has significant expertise in assay development, which includes primer and probe design and SuperMix optimization, as well as the qualification and validation of qPCR and ddPCR methods for both non-GLP and GLP use, respectively. Our lab and subject matter experts have the technology and expertise to deliver quality assay results in the PCR arena.

Our PCR teams work with state-of-the-art equipment to support assay creation, including automation for high-throughout processing and lipid nanoparticle delivery analysis. Our equipment is listed below.

- dedicated PCR hoods and laboratory spaces
- 6 QuantStudio™ 7 Pro real-time PCR systems (thermal cyclers)
- automated droplet generator (for ddPCR)
- 2 QX200 Droplet Readers (for ddPCR)
- QIAcube HT automated nucleic acid extractor
- King Fisher purification instrumentation
- NanoDrop™ One and NanoDrop™ Eight spectrophotometers

We have decades of PCR experience. Our resourceful, innovative scientists work in close collaboration with your team, sharing knowledge and insights as we design and conduct the necessary PCR-based assays to support your drug development needs. We specialize in assay development, assay qualification, validation, and sample testing to meet regulatory, scientific, and operational needs. We have a successful track record supporting stand-alone programs and studies in support of work conducted at our sites in the cell and gene therapy space.

ALTASCIENCES' RESOURCES

Webinar

Uses of Digital Droplet PCR in Preclinical Research

Videos

Take a Look Inside Our PCR Lab

A Virtual Tour of Our Bioanalytical Labs

Poster

Bioanalytical Developments for the Analysis of Antisense Oligonucleotides

Blogs

Test Your Knowledge of PCR Applications

Five Lesser-known Uses for PCR

Webpage

Bioanalytical Methods for Vaccine Development

ABOUT ALTASCIENCES

Altasciences is an integrated drug development solution company offering pharmaceutical and biotechnology companies a proven, flexible approach to preclinical and clinical pharmacology studies, including formulation, manufacturing, and analytical services. For over 25 years, Altasciences has been partnering with sponsors to help support educated, faster, and more complete early drug development decisions. Altasciences' integrated, full-service solutions include preclinical safety testing, clinical pharmacology and proof of concept, bioanalysis, program management, medical writing, biostatistics, clinical monitoring, and data management, all customizable to specific sponsor requirements. Altasciences helps sponsors get better drugs to the people who need them, faster.