White Paper

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Bioanalysis

Recommendations on qPCR/ddPCR assay validation by GCC

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Gene therapy, cell therapy and vaccine research have led to an increased use of qPCR/ddPCR in bioanalytical laboratories. CROs are progressively undertaking the development and validation of qPCR and ddPCR assays. Currently, however, there is limited regulatory guidance for the use of qPCR and a complete lack of any regulatory guidelines for the use of the newer ddPCR to support regulated bioanalysis. Hence, the Global CRO Council in Bioanalysis (GCC) has issued this White Paper to provide; 1) a consensus on the different validation parameters required to support qPCR/ddPCR assays; 2) a harmonized approach to their validation and 3) a consistent development of standard operating procedures (SOPs) for all the bioanalytical laboratories using these techniques.

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Background

The Global CRO Council in Bioanalysis (GCC) is an independent global consortium created in 2010 bringing together CRO leaders to discuss various topics and challenges on scientific and regulatory issues related to bioanalysis while working with many different sponsors, vendors, and regulatory agencies [1]. Since its formation, GCC has held regular meetings and published conference reports to share discussions and opinions [2–10]. White Papers on specific topics of widespread interest in bioanalysis and unified recommendations have also been published and were well received by the global bioanalytical community [11–19].

Introduction

The rapidly rising number of gene therapies, cell therapies and vaccines in development has led to an increased use of quantitative polymerase chain reaction (qPCR) and digital droplet polymerase chain reaction (ddPCR) in bioanalytical laboratories. These unique therapies require diverse bioanalytical support indicated in guidelines published by the EU EMA [20], US FDA [21,22] and Japan PMDA [23]. For example, potential shedding of viral particles produced by viral vector gene therapies into blood, saliva, urine and other excreted matrices should be monitored and/or quantitated to manage the risk of exposure to these infective particles on naive individuals [24]. The environmental risk assessment requires the acquisition of shedding data during early-stage clinical development and is outlined in US FDA Guidance [21,22,25] especially for replication competent vectors. Collection of shedding data from non-pathogenic and replication incompetent vectors such as adeno-associated virus (AAV) is also required. At a minimum, vector shedding should be detected by qPCR/ddPCR making these assays crucial to regulatory approval. Gene therapy treatments also utilize qPCR/ddPCR for quantitative assessments of viral vectors and target gene expression [26]. Moreover, in order to measure low levels of virus in shedding matrices and persistence of cell therapies, more sensitive detections methods such as ddPCR are being employed [27–29].

For cell therapies generated using *ex vivo* genetic medication such as CAR-T cells, the fate (biodistribution and cellular kinetics) as well as the transgene production should be quantitated to establish PK/PD [30,31]. Because of their sensitivity, qPCR, and increasingly ddPCR, assays are the most commonly used methodology due to low quantities of CAR-T cells in long-term studies [29,32].

Vaccine development, including COVID-19 mRNA vaccines, also requires the measurement of gene products [33,34]. Proper validation including the definition of limit of detection (LOD), lower limit of quantitation (LLOQ), and false positive probability are particularly important when using molecular methods for vaccine development.

qPCR is a reproducible, sensitive, and standardizable method to quantitate gene products, and thus is now widely used for bioanalytical support of these therapeutics [29]. More recently, ddPCR has gained popularity for gene and cell therapy applications [30]. ddPCR also uses primers and probes to amplify and quantify target sequences. However, it uses water-oil emulsification to partition single templates into individual droplets to determine the absolute number of target sequences without a standard curve, and increased sensitivity is achievable [30].

Due to the growing number of applications for qPCR/ddPCR in regulated bioanalysis, CROs are increasingly undertaking the development and validation of these qPCR and ddPCR assays. Currently however, there is limited regulatory guidance for the use of qPCR and a complete lack of regulatory guidelines for the use of the newer ddPCR to support regulated bioanalysis. In the absence of a full spectrum of guidelines, laboratories have relied on Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines for qPCR and even for ddPCR [35,36]. Additionally, the continued need for clear recommendations has led to several recent industry/regulators' White Papers [27–29] and industry publications [26,37].

Ongoing discussions between sponsors, regulators and CROs on level of validation (fit for purpose) and acceptance criteria for qPCR/ddPCR assays and the need for harmonization among CROs prompted the issuance of a survey to all the GCC member companies with the aim to assess the current practices used for qPCR and ddPCR in bioanalytical laboratories. The survey received 44 responses, of which 39 respondents use qPCR and ddPCR,

and its results were thoroughly discussed during the 15th GCC Closed Forum on 1 October 2021. These responses were used to determine which existing recommendations are currently applied by CROs, or if other approaches are being used which should be incorporated into a GCC harmonized approach to qPCR/ddPCR assay validation.

The goal of this GCC harmonization White Paper is 1) to provide unified recommendations to all GCC member companies on the validation of qPCR/ddPCR assays in bioanalysis; 2) to facilitate interactions with sponsors and regulators and 3) to develop industry-wide internal standard operating procedures (SOPs) for bioanalytical laboratories.

Table 1 provides a summary of the results of the survey containing questions and answers on the different approaches to qPCR/ddPCR validation, as well as discussion and consensus at the 15th GCC Closed Forum on different validation parameters required to support these assays among CROs.

Discussion

The results of the survey show that most laboratories use qPCR or ddPCR (89%) and the majority of those perform both qPCR and ddPCR assays (71%). These assays are no longer used simply for exploratory purposes as an overwhelming majority (~90%) uses qPCR and/or ddPCR for regulated bioanalysis. In regulated bioanalysis, the primary uses were for gene/cell therapy (cell therapy: 69.23%%, gene therapy: 61.54%, biodistribution: 69.23%, vector copy number: 46.15%, vector shedding: 84.62%). The respondents primarily perform gene expression in a non-regulated environment, but gene/cell therapy assays were also used in the non-regulated environment. Vaccine and Covid-19 related qPCR assays were also performed by approximately 20% of respondents in both regulated and non-regulated environments. Therefore, the survey indicates that recommendations for the application of qPCR/ddPCR assays in regulated environments are urgently needed with a focus on gene/cell therapy bioanalytical assay support.

Respondents were asked several questions about how they perform qPCR method development and validation. There was consensus that primers/probe selection, range of response, specificity, selectivity, and amplification efficiency were important method development assessments. Cycle threshold (Ct) value optimization and Delta Rn with reference genes were performed less frequently.

Biodistribution studies require the analysis of a variety of tissue types, which can create challenges when determining the scope of tissues to be included on a qPCR/ddPCR method development or validation. When respondents were queried about whether they included a representative tissue or a subset of tissues as part of their method development and validation process, 51.85% of respondents included a subset of tissues on both method development and validation, whereas 25.9% of respondents only included one representative tissue for method development and validation. 14% of survey respondents assess a subset of tissues used for sample analysis only in method development and an additional 7% assess one tissue type used for sample analysis only in method development. The remaining respondents perform this assessment only in validation. One respondent noted that they assess extraction efficiency in all matrices but only one tissue type is used in validation. Overall, the majority of the respondents (65.85%) assess a subset of tissues as part of the method development and/or validation process.

There was a near 50/50 split in laboratories assessing the target and reference gene in a single assay versus separate assays. Some respondents clarified that a single assay is used when possible unless there are >2 targets. The GCC survey unanimously demonstrated that positive controls (PC) should be included in each run but there was an even split between using them in duplicate and triplicate wells. Some respondents indicated triplicate PCs are performed at sponsor request or they are assay dependent. One respondent indicated they run two sets of PCs in triplicate.

The majority (>75%) of laboratories perform lot-to-lot bridging of critical reagents. Some laboratories try to use a single lot study design or perform lot-to-lot bridging just for master mix and reference standards. When queried on which reagents are considered critical reagents, an overwhelming consensus (>80%) was reached that these include master mix, primers/probes and calibrator standards (gDNA, synthetic DNA or RNA). Reagent kits for amplification, positive controls (PC) and additional reagents for ddPCR were less frequently considered critical reagents. Finally, a variety of minimal amplification efficiencies above 85% were used ranging from lower 85–90% efficiency (7.41% of respondents) to mid-range 90–100% (48.15% of respondents), and the high-range 100–110% (14.81% of respondents) and other (90–110%, 85–110%): 29.63%.

To determine existing industry practices, respondents were asked to outline what tests and criteria they apply for qPCR/ddPCR validation. Regarding samples that should be used for validations, spike-in samples were used almost unanimously, but currently only approximately half of laboratories use patient samples for validations. The survey demonstrated that there is already a consensus on several validation tests necessary for qPCR. These

Table 1. GCC Survey and 15th GCC Closed Forum Discussions/Consensus on qPCR/ddPCR assays.

Question	Response
Do you perform qPCR/ddPCR assays?	• Yes: 88.64% • No: 11.36%
What PCR technique do you use?	• qPCR only: 28.95% • ddPCR only: 0% • Both qPCR and ddPCR: 71.05%
Do you perform qPCR/ddPCR assays in regulated bioanalysis? (for respondents that do perform qPCR/ddPCR)	• Yes: 89.47% • No: 10.53%
What qPCR/ddPCR assays do you perform in regulated bioanalysis?	• Gene expression: 30.7% • Cell therapy (e.g., CAR-T): 69.23% • Gene therapy (e.g., siRNA): 61.54% • Biodistribution: 69.23% • Vector copy number: 46.15% • Vector shedding: 84.62% • Allele detection (e.g., cancer biomarkers): 11.54% • Other (assays for vaccines and COVID-19, miRNA vaccines, pharmacogenetic): 26.92%
What qPCR/ddPCR assays do you perform in a non-regulated environment?	• Gene expression: 88.89% • Cell therapy (e.g., CAR-T): 59.26% • Gene therapy (e.g., siRNA): 66.67% • Biodistribution: 74.07% • Vector copy number: 51.85% • Vector shedding: 62.96% • Allele detection (e.g., cancer biomarkers): 33.33% • Other (assays for vaccines and COVID-19, miRNA vaccines, pharmacogenetic): 18.52%
What main method development experiments do you perform for qPCR/ddPCR assays?	• Primers/probe selection: 85.71% • Range of response: 96.43% • Specificity and selectivity: 92.86% • Amplification efficiency: 92.86% • Ct optimization: 78.5% • Delta Rn Optimization: 57.1%
Do you assess the method in the tissues you will be collecting for analysis?	• No: 0% • Yes, in method development a subset of tissues: 14.81% • Yes, in method development one representative tissue: 7.41% • Yes, in method development and Validation a subset of tissues: 51.85% • Yes, in method development and Validation one representative tissue: 25.93%
Do you quantify target gene and reference gene in a single assay?	• Yes 46.43% • No 53.57%
Do you use positive controls (PC) for each run?	• No: 0% • Yes, in singlicate well: 10.71% • Yes, in duplicate wells: 46.43% • Yes, in triplicate wells: 42.86%
Do you use lot-to-lot bridging protocols for critical reagents?	• Yes: 78.57% • No: 21.43%
What are you defining as critical reagents?	• Master mix: 82.14% • Primers/probes: 92.86% • Calibrator standards (gDNA, synthetic DNA or RNA): 89.29% • Reagent kits for amplification: 57.14% • Positive controls (PC): 75% • Additional reagents for ddPCR: 14.29% • Other (please specify): 3.57%
Do you use patient samples for validation?	• Yes: 53.57% • No: 46.43%
Do you use spike samples for validation?	• Yes: 96.43% • No: 3.57%
What minimum threshold for qPCR amplification efficiency do you use?	• Lower than 85%: 0% • 85–90%: 7.41% • 90–100%: 48.15% • 100–110%: 14.81% • Other (90–110%, 85–110%): 29.63%

Table 1. GCC Survey and 15th GCC Closed Forum Discussions/Consensus on qPCR/ddPCR assays (cont.).

Question	Response
What validation parameters do you use for qPCR assays?	• Analytical sensitivity: 96.43% • Specificity: 92.86% • Selectivity: 92.86% • Accuracy: 92.86% • Intra-assay precision: 96.43% • Inter-assay precision: 100% • Lower limits of quantitation (LLOQ): 100% • Dilutional linearity: 85.71% • Range of quantitation: 100% • Sample stability (pre-purification): 75% • Sample stability (post-purification): 71.43% • Reproducibility: 85.71% • Robustness: 82.14% • Inhibition assessment: 57.14% • Parallelism: 25% • ISR: 14.29% • Other (ISR only if requested or for regulated studies): 17.86%
What extra validation parameters not listed above do you use for ddPCR assays?	• Do not use ddPCR: 29.63% • No extra validation parameters: 66.67% • No extra validation but manual thresholds set during development: 3.7%
What assay acceptance criteria do you use for qPCR, ddPCR validation?	• Corsaro *et al.* [29]: 10 respondents • Fit-for-purpose: 8 respondents • ±20–25% criteria overall: 6 respondents • US FDA 2015 gene therapy [21] and EU EMA 2009 gene/cell therapy Guidance/Guideline [20]: 4 respondents • Based on assay development and/or client request: 3 respondents • Yang *et al.* [37]: 1 respondent • Stevenson *et al.* [27]: 2 respondents • Rangarajan *et al.* [38]: 2 respondents • Bustin *et al.* [39]: 1 respondent • Ma *et al.* [26]: 1 respondent • Piccoli *et al.* [28]: 1 respondent • FDA BMV [40]: 1 respondent
What assay acceptance criteria do you use for qPCR, ddPCR sample analysis?	• ±20–25% criteria overall: 4 respondents • Corsaro *et al.* [29]: 3 respondents • US FDA 2015 gene therapy [21] and EU EMA 2009 gene/cell therapy Guidance/Guideline [20]: 2 respondents • Ma *et al.* [26]: 2 respondents • Based on validation data or same criteria as validation: 9 respondents • Other fit-for-purpose criteria: 5 respondents

include analytical sensitivity, specificity, selectivity, accuracy, intra-assay precision, inter-assay precision, lower limits of quantitation (LLOQ), and range of quantitation. These are performed nearly 100% of the time. Several of the tests for validation are performed less frequently but still >70% of the time including dilutional linearity, sample stability (pre-purification), sample stability (post-purification), reproducibility, and robustness. Parallelism and inhibition assessment are performed 25% and 57% of the time, respectively. Incurred sample reproducibility (ISR) is rarely performed (<15% of the time). This is likely due to the recent recommendations from Corsaro *et al.* highlighting that "currently, there is no requirement for performing ISR" for qPCR assays [29]. Some respondents stated that they perform ISR only if requested by sponsors or only for regulated studies.

Respondents were asked what acceptance criteria they use for validation of qPCR. Most CRO laboratories are using recommendations from prior White Papers/key publications [26–29,37] and US FDA 2015/EU EMA 2009 gene/cell therapy guidance/guideline. 10 out of 28 respondents are using the recommendations from Corsaro *et al.* [29] probably because it is the most recent Industry/Regulator's consensus on this topic. The recommendations from the above-mentioned papers and regulatory guidance/guideline are summarized in Table 2.

Few other respondents are not aligned with these already published papers and regulatory guidance/guideline, but they use slightly varied criteria such as ±30% of theoretical concentration for accuracy of reference standards and QCs and PCR efficiency of 90–110%. A few respondents provided very detailed custom qPCR assay validation criteria. For precision and accuracy, this included criteria of ≥50% DNA QCs at each level and ≥67% of all DNA QCs with quantity %CV of duplicated wells ≤25% (≤45% for lower and upper limit QCs) and %RE within ±25% (±45% for lower and upper limit QCs). In addition, criteria for reference sample stability was specified to indicate the CV of analytical replicates should be ≤30% (calculated using the experimental quantities) for each

Table 2. Summary of prior recommendations on qPCR assay validation.

	Corsaro *et al.* [29] Piccoli *et al.* [28] Stevenson *et al.* [27]	Yang *et al.* [37]	Ma *et al.* [26] Bustin *et al.* [39] Rangarajan *et al.* [38]	US FDA 2015 and 2020/EU EMA 2009 [20–22]
Replicates per sample	• 3	• 3–6	• Test in duplicate wells	• The DNA samples should be run in triplicate wells 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 qPCR assay sensitivity
Standard calibrators	• Use clinical grade or GMP material if possible • DNA template can be used in development if extraction efficiency demonstrated with encapsulated or cloned DNA • Avoid inhibitory factors • Ct values vs back calculated copy numbers • Perform long term stability	• ≥6 nonzero calibrators, linear curve fit • $R^2 \geq 0.98$ • %CV of Ct values	• $R^2 \geq 0.98$ • Ct %CV <2.0% • Recovery ±25% nominal values • All NTC wells below LOD	• Does not discuss
Primer probe selection and efficiency	• 90–110% • Not lower than 85% • Perform BLAST search and cross reactivity assessment with gDNA in species of intended use to avoid primer dimers • E = [10^(-1/slope)-1) • Efficiency ~100% when slope ~-3.32 • Slope between -3.58 and -3.10 (90–110% efficiency	• $E = [10^{(-1/Slope)}-1]$ • 90–110%	• Optimal primer T_a (gradient PCT 60-65C) • Optimal primer concentration and combination (lowest Ct, absence of dimers) • Primer specificity by melt curve • PCR efficiency determined by standard curve	• Does not discuss
Sensitivity/LOD/LLOQ	• Theoretical LOD is Y intercept of STD curve • ≤50 copies/ug gDNA. 300,000 copies/ug gDNA for reference gene	• Ct <40 and <LLOQ • Positive signal with 95% CI from NTC	• FDA guidance for bio distribution qPCR have a demonstrated LLOQ of ≤50 copies of target per 1 ug gDNA • LOD: lowest standard which gives a positive result (Ct <40) in all replicates through validation runs • LLOQ ≤50 copies/μg gDNA • LOD: lowest amount detected with stated probability • LLOQ: lowest quantified amount with acceptable stated precision and accuracy	• The assay should have a demonstrated limit of quantitation of ≤50 copies/μg genomic DNA, so that assay can detect this limit with 95% confidence
Accuracy and precision	• Perform for both trans gene and reference gene • 3 levels of QCs, plasmid spiked into human gDNA for transgene • CAR-T from normal donors spiked into diseased whole blood • Three replicates per sample (duplicate reactions plus one replicate spiked with internal control)	• LLOC, LQC, MQC, HQC, ULOQ • ≥6 independent runs with 3 replicates/run • Human gDNA isolated from normal human cells such as PBMCs used as external QC • NIBSC human gDNA standard 18/164 [41]	• Precision Ct %CV at each level for STDs and individual and intra-assay %CV for QCs • Accuracy back-calculated % recovery for STDs and individual and intra-assay %CV for QCs	• Does not discuss
Robustness	• Does not discuss	• Lot comparisons of critical reagents • Instrument to instrument • Analyst to analyst	• Does not discuss	• Does not discuss
Critical reagents	• Does not discuss	• Primers/probes • Master mix • Plasmid preparations	• Does not discuss	• Does not discuss

Table 2. Summary of prior recommendations on qPCR assay validation (cont.).

	Corsaro *et al.* [29] Piccoli *et al.* [28] Stevenson *et al.* [27]	Yang *et al.* [37]	Ma *et al.* [26] Bustin *et al.* [39] Rangarajan *et al.* [38]	US FDA 2015 and 2020/EU EMA 2009 [20–22]
Specificity/selectivity	• Gene of interest sequence from other interfering endogenous sequences	• ≥10 gDNA lots isolated from PBMCs of disease indication spiked at LLOQ • Evaluate amplicon size and sequence in isolated gDNA • Confirm amplicon size and sequences	• Required during validation • All naive DNA samples below LOD	• Does not discuss
Stability	• Assess for each matrix and DNA using spike in controls and processing	• QC samples (storage and freeze/thaw) • gDNA and matrices gDNA is isolated from • Critical reagents	• Bench top and storage stability and freeze thaw stability (3 cycles)	• Does not discuss
Incurred sample reanalysis	• Not sufficient data to understand utility and feasibility	• No technical reason to perform ISR or parallelism	• Not feasible in preclinical samples, may be required for clinical samples	• Does not discuss
Linearity	• Does not discuss	• Minimal gDNA needed per PCR reaction	• R^2 and Efficiency of STD curve [26]	• Does not discuss
Factorial optimization	• Examine factors that affect primer probe performance: primer conventions, probe annealing temp, master mix response (Ct, slope, highest fluorescence banding)	• Does not discuss	• Does not discuss	• Does not discuss
Acceptance criteria (validation)	• Some assays can meet LBA criteria (%Bias and %CV at 20%, LLOQ at 25%), define on an assay-by-assay basis • Set criteria before validation based on assay development	• Based on statistical analysis from development	• <25% %Bias and %CV	• Does not discuss
Acceptance criteria (sample analysis)	• Separate criteria not given	• Separate criteria not given	• 2/3 NTC wells below LOD • Efficiency 90–110% • ≥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 ULOQ QC) • ≥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%	• Does not discuss
ddPCR considerations	• Use digital MIQE guidelines [36] as a resource • To set positive/negative threshold: use positive controls as guide and evaluate single from double positives and positives from negatives • LOD is lowest concentrations where 95% of positives are detected • <90% efficiency may be acceptable if FFP and clear positive/negative separation	• Does not discuss	• Does not discuss	• Does not discuss

aliquot and at least two out of three (or 67%) aliquots should be considered acceptable. In addition, the CV of the reference samples should be ≤30% and should be calculated using the experimental quantities. Stability will be considered acceptable if the experimental quantities for at least 67% of the aliquots tested is within ≤30% difference of the overall mean quantity of the reference samples. Beyond these acceptance criteria, one respondent

Table 3. GCC Consensus Recommendations on qPCR assay validation.

Parameter	Recommendations and acceptance criteria
Standard calibrators	• Precision: Ct values • Accuracy: back-calculated copy numbers • ≥6 non-zero calibrators should meet acceptance criteria • Ct %CV ≤2.0% for non-zero standards • Recovery ±25% nominal values • All NTC wells below LOD
Standard curve linearity, primer probe selection and efficiency	• E = [$10^{(-1/Slope)}$-1) • 90–110% • Slope: -3.1 ≤ slope ≤ -3.6 • R^2 ≥0.98
Sensitivity/LOD/LLOQ	• Theoretical LOD is Y intercept of STD curve. At least 95% of samples are positive; i.e., Ct >NTC and Ct <40 • LLOQ ≤50 copies/μg gDNA. 300,000 copies/μg gDNA for reference gene
Accuracy and precision	• Perform for both trans gene and reference gene • 3 levels of QCs, plasmid spiked into human gDNA for transgene. Duplicate reactions plus one replicate spiked with qualified internal control (no need if multiplexing) or multiplexed into each sample
Replicates per sample	• 2 replicates of QC and 3 replicates of NTC
Critical reagents	• Master mix • Primers/probes • Calibrator standards (gDNA, synthetic DNA or RNA) • Reagent kits for amplification • Positive controls (PC)
Specificity/selectivity	• Gene of interest sequence from other similar or relevant interfering endogenous sequences • All NTC wells below LOD
Selectivity	• If possible, at least 10 individual gDNA samples or tissue lysates. Minimum of 3 sample per sex for rare matrices • Tested unspiked and spiked with target DNA • At least 80% of spiked samples should have acceptable accuracy and precision
Stability	• Assess for each matrix and DNA using spike in controls and processing • QC Samples (bench top, storage, and 3 freeze thaw cycles) at least low and high QC levels • %RE of copies relative to freshly prepared QCs
Incurred sample reanalysis	• Not sufficient data to understand utility and feasibility
Factorial optimization	• Examine factors that affect primer probe performance: primer conventions, probe annealing temp, master mix response (Ct, slope, highest fluorescence banding)
Acceptance criteria	• Some assays can meet LBA criteria (%bias and %CV at 20%, LLOQ at 25%), define on an assay-by-assay basis • Set criteria before validation based on assay development. Sample analysis acceptance criteria based on assay validation

also indicated they are waiting for GCC recommendations to harmonize SOPs among other CROs highlighting the utility of the recommendations provided hereafter in this White Paper.

Finally, respondents compared acceptance criteria for validation with acceptance criteria for sample analysis. Most respondents indicated they used acceptance criteria based on the validation results or similar acceptance criteria as used in validation. Other responses included ±20–25% criteria overall, criteria outlined in Corsaro *et al.* [29] or Ma *et al.* [26] and other fit-for-purpose criteria.

For ddPCR assay validation, all CRO respondents who use ddPCR indicated that validation tests performed are overall similar to qPCR, except for some aspects in the experimental and technical design of ddPCR which are fundamentally different from qPCR as highlighted in Table 4 and in line with previously published recommendations [29,30]. This topic was further discussed and clarified during the 15th GCC Closed Forum. However, some differences in experimental and technical design of ddPCR differ from qPCR such as the need to set manual thresholds and the lack of a standard curve requiring the modification of some validation assessments and/or acceptance criteria. Also, these considerations are highlighted in Table 4.

The results of this survey were confirmed and further supported during the 15th GCC Closed Forum. They clearly indicate the urgent need for harmonization among CROs on qPCR/ddPCR assay validation due to the recent increase of gene therapy, cell therapy and vaccines research and development and consequential expanded use of qPCR/ddPCR in a regulated setting.

Table 4. GCC consensus recommendations on ddPCR assay validation.

Parameter	Recommendations and acceptance criteria in agreement with Sugimoto *et al.* [30]
Standard calibrators	• No calibrators during sample analysis. A set of calibrators are needed during validation • Express samples as copies/μg gDNA, copies/μL blood, and/or copies/diploid cells to meet regulatory requirements and accounting for cell expansion and comparison with flow cytometry
Primer probe selection and efficiency	• For cell therapy, duplex ddPCR assay should utilize a single copy gene as the reference gene to normalize the genomic DNA (gDNA) input. • Primer/probe for the reference gene designed to target conserved DNA sequences in the genome (e.g., between mouse and human) for comparison studies (preclinical studies, clinical toxicity and efficacy) • <90% efficiency may be acceptable if fit-for-purpose (FFP) and clear positive/negative separation
Sensitivity/LOD/LLOQ	• LOD: Lowest copy number at which the statistical model predicts the assay can detect 95% of the time and higher than the observed contamination level (mean copy number detected in blank samples or NTC + 3.3-fold standard deviation) • LLOQ: Lowest concentration of an analyte in a sample that can be quantitatively determined with suitable accuracy and precision • To set positive/negative threshold: use positive controls as guide and evaluate single from double positives and positives from negatives
Accuracy and precision	• ±50% bias and ≤80% CV for samples with <50 copies/20 μl ddPCR reaction • ±35% bias and ≤40% CV for samples with ≥50 copies/20 μl ddPCR reaction • Determine nominal value of QCs with the mean of 3 repeat measurements performed in triplicate • QC samples are prepared by spiking plasmid DNA in gDNA • At least two sets of QCs run in triplicate
Robustness	• Intra-laboratory cross validation
Specificity/selectivity	• All NTC wells below LOD
Stability	• Assess for each matrix and DNA using spike in controls and processing • QC Samples (bench top, storage and three freeze thaw cycles)
Incurred sample reanalysis	• Insufficient data to understand utility and feasibility
Linearity	• Calibration curve prepared by the spike-in standard of the linearized plasmid into the gDNA solution
Acceptance criteria (validation)	• >10,000 droplets/well, <2 detectable copies in NTC, ±35% bias and ≤40% CV for the measured gene copy number at each level for high QCs, ±50% bias and ≤80% CV for the measured CAR-T and ±35% bias and ≤40% CV for the measured gene copy number for low QCs
Acceptance criteria (sample analysis)	• As above

Recommendations

Based on the survey results, CROs consensus is driven by prior published recommendations as confirmed at the 15th GCC Closed Forum. Hence, GCC supports the recommendations for qPCR assay validation as presented in Yang *et al.* [37], Ma *et al.* [26], US FDA 2015 and 2020/EU EMA 2009 [20–22] and Corsaro *et al.* [29]. Table 2 summarizes and compares these recommendations. Tables 3 & 4 contain a summary of the GCC consensus recommendations following the survey results and 15th GCC Closed Forum discussions for qPCR and for ddPCR assay validations, respectively.

Conclusion

To harmonize qPCR/ddPCR assays validation, the GCC highly recommends the industry adoption of the parameters and acceptance criteria provided in Tables 3 & 4.

Future perspective

The GCC as a global organization will continue to provide recommendations on hot topics of global interest in bioanalysis. Please contact the GCC [42] for the exact date and time of future meetings, and for all membership information.

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References

1. Premkumar N, Lowes S, Jersey J *et al.* Formation of a Global Contract Research Organization Council for Bioanalysis. *Bioanalysis* 2(11), 1797–1800 (2010).
2. Breda M, Garofolo F, Caturla MC *et al.* Conference Report: The 3rd Global CRO Council for Bioanalysis at the International Reid Bioanalytical Forum. *Bioanalysis* 3(24), 2721–2727 (2011).
3. Lowes S, Jersey J, Shoup R *et al.* Conference Report: 4th Global CRO Council for Bioanalysis: coadministered drugs stability, EMA/US FDA Guidelines, 483s and Carryover. *Bioanalysis* 4(7), 763–768 (2012).
4. Nicholson R, Lowes S, Caturla MC *et al.* Conference Report: 6th GCC focus on LBA: critical reagents, positive controls and reference standards; specificity for endogenous compounds; biomarkers; biosimilars. *Bioanalysis* 4(19), 2335–2342 (2012).
5. Rocci M, Lowes S, Shoup R *et al.* 7th GCC Insights: incurred samples use; fit-for-purpose validation, solution stability, electronic laboratory notebook and hyperlipidemic matrix testing. *Bioanalysis* 6(20), 2713–2720 (2014).
6. Hayes R, LeLacheur R, Dumont I *et al.* 9th GCC closed forum: CAPA in regulated bioanalysis; method robustness, biosimilars, preclinical method validation, endogenous biomarkers, whole blood stability, regulatory audit experiences and electronic laboratory notebooks. *Bioanalysis* 8(6), 487–495 (2016).
7. Islam R, Briscoe C, Bower J *et al.* 11th GCC Closed Forum: cumulative stability; matrix stability; immunogenicity assays; laboratory manuals; biosimilars; chiral methods; hybrid LBA/LCMS assays; fit-for-purpose validation; China Food and Drug Administration bioanalytical method validation. *Bioanalysis* 10(7), 433–444 (2018).
8. Briscoe C, Hughes N, Hayes R *et al.* 12th GCC Closed Forum: critical reagents; oligonucleotides; CoA; method transfer; HRMS; flow cytometry; regulatory findings; stability and immunogenicity. *Bioanalysis* 11(12), 1129–1138 (2019).
9. Bower J, Fast D, Garofolo F *et al.* 8th GCC: Consolidated feedback to US FDA on the 2013 Draft FDA guidance on bioanalytical method validation. *Bioanalysis* 6(22), 2957–2963 (2014).
10. Cape S, Islam R, Nehls C *et al.* The 10th GCC Closed Forum: rejected data, GCP in bioanalysis, extract stability, BAV, processed batch acceptance, matrix stability, critical reagents, ELN and data integrity and counteracting fraud. *Bioanalysis* 9(7), 505–516 (2017).
11. Bower J, Zimmer J, McCown S *et al.* Recommendations for the content and management of Certificates of Analysis for reference standards from the GCC for bioanalysis. *Bioanalysis* 13(8), (2021).
12. Nehls C, Buonarati M, Cape S *et al.* GCC Consolidated Feedback to ICH on the 2019 ICH M10 Bioanalytical Method Validation Draft Guideline. *Bioanalysis* 11(18s), 1–228 (2019).
13. Islam R, Kar S, Ritzén H *et al.* Recommendations for classification of commercial LBA kits for biomarkers in drug development from the GCC for bioanalysis. *Bioanalysis* 11(7), 645–653 (2019).
14. Lowes S, LeLacheur R, Shoup R *et al.* Recommendations on incurred sample stability (ISS) by GCC. *Bioanalysis* 6(18), 2385–2390 (2014).
15. Hougton R, Gouty D, Allinson J *et al.* Recommendations on biomarker bioanalytical method validation by GCC. *Bioanalysis* 4(20), 2439–2446 (2012).
16. Lowes S, Boterman M, Doig M *et al.* Recommendations on bioanalytical method stability implications of co-administered and co-formulated drugs by Global CRO Council for Bioanalysis (GCC). *Bioanalysis* 4(17), 2117–2126 (2012).
17. Boterman M, Doig M, Breda M *et al.* Recommendations on the interpretation of the new European Medicines Agency Guideline on bioanalytical method validation by Global CRO Council for Bioanalysis (GCC). *Bioanalysis* 4(6), 651–660 (2012).
18. Sangster T, Maltas J, Struwe P *et al.* Recommendations on ISR in multi analyte assays, QA/bioanalytical consultants and GCP by Global CRO Council for Bioanalysis (GCC). *Bioanalysis* 4(14), 1723–1730 (2012).
19. Lowes S, Jersey J, Shoup R *et al.* Recommendations on: internal standard criteria, stability, incurred sample reanalysis and recent 483s by the Global CRO Council for Bioanalysis. *Bioanalysis* 3(12), 1323–1332 (2011).
20. European Medicines Agency. EMA General Principles to Address Virus and Vector Shedding (EMEA/CHMP/ICH/449035/2009) (2009). https://www.ema.europa.eu/en/documents/scientific-guideline/international-conference-harmonisation-technical-requirements-registration-pharmaceuticals-human-use_en-10.pdf
21. US FDA Center for biologics evaluation and research. Guidance for industry: design and analysis of shedding studies for virus or bacteria-based gene therapy and oncolytic products (2015). https://www.fda.gov/media/89036/download
22. US FDA Center for biologics evaluation and research. Guidance for industry: long term follow-up after administration of human gene therapy products (2020). https://www.fda.gov/media/113768/download
23. Pharmaceuticals and Medical Devices Agency. PMDA Japan guidance on ensuring the quality and safety of products for gene therapy (2019). https://www.pmda.go.jp/files/000235607.pdf
24. Piccoli S, Mehta D, Vitaliti A *et al.* 2019 White Paper on Recent Issues in Bioanalysis: FDA Immunogenicity Guidance, Gene Therapy, Critical Reagents, Biomarkers and Flow Cytometry Validation (Part 3-Recommendations on 2019 FDA Immunogenicity Guidance, Gene Therapy Bioanalytical Challenges, St. *Bioanalysis* (2019).

25. US FDA Center for biologics evaluation and research. Guidance for Industry: Scientific Considerations in Demonstrating Biosimilarity to a Reference Product (2015). https://www.fda.gov/media/82647/download
26. Ma H, Bell KN, Loker RN. qPCR and qRT-PCR analysis: Regulatory points to consider when conducting biodistribution and vector shedding studies. *Mol. Ther. – Methods Clin. Dev.* 20, 152–168 (2021).
27. Stevenson L, Richards S, Pillutla R *et al.* 2018 White Paper on Recent Issues in Bioanalysis: focus on flow cytometry, gene therapy, cut points and key clarifications on BAV (Part 3 – LBA/cell-based assays: immunogenicity, biomarkers and PK assays). *Bioanalysis* 10(24), 1973–2001 (2018).
28. Piccoli S, Mehta D, Vitaliti A *et al.* 2019 White Paper on Recent Issues in Bioanalysis: FDA Immunogenicity Guidance, Gene Therapy, Critical Reagents, Biomarkers and Flow Cytometry Validation (Part 3 – Recommendations on 2019 FDA Immunogenicity Guidance, Gene Therapy Bioanalytical Challenges, Strategies for Critical Reagent Management, Biomarker Assay Validation, Flow Cytometry Validation & CLSI H62). *Bioanalysis* 2207–2244 (2019).
29. Corsaro B, Yang T, Murphy R *et al.* 2020 White Paper on Recent Issues in Bioanalysis: Vaccine Assay Validation, qPCR Assay Validation, QC for CAR-T Flow Cytometry, NAb Assay Harmonization and ELISpot Validation (Part 3 – Recommendations on Immunogenicity Assay Strategies, NAb Assays, Biosimilars and FDA/EMA Immunogenicity Guidance/Guideline, Gene & Cell Therapy and Vaccine Assays). *Bioanalysis* 13(6), 415–463 (2021).
30. Sugimoto H, Chen S, Minembe JP *et al.* Insights on droplet digital pcr-based cellular kinetics and biodistribution assay support for CAR-T cell therapy. *AAPS J.* 23(2), (2021).
31. European Medicines Agency. Guideline on quality, non-clinical and clinical aspects of medicinal products containing genetically modified cells. https://www.ema.europa.eu/en/documents/scientific-guideline/guideline-quality-non-clinical-clinical-aspects-medicinal-products-containing-genetically-modified_en-0.pdf (2020).
32. Martinez M, Moon EK. CAR T cells for solid tumors: new strategies for finding, infiltrating, and surviving in the tumor microenvironment. *Front. Immunol.* 10 (2019).
33. Roberts CC, Swoyer R, Bryan JT, Taddeo FJ. Comparison of real-time multiplex human papillomavirus (HPV) PCR assays with the linear array HPV genotyping PCR assay and influence of DNA extraction method on HPV detection. *J. Clin. Microbiol.* 49(5), 1899–1906 (2011).
34. Wang Y, Kang H, Liu X, Tong Z. Combination of RT-qPCR testing and clinical features for diagnosis of COVID-19 facilitates management of SARS-CoV-2 outbreak. *J. Med. Virol.* 92(6), 538–539 (2020).
35. Bustin SA, Benes V, Garson JA *et al.* The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. *Clin. Chem.* 55(4), 611–622 (2009).
36. Whale AS, de Spiegelaere W, Trypsteen W *et al.* The digital MIQE guidelines update: minimum information for publication of quantitative digital PCR experiments for 2020. *Clin. Chem.* 66(8), 1012–1029 (2020).
37. Yang TY, Doddareddy R. Considerations in the development and validation of real-time quantitative polymerase chain reaction and its application in regulated bioanalysis to characterize the cellular kinetics of CAR-T products in clinical studies. *Bioanalysis* 13(2), 115–128 (2021).
38. Rangarajan S, Walsh L, Lester W *et al.* AAV5-Factor VIII gene transfer in severe hemophilia A. *N. Engl. J. Med.* 377(26), 2519–2530 (2017).
39. Bustin S, Huggett J. qPCR primer design revisited. *Biomol. Detect. Quantif.* 14, 19–28 (2017).
40. US FDA Center for biologics evaluation and research. Guidance for industry: bioanalytical method validation (2018). https://www.fda.gov/media/70858/download
41. Zhao Y, Stepto H, Schneider CK. Development of the first World Health Organization lentiviral vector standard: toward the production control and standardization of lentivirus-based gene therapy products. *Human Gene Ther. Methods* 28(4), 205–214 (2017).
42. Global CRO Council for Bioanalysis (2021). http://www.global-cro-council.org