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Review

qPCR and qRT-PCR analysis: Regulatory points to consider when conducting biodistribution and vector shedding studies

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Gene and cell therapy fields have experienced remarkable growth during the past decade. Demands for preclinical and clinical safety assessments of these cell and gene therapy test articles (TAs) have effectively increased the necessity for regulated biodistribution, vector shedding, gene expression, and/or pharmacokinetics bioanalysis studies. Guidance documents issued from numerous international regulatory authorities recommend the use of quantitative polymerase chain reaction (qPCR) and/or quantitative reverse transcriptase PCR (qRT-PCR) assays due to their highly sensitive and robust target-specific detection. However, only preclinical biodistribution assay sensitivity is specified in these documents. Criteria such as accuracy, precision, and repeatability are not yet defined. This guidance void has resulted in several conflicting institutional interpretations of essential parameters necessary for the development and validation of robust assays to support safety assessments of gene and cell therapy TAs. There is an urgent need for an ongoing discussion among bioanalytical scientists in this field to generate a "best practice" consensus around preclinical and clinical qPCR/qRT-PCR assay design. With regard to this need, we offer critical points to consider when developing, validating, running sample analysis, and reporting qPCR/qRT-PCR assays.

Although the first clinical trial of gene therapy only started in 1990 for treatment of adenosine deaminase severe combined immunodeficiency disease,[1] 30 cell and gene therapy products had received market authorization worldwide by the end of 2018[2] with numerous others currently in the development pipeline, ranging from the initial stages of research and discovery to phase III human clinical trials.

Gene therapy test articles typically consist of a vector formulation containing a genetically engineered construct that is introduced to the host primarily through injection. These constructs have been designed to affect host cells in highly specific ways, including replacement, introduction, and editing of genetic material. Of the varieties of gene delivery systems available, recombinant adeno-associated virus (AAV) vectors are the most common ones; however, several other types of vector delivery systems have been used, including replication-deficient and replication-competent viral vectors, non-viral vectors, and microbial vectors.[3] In contrast to gene therapy, cell therapy typically involves transplantation of cellular material into the patients. This includes *ex vivo* genetically modified cells, induced pluripotent stem cells derived from previous tissues, or stem cell-derived products sourced from adult, perinatal, fetal, or embryonic tissues.

In the past 15 years, regulatory guidelines have been actively developed and updated by both the US Food and Drug Administration (FDA) and the European Medicines Agency (EMA) describing general principles for assessment of nonclinical and clinical studies as well as monitoring of long-term, serious adverse events of gene and cell therapy TAs.[2–11] Both of these agencies recommend quantitative polymerase chain reaction (qPCR)/quantitative reverse-transcriptase PCR (qRT-PCR) assays to be used for analysis of vector biodistribution, vector shedding, and vector-derived gene expression due to superior sensitivity and specificity toward the detection of administered TA nucleic acid sequences at a wide dynamic range. Biodistribution studies are defined as the distribution, persistence, and clearance of a gene/cell therapy product *in vivo* from the site of administration to target and non-target tissues and biofluids.[12] Numerous preclinical biodistribution studies of gene and cell therapy products have been reported, including several recently published papers.[13–15] While comprehensive bioanalytical guidance documents released by the FDA and EMA focus on methods for nonclinical and clinical pharmacokinetic and toxicokinetic studies for small and large molecules,[16–18] many of those standardized requirements for method development and validation are difficult to apply to qPCR/qRT-PCR due to sophisticated TA-host interaction of gene and cell therapy products and the unique features of the assay.[19]

The FDA has not yet required validation of qPCR/qRT-PCR assays; however, the EMA has imposed such a requirement.[4] Subsequently, many organizations, including the Workshop on Recent Issues in Bioanalysis and the International Pharmaceutical Regulators Programme, have been working to harmonize divergent global practices for method development, qualification/validation, and sample analysis.[12,20]

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Table 1. An example of a TaqMan probe-based qPCR assay components for absolute quantitation of target DNA

Component	Amount
Standard DNA[a]	0–10^8 copies
Forward primer	up to 900 nM
Reverse primer	up to 900 nM
TaqMan probe	up to 300 nM
2× TaqMan universal master mix II or equivalent	1×
Matrix DNA[a]	1,000 ng
Nuclease-free water	to a final volume of 50 μL

[a]Standard and QC wells contain standard and matrix DNA. For sample analysis, standard and matrix DNA will be replaced by up to 1,000 ng of sample DNA.

Currently, there are no outlined recommendations for experimental setup or evaluation processes to determine the acceptance criteria for a validated assay and subsequent sample analysis. Consequently, the approach to conduct clinical and nonclinical qPCR and qRT-PCR method validation and sample analysis can vary by bioanalytical scientists based on their scientific interpretation. Lack of guidance is very real for scientists working daily in academic, biotechnology, pharmaceutical, and contract research laboratories toward development of novel gene and cell therapy TAs to provide interventions for countless unmet medical needs. In an effort to accommodate the need, bioanalytical scientists in this field have tried to provide their insight on development and validation of qPCR/qRT-PCR assays to support analysis of biodistribution and shedding samples.[13,21] Relying on more than 20 combined years of experience on over 250 novel studies, this paper outlines our "best practice" recommendations for qPCR/qRT-PCR assay design, highlights the points to consider during method development, fit-for-purpose validation, and sample analysis, and proposes the acceptance criteria of the assays.

qPCR and qRT-PCR

qPCR and qRT-PCR have become mainstream life science technology for the quantitation of nucleic acids.[22] The focus of this paper is to mainly describe qPCR analysis of DNA vectors as an example to discuss the approach of method development, validation, and sample analysis. qRT-PCR analysis of the RNA viral genome or vector-derived gene expression are discussed briefly later in this paper. Arguably one of the most important aspects of both assays is the design and use of sequence-specific primers and probes. Inclusion of a reporter molecule, such as nonspecific DNA-binding fluorescence dyes (e.g., SYBR Green) or a fluorescently labeled sequence-specific probe allows for real-time detection of PCR products with the use of specialized thermal cyclers such as the QuantStudio 7 flex real-time PCR system. We recommend probe-based qPCR analysis of preclinical and clinical samples due to its superior specificity. Our typical assay development plan includes testing of three uniquely designed sets of primers and probes. Historically, at least one set meets acceptance criteria required for specificity and sensitivity. While probe-based qPCR is more expensive in supply cost than is dye-based qPCR, such as SYBR Green, the additional cost of probe production can easily be offset by fewer labor hours spent on method development. When using dye-based qPCR, more than three sets of primers may be required to ensure that specificity is met due to the assay's proclivity toward false-positive signaling as the fluorescent dye readily binds to non-specific double-stranded DNA. Additionally, melting curve analysis must be performed to ensure that primer dimerization is not occurring within the reaction. While careful primer design can overcome some challenges of dye-based qPCR, detection remains limited to a single target sequence per reaction. Probe-based qPCR, alternatively, has an advantage of multiplexing where probes containing different fluorophores are combined within the same reaction to detect distinct target sequences. This can effectively decrease both the amount of sample required and reagent cost per reaction. An example of TaqMan probe-based qPCR biodistribution assay components is shown in Table 1, although different commercial kits, reaction volumes, or DNA amounts may be chosen by other bioanalytical scientists. Standard curve and quality control (QC) samples are included on each qPCR plate for absolute quantitation of the target DNA's copy number. To mimic biodistribution samples, matrix DNA or genomic DNA (gDNA) extracted from naive animal tissues (animals not dosed by the TA) is included in each standard and QC sample reaction. Reactions for qPCR are loaded into a standard 96-well plate, sealed with optical caps or film, and centrifuged to remove any droplets from the side or top of the wells. After preparation, the plate is then run in a real-time PCR system such as the QuantStudio 7 flex (or equivalent) using the qPCR cycling conditions suggested by the vendor of the master mix.

Table 2. An example of qPCR thermal cycling

	Temperature (°C)	Time	Cycles
Enzyme activation	95	10 min	None
Denaturation	95	15 s	40 cycles
Annealing and extension	60	30–60 s	

An example of thermal cycling on a qPCR instrument includes an initial 10-min heat activation of DNA polymerase, followed by 40 repeated cycles of DNA denaturation, primer annealing, and target elongation (Table 2). Assuming 100% amplification efficiency (E), each cycle doubles the amount of target DNA.[23] For absolute quantitation of vector genome copies, a standard curve is usually generated by serial dilutions of the reference standard DNA and the assay linearity is evaluated by regression analysis of the threshold cycle (Ct) values of each standard DNA concentration compared to the logarithm of their nominal copy value. The slope and y intercept (y_{inter}) derived from the standard curve can be used to calculate the concentration of a target DNA from the reported Ct value as shown in the following equation:[22]

$$DNA\ Quantity\ (copies) = 10^{(Ct\ value - Y_{inter})/slope}$$

This correlation between the Ct value and amount of the standard/target DNA permits accurate quantification of target DNA

concentration over a wide dynamic range. The slope of this line is also used to calculate PCR efficiency (E) using the following equation:

$$E = 10^{\frac{-1}{slope}} - 1$$

If the target DNA is diluted 10-fold, the Ct values between two target DNA concentrations of 10-fold difference should be 3.32 cycles (i.e., the slope of the standard curve is −3.32) assuming E at 100%. On average, the slope of the standard curve falls between −3.6 and −3.1, corresponding to an efficiency of 90%–110%. While research has shown that the presence of sample DNA can inhibit PCR amplification,[24] our experience has shown that the addition of sample/matrix DNA in biodistribution analysis does not appear to have a negative impact, and the E generally ranges between 90% and 100% when the primers and probe are carefully designed to avoid any non-specific cross-reaction with the matrix DNA.

qPCR and digital PCR

The most common form of digital PCR, droplet digital PCR (ddPCR), is a relatively new technology that utilizes microfluidics to partition target DNA into droplets where individual PCR reactions occur. Amplification occurs over 35–45 cycles using probe-based chemistry similar to TaqMan probe-based qPCR. Upon completion, fluorescence is quantified in each sample using a digital reader that establishes amplification thresholds. Poisson statistics are then applied to the ratio of positive droplets to total droplets, ultimately determining the target DNA concentration.[25] This form of absolute quantification analysis is advantageous, as it renders the use of a standard curve obsolete. Other benefits to ddPCR include improved assay sensitivity, precision, accuracy, reproducibility, minimal matrix effect on amplification, decreased susceptibility to many PCR inhibitors, and multiplexing technology.[26–29] The use of ddPCR is ideal for viral vector titration and diagnostic purposes, especially for those diseases involving rare alleles and copy number variation; however, when analyzing biodistribution or vector shedding samples, the use of ddPCR may not be as beneficial. For instance, we have seen the presence of up to 10^9 copies of recombinant AAV vector gDNA per μg of host gDNA in the tissues of AAV-dosed animals. While the upper limit of quantitation (ULOQ) in a traditional qPCR can reach up to at least 10^8 copies per reaction, the QX200 ddPCR from Bio-Rad, for example, limits the assay ULOQ to only 10^5 copies per reaction. Many samples will have to be re-analyzed by dilution of the samples, which will not only decrease the work efficiency but also introduce errors during sample dilution. Additionally, the time involved in ddPCR sample analysis and possibly method development is much longer than that with a traditional qPCR. There are usually between 500 and 2,500 samples to analyze from a typical biodistribution study. Due to additional processes, such as restriction enzyme fragmentation of the gDNA samples, droplet generation, and droplet reading, among others, it can take approximately 7 h to complete a ddPCR run after plate preparation, whereas in typical qPCR, plates can take less than 1 h to finish when TaqMan fast advanced master mix is used. This significant increase in production and run time would make it difficult to meet tight timelines that usually accompany biodistribution studies. Moreover, when using ddPCR it may take additional time to reach the point of sample analysis, as primers and probes are generally designed for a standard 60°C annealing temperature; however, this can negatively affect the resolution of droplet reading.[27] Lowering the annealing temperature can resolve the issue but potentially increase non-specific amplification. Therefore, the length of method development time could increase due to the need for assessment of various conditions not necessarily required for traditional qPCR.

When choosing between ddPCR and traditional PCR a few more things should be taken into consideration. First, research has shown that digital PCR partitioning decreases the negative impact on amplification by matrix effect inhibitors such as SDS, heparin, and co-purified biological compounds such as heme and urea.[30] However, intrinsic properties of the assay components such as GC-rich amplicons and primer melting temperatures can still affect amplification efficiency.[28,31] Therefore, when conducting biodistribution sample analysis, the control reaction should be spiked with a known concentration of target DNA as recommended by FDA guidance to ensure that inhibitors are not causing undetected problems. Furthermore, if reverse transcription digital PCR is required for biodistribution analysis of an RNA viral vector, the RNA standards may still be necessary to evaluate the efficiency and accuracy of the reverse transcription. In the same manner as qPCR, quantification of cellular RNA or RNA viruses reflects only the number of target cDNA molecules converted from the original RNA. This may or may not give an accurate estimate for the original concentration of the RNA molecules of interest and therefore ddPCR may not be advantageous for these assays.

The current disadvantages of ddPCR do hinder its efficacy for use in biodistribution and vector shedding analysis at this time, but as technology evolves it may become the prominent assay within the laboratory. Until then, the primary focus of this paper is aimed toward traditional qPCR.

Method development

Our approaches to qPCR method development, qualification or validation, and sample analysis are based on recommendations by the FDA Guidance for Gene Therapy Clinical Trials and Long Term Follow-Up after Administration of Human Gene Therapy Products,[10,32] which recommends the lower LOQ (LLOQ) of the qPCR assay to be $\leq$50 copies of vector TA per 1 μg of gDNA. We also include validation parameters recommended by the FDA Guidance for Bioanalytical Method Validation[17] whenever applicable.

Prior to undertaking any method development, it is important to note that qPCR is an extremely sensitive assay that, in some cases, can detect a single copy of target DNA, and therefore separation of workstations and control of contamination are crucial for a qPCR

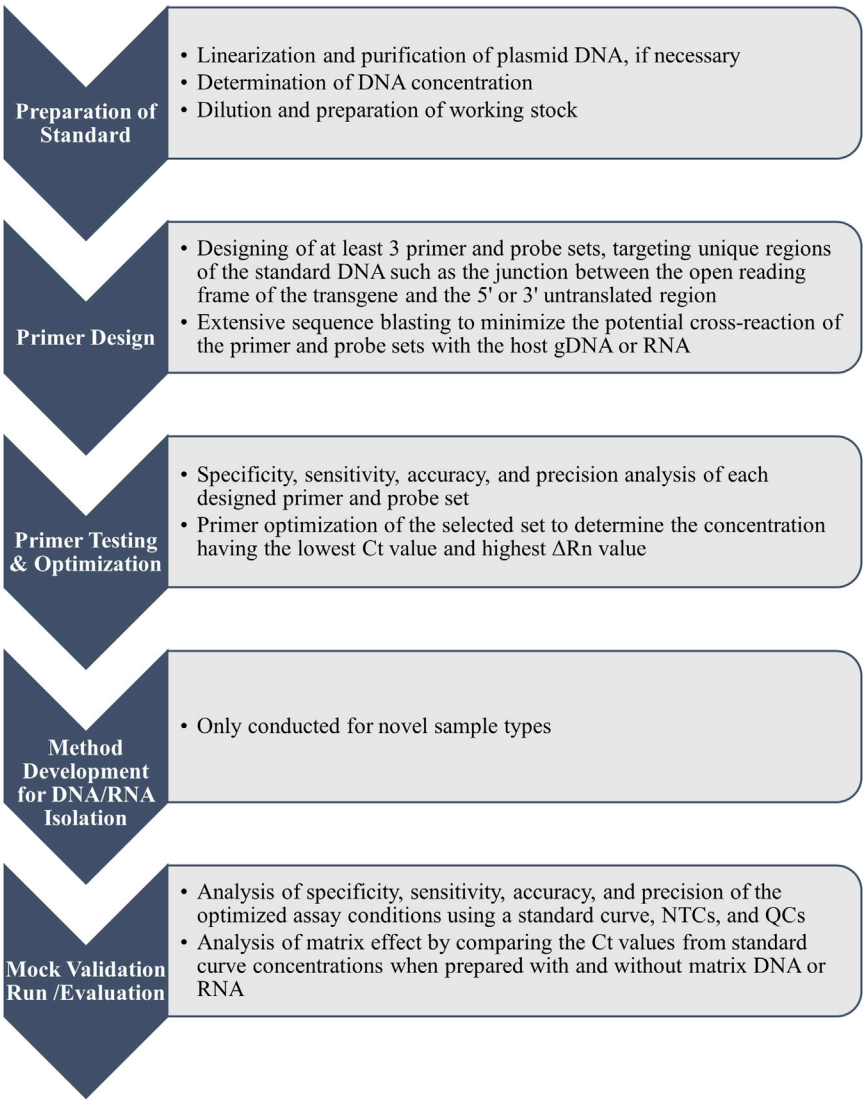

Figure 1. Overview of method development for qPCR analysis

laboratory. The Organization for Economic Co-operation and Development has released guidance outlining the setup of an *in vitro* laboratory to avoid cross-contamination.[33] Although not discussed in this paper, it is assumed that the setup for qPCR analysis always follows good laboratory practices (GLPs).

Method development is intended to provide sound scientific evidence for the final method design and suitability for its intended purpose. Prior to beginning, it is important to gather information regarding the type of cell or gene therapy product (target DNA) to be tested, the host species and strain to be treated with the TA, and the standard DNA to be used for determining the amount of target DNA within each sample. This knowledge will be applied to the design of an appropriate assay for method development/validation and subsequent sample analysis. Importantly, note that the processes involved in method development are not reviewed by quality assurance; however, all activities should be adequately documented to support a reproducible method for validation and sample analysis. An outline for a typical TaqMan-based qPCR assay is shown in Figure 1.

Preparation of standard

Information regarding the nature of the TA and the reference standard, the targeted DNA sequence, the test system(s), and the expected study timelines should be communicated thoroughly with all appropriate personnel. Documentation on the identity, source, lot number, concentration, purity, composition, and stability of the standard DNA should be provided by the sponsor. Identity of the standard DNA should include, at least, the name, the sequence of either the entire or target region of the DNA, the gene map, and the size (in base pairs for DNA) verified by any agarose gel electrophoresis data (or equivalent). Although there is evidence reporting the stability of frozen DNA for more than 7 years,[34,35] the expiration and retest dates are required for standard DNA when used for GLP-compliant studies. If applicable, documentation for any other supporting qPCR methods that have been previously developed or validated for the target gene should also be reviewed.

In general, plasmid DNA carrying the transgene or vector DNA is provided by the sponsor for use in preparation of a standard curve that will be used for absolute quantitation of the target DNA. It is crucial that the conformation of the standard DNA mirrors that of the sample to ensure that E is similar between the two. Studies have shown that PCR using supercoiled plasmids as template DNA yielded higher Ct values (2.65–4.38 more) when compared to equimolar linearized standards. The apparent decrease in sensitivity (higher Ct values) was attributed to the undetected low efficiency of its amplification in the early stage of qPCR when the supercoiled plasmid is the dominant template.[36] It was also reported that plasmid DNA conformation has a significant impact on the accuracy of absolute quantitation by qPCR as indicated by significant shifting of standard curves when prepared with different conformations of plasmid DNA.[37] Since most viral vector gDNAs are linear, the plasmid DNA should also be linearized using a restriction enzyme. Complete digestion of the plasmid DNA should be confirmed by agarose gel electrophoresis and subsequently repurified using a QIAquick PCR purification kit

or equivalent. If working with a plasmid TA, linearization is not required, as the supercoiled plasmid should be used as standard DNA for qPCR analysis to quantify the copies in each sample collected.

The accuracy of the standard DNA concentration is essential for successful qPCR analysis; therefore, regardless of linearization, the concentration of the standard DNA should be analyzed in, at least, quadruplicate, and at a reasonable concentration, using a NanoDrop 8000 spectrophotometer or other fluorescence-based assays for DNA quantitation. The NanoDrop 8000, for example, has an accuracy at ±2.5 ng/μL, and therefore DNA standards of 20 and 100 ng/μL may have a variation of ±12.5% and ±2.5%, respectively. The mean concentration of the standard DNA in ng/μL is converted into copies/μL using the size of the plasmid DNA (N) in base pairs (bp), Avogadro's number (6.022×10^{23} molecules/mol), and the molecular weight of a bp (650 Da),[38] as shown in the following equation:

$$DNA\left(\frac{copies}{\mu L}\right) = \frac{DNA\left(\frac{ng}{\mu L}\right) \text{x } 10^{-9}\frac{g}{ng} \text{x } 6.022 \text{ x } 10^{23}\frac{molecules}{mole}}{N\ (bp) \text{ x } 650\frac{g}{mole\ of\ bp}}$$

Conversion of the concentration of an RNA standard from ng/μL into copies/μL can use the same equation except using the size of the standard RNA (N) in nucleotides (nt) and the molecular weight of a single stranded RNA at [N (nt) × 320.5 + 159.0(nt × g/mol)].[39]

The plasmid DNA is always diluted into working stock and stored frozen at −20°C and below. Aliquots are prepared to avoid extensive freeze-thaw cycling and minimize contamination. It has been shown by research and from our own experience that loss of DNA is more prominent when highly diluted (<0.2 μg/mL) and stored over time due to absorption into tube walls; however, addition of carrier DNA does restore recovery.[40] We recommend dilution of the working stock into buffer containing supplemental nucleic acids such as sheared salmon sperm DNA or yeast tRNA.

If use of the gene or cell therapy product (i.e., use of the TA cells is required for preparation of the standard DNA curve for the qPCR assay) is required for method development and validation, all relevant information about the TA and the study protocol are recommended to be reviewed and approved by the Institutional Biosafety Committee (IBC) prior to the receipt at the analytical site.

Primer and probe design

Target-specific primers and probes should be carefully designed for each assay. The process of designing primers and probes is relatively straightforward with the availability of free (e.g., Primer3, PrimerQuest by IDT, NCBI Primer BLAST) and commercial software (e.g., Primer Express, Oligo.net Primer Analysis). Primer Express is provided with the QuantStudio 7 flex real-time PCR instrument and is typically used in our laboratory due to the design flexibility and ease of use. This program contains an algorithm for the creation of primers and probes specifically optimized for use with TaqMan reagents. The desired target sequence is entered or uploaded into the software and multiple sets of primer and probes are then identified for the given region. Parameters such as primer, probe, or amplicon length and specific melting temperatures can be adjusted manually as necessary. In addition to these features, Primer Express presents a thorough analysis of potential secondary structures (e.g., primer dimers, hairpins) between various primer and probe combinations.

Successful investigational new drug (IND) filings of gene and cell therapy products may require that the preclinical toxicology and biodistribution studies of TAs be performed in several animal species and possibly clinical trials that will require analysis of the same target sequence in human vector shedding samples. Therefore, specificity of primer and probe design should be carefully considered to avoid cross-reaction with any expected matrix DNA types. Additionally, use of the same primer and probe set is expected for vector-derived transgene expression analysis, thus consideration must be given to primer and probe specificity against host mRNA as well. Sequences such as the junction between the open reading frame of the transgene and its 3′or 5′ untranslated region generally contain the most vector-specific sequences and are ideal locations for development of a set of primer and probe as these regions are not only specific to the vector DNA sequence but also to the vector-derived mRNA sequence in the host animal tissues. Regardless of the design location, each primer and probe set should be subjected to a vigorous Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) against the gDNA and RNA databases of all applicable species.

Specificity of a primer and probe set can only be confirmed by experimentation. Due to potential shipping and/or manufacturing delays, it is recommended that a minimum of three sets be designed to increase the success rate of method development and avoid any delay of a timeline. Although a qPCR assay may have been previously developed to detect the gene of interest, the method may not be optimized for biodistribution sample analysis, and thus it is advised that the transferred primer and probe set be re-tested and compared to newly designed primers and probes under the working conditions set for biodistribution sample analysis.

Primers and probes are reconstituted with 10 mM Tris-HCl (pH 8.0), aliquoted to avoid extensive freeze-thaw cycling and minimize contamination, and then stored frozen at −20°C.

For human stem cell- or differentiated cell-derived therapy products, preclinical cell therapy studies require biodistribution analysis of the cell therapy products within the dosed animals. Samples collected from these animals can be analyzed using a primer and probe set specifically targeting the human gDNA and reported as copies of the human haploid gDNA per μg of host gDNA. One set widely used for the detection of human gDNA targets the transposable elements, *Alu*, which have been amplified to over 1 million elements per genome throughout primate evolution, producing a series of subfamilies of *Alu* elements that appear to be of different genetic ages.[41] *Alu*-based qPCR has shown superior sensitivity for the quantitation of human

nuclear DNA from complex sources in forensic casework; however, primers and probes targeting to the *Alu* sequence should be carefully designed to avoid cross-reaction with the host gDNA if the biodistribution DNA samples are analyzed at up to 1,000 ng in each qPCR reaction. One *Alu*-based qPCR assay for analysis of human cell biodistribution in rodent tissues has been reported.[13,42] We have successfully developed several sets of primers and probes that specifically target to human gene β-globin and *APOB*, and both present as a single copy per haploid human genome. We have found that at least one set of primers and probe to β-globin shows excellent sensitivity and specificity to human cell DNA not only in rodents but also in nonhuman primates.

Primer testing and optimization

Three primer and probe sets are screened for specificity against the matrix DNA to determine which is most suitable for biodistribution and vector shedding analysis. We usually include one set of standards (from 0 up to 10^8 copies/reaction) and two sets of QCs (minimal four levels) using 600 nM forward and reverse primer concentrations and 300 nM for the probe concentration as a starting point. The chosen primer and probe set will be determined based on the assay efficiency, sensitivity, specificity, precision, and accuracy upon completion of the initial screening.

There are multiple strategies used for optimizing qPCR assays such as altering the magnesium concentration and changing the annealing temperatures; however, these approaches have become obsolete and/or inefficient, especially when dealing with tight timelines. Commercial master mixes usually contain the optimal concentration of magnesium as well as other assay components, and tasks such as modifying the annealing temperature to improve the qPCR assay performance can become costly and time-consuming. We choose to optimize an assay by testing various combinations of primer and probe concentrations in an effort to improve the assay sensitivity and specificity.[22] In general, primer concentrations of 100, 300, 600, and 900 nM and probe concentrations from 100 to 300 nM should be tested using two concentrations of the standard DNA, 200 copies and a high copy number, typically at one-tenth of the ULOQ. Every combination of the primer concentrations can be assessed on a single qPCR plate to determine the appropriate amount of forward and reverse primer to be used for the assay. The primer concentrations are chosen based on variables reflecting the best E such as having the lowest Ct values and highest ΔRn. With the selected forward and reverse primer concentrations, the probe concentrations from 100 to 300 nM can then be evaluated on the next plate.

Method development for DNA isolation

FDA guidance recommends the following panel of tissues, at minimum, to be analyzed during preclinical biodistribution studies of gene and cell therapy products: blood, injection site(s), gonads, brain, liver, kidneys, lung, heart, and spleen.[10,43] Additional tissues should be evaluated, depending on the TA, vector type and tropism, and transgene(s), as well as the route of administration (e.g., draining lymph nodes and contralateral sites for subcutaneous/intramuscular injection, bone marrow, eyes). DNA extraction from these tissues and biofluids should be well established prior to sample analysis by qPCR. Biodistribution data are presented as copies of the target DNA per μg of host gDNA. It is important to limit the RNA contamination in the extracted DNA samples to ensure an accurate DNA quantitation. One way to check for RNA contamination is through electrophoresis of the extracted gDNA (e.g., 1 μg) on an agarose gel. If there are no observable smeared RNA bands around 1.5–3 kb, the RNA contamination is minimal. Otherwise, RNase A treatment should be included in DNA extraction or the DNA sample should be analyzed using fluorescence-based assays specifically binding to DNA. Generally, the same purification method used for a specific sample type can be applied to all animal species and humans; therefore, additional method development is likely not required. Carrier DNA may be included for nucleic acid extraction from tiny tissue (<5 mg) or biofluid samples when low DNA concentration is expected.[44]

If a new type of tissue will be collected for sample analysis, a recovery test of the target DNA from the new tissue and the matrix effect of the new tissue DNA (such as impurities in DNA extraction that may cause amplification inhibition) should be conducted to ensure that the purification method is reliable and that the extracted product is suitable for downstream use. As shown in Figure 2, recovery is performed by spiking a known concentration of TA or reference standard into tissue lysate or biofluid samples prior to DNA extraction. After subsequent qPCR analysis of the purified product, calculation of the recovery is easily determined. If a TA is spiked into the lysate for a recovery test, to eliminate variation introduced by different qPCR assay methods or laboratory instruments, the nominal concentration of spiked TA vector should be determined with the same qPCR assay in the same laboratory setting rather than using a nominal titer provided in the certificate of analysis of the TA. Importantly, note that loss of some target DNA is an inherent attribute of nucleic acid purification, dependent on the sample type, the volume of elution buffer, and the method used for purification. We have seen the recovery range between 30% and 80% in general when we purify DNA using various extraction kits such as the QIASymphony DSP DNA kit or others following the vendors' procedures. Performing matrix effect evaluations are similar to the recovery test; however, the reference standard is spiked directly into purified DNA for each sample type followed by qPCR analysis in duplicate. Each spiked sample must meet the acceptance criteria including quantity (Qty), percent coefficient of variation (%CV) $\leq$25%, and a mean percentage of relative error (%RE) within ±25%. If %RE falls outside ±25%, it could be indicative of possible inhibitory or enhancer elements that have copurified with DNA and may have affected qPCR amplification.

Tissue used for the preparation of matrix DNA should be obtained from the same animal species and strain (e.g., C57BL/6 mouse, nude rat, New Zealand White rabbit, beagle dog, Göttingen minipig, cynomolgus monkey) used in the toxicology program. For human clinical sample analysis, matrix DNA should be extracted from the same type of clinical samples collected from donors. The process

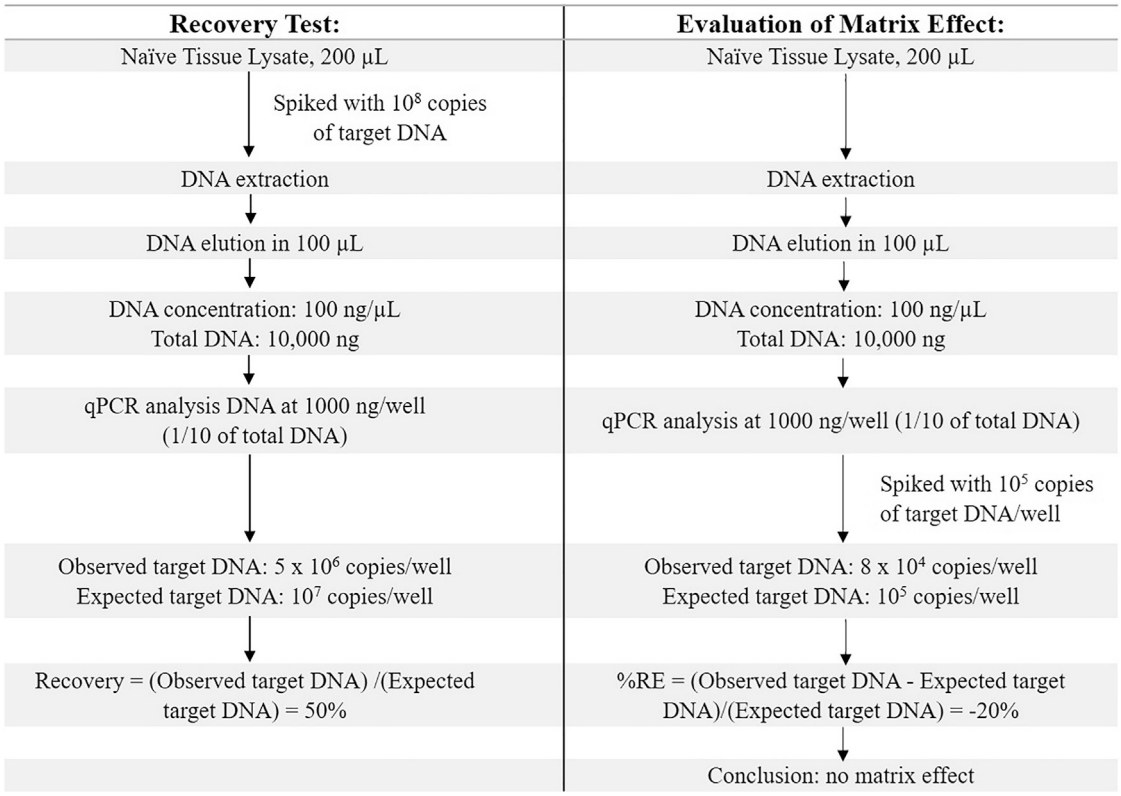

Figure 2. Analysis of target DNA recovery from the tissue lysate and the potential matrix effect of purified DNA

for matrix DNA extraction should mimic sample DNA extraction. If tissue from the target animal strain is limited (e.g., specific mouse models), additional tissues from a related strain of the same animal species may be used; however, the matrix DNA from both strains should be compared and bridged during method validation. Eluted products from different DNA purification kits may contain differential impurities that have an impact on qPCR, and thus it is recommended that the preparation of matrix DNA from the naive animals follow the same method to be used for sample DNA extraction. If the matrix DNA and sample DNA are prepared using different methods, additional tests should be performed to evaluate any potential matrix effect that may be introduced by using different extraction approaches.

Clinical samples for qPCR analysis, including urine, stool, whole blood, plasma, saliva, semen, and swab materials, may contain several transmissible infectious agents, including hepatitis viruses and human immunodeficiency virus. Furthermore, samples collected from dosed animals used in some preclinical studies may contain potentially harmful gene and cell therapy products. At minimum, these samples should be handled following procedures in accordance with the biosafety level 2 policy until completion of sample lysis. Prior to study initiation, all relevant information including the study plan are recommended to be reviewed and approved by the Institutional Biosafety Committee (IBC).

Evaluation run

As a final approach to method development, an evaluation plate, or mock validation run, containing the standard curve and several sets of QCs may be tested to evaluate the assay performance.

FDA guidance recommends the LLOQ of a qPCR assay to be at least 50 copies per µg of host gDNA with consideration given to the sample size used for analysis relative to the size of the tissue in its entirety.[32] Development of a qPCR assay that can analyze up to 1,000 ng of sample DNA per reaction helps meet FDA expectations. Although a reaction volume of 20–25 µL is widely used for qPCR assays, a reaction volume of 50 µL is recommended for analysis of 1,000 ng of biodistribution sample DNA to improve the assay precision and accuracy. Additives such as dimethyl sulfoxide (up to 3%) and/or bovine serum albumin (up to 2.5 µg/reaction) may be included in the reaction if the standard DNA carries GC-rich sequences or when high assay variation is observed.

If all of the above aspects of method development have been carefully considered and experiments have been properly executed with favorable results, then the resultant qPCR assay should be deemed suitable to proceed with validation. Note that Clinical Laboratory Improvement Amendments certification is not required for clinical sample analysis, as the data obtained are not used by physicians for diagnosis or treatment-management decisions; however, compliance with GLPs

Table 3. Example of method validation: plate setup and assay evaluation parameters

Validation plate (copies of target DNA per μg of matrix DNA)		One standard curve (10^8, 10^7, 10^6, 10^5, 10^4, 10^3, 10^2, 50, 25, 10, 5, and 0)	Four sets of QCs (10^8 [optional], 10^7, 10^5, 10^3, and 10^2)
Parameters for assay evaluation	sensitivity	LOD, LLOQ	
	specificity	no template control (NTC)	
	linearity	R^2 and E (from LLOQ to ULOQ)	
	precision	Ct %CV at each level	individual and intra-assay Qty %CV
	accuracy	back-calculated Qty % RE at each level	individual and intra-assay %RE
	reproducibility	inter-assay Ct %CV at each level across all five validation plates	inter-assay Qty %RE and Qty %CV

is recommended to ensure that quality and integrity of safety data are maintained when filed in support of investigational new drug applications.

Method validation

Method validations are instrumental in ensuring the accuracy, precision, and repeatability of sample analysis. qPCR method validations are conducted following the pre-defined, fit-for-purpose validation parameters and acceptance criteria stated in the study-specific protocol and/or method and in compliance with standard operating procedures (SOPs) designed to be consistent with FDA Guidance for Bioanalytical Method Validation, as applicable, and in accordance with FDA GLP Regulations[45] and 21 Code of Federal Regulations Part 58.[46] The laboratory tests, data, and draft and final reports are audited for compliance with the protocol, method, and any applicable SOPs. Experiments to demonstrate the assay specificity, accuracy, precision, repeatability, sensitivity, linearity, dynamic range, limit of detection (LOD), LLOQ, and ULOQ are routinely included in a validation study.

Core validation

For preclinical biodistribution and vector shedding studies, a fit-for-purpose validation is performed by a minimum of two operators, on multiple real-time PCR instruments of the same model, using at least two lots of assay components. In some instances, when only a limited lot of an assay component is available, and assay robustness cannot be evaluated during method validation, a bridging, or partial, validation should be performed before the second lot of an assay component is used in sample analysis. Additionally, a bridging validation should be performed when the sample preparation procedure is significantly modified, the dynamic range of the standard curve requires adjustment, or when the model of the qPCR instrument has changed.

An example of a core validation includes five qPCR runs/plates performed over a minimum of 3 days to evaluate the assay characteristics. Each plate contains one set of standards and four sets of QCs, all independently prepared, plated in the presence of 1,000 ng of matrix DNA, and tested in duplicate qPCR wells as shown in Table 3.

A typical assay standard curve for analysis of AAV vector biodistribution is prepared by serially diluting the reference standard DNA (e.g., 10^8, 10^7, 10^6, 10^5, 10^4, 10^3, 100, 50, 25, 10, 5, and 0 copies per reaction). The standard points at 50 copies and below may be tested in triplicate or quadruplicate qPCR wells to facilitate an establishment of more robust LOD. The lowest concentration level can be as low as three or five copies per reaction, and the greatest concentration should cover the predicted highest target DNA concentration per reaction, if possible. For analysis of human stem cell biodistribution samples, if human gDNA is used as the standards and the qPCR assay is developed to detect a gene with single copy on the haploid human genome, the highest standard concentration in 1,000 ng of DNA is 3.1×10^5 copies of haploid human gDNA (given the haploid human genome mass at 3.2 pg[47]). Therefore, adjustment of the ULOQ may be lowered (10^5 copies) for these studies.

Standard curves prepared for biodistribution validation runs include the addition of 1,000 ng of matrix DNA into each reaction; however, lower amounts of matrix DNA may be used when validating studies for vector-shedding analysis, as many of these samples, such as feces and urine, are rich in organic matter that may copurify with extracted DNA and could possibly inhibit subsequent qPCR amplification. The standard curve is used to evaluate the linear range, specificity, sensitivity, and repeatability of the qPCR assay by evaluating the LOD, LLOQ, ULOQ, linearity, and E. The linearity of the Ct curve is determined using a suitable linear regression analysis of the Ct value versus log nominal DNA concentration. The specificity will be demonstrated by the lack of amplification, or below the LOD, in the absence of standard DNA, or wells containing only matrix DNA (no template control [NTC]). If vector shedding samples such as saliva or feces are to be analyzed, the specificity of the qPCR assay against the matrix DNA extracted from naive host saliva or feces samples should be demonstrated, as DNA purified from these sample types contains a significant percentage of microbial DNA.

FDA guidance recommends that qPCR assays for biodistribution have a demonstrated LLOQ of $\leq$50 copies of target vector per 1 μg of host gDNA with 95% confidence.[10,32] Consequently, the LOD is defined as the minimum concentration of target DNA that significantly (more than 95% of the reactions tested) yields a positive qPCR result in all replicate wells, yet is not necessarily quantified as an exact value. There are many ways to determine the LOD of a qPCR assay[48,49]. Based on the Poisson distribution, the LOD for qPCR cannot be lower than three copies of nucleic acid targets.[19,49,50] We define the LOD as the lowest standard level, which gives a positive qPCR result (Ct value $\leq$40) in all replicates tested throughout the five validation runs. Since the five validation plates will be performed by a minimum of two laboratory operators, on different real-time PCR instruments of the same model, and using at least two lots of assay components, the LOD value obtained using this method is more reliable

and robust than the one obtained from a single plate. The LLOQ is the lowest standard level that not only shows positive amplification in all replicates of the five validation runs, but it also has acceptable assay precision and accuracy (i.e., individual standard's duplicate wells with Ct %CV $\leq$2.0% and back-calculated Qty %RE within ±45%, inter-assay Ct %CV among all validation runs $\leq$3.0%). The ULOQ is the highest standard level showing an acceptable assay precision and accuracy (i.e., individual standard's duplicate wells with Ct % CV $\leq$2.0% and back-calculated Qty %RE within ±25%, inter-assay Ct %CV among all validation runs $\leq$3.0%). The dynamic range of a standard curve in qPCR usually spans eight or nine $\log_{10}$ units. The highest standard level at 10^8 copies per reaction is well within the dynamic range and usually shows good precision and accuracy; however, standard levels at 100 copies per reaction and below tend to have more variable amplification due to stochastic effects that occur during both sampling and the PCR process itself.[51]

Four sets of QC samples are individually prepared and included in each validation run to assess the accuracy, precision, and repeatability of the assay. Each QC set contains an ultra-low (UL, e.g., 10^2 copies), low (L, e.g., 10^3 copies), middle (M, e.g., 10^5 copies), high (H, e.g., 10^7 copies), and ULOQ (optional, e.g., 10^8 copies) concentration of standard DNA that test within the assay's dynamic range. All QCs are tested in duplicate qPCR wells and run in the presence of matrix DNA to mimic sample analysis. The accuracy and precision of the method is evaluated by Qty %RE, a comparison of the measured value of each individual QC with its nominal value, and Qty %CV of each QC's duplicate wells, respectively. In addition, the mean Qty %RE and Qty %CV of all replicate wells at each concentration level on one plate (i.e., all 8 replicates of 4 QC sets) determine the intra-assay accuracy and precision, while the mean Qty %RE and Qty %CV of the replicate wells at each concentration level of all five plates (i.e., all 40 replicates of total 20 QC sets) determine the inter-assay accuracy and precision.

During actual sample analysis, DNA samples are usually stored frozen after extraction and prior to qPCR analysis. Therefore, assessment of freeze-thaw stability is necessary. In our practice, two out of four QC sets undergo three freeze-thaw cycles between room temperature and −80°C after preparation prior to being analyzed along with two freshly prepared QC sets on at least one of the five validation plates.

Matrix effect

DNA samples purified from blood or various types of tissues of the same animal strain contain the same host gDNA. Therefore, matrix DNA that will be added to the standard curve and QC sample reactions is prepared from tissues (e.g., liver) of naive animals using the same purification method used for DNA extraction of study samples. It is well known that impurities, such as phenol, ions, salts, or acids, can copurify with DNA and potentially interfere with qPCR amplification, thus impacting the quality of data obtained from the assay.[52–54] Consequently, any possible matrix effects are evaluated during biodistribution and vector shedding analysis. While PCR inhibition is rarely observed in most tissues, matrix effects are prevalent in others such as blood, injection sites, and skin when evaluated under the laboratory's qPCR conditions (e.g., 1,000 ng of DNA per well). When preparing qPCR plates for biodistribution sample analysis, each DNA sample is run in triplicate; however, the third well is spiked with a known copy number of the target DNA to monitor any amplification inhibition. Spiking standard DNA provides the advantage of effectively indicating false-negative data. When a DNA sample obtained from a TA vector-dosed animal tissue returns a negative result, the spiked replicate can be used to determine whether the data are the result of amplification inhibition or whether the target is truly absent in the sample. If inhibition is observed, the affected DNA sample will be reanalyzed at one-tenth of the original loading amount. Despite the beneficial use of spiking standard DNA into each sample, note that mild inhibition of qPCR may not be detected in samples containing high levels of TA vectors. Therefore, special attention should be paid to the DNA purified from blood, biofluid, tissue, and vector shedding samples of preclinical vehicle-control animals, which should contain no TA vector DNA. Data provided by the addition of the third spiked well in these samples may provide a good indication of potential matrix effects, even mild ones, associated with each sample type. When PCR inhibition occurs frequently in a specific sample type (e.g., more than 2 out of 10 control skin DNA samples), subsequent analysis of the affected DNA samples should be performed at a lower loading amount.

It is recommended that the analytical site performs a thorough evaluation of the potential matrix effects of the commonly tested tissues, blood, and biofluids using an internal established assay when the analytical site establishes its qPCR services. DNA purified from urine, serum, plasma, or other sample types that require plate loading by volume rather than concentration should be evaluated to ensure that the loading volume will not impact amplification. Furthermore, potential matrix effects should be evaluated if a novel tissue is to be tested or a new purification method is implemented prior to the start of a new study sample analysis. Otherwise, historical data and the inclusion of the third spiked well during sample analysis should provide enough data to assess any possible issues with amplification.

Clinical studies often require the analysis of various types of vector shedding samples such as blood, feces, urine, and saliva. One cost-effective approach to analyzing these samples is to use a standard curve prepared in the presence of blood matrix DNA rather than using a standard curve for each matrix type. In this approach, method validation should assess various QC sets containing matrix from blood and each of the remaining sample types (e.g., QC sets 1–2 contain blood DNA, QC sets 3–4 contain saliva DNA, QC sets 5–6 contain fecal DNA) to confirm the assay accuracy and precision. Complex organic matter found in feces and urine or microbial DNA found in saliva and feces may be potential sources of qPCR inhibition. Therefore, potential matrix effects of these clinical samples should be investigated extensively during method validation.

Recovery testing

Thorough recovery testing of the target DNA from commonly analyzed tissues and biofluid samples should be assessed when the analytical site establishes its qPCR services. In our experience,

30%–80% recovery is generally expected from a well-developed assay. This rate typically reflects the loss of nucleic acids during DNA extraction. If the data are expressed as copies of the target DNA per specified concentration of host gDNA (e.g., 1,000 ng), the data are presented as a ratio of the target DNA over host gDNA, and therefore should not change significantly throughout extraction. For biofluid samples, however, the data are presented as copies of the target DNA per volume, and therefore the recovery rate will affect the data and should be evaluated during method development and/or validation for each assay. When analyzing clinical vector shedding samples, FDA guidance recommends that for each study, TA recovery be performed for all clinical matrices that will be investigated.[11] This requirement is reasonable, as the data for vector shedding samples are reported as copies of target DNA per volume or mass of the clinical sample.

Stability testing

In the absence of any contaminating nuclease, elution of pure DNA in water or Tris-EDTA (pH 8.0) buffer is considered stable for up to 16 years when stored at −20°C and below and up to 2 hours when stored at room temperature.[34,35] Furthermore, RNA eluted in water is considered stable for at least 1 year when stored at −80°C and below.[55] These reports support that short- and long-term stability evaluations of the standard DNA and RNA, when stored frozen at −80°C, are not necessary and therefore not included in our standard method validations. However, it is recommended that the analytical site establishes historical data for the storage stability of DNA and RNA both in purified form and in tissues or biofluid samples at room temperature, 4°C, −20°C, and −80°C. A well-recognized approach to evaluate the stability of DNA is agarose gel electrophoresis, which can provide reliable data regarding degradation. Alternatively, the storage stability of both DNA and RNA may be assessed using the Agilent BioAnalyzer 2100 or an equivalent instrument, or an established qPCR assay.

The stability of target DNA in prepared QC samples is routinely assessed by performing up to three freeze-thaw cycles during method validation. Furthermore, for clinical sample analysis, benchtop and storage stability of the TA vectors in qPCR should be assessed during method validation unless stability information has been provided by the clients.

Sample analysis

Below are the recommendations for biodistribution sample analysis by qPCR in the FDA guidance issued in 2020.

The assay should have a demonstrated LOQ of $\leq$50 copies of vector per 1 μg of gDNA, so that your assay can detect this limit with 95% confidence.

The DNA samples should be run in triplicate for each tissue. To aid the interpretation of the qPCR assay results, one replicate of each tissue sample should include a spike of control DNA, including a known amount of the vector sequences. The spike control will determine the specified PCR assay sensitivity.

In the final study report, individual animal data should be provided. The method for how values below the LOQ of the assay are categorized and calculation of the median or mean value should be specified.

In addition, the FDA guidance issued in 2006 recommends consideration of the sample size relative to the tissue being tested for biodistribution sample analysis.[10] In our practice, up to 200 mg of tissue (or the whole organ if smaller) are collected, snap-frozen, and stored at −60°C to −90°C until sample lysis and DNA extraction. Each sample is then analyzed at up to 1,000 ng per qPCR well in triplicate using the validated qPCR assay; however, if the DNA sample concentration is too low, the maximal amount of DNA will be analyzed. The mean value of the first two replicate reactions are used to report the sample copy number, while the third well is spiked with a known copy number of the target DNA to monitor potential PCR inhibition and false-negative data introduced by the sample DNA, as previously mentioned. If data provided by the third well indicates a presence of PCR inhibition (e.g., a recovery of the spiked target DNA less than 55%), the DNA sample will be re-analyzed in triplicate qPCR at lower amount, such as one-tenth to one-fourth of the original DNA sample. Samples containing a well-known potential PCR inhibitor, such as blood, are loaded at smaller initial concentrations (e.g., 100 ng).

Biodistribution of gene and cell therapy products in various animal tissues can vary from negative to above 10^8 copies per μg of host tissue gDNA. Although the extremely high dynamic range of a qPCR assay makes it feasible to obtain reportable data from the initial test, there may be some tissues (e.g., liver) that contain high copy numbers of target DNA and test above ULOQ (or $>10^8$ copies per μg of host gDNA). In this case, the sample is re-analyzed at one-hundredth or one-thousandth of the original loading amount. To ensure the assay accuracy, matrix DNA should be supplemented in these samples to obtain a final concentration of 1,000 ng of total DNA per qPCR reaction.

In the final study report, both individual animal data and the calculated group mean values of the investigated tissues collected at each time interval are presented. Data tested below LOD and LLOQ are reported as BLOD and BLOQ, respectively. Additionally, reported data should be clearly labeled as either double or single stranded. Generally, linearized plasmid DNA encoding the full-length or partial vector gDNA is used as standard, and therefore the biodistribution data are reported as double-stranded copies of the target DNA per μg of host gDNA or are converted into copies of the target DNA per volume or mass of the sample (i.e., copies of target per mL of urine, or copies of target per mg of feces). Furthermore, although recombinant AAV vectors contain single-stranded gDNA, it is present as double stranded after entering the cells.

Recommendations and acceptance criteria for assay validation and sample analysis

Table 4 provides our testing recommendations and acceptance criteria for qPCR method validation and sample analysis. Each parameter is discussed and an evaluation of its inclusion in the list

Table 4. Recommendations and acceptance criteria for qPCR analysis of biodistribution and vector shedding samples

	Validation		Sample analysis	
	Testing	Acceptance criteria	Testing	Acceptance criteria
Standard curve for sensitivity, selectivity, and linearity	as described in the section of core validation	● all NTC wells should test BLOD	● at least eight non-zero standard levels are plated in duplicate wells, from the validated LLOQ to ULOQ; NTCs are plated in triplicate wells	● at least two-thirds of the NTC wells should test BLOD
		● E should be between 90% and 110%	● all NTCs and standards are prepared in matrix DNA extracted from naive tissues of the same animal strain and species, or human donors, to mimic the study samples	● E should be between 90% and 110%
		● the standard curve should show a coefficient $R^2 \geq 0.980$		● the standard curve should show a coefficient $R^2 \geq 0.980$
		● the LLOQ should be ≤50 copies of target DNA per µg of host gDNA for biodistribution sample analysis, or per reaction well for vector shedding sample analysis		● at least 75% and a minimum of six non-zero standard concentrations should have a Ct %CV ≤2.0% and back-calculated standard concentrations within ±25% of nominal value (±45% for standards between LLOQ and QC-UL)
		● back-calculated non-zero standard levels at LLOQ and above should have a %RE within ±25% of nominal value (±45% for standards between LLOQ and QC-UL)		● if the LLOQ (or ULOQ) fails the acceptance criteria on a plate, the next lower (or higher) standard level can be selected as a plate-specific LLOQ (or ULOQ), provided the resulting standard curve meets acceptance criteria; samples with values between a validated and the plate-specific LLOQ (or ULOQ) on the plate will be re-analyzed
		● the non-zero standard levels, from LLOQ to ULOQ, should have a Ct %CV ≤2.0% of their duplicate wells		
		● at least 75% and a minimum of seven non-zero standard concentrations, from LLOQ to ULOQ, should meet the above criteria in each validation run		
QCs for assay precision, accuracy, and reproducibility	four or more QC sets per validation run, as described in the section of core validation	● ≥50% of the QCs at each level and ≥67% of all QCs have an individual Qty %CVs of their duplicate wells ≤25% (≤45% for the QC-UL) and individual Qty %REs within ±25% (±45% for the QC-UL)	● two or more QC sets per qPCR plate and each set includes low (L), medium (M), and high (H) tested in duplicate qPCR wells and prepared in the presence of matrix DNA	● ≥50% of the QCs at each level and ≥67% of all QCs have an individual Qty %CVs of their duplicate wells ≤25%, and individual Qty %REs within ±25%
		● for each validation run, the Qty %CV of all QCs at each level (intra-assay precision) should be ≤25% (≤45% for QC-ULs); the mean %RE of all QCs at each level (intra-assay accuracy) should be within ±25% (±45% for QC-ULs)		
		● the Qty %CV of all QCs at each level from all five validation runs (inter-assay precision) should be ≤25% (≤45% for QC-ULs); the mean %RE of all QCs at each level from all five validation runs (inter-assay accuracy) should be ±25% (±45% for QC-ULs)		

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Table 4. *Continued*

	Validation		Sample analysis	
	Testing	Acceptance criteria	Testing	Acceptance criteria
Specificity	● analyze the DNA samples or a pool of the matrix DNA extracted from naive tissues of at least three individual animals of the relevant species/strain, including at least one male	all naive DNA samples should test BLOD	● all NTCs should include matrix DNA collected from the same strain and species as study samples	● At least two-thirds of the NTC replicate wells should test BLOD
	● for vector shedding studies, analyze the DNA samples of each type of shedding sample, collected from three to six naive hosts (animals or human donors), including at least one male		● pretest samples such as blood or vector shedding samples from the dosed individual species, samples from vehicle-control animals, or both should be included in the study as negative controls	● all pretest and vehicle-control samples should test BLOD; if not, an investigation should be executed to ascertain the source of contamination
Matrix effect	● test procedures are described in Figure 2; it is recommended that the matrix effect from each type of tissue/sample is evaluated for each new strain and species tested at the analytical site	● the DNA samples of a same type should have a mean Qty %CV $\leq$25% and mean Qty %RE within ±25% of the spiked copies of the target DNA ($\leq$45% and ±45%, respectively, if the spiked copies are at QC-UL and below) to be considered as having no matrix effect	● each DNA sample (e.g., tissues, biofluids, excreta/secreta) is tested in triplicate at up to 1,000 ng of sample DNA per well; DNA samples from biofluid samples may be loaded at a fixed volume per qPCR well; biodistribution/vector shedding data will be obtained as a mean of the first two replicate wells; ghe third well will be spiked with a known amount of target DNA (e.g., 200 copies) to monitor the potential qPCR inhibition	the third spiked well should test $\geq$55% of the spiked nominal copies of the target DNA to exclude the presence of a PCR inhibitor; if not, the sample DNA will be re-analyzed at a lower concentration (e.g., one-fourth or one-tenth of the original reaction) to dilute out the effect of the inhibitor
	● analyze the DNA samples extracted from each type of tissue/sample of at least three individual naive animals or human donors in duplicate qPCR wells by spiking a known target DNA copies into each well; this test may be optional if the analytical site has established historical data for the sample type of tissue/sample	● if the acceptance criteria for %RE from one type of tissue is not met, the amount of the affected sample DNA loaded to the qPCR wells should be adjusted and re-established until the Qty %CV and %RE meet the acceptance criteria; the DNA loading amount showing no matrix effect should be used for sample analysis	● if one type of DNA sample consistently shows the matrix effect from the third spiked wells of all vehicle control or pre-test samples in a study, the same type of sample from all dosed animals should be loaded at lower DNA amount to each qPCR well	
Recovery test	● test procedures are described in Figure 2	recovery between 30% and 80% can be expected from most of the sample types		
	● recovery testing of viral vector TA from biofluid samples and clinical shedding samples should be demonstrated using the validated assay; data are reported as copies per the final volume or mass of the clinical sample			
	● recovery of target DNA from various animal tissue and blood samples should be established when setting up the qPCR services; it is not necessary to evaluate every sample type for every study if the analytical site has an established DNA extraction method with historic data of DNA recovery from the sample type			

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Table 4. *Continued*

	Validation		Sample analysis	
	Testing	Acceptance criteria	Testing	Acceptance criteria
Stability	● storage stability of target DNA in both purified DNA samples and tissue/biofluid/vector shedding samples is not routinely included in validation testing for preclinical biodistribution analysis	● stability of QC freeze-thaw cycling is acceptable if all QCs of each level have mean Qty %CV ≤25% and mean Qty % RE within ±25% (≤45% and within ±45%, respectively, for the QC-UL)		
	● freeze-thaw stability of the target DNA is assessed by including at least two sets of DNA QC samples with up to three cycles of freeze-thaw on at least one validation plate to mimic the status of samples prior to qPCR analysis	● the TA vectors stored in clinical matrices over specified storage conditions and time periods should range between 30% and 170% when compared to time zero to be considered stable		
	● benchtop and storage stability of TA vectors should be investigated in all clinical matrices to support clinical sample collection and storage			
Repeat analysis			● if the standard curve or QCs fail to meet acceptance criteria on a sample analysis plate, the run will be repeated; if the plate fails a second, consecutive run, the Study Director will be notified to determine a course of action to determine the root cause	repeat analysis if any of the following is not met:
			● if the result of a pretest or vehicle control sample is not BLOD, the sample will be re-analyzed from the remaining tissue/tissue lysate, or the remaining DNA if there is no more remaining tissue/tissue lysate; if the second run returns a result that is BLOD, indicating contamination may have occurred during initial DNA extraction or qPCR analysis, BLOD will be reported based on the data from the second run; if the second run returns another positive value, the average result of the two runs will be reported	● the standard curve (including NTC wells) and QCs included in each sample analysis run should meet acceptance criteria
			● if a DNA sample shows evidence of PCR inhibition (i.e., the third well returns a value that is <55% of the spiked nominal value), it will be re-analyzed at a lower amount (e.g., one-fourth or one-tenth of the original reaction) to dilute out the inhibitor; if inhibition is seen in the second run, the sample may be further diluted, or the value may be reported as "non-reportable"	● all samples collected from pretest and vehicle control animals should test BLOD

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Table 4. *Continued*

	Validation		Sample analysis	
	Testing	Acceptance criteria	Testing	Acceptance criteria
			• if a sample tests greater than ULOQ, it may be diluted and reanalyzed; however, the sample should be supplemented with matrix DNA to an amount equal to the original loading amount (e.g., 100 ng of sample DNA mixed with 900 ng of matrix DNA to final 1,000 ng of total DNA)	• the third spiked well should test >55% of the nominal copy value
				• all reportable samples should test within the dynamic range of the standard curve (i.e., values cannot be extrapolated)
				• all reportable samples should have individual Qty %CV $\leq$25% ($\leq$45% at the level of QC-UL and below)
Incurred sample reanalysis (ISR)			due to limit of many biodistribution samples collected from the animals, it is not feasible to perform ISR at least for tiny tissues; however, this may be required for clinical studies in which a larger sampling pool is accumulated	at least 67% of the samples tested during ISR should be within ±45% of the mean value

is made based on industry practice, our field experience, and the feasibility of the experiments.

qRT-PCR method development, validation, and sample analysis

Analysis of RNA virus biodistribution, vector shedding, and vector-derived transgene expression can be performed using RNA standards in a one-step qRT-PCR assay, where reverse transcription of RNA into cDNA and subsequent qPCR amplification of the target cDNA can occur in the same reaction. In this assay, a standard curve and QC sets are prepared using RNA, and thus they are subjected to the same reverse transcription as each target sample. One-step qRT-PCR has the benefits of limited contact with samples and less pipetting, which effectively decreases the possibility for cross-contamination and technical errors. An example of one-step qRT-PCR analysis of RNA virus biodistribution and vector shedding is shown in Table 5. Matrix RNA is total RNA extracted from the naive animal tissues and included in the standards and QCs to mimic RNA samples. Both the qScript XLT one-step qRT-PCR ToughMix and the AgPath-ID one-step RT-PCR kit work well for one-step qRT-PCR assays using the conditions shown in Table 6. While the strategy for a one-step qRT-PCR assay development, validation and sample analysis mirrors those described for qPCR in general, some notable differences should be mentioned.

RNA standards will be plated in the presence of RNA matrix (usually total RNA) with both reverse transcription and qPCR amplification of the cDNA occurring in the same reaction well/tube, and thus reaction conditions will vary. The data will be reported as copies of the target RNA per 1,000 ng of sample RNA. If the assay is for biodistribution sample analysis, the assay is expected to have an LLOQ $\leq$50 copies per 1,000 ng of RNA.

When RNA is analyzed using one-step qRT-PCR, the matrix effect and specificity should be performed on total RNA extracted from all sample types such as blood and various tissues during method development and validation, as differential gene expression is expected among different types of tissues.

Specific to DNA viral vector-derived gene expression, there is potential contamination of viral vector DNA in the extracted RNA samples even after DNase I treatment is included during RNA extraction. Therefore, each RNA sample should be analyzed by qRT-PCR with the reverse transcriptase removed from the reaction mixture to ensure the values obtained from qRT-PCR analysis truly reflect the vector-derived mRNAs rather than the contaminated vector DNAs.

Furthermore, handling standard and sample DNA can be conducted at room temperature; however, standard and sample RNA should be handled on wet ice.

Finally, tissues and blood should be collected into RNAlater or equivalent reagents to minimize any potential RNA degradation. Relative quantitation of a host housekeeping gene may be performed accompanying the absolute quantitation of the viral vector RNA copies by

Table 5. Example of one-step qRT-PCR for absolute quantitation of target RNA

Component	Final concentration
Standard RNA[a]	0–10^8 copies
Forward primer	up to 900 nM
Reverse primer	up to 900 nM
TaqMan probe	up to 300 nM
2× qScript XLT one-step qRT-PCR ToughMix or equivalent	1×
Matrix RNA[a]	up to 1,000 ng
Nuclease-free water	to final 50 µL

[a]Standard and QC wells contain standard and matrix RNA. For sample analysis, standard and matrix RNA will be replaced by up to 1,000 ng of sample RNA.

one-step duplex qRT-PCR to monitor the integrity of the RNA samples.

Although the lentiviral vectors contain RNA genome, the vectors are present as integrated DNA upon entry into host cells. Therefore, DNA standards, rather than RNA, are generally used for analyzing the biodistribution of lentiviral vectors by qPCR assays.

Conclusions

Currently, analytical laboratories and sponsors have different assumptions of what constitutes a validated biodistribution or vector shedding assay. Unfortunately, with lack of direction, many individuals and companies reference the FDA guidance on bioanalytical method validation. While some testing parameters and acceptance criteria may be applicable to qPCR, there are certainly others that are not necessary, or even beneficial, when using this assay. The main difference lies within the properties of the sample being tested. Most bioanalytical assays require the use of crude lysate, which increases the likelihood of off-target results, degradation, and overall variation in data. Real-time qPCR offers the advantage of using purified DNA or RNA as the testing sample. The stability of nucleic acids has been researched for years and should not be subjected to the same testing recommendations as other sample types. Additionally, many PCR inhibitors are inherent to the sample type and can be anticipated between studies. Ultimately, method development is as aimed toward optimizing the detection of a specified sequence rather than optimizing the assay for the sample input. The primary purpose of this paper is to help the new bioanalytical scientists quickly grasp the essentials of biodistribution and vector shedding analysis, and to provide a set of recommendations that promote efficient and thorough design and validation of the robust qPCR and qRT-PCR assays. As technology advances in this area, it is important that members of the scientific community collaborate to establish routine guidelines and acceptance criteria when evaluating gene and cell therapies in order to set a standard level of expectations to be evaluated prior to releasing these novel therapeutics into routine use. Overall, a robust, well-documented methodology is necessary, and, by thorough explanation, the authors have attempted to provide this standard for the evaluation of future cell and gene therapies.

Table 6. Example of one-step qRT-PCR thermal cycling

	Temperature (°C)	Time	Cycles
Reverse transcription	50	10 min	none
Enzyme activation/deactivation	95	1 min	none
Denaturation	95	10 s	40 cycles
Annealing and extension	60	30–60 s	

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AUTHOR CONTRIBUTIONS

H.M. wrote and revised the manuscript, including designing the tables and one figure. K.N.B. wrote and revised the manuscript, including designing one figure. R.N.L. scientifically reviewed the manuscript based on contributions to process development.

DECLARATION OF INTERESTS

All authors are employees of Northern Biomolecular Services, and may own respective stock of the company. The authors declare no additional competing interests.

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