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Meeting Report

Best Practices in qPCR and dPCR Validation in Regulated Bioanalytical Laboratories

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Abstract. The use of molecular techniques in the bioanalytical laboratory is becoming more common as the number of gene and cell therapy products continues to increase. Currently, there is no bioanalytical regulatory guidance for these techniques, and contract research organizations are dependent on scientific judgment and best practice to execute this work to GxP compliant status for preclinical and clinical studies supporting biodistribution and vector shedding. This manuscript describes the process and rationale for development and validation of quantitative polymerase chain reaction (qPCR) and digital PCR (dPCR) assays as presented in a 2021 AAPS two-day workshop on the harmonization of qPCR. The scope, herein, includes bioanalytical validation parameters and acceptance criteria utilizing these technologies. Additionally, pros and cons of these molecular techniques will be highlighted, and the common pitfalls to avoid will be illustrated. The aim of this manuscript is to provide best-practice, working recommendations, and the facilitation of future regulatory guidance.

INTRODUCTION

Cellular and gene therapies represent a new wave of rational therapeutic design by genetically modifying cells to carry out therapeutic tasks. While gene therapies use nucleic acids, cell therapies involve use of cellular material to replace missing functional proteins or alter cellular networks responsible for a diseased state. An increased understanding of disease-associated biological pathways and our ability to genetically encode cell and gene therapeutic programs have made treating monogenic disorders and hematological malignancies possible over the past two decades [1].

BACKGROUND

Examples of these novel drug modalities are the chimeric antigen receptor (CAR) cell therapies that utilize an *ex vivo* reprogramming of immune cells of cancer patients by inserting a gene for CAR; these cells are then transplanted back into the patient for effective targeting and killing of tumor cells [2]. Similarly, an *ex vivo* viral transduction of the functional β-globin locus into $CD34^+$ hematopoietic stem and progenitor cells has demonstrated remarkable clinical success in treating β-thalassemia patients [3]. Gene-editing tools based on zinc-finger nucleases and transcription activator-like effector nucleases (TALEN) have also surfaced for treatment of patients infected with HIV [4]. The recent advances in CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) systems are generating encouraging results in animal models [5] and will continue to propel the field of cell and gene therapy for development of novel treatment options for previously intractable diseases.

Although cell and gene therapies are demonstrating promising clinical results, an important limitation of many current approaches is that they provide little control over the dosage and cellular context of the therapeutic effect [6]. For example, clinical trials using CAR-T (chimeric antigen receptor T cells) have reported several fatal or life-threatening adverse events, including cytokine release syndrome and neurotoxicity related to excessive activation of engineered T cells [7].

There is a critical need for method development, validation and life cycle management of molecular methods, specifically quantitative PCR (qPCR) and digital PCR (dPCR) or NGS (Next Generation Sequencing), which are being used to support bioanalytical workflows for persistence monitoring, biodistribution, viral shedding, copy number and gene expression changes to support effective and reproducible therapies in this area. While emerging NGS promises to monitor both the efficacy of editing, as well as potential off-target gene edits with high sensitivity, the technology requires extensive infrastructure and is challenging to validate. Both qPCR and dPCR have currently emerged as a method of choice for bioanalytical assays and have their own pros and

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Table I. Comparison of digital and quantitative PCR

	Digital PCR (dPCR)	Quantitative PCR (qPCR)
Quantitative (with standard curve)	+	+
Absolute quantitative (without standard curve)	+	-
Susceptible to interferents	+/-	++
Multiplex capable	+	++
Assay dynamic range	**++**	**+++**
Sensitivity	++	++
Precision for rare events	++	+
Reverse transcriptase-incorporated workflow	+	+
Cost of instrumentation	$$/$$$	$/$$
Cost of reagents/consumables	$$	$
Average run throughput	5h	1.5h
Reactions per plate	96	96/384

cons that are described in the qPCR platform comparison section and illustrated in Table I.

The emergence of novel cell and gene therapies combined with the evolution of molecular platforms that can be used to support drug development studies have made it imperative to understand the best practices and recommendations for developing and validating qPCR assays that could be used in regulatory submissions. Recently, global regulatory agencies including US Food and Drug Administration (FDA) have developed guidelines for monitoring and assessment of nonclinical and clinical studies to avoid adverse events [8]. These agencies have recommended and provided framework for use of molecular assays to assess long-term efficacy and safety outcomes. However, these guidelines do not address the bioanalytical requirements for characterizing and properly validating qPCR and dPCR assays. Thus, relying on the combined experience of contract research organizations, this paper outlines the recommendations for qPCR/dPCR assay design, method development, assay validation, acceptance criteria and subsequent sample analysis as recently discussed at an AAPS workshop. In the remainder of this manuscript, PCR refers to either traditional quantitative and digital PCR unless specifically noted. RNA targets and RT-qPCR are outside of the scope of this discussion.

QPCR AND DPCR PLATFORM COMPARISON

Digital PCR (dPCR) is beginning to emerge as a preferred technology over qPCR, for both CMC (Chemistry, Manufacturing, and Control) and bioanalysis, because of several analytical advantages. The premise behind digital PCR relies on setting up a reaction very similarly to TaqMan qPCR and then partitioning each sample into tens of thousands of unique reactions. These unique, individual partitions are cycled to end-point, and fluorescence data for each partition is collected. These partition data are evaluated in a binary fashion (positive or negative) and analyzed using Poisson distribution. This analysis allows for absolute quantification of copy number in a sample without the need for a calibration curve.

Absolute quantification has emerged as the key advantage of dPCR relative to qPCR, but other aspects of the technology make it very attractive for drug development as well. Unlike qPCR which quantitates based on cycle threshold (Ct), dPCR does not measure signal after each cycle; all partitioned samples are run to 40 cycles and then analyzed. Even in the presence of a moderate polymerase inhibitor, the target will be amplified within positive partitions to generate a fluorescent signal. Due to the finite number of partitioning events per sample, the quantitative range of dPCR is typically smaller than that of qPCR, but sensitivity is typically similar to or improved relative to qPCR. This technology is equally well adapted to tolerate complex drug formulation buffers for CMC testing because it allows for use of a wide array of biological matrices for biodistribution and shedding studies.

While dPCR is highly accurate and sensitive, it does have some drawbacks compared to qPCR. Historically, the partitioning was done manually and variations in this process led to quantitative inaccuracy. Modern dPCR platforms have made this process more automated, increasing throughput and accuracy. The platforms and software available today are also 21 CFR Part 11 compliant. The ability of dPCR to multiplex is limited compared to qPCR. However newer platforms are narrowing that gap. With the latest dPCR platforms, the biggest cons are cost and throughput. The generation of a partitioned sample requires additional reagents and consumables that are not needed in a qPCR workflow, and the technology is more sophisticated with platform costs considerably higher than qPCR systems. The sample throughput and quantitation of dPCR is also decreased with typical run times around 5 hours for a 96-well plate compared to 96- or 384-well runs of <90 minutes for qPCR.

Each technology has utility in support of bioanalytical workflow for persistence monitoring, biodistribution, viral shedding, copy number, gene expression, and numerous other applications. dPCR is gaining popularity for use in liquid biopsies to identify rare polymorphisms in cancer monitoring and functioning as an orthogonal method for certain next-generation sequencing applications [9–11]. Obviously, qPCR

and dPCR are both valuable tools for drug developers and CROs alike.

Method Development Considerations

Defining Assay Design Specifications

Method development, by definition, is the exercise to provide sound scientific evidence that a method is suitable for the context of use (COU). The COU for PCR (qPCR and dPCR) assays in regulated bioanalytical labs include biodistribution, vector shedding, gene expression, and pharmacokinetics. A fit-for-purpose (FFP) approach should be utilized for the development and validation of qPCR assays based on the COU. The first step in method development is to define the design specifications of the method that will qualify it for the intended COU [12]. The design specification includes a selection of target(s) sequence, reference gene, singleplex versus multiplex, reference materials, instrumentation requirement, specimen type, and throughput requirement. In addition, the minimum required performance specifications for assay parameters for any bioanalytical method such as specificity, accuracy, precision, selectivity/ matrix interference, linearity, the limits of quantitation (LOQ), limit of detection (LOD), robustness and ruggedness are also taken into consideration.

Selection of Target Sequence and Reference Gene

Most molecular studies, including cell and gene therapy, require two qPCR assays – one for the measurement of the target gene and the other to measure the selected reference gene. It is important to choose a target sequence that is suitable for the intended purpose of the assay. Generally, primer and probe design should be approached in a systematic manner with attention to the number of nucleotide bases, the melting temperature, the annealing temperature, the GC (guanine and cytosine) content and consideration for the probe location. Once designed, the primer sequences should be checked for specificity related to both the organism as well as the target sequence. It is critical to not consider a primer sequence universal across animal models and for use in clinical trials. It is equally important to select a reference gene that can be reliably measured regardless of a disease condition or genetic polymorphism. Given the difficulty in selecting an appropriate reference gene and the challenge of differential expression, there are several methods that can be used to select sets of reference genes including software algorithms like geNorm™, NormFinder™ and others [13–15]. The copy number for the reference gene is used to normalize the copy number for the target gene.

If both the target gene and the reference gene will be measured in a multiplex assay, any potential interference in amplifying both genes should be investigated. Due to the high abundance of the reference gene, amplification of it can deplete the master mix enzyme. This in turn will cause low amplification efficiency for the target gene, which in general is present in lower concentrations. The presence of multiple probe and primer sets can also have an impact on assay performance. To ensure the suitability of a multiplex format, the following assay parameters may be evaluated in both singleplex format and multiplex format: sensitivity, linearity, PCR efficiency, and quantification cycle (Cq) values [16].

Nucleic Acid Extraction

qPCR assays require the isolation of nucleic acid from various matrices. In the case of biodistribution and viral shedding assays, extraction of nucleic acid is performed on various tissues of human and animal species. There are several protocols and commercial kits available for the isolation of nucleic acids. The pros and cons of each of these procedures are out of the scope of this meeting report. The extracted nucleic acids should be evaluated for quantity, purity, and integrity during method development. Different instrumentation and technology can be used to evaluate and quantitate extracted nucleic acids. Nucleic acid quantification can be determined by measuring absorbance at the 260nm wavelength using a spectrophotometer-based system. Alternative instruments utilize fluorescent based dyes that bind nucleic acids for quantitation. Nucleic acid purity can be evaluated by assessment of the 260/280nm ratio and should ideally be 1.8 for extracted DNA. Assessing integrity is a measure for breaks or degradation of nucleic acid and can be determined using bioanalyzer instrumentation that produces a DIN (DNA integrity number) score, which is indicative of nucleic acid integrity. Once an extraction method performance is established during method development, purity and integrity assessment may not be necessary during validation and routine sample analysis. However, it should be noted that the performance of qPCR assays should be verified when an extraction procedure is changed, and a partial validation of the qPCR method is required on a case-by-case basis. In general, if extraction chemistry is changed, then a full validation would be required. The scope of the partial validation is dependent on the nature and extent of the change. The partial validation should, at minimum, assess recovery and matrix effect assessment using a minimum of 6 lots of each matrix.

When evaluating selectivity for biodistribution assays, an extensive evaluation is performed in method development with a larger subset of tissues to characterize the assay for each tissue in each species. For validation, a smaller subset of tissues can be used for evaluating matrix interference using the same extraction process.

The amount of nucleic acid needed for dPCR assay will vary with expression level of the target DNA. Another variability to consider are inhibitors and their impact on the reaction. For example, when quantifying FFPE (Formalin-Fixed Paraffin-Embedded) tissues, saliva, urine, or feces samples, it is important to note that these samples may have a difference in the amount of quantifiable DNA and amount of DNA able to be amplified [17].

Critical Reagent Selection

For qPCR assays, in general, the following reagents are considered critical: reference standard, primers/probes, matrix DNA, and master mix.

Primers and probes should be carefully selected to ensure the specificity of the target. Over the last decade, with the introduction of numerous sophisticated software tools

(e.g. NCBI primer BLAST, IDT PrimerQuest™, Primer Express™, SnapGene™), primer design has become straightforward. Primers can be designed for a target length and specified melting temperature. Some tools also allow for the evaluation of potential secondary structures such as primer dimers, hairpins, etc. The specificity of the primers/probe must be confirmed during method development to ensure that the sequence of the gene of interest can be distinguished from other interfering endogenous or similar sequences.

With the availability of various high-quality "ready to use" master mixes, it is unlikely that a master mix needs to be formulated in-house. The selection of master mix is determined experimentally during method development. Master mixes should be handled as per the manufacturer's specification. The robustness of qPCR assays is highly dependent on the integrity of the master mix.

The critical reagents used in qPCR assays are manufactured at various quality levels (e.g., diagnostics grade, cGMP (current Good Manufacturing Practices), research-grade) and undergo a varying degree of characterization. It is important to identify and document minimum performance specifications for each critical reagent. The certificate of analysis for critical materials documenting at minimum the identity, source, concentration, purity, and stability of the material should be available. Regardless of the manufacturer's characterization, all critical reagents must be qualified for functionality to ensure robust assay performance. Any changes in lots or manufacturer should not be considered interchangeable and must be qualified before use.

All critical reagents should be handled and stored as per the manufacturer's specifications. If no stability information is provided by the manufacturer, the bioanalytical laboratory must establish the stability that is consistent with their practices (e.g., freeze-thaw stability, various storage temperature stability).

Calibrator and Quality Control Samples

Like most bioanalytical assays, it is strongly recommended to use the test article/clinical-grade material manufactured under cGMP. When the cGMP material is not available, research-grade material may be used for method validation. In this case, bridging of the research lot to the test article/clinical-grade material is required. Bridging should involve, at minimum, the assessment of extraction recovery and linearity assessment.

In some cases, surrogate reference materials may be used with appropriate scientific justification. It should be noted that when absolute quantitation is required or reference material is poorly characterized, dPCR is preferred. Otherwise, it is important to have the confirmation of surrogate standard and sample template equivalent, if not identical. Conformational differences of plasmid DNA can cause a significant difference in standard curves resulting in inaccurate quantitation [18]. If linearization of plasmid DNA is required, it should be tested during method development for complete digestion. If restriction digest is used for the plasmid it needs to be used in sample treatment as well.

For qPCR assays, gDNA (genomic DNA) is used to prepare standards and QCs to mimic test samples. For reference gene measurement, a surrogate gDNA can be used. The gDNA source should be tested to ensure there is no cross-reactivity to primers/probe pairs. The validity of the non-clinical and clinical qPCR and dPCR methods are dependent on the use of PCR quality control samples (QCs), such as positive and negative (NTC; no template control) controls. In addition to the PCR controls, extraction controls are required to ensure performance of the method. In routine operation, it is suggested to have a low positive control, approximately five times the LOD and a high positive control. Ideally, the positive QCs would be large batch of incurred sample and/or a known positive tissue sample to match the matrices used during validation and sample analysis. However, most often the QCs to be used in qPCR/dPCR methods are mock samples. Mock samples are negative matrix samples that are spiked with the target DNA.

First and foremost, QCs are used during method validation to assess parameters and to assess stability as well as set-up plate validity criteria for the positive QCs followed by long term use for control during sample analysis. The QCs should be treated in the same manner as test samples, with the exception of PCR QCs. These QCs do not go through nucleic acid purification steps and are added directly to the qPCR or dPCR plates.

Critical Reagent Bridging and Assay Trending

Not only are the QCs used during sample analysis, they should also be used during critical reagent qualification/bridging experiments. As critical reagents utilized in assays are depleted or reach expiration, new (candidate) reagent lots need to be qualified. To qualify candidate lots, experiments are performed to assess the impact on assay performance. Upon demonstrating that the candidate lots being qualified produce similar results to those of the current qualified lots, testing will resume using the newly qualified lot. In some cases where assay trending parameters have already potentially been set, the QCs not only provide assay validity, but also an assessment of the performance of the new reagent lot as compared to the historic lot. In the event new QCs needs to be qualified, it is recommended to run a minimum of six independent purifications and qPCR/dPCR plates with the new candidate lot of QCs tested in replicates as unknown samples. This will allow for new QCs to be set and adjust the method trending profile.

It is recommended to perform method monitoring or trending of QCs to monitor assay performance over time for studies that are expected to be run for many years. Ideally, method trending will be retrospectively introduced starting with the assay QCs limited data set from the method validation. A data set and graph for each parameter, including the target (mean), specification limits (two standard deviations from the mean), and trending/monitoring limits (three standard deviations from the mean), for each parameter will be created at a time period set during the course of testing. If more than ten runs are performed weekly, it is suggested to trend on a weekly basis. Every run is subject to trending and a daily value will be computed for each parameter as defined in the trending plan. Assays are considered out-of-trend if ≥ 4 of the last 6 consecutive daily values are outside of the trending limits in the same direction. Although, different trending analysis and parameters can be considered and outlined based on assay specifics. Other trending criteria can also be used based on the intended use of the data.

Table II. Recommendations and Acceptance Criteria for qPCR and dPCR Method Validation

Parameter	Validation Recommendations	Acceptance Criteria Recommendations
Calibration Curve/Dynamic Range	At least six, non-zero calibrator levels covering the quantitation range, including ULOQ and LLOQ per validation run. The concentration-response relationship is fit with a linear curve fit with R^2 determination.	Non-zero calibrators should be 20-25% of relative concentrations and %CV in each validation run. 75% and a minimum of six non-zero calibrator levels should meet the above criteria in each validation run.
Linearity	Calibration curve data is used to assess linearity	R^2 value of > 0.98 PCR efficiency 90-110%
Reproducibility (Precision and Accuracy)	Positive Controls: Five QC levels (LLOQ, LQC, MQC, HQC and ULOQ) and ≥ two replicates per QC level in each analytical run. Negative Control: At least three NTCs per plate.	QCs should be within ± 20% of the relative concentration and %CV, or within acceptance as determined in method development. NTCs should pass acceptance criteria or be >LOD
Analytical Specificity	One run that demonstrates specificity of the primers/probes by spiking nucleic acid from a non-specific related target in extracted matrix to ensure the specificity for the target sequence.	No amplification seen with the non-specific target spiked.
Limit of Quantification (LOQ)	The lowest nonzero standard on the calibration curve defines the sensitivity (LOQ).	The replicates of the LOQ in the calibrator curve should consistently back calculate to a reportable concentration on the curve and give positive result (Ct ≤ 40) in all replicates throughout the validation runs. The LOQ should demonstrate acceptable relative accuracy and precision in all validation runs.
Sensitivity (LOD)	The LOD is the lowest concentration at which 95% of positive samples can be detected in the assay. The LOD is determined experimentally for the assay by preparing a serial dilution of a spiked positive sample and analyzing multiple replicates at each dilution point.	The last dilution where 95% of replicates give a positive and specific amplification is considered the LOD.
Matrix Interference	Samples from at least 10 individual gDNA samples or representative tissues are left unspiked or spiked with target DNA into each extracted sample at 3 times the LOQ level and assessed in the PCR assay.	At least 80% of unspiked or spiked samples should have acceptable precision and accuracy in the assay.
Recovery	At least 3 liquid matrices or lysate samples and buffer control are spiked with target DNA prior to extraction and assessed in the PCR assay.	The recovery range is reported for each tested matrix. Ideally > 50% RE
Stability (in matrix)	For bench-top and freeze-thaw, subject at least three replicates of the LQC and HQC to short term bench top stability conditions (e.g. 6 and 24 hours) or freeze/thaw cycles (e.g. 1, 3 and 5 cycles) and evaluate all stability samples in a single run.	The accuracy and precision at each level should be within QC criteria when not subjected to benchtop and freeze-thaw stability conditions.

Method Development and Optimization

The first step in method development is the selection of a primer/probe set by experimental means. It is recommended to design at least 3 sets of primer/probe per target. A multifactorial design of experiments (DOE) approach may be utilized for this purpose. The experiment, in general, involves a set of standards and at least two levels of QCs (near anticipated LQC and HQC) run with various concentrations of different primers and probes. The correct combination of primer and probe concentration is chosen based on

assay sensitivity (e.g., lowest Cq value for LOQ) and specificity (e.g., NTC control).

The performance of qPCR-based methods is influenced by the following parameters: master mix composition, buffer composition, and qPCR program parameters such as time and temperature for preincubation, initial denaturation, denaturation, annealing/amplification, and cooling. With the availability of highly optimized commercial master mixes, the optimization of different component of master mixes may not be necessary. Multifactorial design of experiments (DOE) approach may be utilized to select optimal master mixes from different commercial sources and for the optimization of qPCR program parameters. All assay parameters should be evaluated in presence of matrix DNA. The matrix DNA should be collected from the same animal species and strain.

Upon selection of primer/probes and their optimal concentrations, a set of standards and QCs should be utilized to evaluate assay performance before validation and includes precision, accuracy, sensitivity, linearity, and dynamic range. The experimental approach for the evaluation of the above parameters are described under the validation section.

Method Validation

The recommendations for validation of qPCR and dPCR assays are listed within this section. In each validation parameter, we include the purpose for conducting these experiments, our recommendations on how to conduct these experiments and general acceptance criteria for each parameter. The information in this section is summarized in Table II.

Specificity

Specificity refers to the qPCR assay detecting the appropriate target sequence rather than other nonspecific targets that could also be present in a sample. Specificity can be done via *in silico* or empirical evaluations of the sequence. This type of specificity analysis is typically done in method development. *In silico* methods include evaluation of the primer sequence in BLAST or a similar program to demonstrate sufficient specificity of the primers for the target. Empirical evaluations include demonstrating specificity with direct experimental evidence such as gel electrophoresis, melting profile, DNA sequencing, amplicon size, or restriction enzyme digestion to demonstrate no evidence of off target binding that would lead to inaccurate reporting.

In validation, analytical specificity is performed by spiking nucleic acid from a non-specific related target in extracted matrix to ensure the primers and probes are specific for the target sequence. The recommended acceptance criteria would be no amplification seen with the non-specific target spiked with results of Cq > LOD. For dPCR, target specificity is performed similarly by spiking non-specific nucleic acid in extracted matrix. Acceptance criteria would be a negative partition result using the non-specific target.

Linearity, Dynamic Range and LOQ

The linear dynamic range of the assay is defined by the linear range established by the low and high limits of quantification. To evaluate the dynamic range of the assay, for quantitative PCR assays, a calibration curve is prepared fresh by spiking linearized plasmid in matrix and assayed at each calibrator level. The calibration curve should contain preferably eight, but at minimum six non-zero calibrators that include the defined ULOQ (upper limit of quantitation) and LLOQ (lower limit of quantitation) of the assay. The dynamic range of the assay is ideally at least 6-8 orders of magnitude and 3-4 log range for cDNA (complementary DNA) or genomic DNA. However, each method should be validated for a dynamic range that is relevant to the application of the assay. The curve should be plotted with a semi log (concentration) linear (response) curve fit. The R^2 value and Cq values for the entire dynamic range are reported in the validation.

It is recommended for acceptance criteria that 75% of calibrators have precision and accuracy within the established acceptance criteria for assay reproducibility as well as an R^2 value of >0.98. In addition to assessing dynamic range of an assay, PCR efficiency can be determined from the slope of the linear regression line according to the equation (Efficiency = (10^(–1/slope)–1) *100). Efficiency is recommended to be between 90–110% and a slope between -3.1 and -3.6 (at 100% efficiency, the slope of the standard curve is -3.32).

For dPCR, the analysis of absolute quantification of copy number in a sample does not require the need for a calibrator curve. Thus, a working range can be verified by measuring different levels of reference PCR copy number concentrations with upper and lower limits.

LOQ is the lowest amount of target that can be quantified with accuracy and reproducibility and is the lowest concentration of the linear dynamic range of the assay, which is included as a calibrator in the standard curve and assessed during the linear dynamic range assessment. The replicates of the LOQ in the calibrator curve should consistently back calculate to a reportable concentration on the curve and should give a positive result in all replicates throughout the validation runs. In addition, the LOQ should demonstrate acceptable relative accuracy and precision in all validation runs.

For dPCR, acceptance criteria would apply in a similar manner, where all replicates of the LOQ are positive with acceptable precision and accuracy.

Sensitivity and LOD

Assay sensitivity is often determined by defining the assay LOD. Currently, there is only one mention of regulatory guidance that dictates that assay sensitivity should be established for gene therapy products at ≤ 50 copies vector genome/µg of gDNA [9] and refers to preclinical biodistribution assays. However, this sensitivity level is used as a basis for most qPCR assays, so it is important to establish sensitivity of the assay based on the assay's fit for purpose requirements.

The LOD is the lowest concentration at which 95% of positive samples can be detected in the assay. The LOD is determined experimentally by preparing a serial dilution of a spiked positive sample and analyzing each dilution point in several replicates over multiple runs. The

last dilution where 95% of the total replicates give a positive and specific amplification is considered the LOD_{95}. The LOD can be determined empirically or with other statistical means or available software (e.g., Probit analysis) [19]. Although LOD is not typically determined for other common bioanalytical technologies like chromatographic or ligand binding assays, it provides utility in qualitative assays, whereas if a sample were to have signal above the LOD and below the LLOQ, the sample result could be reported as "detected" but not quantifiable.

Reproducibility (Accuracy and Precision), Robustness and Ruggedness

Assay reproducibility should be evaluated during method validation. Within run reproducibility may be assessed by evaluating precision and accuracy of independent preparations of five positive control levels that are evenly distributed in logarithmic scale along the standard curve (ULOQ, HQC, MQC, LQC and LLOQ) and assayed in triplicate within a single assay assessment. Between run reproducibility may be assessed by using data from six or more independent validation runs with at least three replicates per positive control level in each run. It is recommended that reproducibility runs be conducted by at least 2 analysts over multiple days using multiple instruments for ruggedness assessment.

For evaluating reproducibility, it is expected that the precision and accuracy of the positive controls meet the established acceptance criteria. Precision can be determined using standard deviation or %CV of output values. The acceptance criteria are typically set based on the observed performance of the assay during method development and should ultimately support the context of use of the assay. For example, in clinical vector shedding studies, qPCR assays extreme precision is often not necessary given that in those studies, patient's release criteria are dependent on their data values to fall below the assay LOQ, and thus making sensitivity important for these assays. In general, acceptance criteria for fit-for-purpose qPCR assays can range from 20%-25% CV. Tighter criteria are used if calculations are based off raw Cq values, for example, qualitative values use ≤ 2% between duplicates and ≤ 5% for inter-assay acceptance.

In addition to positive controls, negative controls should be included in method validation and are usually represented as the NTC, which includes all PCR reaction reagents with the exception of template. In this case, template is often substituted with the same volume of DNase/RNase free water. This is a standard negative control used to identify set-up contamination and demonstrates assay specificity. The NTC control Cq value should not be lower than the Cq value of LOD. In the case that the NTC fails in a reproducibility run, the data is not included in the overall reproducibility calculation.

For dPCR assays, similar procedure is used to set up the experimental analysis of reproducibility by evaluating precision and accuracy of the QCs. Acceptance criteria include clear positive results of the positive controls and negative readout for the NTC wells.

Robustness is evaluated in method development and can be confirmed in method validation as needed. These experiments are performed to demonstrate that changing certain parameters (e.g., critical reagent input, temperatures, and incubation times) in the assay does not affect the overall assay performance, indicating robustness of the method.

Matrix Interference and Recovery

Matrix interference is defined as the ability of the assay to measure the analyte of interest regardless of the presence of interfering components in the sample matrix. To determine assay matrix interference, it is recommended that samples from at least 10 individual gDNA samples are used for assessment. In some cases, obtaining 10 matrices is difficult and thus this assessment can be justifiably performed with a lower number of samples. To assess matrix interference, target DNA is spiked into each extracted sample at 3 times the LOQ level and assessed in the qPCR assay. Unspiked samples or blank samples should also be included in the assessment. Matrix interference is evaluated in a single validation run. Acceptance criteria include at least 80% of unspiked or spiked samples with acceptable precision and accuracy in the assay. It is important to note that depending on the extraction process, there may be PCR inhibitors that affect the matrix interference assessment but may not be inherent to the matrix itself, highlighting the importance of a deeper evaluation during method development.

For recovery, tissue lysate is spiked with target DNA prior to extraction. Pre-spiked samples are run undiluted and % recovery is calculated as a ratio to the spiked controls. (e.g., spike control of 250 copies/µL = (50,000 copies spike/200µL elution). The target acceptance criteria for recovery should be 30 to 100% of the spiked concentration and ideally be >50%.

Stability

Stability is defined as the amount of time that sample integrity is upheld during sample handling**,** transit, and storage prior to being assessed in the assay pre- and post-extraction. Sample stability is performed by assessing the high and low positive controls prepared in assay matrix in the assay to demonstrate sample stability for multiple freeze/thaws and short term or benchtop stability, as needed. Stability should be performed by assessing triplicate aliquots of the positive controls, LQC and HQC after subjection to the stability condition being assessed. The results are compared to a reference QC (LQC and HQC) that has not been subjected to the stability condition. In some instances, if instability is suspected in the assay, stability can be evaluated on a case-by-case basis and can be determined by gel electrophoresis to check degradation of the QC sample as evidence of loss in stability.

Long term stability of nucleic acids is typically not assessed for qPCR assays given the historical evidence for stability of nucleic acids when frozen for greater than one year at -80^0C or -20^0C [20].

Table III. Recommendations and Acceptance Criteria for Sample Analysis

Parameter	In-Study Analysis Recommendations	Acceptance Criteria Recommendations
Calibration Curve/Dynamic Range	At least six, non-zero calibrator levels covering the expected range, including LLOQ per analytical run. The in-study analysis should use the same linear regression model as used in validation.	Non-zero calibrators should be within precision and accuracy determined in validation. 75% and a minimum of six non-zero calibrator levels should meet the above criteria in each run.
Linearity	Calibration curve data is used to assess linearity.	R^2 value of >0.98 of calibration curve. PCR efficiency 90-110%.
Quality Controls	≥ three QC levels (LQC, MQC & HQC) and ≥ two replicates per QC level in each analytical run and ≥ three NTCs Total QCs should be 5% of unknown samples or ≥ six, whichever number is greater. If the analytical runs consist of distinct processing batches, the QC acceptance criteria should be applied for the whole run and for each distinct batch within the runs.	≥ 67% of QCs should be ± 20% of the relative concentration and %CV, and ≥ 50% of QCs per level should be ± 20% of their relative concentration and %CV or within acceptable precision and accuracy as determined in validation. NTCs should pass acceptance criteria or be >LOD.
Incurred Sample Reproducibility (ISR)	Not applicable	

Sample Analysis

The recommendations for sample analysis to determine run acceptance is summarized in Table III.

Quality Controls

Once in production, it is acceptable to use three QC levels to determine run acceptance, typically the high, mid and low QC in duplicate. For run acceptance in sample testing, the 4/6 rule is recommended, where 4 out of 6 QCs should have precision and accuracy within criteria that was determined in validation with at least 50% of QCs within acceptance criteria.

Incurred Sample Reanalysis (ISR)

The current consensus in the industry is to not assess ISR in qPCR and dPCR assay in-study validations due to limited availability of the data to understand suitable criteria on how to conduct this assessment [21]. Additionally, certain limitations may exist for pre-clinical biodistribution assays in small tissues that may not allow for reanalysis [22]. One way to evaluate assay reproducibility over time is reliance on QC trending to be monitored throughout assay validation and sample analysis. Trending and monitoring of QCs provides justification for reproducibility of the assay. Traditional ISR as described in the BMV PK guidance [23] may not be the best approach for understanding sample reproducibility in qPCR assays. Important discussions remain on whether ISR is applicable to qPCR assays and may be defined on a case-by-case basis as related to specific studies.

CONCLUSION

The aim of this manuscript is to provide best-practice, working recommendations and the facilitation of future regulatory guidance as an outcome of the 2021 AAPS workshop on harmonization of qPCR. The authors summarized recommendations for developing and validating qPCR and dPCR assays by evaluating core performance characteristics of bioanalytical methods that are used in support of regulated bioanalysis. Although the current regulatory guidance on bioanalytical method development is specific to chromatographic and ligand binding methods and can be used as a roadmap for other technologies [23], the authors ultimately recommend providing sound scientific evidence that the method is suitable for its COU and establishing criteria that support a fit-for-purpose validation in support of the EBF's viewpoint [24]. There remains a need for continued discussion and collaboration amongst the bioanalytical community and health authorities to refine considerations and best practices for qPCR in regulated bioanalysis.

AUTHOR CONTRIBUTIONS

A.H, R.I, K.M, and D.W contributed equally in writing of this manuscript.

DECLARATIONS

Conflict of Interest Statement The authors are employed by and receive compensation from companies that are involved in development and validation of qPCR methods and are listed on the title page of the manuscript. The authors have no other relevant affiliations or financial involvements with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from

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