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Challenges in Development and Qualification of PCR/dPCR Assays for Gene Therapy Biodistribution and Viral Shedding Assessment

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Abstract

Gene therapies are part of a larger class of advanced therapies that aim to treat disease via delivery of recombinant genetic material. A gene therapy product has two components, the delivery system (viral vector or non-viral) and the transgene (DNA or RNA). These therapies act via replacement of a non-functional gene, silencing of a disease-causing gene, or introduction of a new or modified gene with the goal of generating a therapeutic response in patients. Gene therapy biodistribution and viral vector shedding must be evaluated during non-clinical testing. Polymerase chain reaction (PCR) has emerged as the technique of choice to quantify the gene therapy product and the transferred genetic material in study samples. With increasing numbers of gene therapies in pre-clinical development, there has been a concomitant increase in the use of PCR in bioanalytical laboratories. A major challenge in this space is the lack of formal guidance for the development, characterization, and validation of PCR assays. This article will focus on the opportunities and challenges in developing and characterizing non-GLP, digital PCR assays for AAV gene therapy products. AAV vectors are currently the most common viral delivery system, however many of the insights presented will be applicable to other delivery systems.

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1.0 Context of Use; Biodistribution and Shedding

Gene therapy biodistribution studies evaluate the in vivo distribution, persistence, and clearance of the gene therapy (product and/or transgene) within a biologically relevant species. Evaluation of gene therapy viral vector shedding via secreta and excreta is an additional end point that can be incorporated into these non-clinical studies. Sample analysis requires a sensitive and specific method to quantify the delivered transgene material inside cells as well as the gene therapy product itself in biofluids, secreta, and excreta. Robust assay performance across these diverse target and non-target tissues and biofluids is required. Assays must be selective for the delivered transgene sequence and not cross-react with the test species' endogenous nucleic acid sequence. The context of use and the assay performance specifications for biodistribution and shedding bioanalysis are different, therefore PCR assay qualification and/or validation may not be the same. PCR can also be used to measure the transgene expression product, for example if the functional transgene product is an RNA molecule, or if there is no suitable protein assay and transgene mRNA is measured as a surrogate for expression. These uses for PCR and the relevant assay performance criteria fall into the biomarker space and are out of scope for this discussion.

The route of administration (targeted or systemic), vector tropism, and dose will all influence the biodistribution and shedding profile of the gene therapy product [1-6]. It is challenging to predict the levels of transgene that will be quantified across study samples. PCR workflows must efficiently recover target nucleic acids during extraction, demonstrate acceptable accuracy and precision across a wide dynamic range, and achieve robust assay performance in diverse matrices to support these studies.

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Non-regulatory biodistribution studies generate information on levels of transgene expected in target and non-target organs and biofluids and these key learnings should be considered during transfer of methods for validation in support of GLP toxicology studies.

2.0 PCR technologies: value of digital PCR

The most widely used PCR technology platforms for quantification of nucleic acids are quantitative PCR (qPCR) and digital PCR (dPCR). While qPCR is the more established technology, there is increasing use of dPCR in many bioanalytical laboratories. Both technologies use primers and a fluorescently labeled probe to amplify and quantify a target sequence, however the methods of quantification are different. qPCR measures fluorescence in the reaction following each cycle of PCR. The cycle number where the fluorescence crosses a defined threshold can be compared to a standard curve to quantify the amount of target in a sample. In contrast, dPCR uses specialized methods to partition the reaction into thousands of micro-reactions and allows PCR cycling to proceed to completion before measuring the fluorescence of each partition. Absolute quantification is achieved without a standard curve through the application of Poisson statistics on the ratio of positive partitions to total partitions in the reaction. Different partitioning strategies are used across available dPCR systems [7]. Both PCR platforms are routinely used in the bioanalytical space and have their own pros and cons which have been compared in detail elsewhere [8, 9].

The value of absolute quantification without the need of a standard curve is, perhaps, the key advantage of dPCR over qPCR. Copy numbers are measured directly for each sample, removing precision and accuracy error due to standard curve preparation from impacting sample quantification. Run acceptance during sample testing is no longer dependent on standard curve performance, only plate quality controls (QCs). In our experience, this improved run pass rates and reduced re-analysis during sample testing.

Assay sensitivity and precision are equivalent to, if not better than qPCR. By partitioning the reaction, competition for reaction components is reduced and primers/probe have improved template access, allowing for better detection of low copy numbers in samples. A unique feature of dPCR compared to qPCR is the ability to combine the data from replicate wells and treat them as a single reaction within the analysis software, effectively doubling the number of partitions analyzed and improving the Poisson statistical precision. This strategy can improve assay sensitivity as a larger volume of sample can be interrogated across multiple reactions to detect very low copy numbers and can be particularly effective when analyzing gene therapy vector shedding samples, for example. By increasing or decreasing the number of wells analyzed, assay sensitivity can be adjusted to the context of use. Merging wells effectively generates singlicate measurements for sample analysis and qualification/validation strategies need to be adapted appropriately. These decisions will impact sample testing throughput and need to be balanced against study requirements and resources.

The impact of inhibitory components co-purified with sample DNA on target quantification is reduced as PCR cycling is completed prior to fluorescence measurement. This is a significant benefit when working with the diverse matrices anticipated in biodistribution and shedding studies. High assay sensitivity combined with tolerance to PCR inhibitors makes dPCR an appropriate technology for analysis of biodistribution and shedding samples. However, there is a technical limit to the maximum number of copies per reaction that can be quantified due to the number of partitions assessed and the statistical methods used for absolute quantification. This results in a reduced dynamic range for dPCR (around 1e5 copies/reaction) compared to qPCR (at least 1e8 copies/reaction). Samples with measured copies above the ULOQ of the assay will require dilution and re-assessment. Considering the route of administration and the expected target and non-target biodistribution of the gene therapy can anticipate which samples may contain the highest target copies. Experience gained during non-regulatory studies can be used in subsequent GLP studies to make decisions on sample dilutions to avoid re-analysis and mitigate the impact of the reduced dynamic range on project delivery.

A final consideration for using dPCR is the increased cost of materials and the reduced throughput of the technology. The extra steps of partitioning the reaction and analyzing the partitions requires sophisticated and expensive equipment compared to qPCR in addition to the costs of specialized consumables. Current dPCR technology platforms support up to 96-well format runs with variable run times depending on the system, restricting sample throughput. Some of the negative impact of reduced throughput can be mitigated during sample testing as reaction wells are not needed for standard curve levels. Increasingly automated platforms are becoming available and help to reduce operator burden and improve throughput by allowing processing to occur at all hours.

3.0 Method Development

The fundamental difference between quantification relative to a standard curve and absolute quantification is critical for understanding how to develop and characterize an assay. Guidance documents from the global regulatory agencies outline recommendations for the use of molecular assays in non-clinical testing, but there is currently no formal guidance on how to develop, qualify, and validate PCR assays (qPCR or dPCR) for bioanalysis [4-6, 10-13]. A guideline from the FDA presents a sensitivity threshold for vector quantification by qPCR, but there are concerns that this performance criteria does not sufficiently consider the

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context of use and cannot be applied in all cases [10]. Growing consensus in the bioanalytical community supports an approach that emphasizes development of fit-for-purpose assays focusing on the context of use. Several white papers and articles have been published from the EBF, AAPS, and other organizations to harmonize the approach to assay validation [8, 9, 14-19]. Most of these articles have a focus on qPCR/qRT-PCR technology as there is more collective experience with this established technology. As dPCR becomes more broadly used in bioanalysis, we anticipate further specific considerations for this technology will be proposed.

3.1 Nucleic acid extraction

PCR workflows are surprisingly complex and begin long before setting up the PCR reaction. The first step is extraction of nucleic acids from the tissue samples, biofluids, and/or feces. The ICH S12 guideline outlines a core panel of 11 different matrices to evaluate in biodistribution studies [10]. The EMA has a comprehensive list of over 40 possible samples that should be considered for evaluation [20, 21]. While not necessarily a dPCR specific consideration, the quality of the extraction method(s) will directly impact PCR assay performance. For example, viscosity of the eluted DNA could have a negative impact on reaction partitioning. There is a delicate balance between developing an "all purpose" extraction method and developing bespoke workflows for challenging tissue types and choices should be made considering the context of use. Perhaps the most challenging extraction methods to develop are for biofluids and shedding matrices. Secreta and excreta samples (urine, saliva, feces, etc.) are complex matrices that are known to contain inhibitors that can interfere with PCR performance like proteases, nucleases, ions, and salts. Feces samples additionally contain nucleic acid content from the natural flora increasing potential for non-specific amplification. This added complexity likely requires unique workflows to be optimized for each matrix. Extraction method development activities represent a major effort of the bioanalytical team and the time required should not be underestimated.

Standardization of extraction methods across the bioanalysis community is not feasible given the variety of kits available and possibilities for optimization. Extracted nucleic acid material should be evaluated for purity, quantity, and integrity. Concentration can be measured by spectrophotometry-based or fluorescent dye-based methods. Fluorescent dye-based methods have higher tolerance to impurities in the sample and provide more accurate concentration measurements. Spectrophotometry-based assays should be included during method development as sample purity can be investigated by assessing the 260/280nm ratio (indicative of contaminants and potential inhibitors of PCR reactions). Gel electrophoresis or bioanalyzer assessment can be used to establish sample integrity.

The true test for the quality of extraction methods is the evaluation of matrix interference and recovery efficiency. Matrix interference evaluation defines the ability of the PCR assay to measure the target accurately and precisely in the presence of inhibitory components co-purified with the sample DNA. Recovery efficiency defines the ability of the extraction method to recover the target DNA sequence from the sample. These criteria can only be evaluated following development of a well characterized PCR assay and an iterative method development program should be used to improve extraction parameters and evaluate their effect on PCR assay performance. Once a method has been established, comprehensive evaluation of the method performance is not required for every assay qualification. However, modifications to existing extraction methods should trigger a complete or partial re-qualification of assay performance.

3.2 Assay Development

The target assay should recognize a unique sequence within the delivered transgene that is not present within the endogenous genome. The 5′ and 3′ junctions of the functional sequence with other elements of the transgene cassette are the most obvious locations for designing assays. Numerous software tools are available for assay design and most tools allow for specification of parameters such as amplicon length, melting temperatures, GC content, and others. The MIQE guidelines for qPCR and dPCR are excellent references for assay design considerations [22, 23]. In silico specificity testing should be performed to screen for cross reactivity of assays against the study species genome. Primers and probe cross reactivity against the human genome sequence should be considered to facilitate transition of target assays from the nonclinical to clinical space whenever possible. Where relevant, a genomic reference assay can be developed against a single copy per haploid genome gene in the study species to normalize target copies against the input gDNA assessed for tissue samples. Biofluid and secreta/excreta data is generally normalized against the volume or weight of input material. Other reportable units should be considered on a case-by-case basis.

Multiple assays should be evaluated for linearity, efficiency, sensitivity, selectivity, accuracy, and precision to identify the optimal assay to move forward into qualification. When using a multiplex reaction format for data normalization, method development should establish whether there is interference between assays through comparison of parameters in singleplex and multiplex reaction formats. Multiplex reactions can be easier to develop for dPCR as the impact of assay competition is reduced by reaction partitioning and end-point fluorescence measurement. Optimizing the annealing temperature is particularly important for dPCR to yield the greatest resolution in fluorescence amplitude between positive and negative partitions for all multiplexed assays.

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3.3 Reference Material and Calibrators

Assay calibrators and quality controls (QCs) used during method development and validation should adequately represent study samples. Surrogate gDNA from the study species is reasonable to use for QC sample preparation and various commercial suppliers are available. Assay development is generally performed on QCs prepared from surrogate gDNA spiked with target DNA reference material. Synthetic double stranded DNA fragments and DNA plasmids are the two most common reference materials used in PCR. The biology of study samples should be considered when choosing an appropriate reference material format. Transgene DNA inside cells transduced by AAV vectors generally persists as circular, extra-chromosomal episomes, and structurally, plasmid DNA is more representative of target copies in tissue samples obtained in AAV gene therapy biodistribution studies. A common practice when working with plasmid DNA standards is to linearize the plasmid prior to PCR assessment to reduce the tertiary structure of the template and improve primer/probe access and amplification efficiency. This method has been used successfully to validate fit-for-purpose dPCR methods [24, 25]. However, a specific technical consideration for dPCR is the recommendation to include a restriction enzyme into the PCR reaction master mix to fragment the chromosomal DNA to allow for better reaction partitioning. Selecting a restriction enzyme that will also linearize the delivered transgene DNA sequence can help achieve optimal assay efficiency. Including the restriction enzyme in the PCR reaction mix eliminates the need to linearize the reference material ahead of QC preparation reducing the number of processing steps. Multiple restriction enzymes, when possible, and concentrations should be tested on QCs during method development. In our experience, using circular plasmid in QC samples is critical for fully optimizing PCR assay conditions during method development. Insufficient units of restriction enzyme in the dPCR reaction prevents full linearization of the delivered vector genomes in study samples. Incomplete template linearization can result in positive partitions with intermediate fluorescence amplitude indicating reduced amplification efficiency and suboptimal performance.

3.4 Assay Criteria

The lack of formal regulatory guidance for the qualification and validation of PCR assays combined with their increasing use within bioanalytical labs has prompted several recent publications and white papers aimed at harmonizing best practices and building consensus within the community [8, 9, 14-19]. Across publications, there is broad consensus that existing bioanalytical method validation guidelines written for chromatographic and ligand-binding assay technologies to assess pharmacokinetics are not suited for PCR assays as the technologies are fundamentally different. We argue that even between PCR technologies (qPCR and dPCR) that there are sufficient differences that recommendations should not be blindly applied to all PCR assays without consideration of the scientific rationale.

Applying bioanalytical vocabulary and concepts to an absolute quantification method is not straightforward. QCs concentrations in dPCR do not require back-calculation against a standard curve complicating how to define the nominal concentration of the prepared sample and to set performance criteria for accuracy. Establishing the limit of blank (LOB), limit of detection (LOD), and lower limit of quantification (LLOQ) is also challenging. Articles state that “appropriate statistical methods” should be used to establish the LOB and LOD, however there is no consensus on how this should be evaluated [14, 18]. Classically, an LLOQ is defined as the lowest QC with acceptable accuracy and precision, but it is still unclear how to apply this definition to an absolute quantification method.

A dPCR specific consideration is setting a threshold to distinguish positive and negative partitions ahead of data generation and is an overlooked point of discussion within assay validation articles. The threshold value will be unique for every assay based on partition fluorescence amplitude and can be set manually or automatically within the analysis software. The impact of manual threshold setting on the data should be evaluated during method development. A properly developed assay should have a minimal amount of intermediate amplitude partitions and modifying the threshold will not impact the measured copies. Automatic threshold options based on a positive control sample (plate QCs for example) reduces operator bias compared to manual thresholding and should be used for qualification/validation and sample testing. Threshold trending should be evaluated during sample testing to ensure consistent assay performance and should be evaluated when bridging lots of primers/probe.

4.0 Conclusions

The introduction of new technologies in the bioanalytical laboratory brings exciting opportunities and challenges. Significant work remains to align on best practices for the development and validation of dPCR assays for gene therapy biodistribution and viral vector shedding studies. As discussions around validation parameters and establishing acceptance criteria continue within the community, method performance evaluation should be scientifically justified with a mind towards establishment of fit for purpose methods considering the context of use. In our experience, learnings during assay development, qualification, and non-regulated sample testing can anticipate challenges that will arise during subsequent GLP studies. It is the accumulation of this collective experience within the community that will ultimately define best practices for dPCR bioanalysis.

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