qPCR in Regulated Bioanalysis – Current Discussions in the Industry

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On Behalf of the AAPS qPCR Working Group

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qPCR Applications in Regulated Bioanalysis

- TK/PK Studies
- Biodistribution
- Vector shedding, persistence/clearance
- CMC, residual DNA detection
- CG&T
  - transduction efficiency
  - vector copy number
  - insertion site quantification
- Biomarkers
  - Gene expression
- Mutation detection
- Pathogen and infectious detection

Recommendations and Best Practices for qPCR Assays in Regulated Bioanalysis

Harmonization Workshops

Collaborations, sharing experiences, presentations

Context of Use

Different applications, sensitivity, precision, defining what makes sense

Sample Analysis

Data analysis, sample reporting, Duplicate, triplicate, criteria for run acceptance

Industry-Wide Discussions

Recommendations, best practices, regulatory feedback, white papers

Method Development

Designing format, choosing platforms, primer design, COU

Method Validation

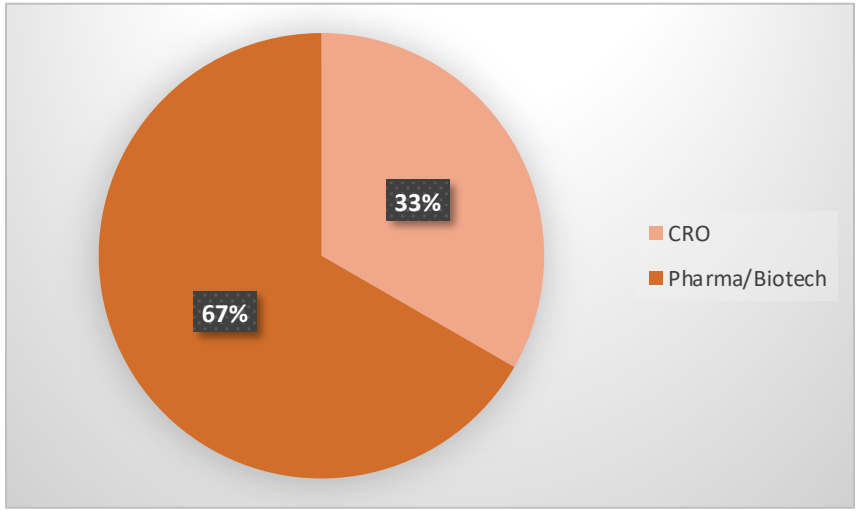
Experimental design, criteria for acceptance, Best practice and practical approach

Critical Reagents

Defining, Bridging, acceptance criteria, normalization, etc.

AAPS qPCR Working Group

- Established December 2021, under auspices of the AAPS Bioanalytical Community
- Mission: To provide an opportunity for scientific exchange and collaboration among scientists for the harmonization in the development, validation, and analysis of qPCR in regulated bioanalysis
- In Discussion Scope: qPCR, RT-qPCR, dPCR
- Sub-Teams
  - Assay Design
  - Critical Reagents
  - Method Development and Validation
  - Sample Analysis



Discussion Topics

- Assay Design and COU
  - Platform selection
  - Clinical *versus* non-clinical
  - Throughput considerations
  - Automation
  - Quantitative *versus* qualitative

- Method Development and Validation
  - Understanding Assay sensitivity
  - LOD *versus* LLOQ
  - Regulatory considerations
  - Acceptable acceptance criteria

- Critical Reagents
  - Lifecycle management
  - Trending and monitoring performance
  - Partial validation *versus* bridging

- Sample reporting and run acceptance
  - Calibration curve acceptance criteria
  - QC acceptance
  - ISR appropriateness
  - Reportable units

How Should I Design my qPCR Assay?

Understanding Context of Use -

The first step in assay design is to define the design specifications of the method that will qualify it for the intended COU

Design Specifications/Checklist

- Selection of target(s) sequence
- Reference gene
- Critical reagents
- Single plex vs. multiplex
- Instrumentation requirements
- Specimen type(s)
- Throughput requirement
- Minimum required performance specifications for assay parameters

How Should I Design my qPCR Assay?

- Platform selection
  - qPCR versus dPCR-
    - What kind of throughput do I need?
    - What kind of dynamic range and sensitivity am I targeting?
    - What level of precision is acceptable for my assay?
      - Variability reduction with absolute quantitation
    - Is there a superior instrument for measuring my nucleic acid?
    - Does my curve need to be in duplicate or triplicate wells?

	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

Hays *et al.* AAPS J (2022)

How do you Determine Sensitivity for qPCR Assays?

- **Long Term Follow-Up After Administration of Human Gene Therapy Products (Jan 2020) Guidance for Industry**

"Specifically, we recommend the polymerase chain reaction (PCR) assay for determining vector persistence in biodistribution studies. Following administration of the product, persistence is indicated by detectable levels of GT product sequences above the threshold level (<50 copies/ug) of the PCR assay, and absence of an apparent downward trend over several timepoints.

The assay should have a demonstrated limit of quantitation of <50 copies/ug so that your assay can detect this limit with 95% confidence."

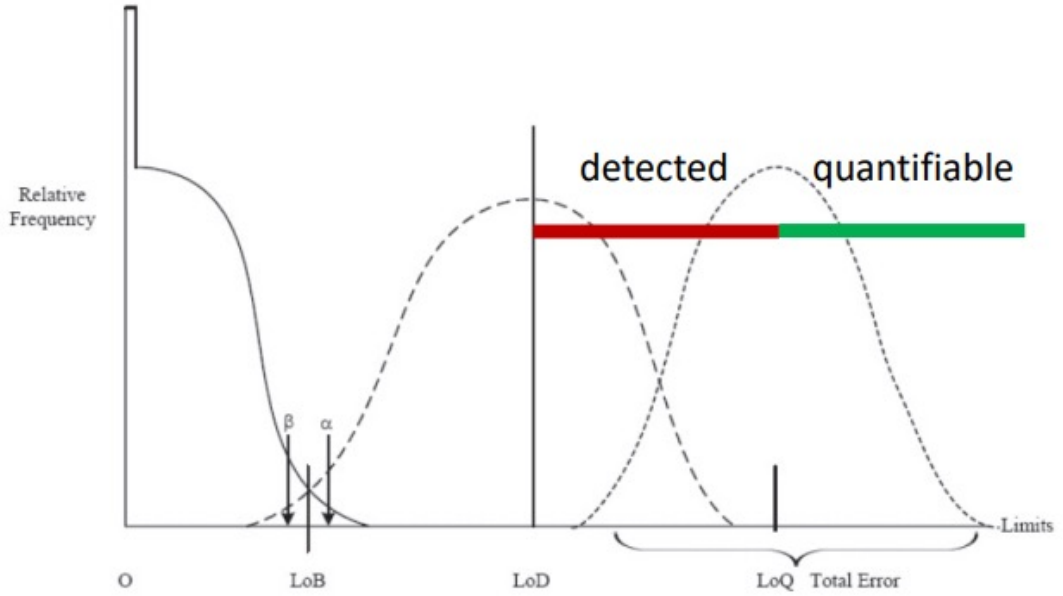
Is this recommendation referencing LLOQ or LOD determination? How do you characterize assay sensitivity in qPCR?

Adapted from Kelly Colletti, AAPS PharmSci 2022 Symposium Presentation

How do you Determine Sensitivity for qPCR Assays?

- **Limit of Blank (LOB)** – not applicable in qPCR
- **Lower Limit of Quantification (LLOQ)** - is the lowest amount of target that can be quantified with accuracy and reproducibility
  - Included as the lowest calibrator on the standard curve
  - Within precision and accuracy acceptance criteria

- **Limit of Detection (LOD)** - is the lowest concentration at which 95% of positive samples can be detected in the assay.
  - Empirical determination of the LOD_{95}
  - Probit analysis



Taken from Kelly Colletti, AAPS PharmSci 2022 Symposium Presentation
Clin Biochem Rev Vol 29 Suppl (i) August 2008 I S49

How do you Determine Sensitivity for qPCR Assays?

- **Why does defining the LOD/LLOQ matter?**

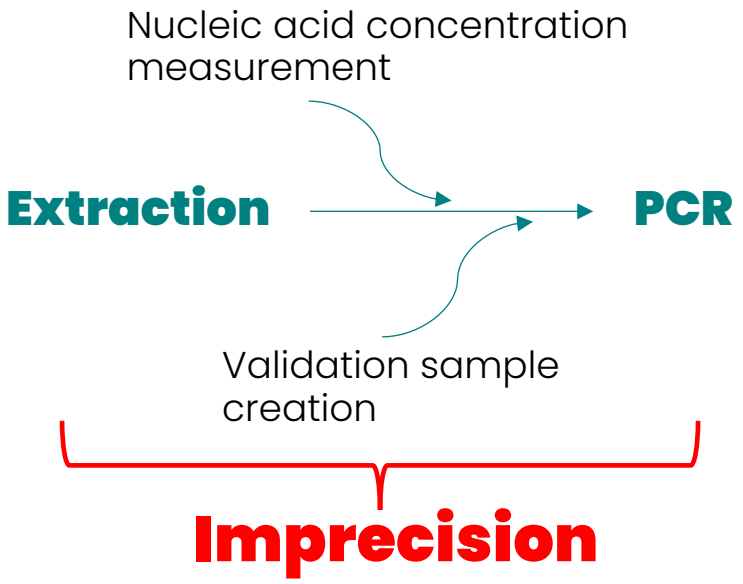
- It depends on the Context of Use
  - Is the objective of your assay to detect levels of a given target without a need for quantifiable results?
  - E.g., viral shedding studies, cell therapy persistence
  - Is it crucial for biodistribution or toxicology studies with high expected doses of target? Off target exposure in tissues?
  - Is it a qualitative assay? LOD becomes important

❖ Lower Limit of Quantification (LLOQ) - is the lowest amount of target that can be **quantified** with accuracy and precision.
❖ What is acceptable precision??

Adapted from Kelly Colletti, AAPS PharmSci 2022 Symposium Presentation

What Acceptance Criteria Should I Set for Precision?

What do we mean by precision?	
Biological replicates- extraction and qPCR for each sample	Mimics sample analysis but contributes to imprecision
Technical replicates- single extraction and multiple qPCR replicates	Replicates are more homogenous Good assessment of qPCR but leaves out extraction imprecision
Coefficient of variation of interpolated units (e.g., copies/mL)	%CVs should not be calculated on Ct/Cq values (Log2 values)



Adapted from Mark Wissel, AAPS PharmSci 2022 Rapid Fire Presentation

What Acceptance Criteria Should I Set for Precision?

- Precision acceptance criteria and COU
  - Precision of the LLOQ important for
    - VCN – cellular kinetics
    - Safety testing
    - Viral load
- Biodistribution may require less precision stringency

- Context of Use is essential in determining acceptable precision for validation replicates and for run QC's

- %CV acceptance of 40+% is not uncommon in qPCR

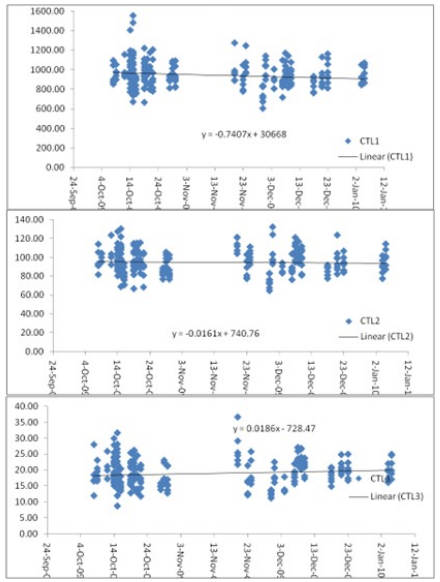
Adapted from Mark Wissel, AAPS PharmSci 2022 Rapid Fire Presentation

What Constitutes a Critical Reagent in qPCR Assays?

- Life Cycle Management
  - Monitor assay performance by trending QCs, Levey-Jennings plots
  - Bridging lots of reagents- partial validation?

- Extraction
  - Extraction method selected must be able to accommodate the matrix/matrices that are to be tested, but also able to reach acceptance criteria for the given assay.

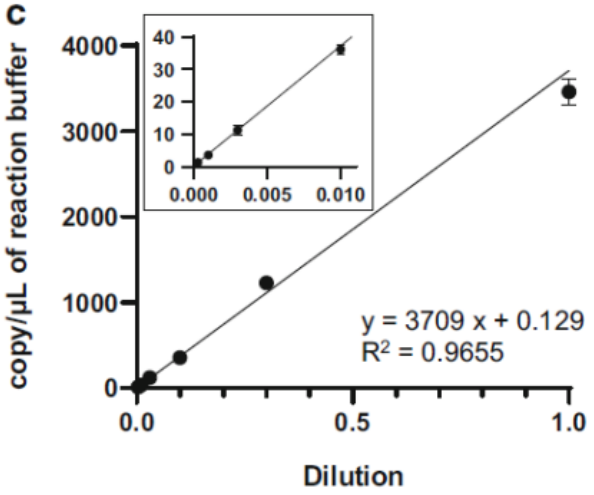
- Reference Material
  - The number of matrices, timepoints and total samples should be considered when choosing spike-ins/surrogates and how to develop your standard curve.
  - Calibrator material
    - Plasmid DNA is acceptable for biodistribution and/or vector copy number
    - Encapsidated material preferred for vector shedding assays
  - No standard/calibrator curve for dPCR/ddPCR



What Criteria Should I Use to Accept a Run and Report my Data?

Calibration Curve Acceptance Criteria

- Preferably 8, a minimum 6 non-zero calibrators
- Ideally 6-8 orders of magnitude and 3-4 log
  - Should be validated for a dynamic range that is relevant to the assay application
- Includes the defined ULOQ and LLOQ of the assay, only if quantitative
- The R^2 value (typically >0.98) and PCR efficiency (90-110%) values are reported
  - Some cases where acceptance criteria are used
  - No acceptance criteria, case-by-case basis depending on variability observed in development/validation, clinical vs nonclinical, monitored and used if investigation of an odd curve
- Masking wells
  - Good practice for masking calibrators or wells (e.g., Dixon test)
    - Triplicate per calibrator level (for ability to exclude single well and for robustness)
    - If quantitative, at least one well represented for LLOQ on the plate

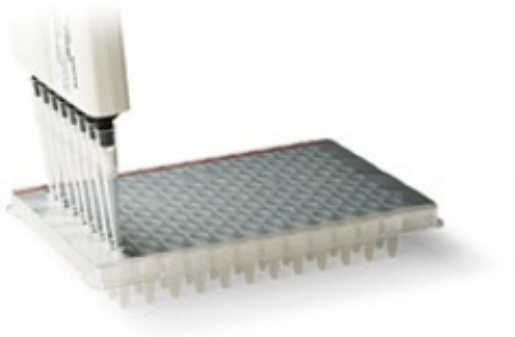


Sugimoto *et al.* AAPS J (2021)

What Criteria Should I Use to Accept a Run and Report my Data?

Quality Controls Acceptance Criteria

- Positive Controls
  - Two sets of High, Mid, Low
    - Acceptance criteria depends on the application and confidence that the assay is robust
      - Ct/Cq just need to fall within the curve
      - 4/6 pass (67%) with precision and accuracy
      - Fall within an established range
- Negative Control
  - NTCs per plate: 3 wells (2/3 not detected)
- Other considerations
  - dPCR merged well analysis acceptance criteria
  - Need to establish a plan for acceptance



Summary and Conclusions

- There are many different applications for qPCR in regulated bioanalysis
- With no regulatory guidelines, experience with qPCR is key to understand the best practices for development and validation of qPCR assays
- It is critical to understand the assay's context of use to ensure a proper fit-for-purpose characterization.
- There is still a need for collaboration and harmonization in among industry scientists to find the best practices for qPCR in regulated bioanalysis

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