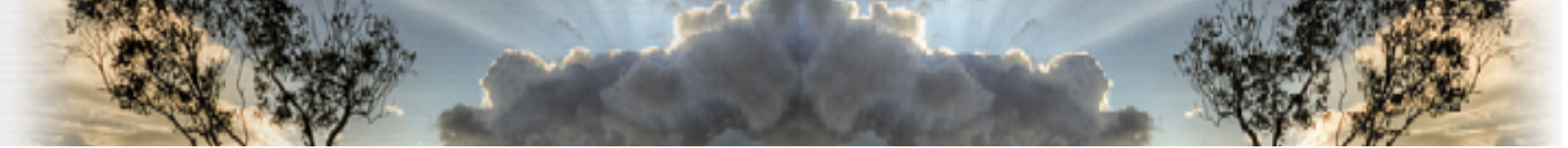


qPCR METHOD DEVELOPMENT & VALIDATION CONSIDERATIONS

Presenter: Milena Blaga
on behalf of the EBF

Cell & Gene Therapy Training Day
17th September 2020

OVERVIEW

1. (RT-)qPCR Applications
2. Technical Background
3. Assay Development Parameters
4. Method Validation Parameters
5. Implementation – Sample Analysis Considerations

(RT-)qPCR Applications

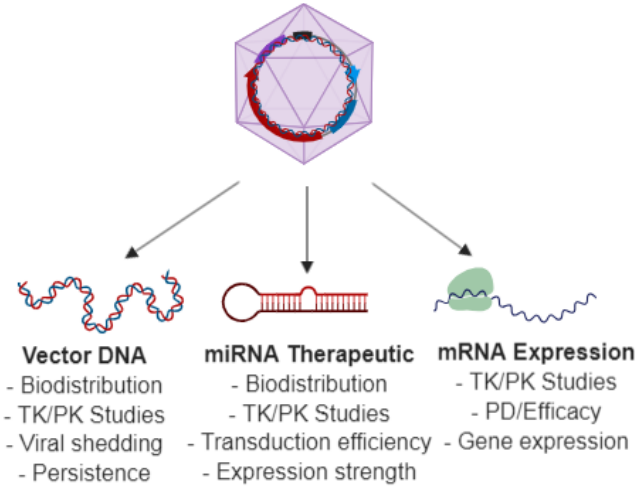
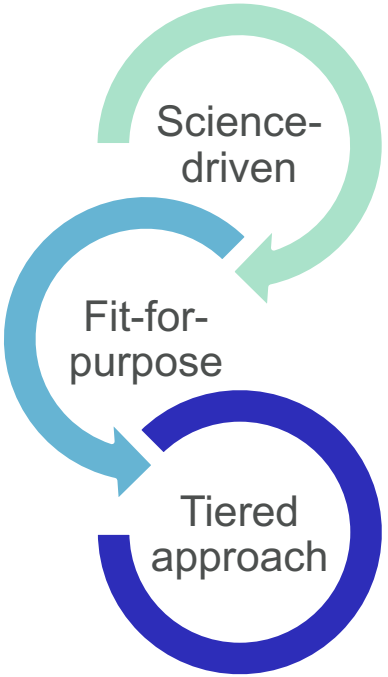


Image created using Bio-Render

- Therapeutic modalities
  - ✓ Gene therapies (viral delivery systems e.g. AAV, lentivirus, etc)
  - ✓ CAR-T Therapeutics
  - ✓ RNA & Oligonucleotide therapeutics
- Biodistribution (non-clinical)
- TK/PK, viral shedding and persistence/clearance
- Efficacy (PD) endpoints
- Biomarkers (gene expression) e.g. immune response, liver damage, gene expression studies
- Model organism validation (e.g. conditional KO)



qPCR Technical Background

qPCR TECHNICAL OVERVIEW

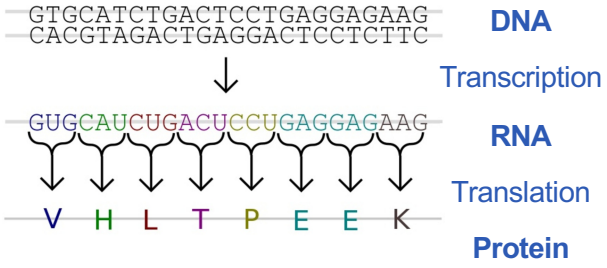
PCR = Polymerase Chain Reaction
Amplification of specific sequences: DNA, mRNA, miRNA, oligos

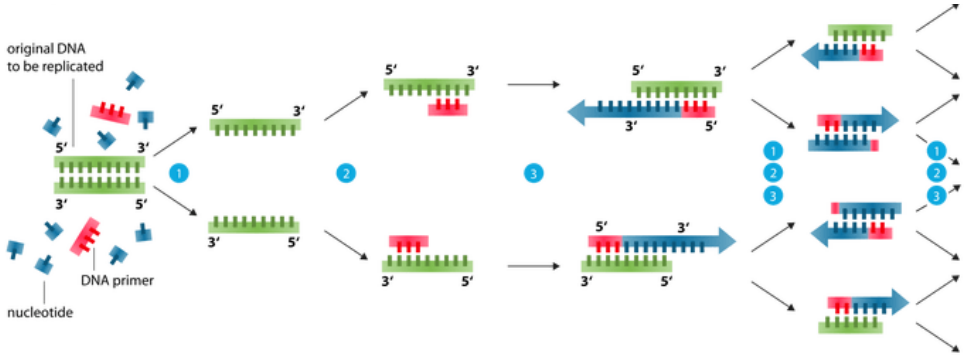
Real-time PCR:

1. Denaturation
2. Annealing
3. Extension
4. Fluorescence read
5. Repeat steps 1-4 x 40 cycles

Components:

- Reverse Transcriptase (RNA targets)
- dNTPs
- DNA Polymerase
- Primers and Probe
- Detection (SYBR, fluorescent dye)





https://microbeonline.com/polymerase-chain-reaction-pcr-steps-types-applications/

Assay Development

METHOD DEVELOPMENT CONSIDERATIONS

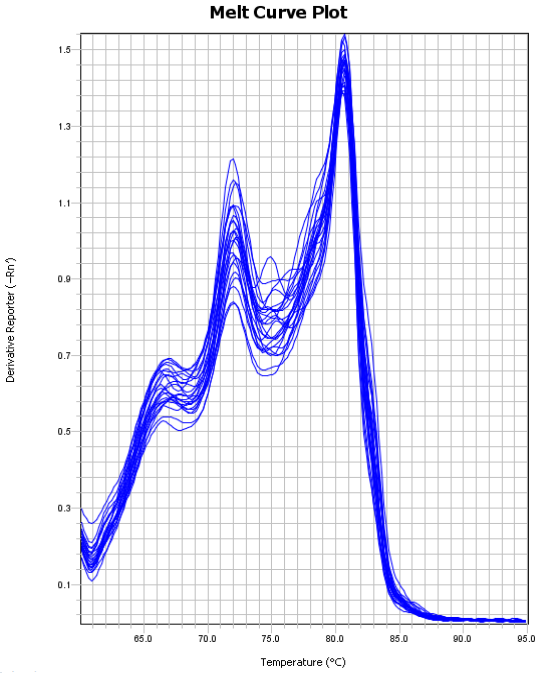
TIER 1 – qPCR ASSAY: PRIMER DESIGN AND SELECTION

Step 1: Design

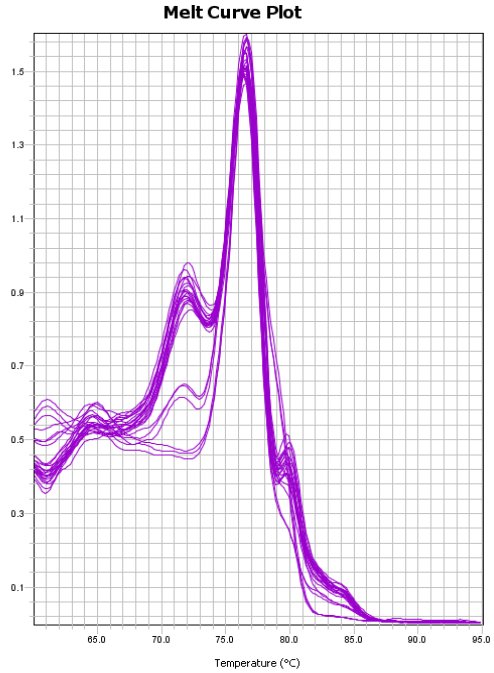
- Primers
- Probe
- Check against all species of interest

Step 2: SYBR Green

- Calibration Standards
- + & - gDNA
- All species + human
- Matrix blank
- Water blank



Set A: 10^8 to 10 copies plasmid in Cyno gDNA



Set B: 10^8 to 10 copies plasmid in Cyno gDNA

METHOD DEVELOPMENT CONSIDERATIONS

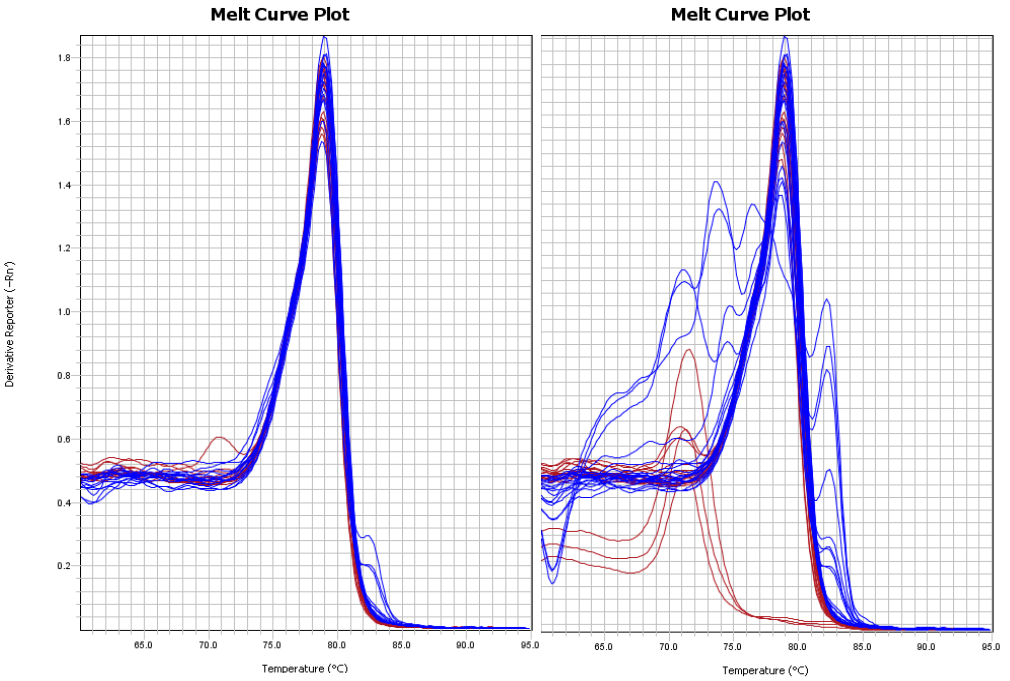
TIER 1 – qPCR ASSAY: PRIMER DESIGN AND SELECTION

Step 3: Selection

- Specificity & Selectivity
  - Secondary products in matrix blanks
  - Primer-dimer in water blank
- Sensitivity
- Amplification efficiency

Step 4: Probe Assay

- Calibration Standards
  - 6-8 orders of magnitude (copy number)
  - Ct ranging from 10-35
- +/- gDNA
- Matrix blank
- Water blank



Set A: 10^8 to 10 copies plasmid in Cyno gDNA

Set B: 10^8 to 10 copies plasmid in Cyno gDNA

Method Validation
Fixed parameters

METHOD VALIDATION PARAMETERS

TIER 2 – FIXED VALIDATION PARAMETERS

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%

✓ $R^2 \geq 0.990$

	1	2	3	4	5	6	7	8	9	10	11	12
A	10^8 0 gDNA	10^8 0 gDNA	10^8 0 gDNA	Empty	10^8 0.5 µg	10^8 0.5 µg	10^8 0.5 µg	Empty	10^8 1 µg	10^8 1 µg	10^8 1 µg	Water Blank
B	10^7 0 gDNA	10^7 0 gDNA	10^7 0 gDNA	Empty	10^7 0.5 µg	10^7 0.5 µg	10^7 0.5 µg	Empty	10^7 1 µg	10^7 1 µg	10^7 1 µg	Water Blank
C	10^5 0 gDNA	10^5 0 gDNA	10^5 0 gDNA	Empty	10^5 0.5 µg	10^5 0.5 µg	10^5 0.5 µg	Empty	10^5 1 µg	10^5 1 µg	10^5 1 µg	Empty
D	10^4 0 gDNA	10^4 0 gDNA	10^4 0 gDNA	Empty	10^4 0.5 µg	10^4 0.5 µg	10^4 0.5 µg	Empty	10^4 1 µg	10^4 1 µg	10^4 1 µg	Matrix Blank 0.5 µg
E	10^3 0 gDNA	10^3 0 gDNA	10^3 0 gDNA	Empty	10^3 0.5 µg	10^3 0.5 µg	10^3 0.5 µg	Empty	10^3 1 µg	10^3 1 µg	10^3 1 µg	Matrix Blank 0.5 µg
F	10^2 0 gDNA	10^2 0 gDNA	10^2 0 gDNA	Empty	10^2 0.5 µg	10^2 0.5 µg	10^2 0.5 µg	Empty	10^2 1 µg	10^2 1 µg	10^2 1 µg	Empty
G	50 0 gDNA	50 0 gDNA	50 0 gDNA	Empty	50 0.5 µg	50 0.5 µg	50 0.5 µg	Empty	50 1 µg	50 1 µg	50 1 µg	Matrix Blank 1 µg
H	10 0 gDNA	10 0 gDNA	10 0 gDNA	Empty	10 0.5 µg	10 0.5 µg	10 0.5 µg	Empty	10 1 µg	10 1 µg	10 1 µg	Matrix Blank 1 µg

METHOD VALIDATION PARAMETERS

TIER 2 – FIXED VALIDATION PARAMETERS

Sensitivity

Limit of Detection/Quantification

- ≥ 20 replicates
- 3 levels
- + gDNA

✓ LOD = lowest concentration with ≥ 95% positive wells

✓ LLOQ = lowest concentration with acceptable accuracy and precision (A&P)

	1	2	3	4	5	6	7	8	9	10	11	12
A	10^8	10^7	10^6	10^5	10^4	10^3	10^2	50	25	10	Matrix Blank	Water Blank
B	10^8	10^7	10^6	10^5	10^4	10^3	10^2	50	25	10	Matrix Blank	Water Blank
C	10^8	10^7	10^6	10^5	10^4	10^3	10^2	50	25	10	Empty	Empty
D	50 QC1	50 QC1	50 QC6	50 QC6	25 QC1	25 QC1	25 QC6	25 QC6	10 QC1	10 QC1	10 QC6	10 QC6
E	50 QC2	50 QC2	50 QC7	50 QC7	25 QC2	25 QC2	25 QC7	25 QC7	10 QC2	10 QC2	10 QC7	10 QC7
F	50 QC3	50 QC3	50 QC8	50 QC8	25 QC3	25 QC3	25 QC8	25 QC8	10 QC3	10 QC3	10 QC8	10 QC8
G	50 QC4	50 QC4	50 QC9	50 QC9	25 QC4	25 QC4	25 QC9	25 QC9	10 QC4	10 QC4	10 QC9	10 QC9
H	50 QC5	50 QC5	50 QC10	50 QC10	25 QC5	25 QC5	25 QC10	25 QC10	10 QC5	10 QC5	10 QC10	10 QC10

METHOD VALIDATION PARAMETERS

TIER 2 – FIXED VALIDATION PARAMETERS

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

	1	2	3	4	5	6	7	8	9	10	11	12
A	10^8	10^7	10^6	10^5	10^4	10^3	10^2	50	25	10	Matrix Blank	Water Blank
B	10^8	10^7	10^6	10^5	10^4	10^3	10^2	50	25	10	Matrix Blank	Water Blank
C	10^8	10^7	10^6	10^5	10^4	10^3	10^2	50	25	10	Empty	Empty
D	10^8 ULOQ1	10^7 HQC1	10^4 MQC1	50 LQC1	25 LLOQ1	Empty	Empty	Empty	Empty	Empty	Empty	Empty
E	10^8 ULOQ1	10^7 HQC1	10^4 MQC1	50 LQC1	25 LLOQ1	Empty	Empty	10^8 ULOQ2	10^7 HQC2	10^4 MQC2	50 LQC2	25 LLOQ2
F	Empty	Empty	Empty	Empty	Empty	Empty	Empty	10^8 ULOQ2	10^7 HQC2	10^4 MQC2	50 LQC2	25 LLOQ2
G	Empty	Empty	10^8 ULOQ3	10^7 HQC3	10^4 MQC3	50 LQC3	25 LLOQ3	Empty	Empty	Empty	Empty	Empty
H	Empty	Empty	10^8 ULOQ3	10^7 HQC3	10^4 MQC3	50 LQC3	25 LLOQ3	Empty	Empty	Empty	Empty	Empty

METHOD VALIDATION PARAMETERS

TIER 2 – FIXED VALIDATION PARAMETERS

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CHALLENGES AND CONSIDERATIONS

qPCR ASSAY

Challenges:	Considerations:
▪ Plasmid stability (and sublimation due to storage) at low concentrations	▪ Testing stability if using stored Cal Std and QCs Single-use working stock aliquots with Cal Std and QCs freshly prepared
▪ Dilution buffer e.g. nuclease-free water, TE buffer, total genomic DNA. Does genomic DNA source matter?	▪ Pooled genomic DNA from various blank matrices for QC and Matrix Blanks. Determine matrix effects/interference.
▪ **Accuracy:** The linear response is in log copies vs Ct. Copy number is on a logarithmic scale	▪ **Accuracy:** For log copies – tighten acceptance criteria. For copy number – relax acceptance criteria. Address low copy number issues.
▪ **Precision:** Ct signal may be affected by technical inhibitory effects and should not be used to calculate precision	▪ **Precision:** Ct signal provides a useful measure of the inter-assay variation. Adjust acceptance criteria to detect outliers.

Method Validation
Adjustable parameters

METHOD VALIDATION PARAMETERS

TIER 3 – ADJUSTABLE VALIDATION PARAMETERS

Recovery

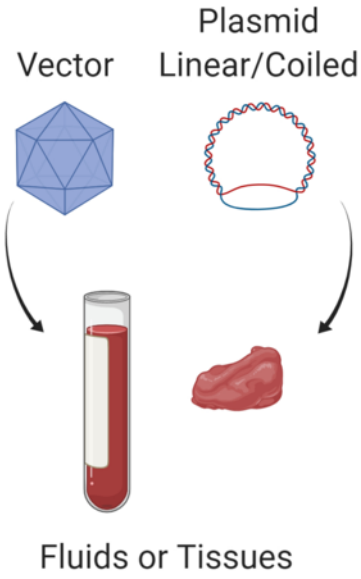
Clinical:

- All matrices
- Vector spike into fluids
- Collection tubes +/- stabilisation buffer

Pre-clinical:

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

Extraction/recovery efficiency



Challenges:

- Cannot mimic vector transfection into target matrix cells
- Control tissues difficult to obtain (e.g. NHP)
- Acceptance criteria?
- Degradation may occur immediately upon spiking

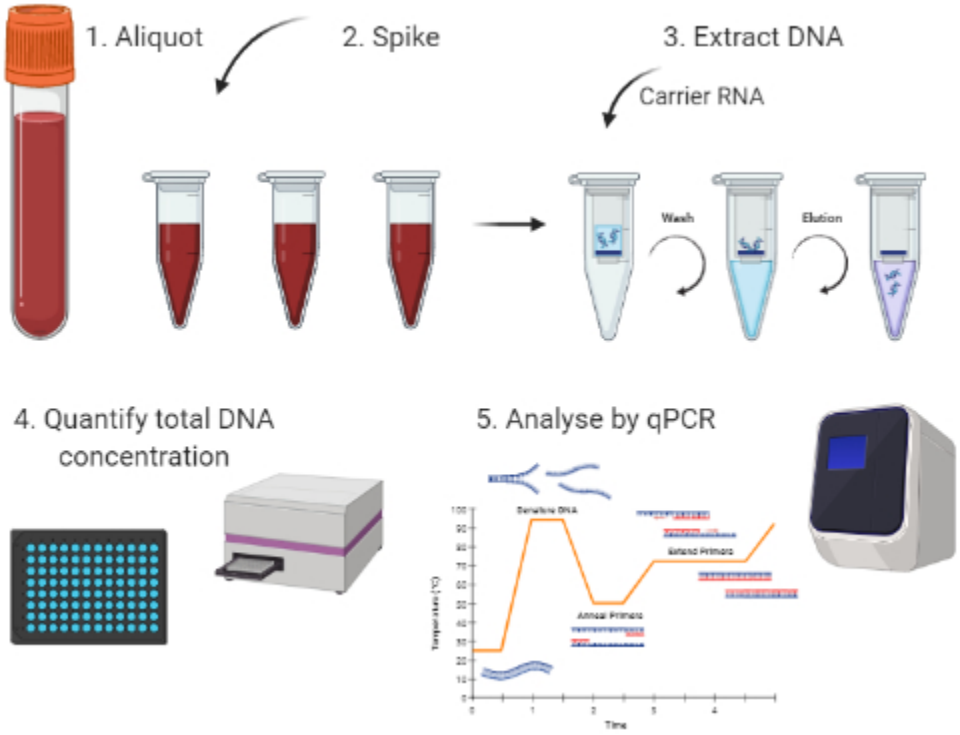
METHOD VALIDATION PARAMETERS

TIER 3 – ADJUSTABLE VALIDATION PARAMETERS

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



METHOD VALIDATION PARAMETERS

TIER 3 – ADJUSTABLE VALIDATION PARAMETERS

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- Freeze-thaw stability
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- Complete sample processing workflow

Calculations

Vector copy per:

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

Normalise based on:

- Vol. of sample isolated
- Vol. of DNA eluted
- Vol. of DNA analysed

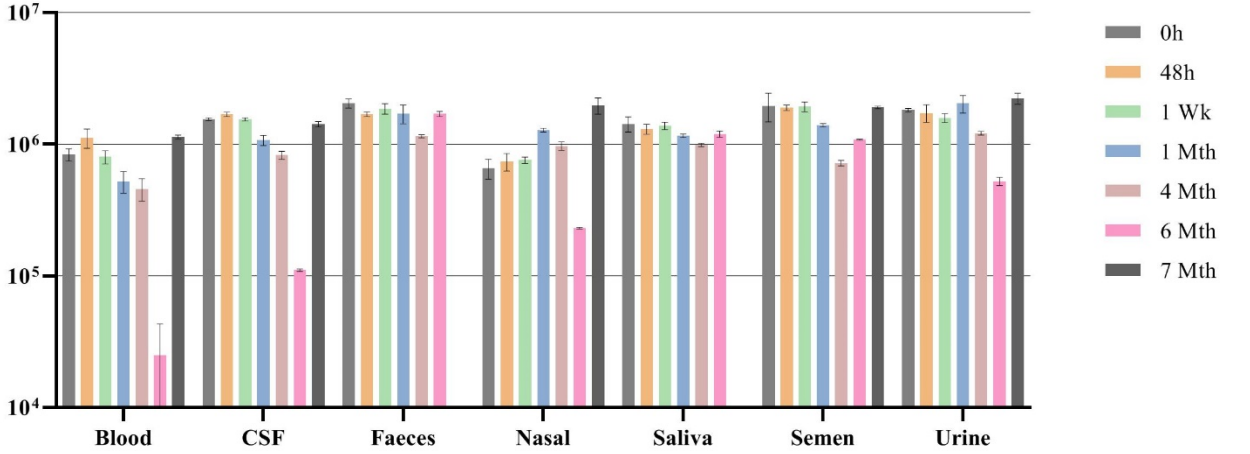
CHALLENGES AND CONSIDERATIONS

STABILITY ASSESSMENTS

- **Challenges:**
  - Variability between occasions introduced by extractions How to set acceptance criteria?
  - For trends, potentially large number of samples required
- e.g. For 8 matrices x 2 levels x 3 aliquots x 5 timepoints = 240

Considerations:

- Assess trends rather than absolute recoveries at each timepoint
- Test a limited no. of matrices if similar



Blood, CSF, Nasal & Urine – isolated using the same method on each occasion

Note:

- 6 months low recovery
- 7 months normal

Sample Analysis Considerations

SAMPLE ANALYSIS

NUCLEIC ACID QUANTIFICATION ASSESSMENT

- **Fluorscence-based methods**
  - Using a Lambda DNA or Ribosomal RNA calibration standard
  - DNA sample used between 1-7 µL
  - Specific to DNA or RNA
  - Reliable, robust, low inter-assay variability
  - However does it require a validation?

- **UV Spectrophotometer (e.g. Nanodrop)**
  - A260/280 ratio
  - Fast, no calibration standards
  - Low sample volume used 1-2 µL
  - Non-specific to DNA/RNA
  - May result in higher variability during analysis

Nominal Conc. (ng/mL)	2	10	20	100	200	2000
n	33	33	32	33	33	33
Mean Conc.	1.99	10.22	20.01	99.13	199.56	1985.02
CV (%) Conc.	1.4	6.3	3.5	2.9	3.8	4.6
Bias (%) Conc.	-0.4	2.2	0.0	-0.9	-0.2	-0.7
Mean Signal	0.17	0.84	1.64	8.04	16.15	160.47
CV (%) Signal	34.4	10.4	8.9	7.8	7.6	6.7

SAMPLE ANALYSIS

qPCR INHIBITION ASSESSMENT

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

Guidance for Industry

Approach 1:

- Spike samples with plasmid standard
- Measure spiked vs unspiked sample
- Recommended in FDA document

Challenges:

- High plasmid concentrations in samples may not show inhibitory effects

Approach 2:

- Use exogenous spike for samples vs control spike

Challenges:

- Does not detect target-specific inhibitory effects
- Critical mostly in confirming false negatives

Considerations:

1. Diluting samples at high concentrations

- will also dilute inhibitors?
- replicate wells for both spiked and unspiked samples?

2. qPCR inhibitors are rarely (never) target-specific

- Multiplex exogenous spike and target => reduce technical variability, time and costs

FUTURE OUTLOOK

BIOANALYTICAL METHOD VALIDATION

- There are multiple approaches for evaluating the same parameter
- No regulatory guidelines for qPCR-based assay validations
  - Is there a need for specific qPCR assay validation guideline?
  - We need to consider the risk of current industry practices (= copy/past BA assay criteria from BMV into qPCR assays) *vs.* creating a (non-science based and premature) specific qPCR guideline which may not include all scientific challenges and nuances required.
  - Requires continued open discussion including all stakeholders rather than immediate action.
  - Learn from Biomarker discussion and confusion created by FDA 2018
  - EBF ready to join the discussion and share experience

- Until we see clear, build on best practice:
  - Understand context of use
  - Adapt LBA guidance where possible
  - Adapt acceptance criteria to reflect assay features and decisions taken
  - Account for specific challenges
    - e.g. spiked vectors will not be incorporate into cells => stability assessments?

Acknowledgments

- Philip Timmerman and SC
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