EBF

14th EBF Open Symposium

Science – Our Universal Language

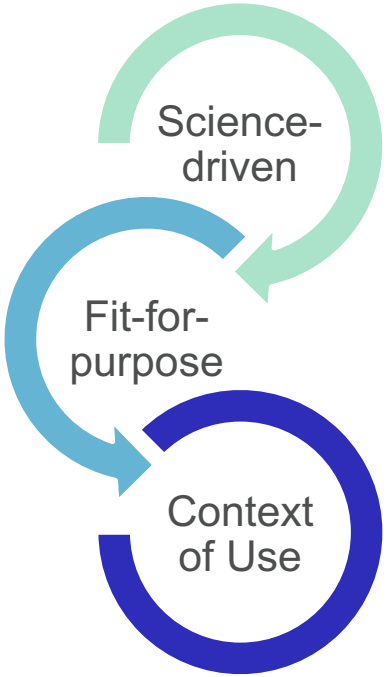
Applying Context of Use to qPCR Method Validation and Analysis: A Recommendation from the European Bioanalysis Forum

Anna Laurén, on behalf of the EBF

24-26 November 2021, Barcelona

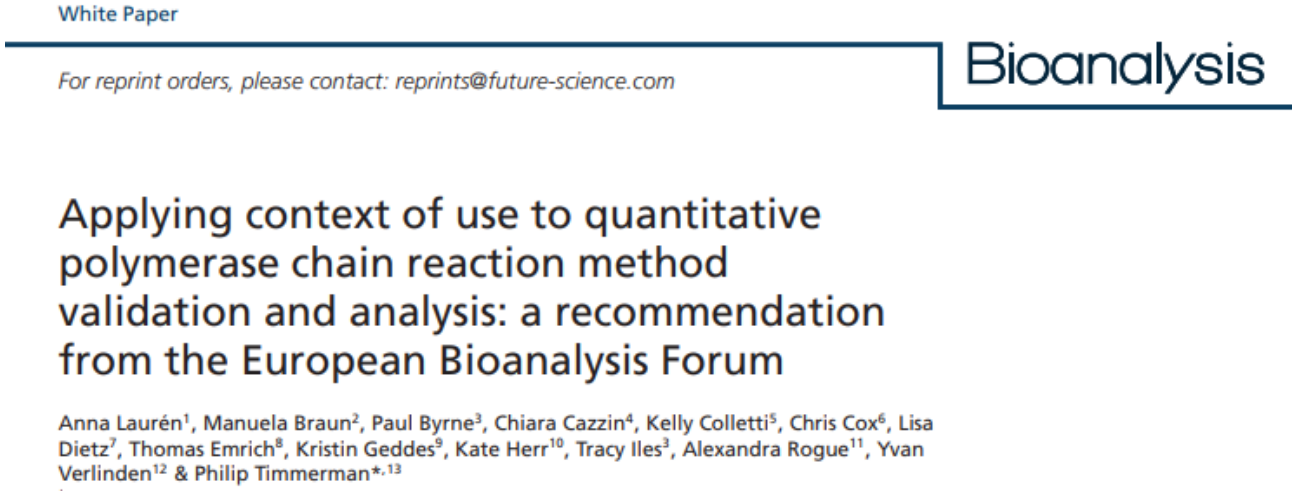
The EBF qPCR team

- Aim is to share considerations on qPCR assay strategies from the EBF member companies
- Vision is to use a Scientific approach for qPCR in regulated studies
- Team members are a mix of experts on regulated BA and qPCR applications
- Both CRO and Pharma
- Formed in 2019



The EBF qPCR team activities

- Several surveys has been performed to harmonize current practices
- Presentations OS 2018, C&GT Training day 2020 and Virtual Workshop in 2021
- Publication in Bioanalysis:
  online: 28 October 2021



White Paper

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Bioanalysis

Applying context of use to quantitative polymerase chain reaction method validation and analysis: a recommendation from the European Bioanalysis Forum

Anna Laurén[1], Manuela Braun[2], Paul Byrne[3], Chiara Cazzin[4], Kelly Colletti[5], Chris Cox[6], Lisa Dietz[7], Thomas Emrich[8], Kristin Geddes[9], Kate Herr[10], Tracy Iles[3], Alexandra Rogue[11], Yvan Verlinden[12] & Philip Timmerman*,[13]

When is qPCR used in the bioanalytical laboratory?

Analyte

Gene Therapy (GT) product

Examples:

- Vector delivery systems AAV, lentivirus
- Naked Plasmids
- Oligonucleotides
- Integrated gene

Cell Therapy (CT) product

Examples:

- Stem cell
- CAR-T cells

Gene expression

May be relevant in all drug modalities

Examples:

- mRNA expression and changes
- Model organism validation (i.e. conditional knock out)
- miRNA

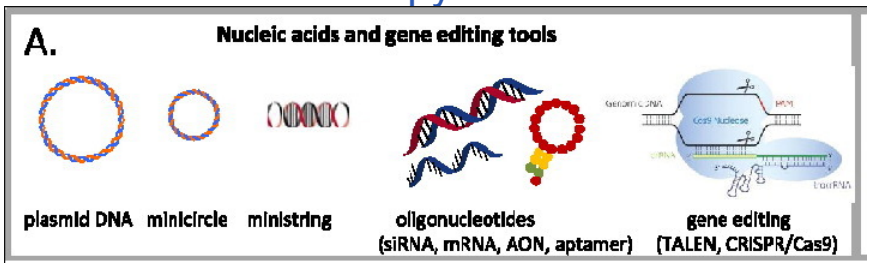
Purpose

- Exposure of drug product
- Biodistribution
- Viral shedding and persistence/clearance
- Effect Biomarker (eg TE/PD)
- Safety Biomarker

Bioanalytical Method Validation (BMV) guidelines focus on LC-MS and LBA – not relevant for PCR

Many examples of qPCR applications

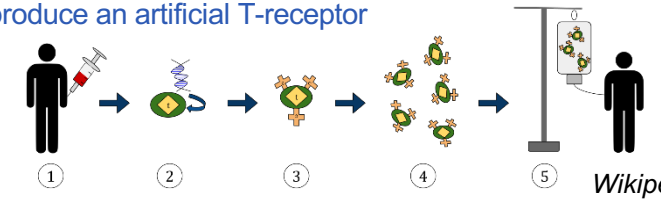
Gene Therapy



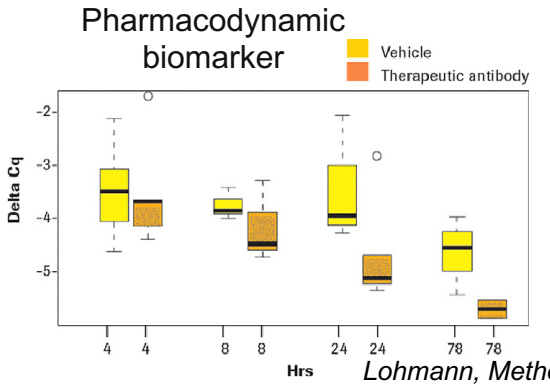
Foldvari, J. Controlled Release, 2016

Delivery systems:
Viral capsids
Non-viral gene therapy

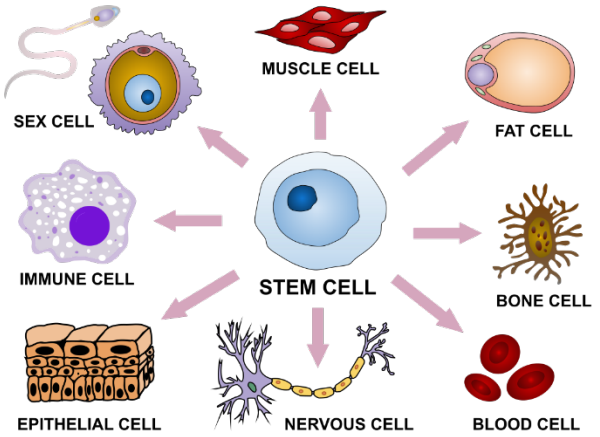
CAR-T: T cells that have been genetically engineered to produce an artificial T-receptor



Wikipedia



Lohmann, Methods, 2013



Wikipedia

Guidelines?

Gene Therapy (GT)

A few GT guidelines present on biodistribution, long-term persistence, plasmid-DNA vaccines, and shedding etc.

Limited recommendation around qPCR assay characterization, validation and sample analysis.

Cell Therapy (CT)

A few CT guidelines present.

None give recommendation around qPCR assay characterization, validation and sample analysis

Gene expression

No guidelines present.

Assay is used as a biomarker assay. qPCR assay characterization, validation and sample analysis based on the intended use of the data.

Current GT guidelines and PCR requirements

US FDA. Guidance for Industry: Long Term Follow-up After Administration of Human Gene Therapy Products (2020)	ICH Considerations: general principles to address virus and vector shedding (2009) US FDA. Guidance for Industry: Design and Analysis of Shedding Studies for Virus or Bacteria-Based Gene Therapy and Oncolytic Products (2015)
Persistence is indicated by detectable levels of GT product sequences above the threshold level (<50 copies/µg genomic DNA) of the PCR assay, and absence of an apparent downward trend over several time points A limit of quantitation (LOQ) of ≤50 copies/µg genomic DNA with 95% confidence for nonclinical biodistribution Samples should at least be run in triplicate for each tissue To aid the interpretation of the qPCR assay results, one replicate of each tissue sample should include a spike of control DNA, including a known amount of the vector sequences	Shedding assays are requested to be qualified and meet minimal performance capabilities. Sensitive, accurate, reproducible, specific and 'fit for purpose'. Recommend to use of an interference control to exclude shedding underestimation due to inhibition of the qPCR reaction Addition of a reference standard or an internal positive control to determine the extraction recovery is recommended

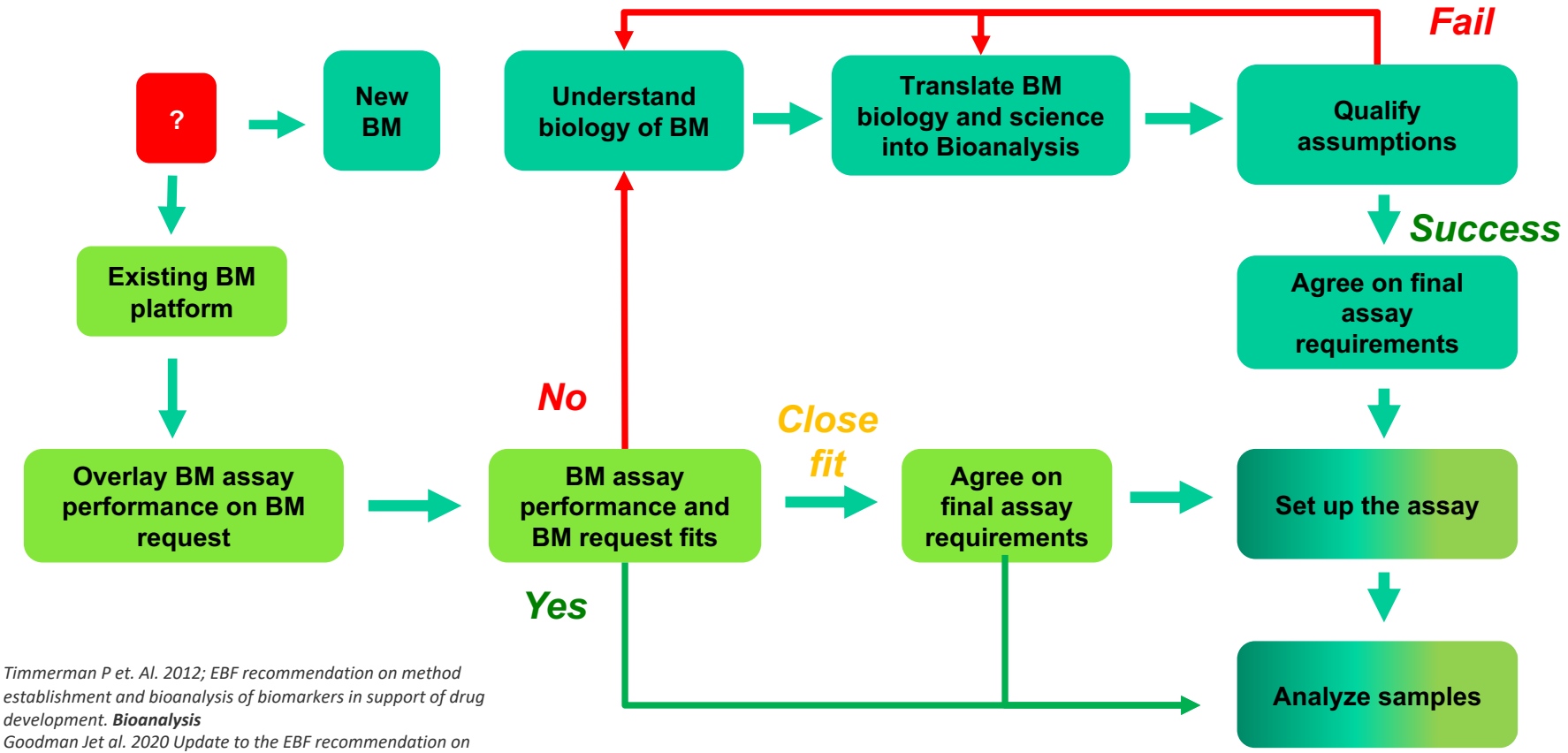
Scope of these guidelines: GT products

Addional GT guidelines PCR requirements

ICH DRAFT Guideline S12 on nonclinical biodistribution considerations for gene therapy products (2021)	US FDA. Guidance for Industry: Considerations for Plasmid DNA Vaccines for Infectious Disease Indications (2007)
Focus on characterization of assay in biofluids and tissue A minimal sensitivity value, is not given in this new draft ICH guideline More later in the session by Johannes Stanta	Include recommendation for a quantification of <100 copies of plasmid per µg of host DNA

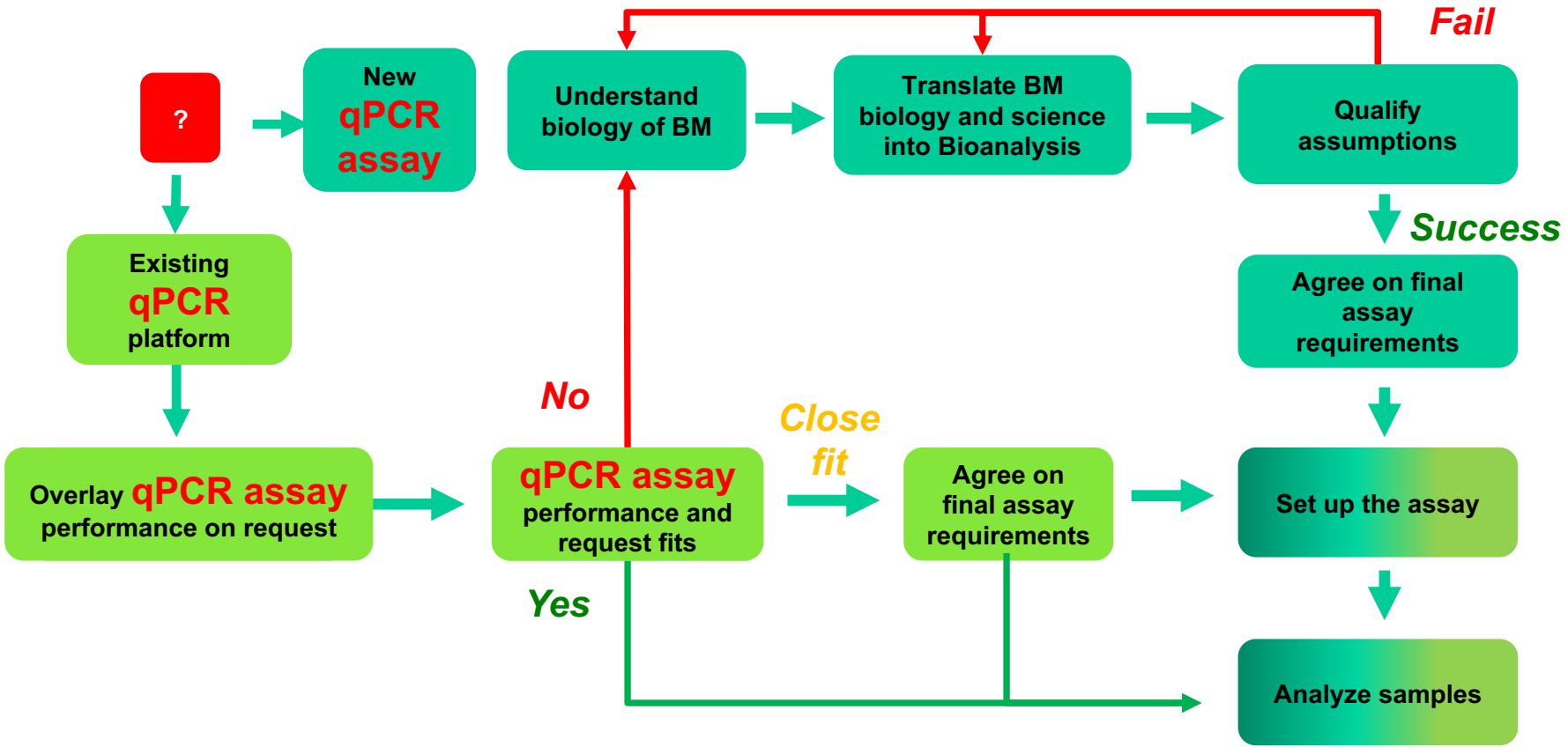
Scope of these guidelines: GT products or Plasmid DNA vaccines

CoU and Biomarkers

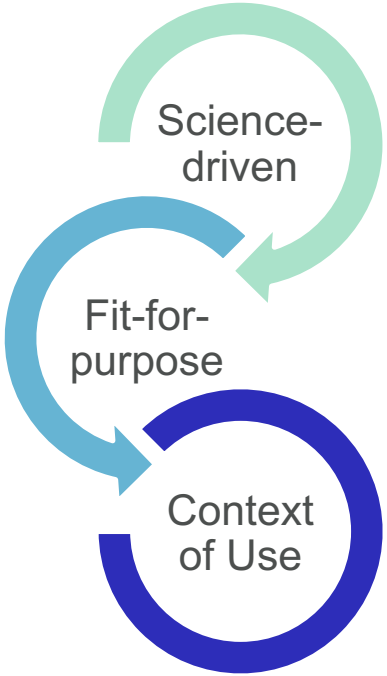


Timmerman P et. Al. 2012; EBF recommendation on method establishment and bioanalysis of biomarkers in support of drug development. ***Bioanalysis***
Goodman Jet al. 2020 Update to the EBF recommendation on biomarkers assays; bringing context of use into practice. ***Bioanalysis***

qPCR considerations should be the same as for Biomarkers



Use a Scientific approach for qPCR assays



- One size will not fit all
- Current C&GT guidelines are written for specific scopes of cell, gene therapy or plasmid DNA vaccine products
- Bioanalytical method validation (BMV) guidelines were written for chromatographic methods (eg LC-MS) and ligand binding assays (LBA) and focus on Drug Product Exposure.
- EBF recommends applying Context-of-Use to each individual qPCR Method Validation and Sample Analysis

SPECIFIC CONSIDERATIONS: GT/CT TISSUE BIODISTRIBUTION/SHEDDING

Context of use

- Proof of exposure
- Biodistribution and persistance = ie as a proof of limited exposure to relevant organs
- Regulated requirement

Analyte and Calibrator Standard

- Drug as reference standard
- Synthetic DNA or RNA standard
- Plasmid DNA
- CoA required
- Virus for recovery assessment (if applicable)

Matrix

- High number of tissues, biofluids
- Risk of qPCR inhibition
- Not always all matrices available for validation
- Different LOD/LOQ in different tissues
- Precision variable between tissue
- Critical aspect is contamination from operator

Reporting

- Absolute quantification
- Normalized values for µg DNA/RNA
- Normalized values for amount processed sample
- Copy number/µg of species gDNA
- Cell copies/ µg of gDNA

SPECIFIC CONSIDERATIONS: GENE EXPRESSION APPLICATION

Context of use	Analyte and Calibrator standard	Matrix	Reporting

• Gene expression e.g.:
  • safety parameter (i.e. tissue specific promoter in biodistribution studies
  • efficacy endpoints
• Genotyping for clinical study enrollment
• Genotyping for animal models
• Gene amplification (oncology)
• Methylation variation
	• Synthetic DNA or RNA standard
	• Usually no reference materials
		• Blood or biofluids
		• Tissue
		• Different expression in different tissues/biofluids
		• Critical aspect purity: and yield of gDNA/RNA/DNA
			• Relative quantification (fold change vs vehicle or control group, delta-delta Ct)
			• Yes or no result
			• Absolute quantification (rare)

PLAN THE SAMPLE ANALYSIS BEFORE VALIDATION

SPECIAL CONSIDERATIONS

qPCR INHIBITION ASSESSMENT

- Regulatory requirement for GT: One of three samples from study sample spiked with STD, spiked sample shall be positive

Long Term Follow-Up After Administration of Human Gene Therapy Products

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Guidance for Industry

Why?

- qPCR inhibition is a well known phenomenon
- Due to risk of inhibiting factors extracted together with RNA/DNA

- In study assessment: Will show that negative result are not false negative due to qPCR inhibition

PLAN THE SAMPLE ANALYSIS BEFORE VALIDATION

EBF RECOMMENDATION

qPCR INHIBITION ASSESSMENT

- Regulatory requirement for GT: One of three samples from study sample spiked with STD, spiked sample shall be positive
- Alternative A: qPCR inhibition assessed during validation with all tissues/biofluids?
- Alternative B: qPCR inhibition assessed during validation with most relevant tissues/biofluids? Rare tissue can follow A)
- Alternative C: assessment of a reference gene or exogenous spike (multiplex reaction)?
- Alternative D: Internal-controlled qPCR assay

- A carefully planned assay characterization will indicate need for In study assessment of assay performance
- Risk for qPCR inhibition may only be apparent in larger subject populations (ie In study)
- Reference gene/Internal control should be considered as part of assay characterization/validation

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EBF RECOMMENDATION

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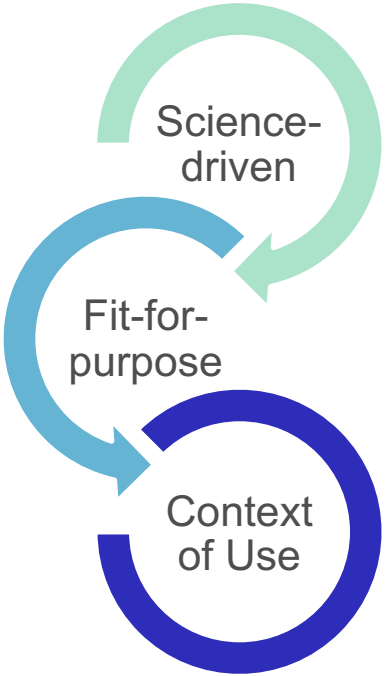
CONTEXT OF USE
CASE BY CASE

ISR/Parallelism and qPCR
EBF RECOMMENDATION

- BMV focus on LC-MS and LBA
- Parallelism: Tissue/DNA/RNA amount and dilutional linearity is part of development/characterisation
- Absolute copy number not a critical aspect
- Not a real quantitative method for certain context of use

ISR not applicable for (RT-)qPCR

qPCR inhibition experiments when relevant will replace ISR/parallelism



METHOD VALIDATION PARAMETERS

FOCUS on the qPCR reaction and include COU!

General experience for acceptance criteria on qPCR reaction from the EBF qPCR team

Calibration Curve

Calibration Standards

- Duplicate/Triplicate
- 6-10 levels
- Linear regression Log Copies vs Ct response
- + & - genomic DNA for dilution effects

✓ Amplification efficiency 90-110%

✓ Dilution Linearity $R^2 \geq 0.98$

Sensitivity

Limit of Detection/Quantification

- LOD/LOQ = lowest detected concentration with ≥ 95% consistency
- LLOQ = lowest concentration with acceptable accuracy and precision (A&P)

Accuracy and Precision

Quality Controls

- 3 sets, 5 levels, duplicate wells
- 6 occasions, 2 analysts

Accuracy:

✓ ± 10% of nominal log copies

✓ ± 25-45% of nominal copies

Precision:

✓ ≤ 3% based on Ct values

✓ ≤ 25-45% based on copies

METHOD VALIDATION PARAMETERS

ADDITIONAL VALIDATION PARAMETERS BASED ON CONTEXT OF USE

Recovery and Matrix Effect

Extraction/recovery efficiency

Clinical:

- All matrices
- Vector spike into fluids

Pre-clinical:

- Selected matrices
- Plasmid spike into tissues
- Tissues frozen or homogenised

Matrix effect (spike after extraction)

Stability

Spiking Experiments

- 1-2 concentration levels
- 3 aliquots of matrix/level
- Long-term storage stability
- Freeze-thaw stability
- Biological matrix and DNA stability
- Complete sample processing workflow

Calculations

Vector copy per:

- µg total DNA/RNA
- mL of fluid
- mg of faeces

Normalise based on:

- Vol. of sample isolated
- Vol. of DNA/RNA eluted
- Vol. of DNA/RNA analysed
- Internal control/Reference gene
- Delta-delta Ct

SUMMARY

- qPCR is used for many different Context of Use
  - Variety of Cell and Gene Therapy aspects
  - Variety of Gene Expression aspects
- Vision of the EBF team is a science driven approach for using qPCR in regulated studies – Context of Use
- The team have focused on aspects where harmonization can be done
- Communicate with stakeholders on Context of Use and pre-existing considerations
- qPCR Context of Use recommendations published
- Aim to publish additional experiences from EBF qPCR team

Acknowledgements

Current members of the team

- Manuela Braun – Bayer
- Paul Byrne – Labcorp Drug Development
- Chiara Cazzin - Aptuit Srl, an Evotec Company
- Kelly Colletti - Charles River Laboratories
- Chris Cox - PsiOxus Therapeutics
- Lisa Dietz - Bayer
- Thomas Emrich - F Hoffmann La Roche
- Kristin Geddes - Merck & Co
- Kate Herr - Janssen BioTherapeutics
- Tracy Iles - Labcorp Drug Development
- Alexandra Rogue - Charles River Laboratories
- Yvan Verlinden - Janssen BioTherapeutics
- Anna Laurén – Novo Nordisk

Previous members of the team

EBF community

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