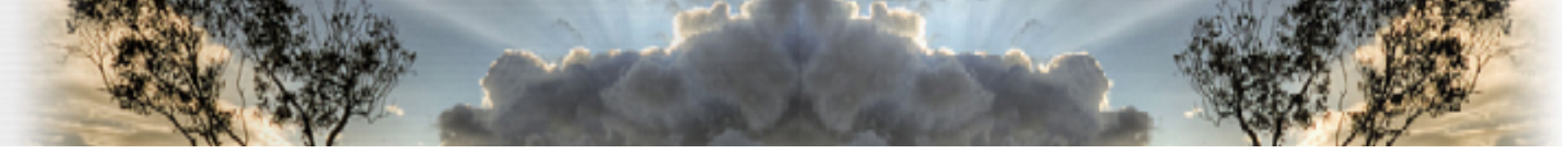


Workshop on qPCR in Regulated Bioanalysis

12th EBF Open Symposium
Imagine! A new bioanalytical Earthrise

http://www.e-b-f..eu

Workshop Agenda

- Update from the EBF qPCR team
  - Milena Blaga, on behalf of EBF
    - Introduction to qPCR & applications
    - qPCR team survey results

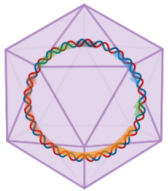
- Validation expectations for shedding and biodistribution studies
    - qPCR assay validation parameters
    - Recovery and stability
    - Nucleic acid quantitation
    - Inhibition assessments

- Future directions

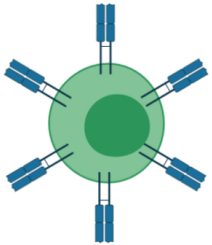
(RT-)qPCR Applications

- Pre-clinical and clinical studies
- PK and PD endpoints
- Biodistribution and shedding assessments
- (Trans)Gene expression, distribution or knockdown
- CMC packages: infectivity, replication competence, potency assays

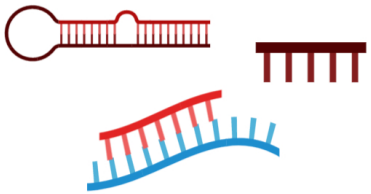
Gene Therapy



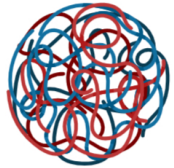
Cell Therapy



Oligonucleotide Therapeutics



Nanoparticle Therapeutics



Disease Models + Biomarkers

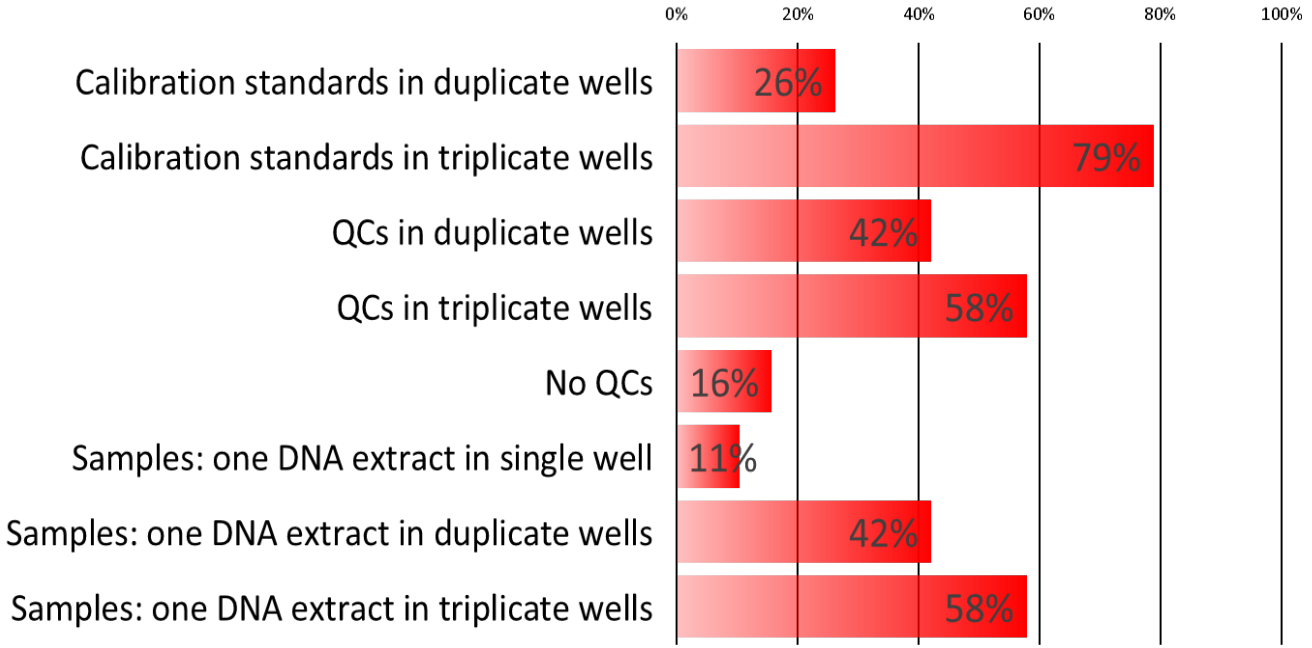
2019 Survey – Method Validation Parameters 1

Parameter	Cell Therapy	Gene Therapy	Oncolytic Virus	Gene expression
Calibration standards dynamic range	100%	100%	100%	75%
Amplification efficiency	100%	100%	100%	100%
Assay accuracy and precision	100%	100%	100%	75%
LOD in addition to LLOQ	80%	86%	100%	50%
Species-specific total DNA/RNA interference with linearity	60%	57%	100%	50%
Sample type-specific DNA/RNA interference with linearity	60%	43%	50%	25%
Recovery efficiency from spiked fluid/tissue	100%	100%	100%	0%
Recovery efficiency from spiked total DNA/RNA	80%	71%	75%	0%

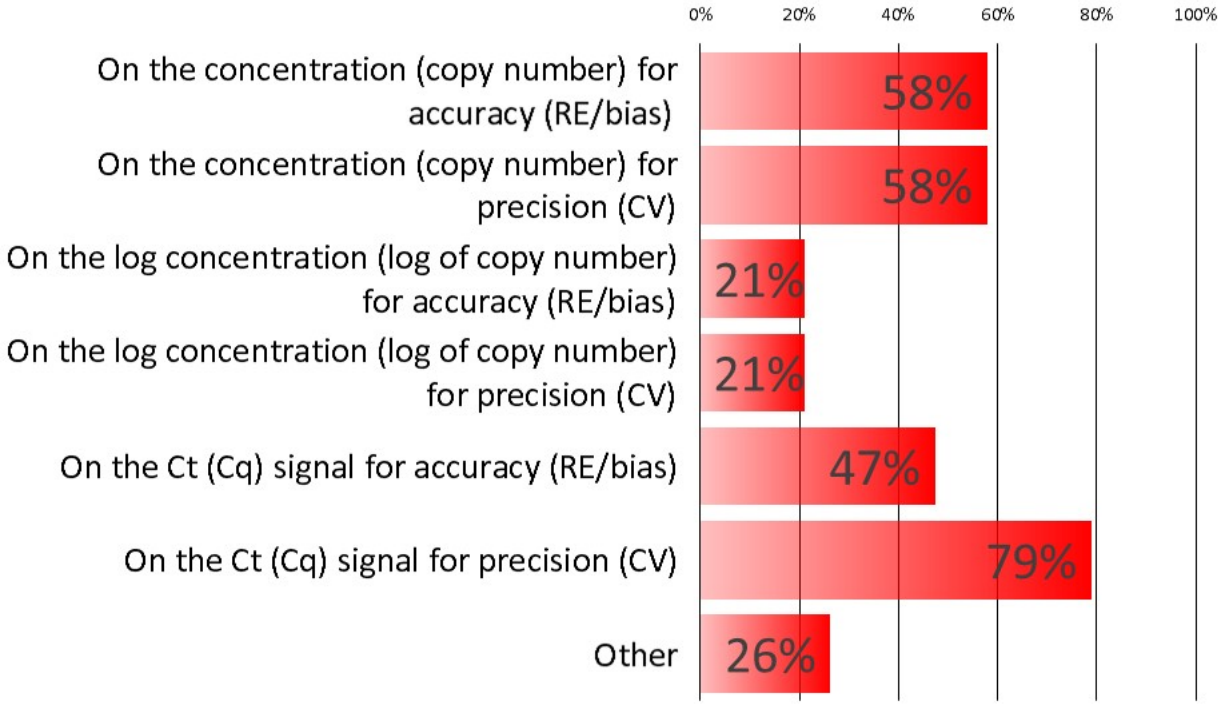
2019 Survey – Method Validation Parameters 2

Parameter	Cell Therapy	Gene Therapy	Oncolytic Virus	Gene expression
Stability of test item/reference in species-specific fluid or tissue	80%	57%	75%	25%
Stability of test item/reference in species-specific genomic DNA	40%	43%	25%	25%
Freezer storage stability	60%	57%	75%	50%
Room temperature stability	80%	71%	50%	50%
Freeze-thaw stability	40%	43%	75%	25%
Critical reagents stability	20%	14%	25%	25%

2018 Survey – Replicate Wells
Raise of hands



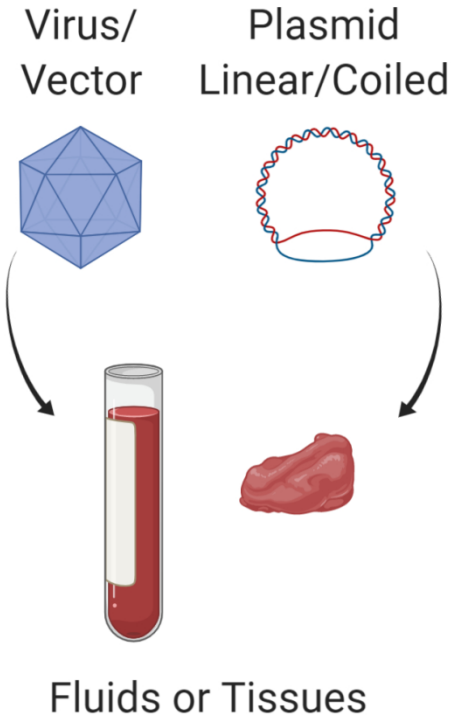
2018 Survey – Acceptance Criteria



DISCUSSION
Reference Standards, Calibration Curve and QCs

- How representative is the calibration standard of the target DNA/RNA in samples?
- What is the matrix for calibrator and QCs – buffer vs extract?
- Are QCs dependent on the end use of the assay? e.g limit test, PK type
- What controls do we need to run in each assay to demonstrate acceptable performance? DNA controls in buffer? Extraction controls?
- What **acceptance criteria** do we apply?
- How do we assess **specificity**? Is it acceptable to rely on in silico primer/probe design or do we need to physically test with related target? Is species matrix DNA sufficient? If related target needed, how closely should it be related to the intended sequence?
- How do we assess stability of standards, controls and qPCR critical regents?

Recovery and Stability – Approaches 1



Spike fluid/tissue with test item/reference

Advantages:

- Most representative of study samples
- Covers complete process from extraction to analysis

Disadvantages:

- Highly variable
- Test item/reference prone to immediate degradation
- Different spike-in vector vs plasmid for fluids vs tissues
- Difficult to set acceptance criteria
- Unable to assess RT and FT stability

Recovery and Stability – Approaches 2

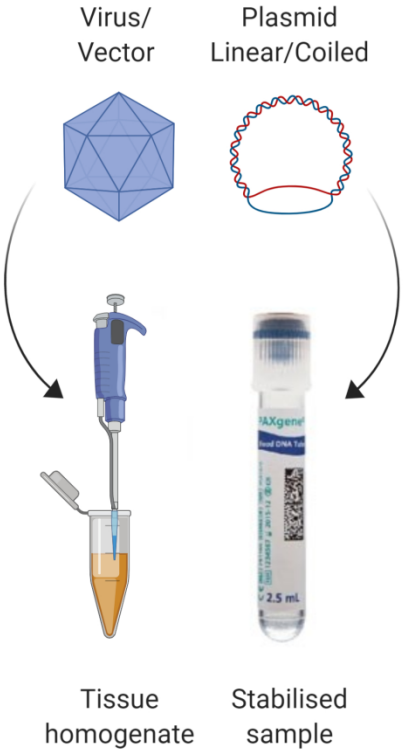
Spike stabilised fluid/tissue homogenate with test item/reference

Advantages:

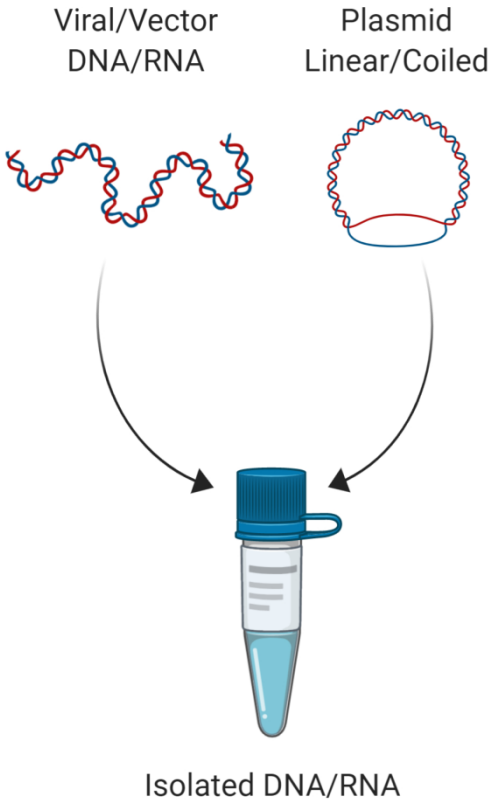
- Reduced likelihood of degradation
- Reduced variability
- May assess RT and FT stability

Disadvantages:

- May not reflect sample storage post collection
- Difficult to set acceptance criteria



Recovery and Stability – Approaches 3



Spike isolated DNA/RNA with test item/reference

Advantages:

- Lowest variability
- High accuracy and precision
- Simple acceptance criteria (as for QC samples)

Disadvantages:

- Does not reflect complete sample processing

2019 Survey – Recovery Approaches 1

Approach	Cell Therapy	Gene Therapy	Oncolytic Virus
Spike fluid/tissue with test item/reference: 1 aliquot for each sample type, 1 concentration level	33%	14%	0%
Spike fluid/tissue with test item/reference: 1 aliquot for each sample type, ≥ 2 concentration levels	0%	14%	0%
Spike fluid/tissue with test item/reference: 3 aliquots for each sample type, 1 concentration level	33%	14%	25%
Spike fluid/tissue with test item/reference: 3 aliquots for each sample type, ≥ 2 concentration levels	50%	43%	50%

2019 Survey – Recovery Approaches 2

Approach	Cell Therapy	Gene Therapy	Oncolytic Virus
Spike stabilised/homogenised fluid/tissue with test item: 1 aliquot for each sample type, 1 concentration level	33%	0%	25%
Spike stabilised/homogenised fluid/tissue with test item: 1 aliquot for each sample type, ≥ 2 concentration levels	0%	14%	0%
Spike stabilised/homogenised fluid/tissue with test item: 3 aliquots for each sample type, 1 concentration level	33%	14%	25%
Spike stabilised/homogenised fluid/tissue with test item: 3 aliquots for each sample type, ≥ 2 concentration levels	33%	29%	50%

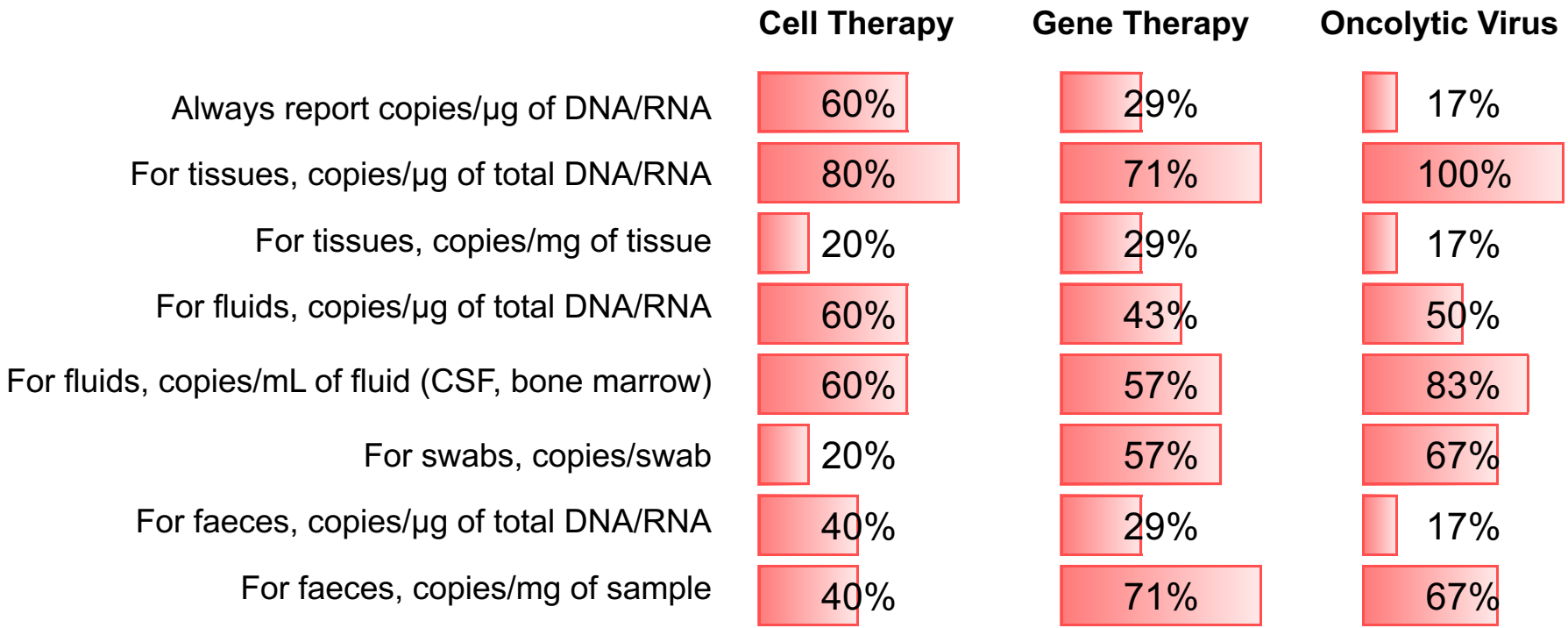
2019 Survey – Recovery Approaches 3

Approach	Cell Therapy	Gene Therapy	Oncolytic Virus
Spike total DNA/RNA with test item/reference: 1 aliquot per sample type, 1 concentration level, qPCR-only	33%	43%	25%
Spike total DNA/RNA with test item/reference: 1 aliquot per sample type, ≥ 2 concentration levels, qPCR-only	0%	29%	25%
Spike total DNA/RNA with test item/reference: 3 aliquots per sample type, 1 concentration level, qPCR-only	0%	0%	0%
Spike total DNA/RNA with test item/reference: 3 aliquots per sample type, ≥ 2 concentration levels, qPCR-only	33%	14%	25%
Recovery efficiency is validated internally/within SOP, not test item/reference-specific	50%	29%	25%

2019 Survey – Acceptance Criteria for Recovery

Criteria	Cell Therapy	Gene Therapy	Oncolytic Virus
We monitor trends, no acceptance criteria	60%	71%	50%
≥50% of spiked expected copies	60%	57%	67%
≥60% of spiked expected copies	0%	0%	0%
≥70% of spiked expected copies	0%	0%	0%
≥80% of spiked expected copies	0%	0%	0%
Bias (%) within ±10% of log copies	0%	0%	0%
Bias (%) within ±15% of log copies	20%	14%	17%
Ct values within ±1 of control	0%	0%	17%
Ct values within ±2 of control	0%	0%	0%
Ct values within ±3 of control	0%	0%	0%

2019 Survey – Reporting Units



DISCUSSION
Recovery, Sensitivity, Selectivity

- Do we need to validate the extraction methodology?
- Do we need to adapt extraction methodology to tissue type for best recovery?
- In view of the highly variable extraction recovery within and between matrices, how do we assess the **sensitivity** of the assay?
- How do we assess **selectivity**? Are we measuring differences in the qPCR assay performance between individuals or is this variability in extraction recovery?

DISCUSSION
Stability

- How many matrices should you assess (for a biodistribution study)?
- What are **stability samples?**
- What **storage conditions**?
  - Short-term at RT, refrigerated, additional F/Ts, Long term (-20C, -80C)
  - What do we do if standards and primer/probe sets have changed?

Nucleic acid quantification methodologies

- UV spectrophotometer (e.g. Nanodrop)

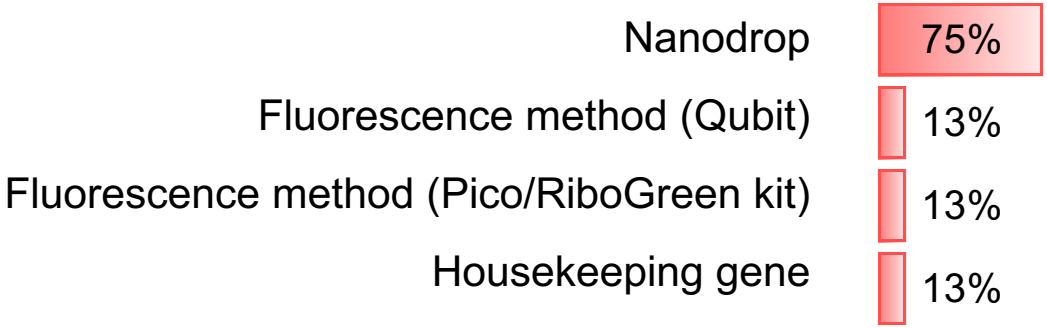
- DNA and RNA
- Concentration
- Purity

- Fluorescence (e.g. Qubit, Pico/Ribo/OliGreen Assays)

- NA-specific kit
- NA-specific binding
- Concentration

- Ref Standard provided
- 2 or ≥ 6-point calibration curve

2019 Survey – Total Nucleic Acid Concentration



UV vs Fluorescence Methods

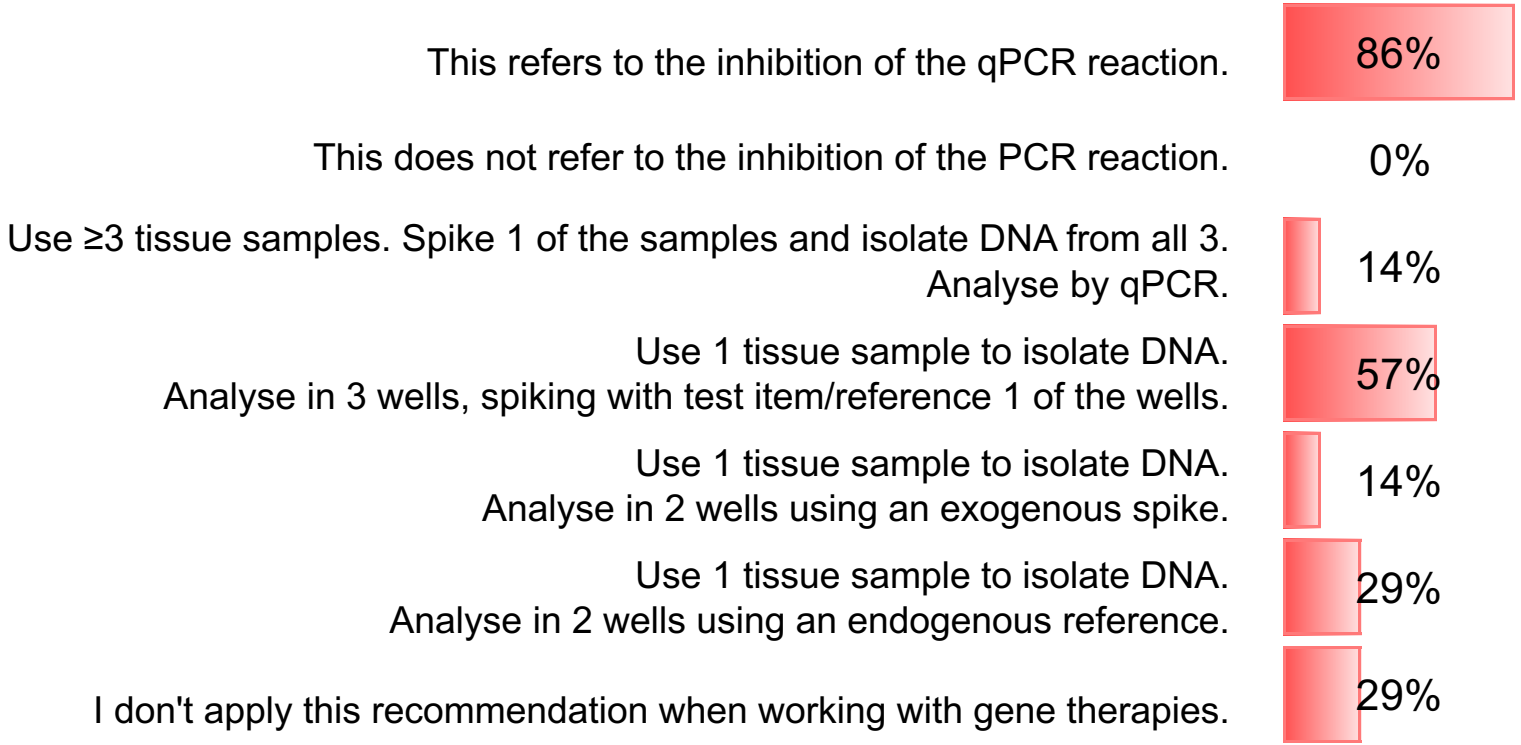
	UV	Fluorescence
Sensitivity	2 ng/µL	10 pg/µL
Selectivity	DNA, RNA, Protein	Dye binds specifically
Interference	Free-nucleotides, Salts, Organic solvents	
Accuracy and Precision		

FDA GT Clinical Trials – Inhibition Assessment

Use a quantitative, sensitive PCR assay to analyze the samples for vector sequences. You should submit data to your IND to demonstrate that your assay methodology is capable of specifically detecting vector sequence in both animal and human tissues. We recognize that PCR technology is constantly changing, and encourage you to discuss the assay methology with us before initiating sample analysis. Current recommendations include the following:

- The assay should have a demonstrated limit of quantitation of ≤50 copies of vector/1 µg genomic DNA, so that your assay can detect this limit with 95% confidence.
- Use a minimum of three samples per tissue. One sample of each tissue should include a spike of control DNA, including a known amount of the vector sequences, in order to assess the adequacy of the PCR assay reaction. The spike control will determine the specified PCR assay sensitivity.
- Provide a rationale for the number of replicates for testing per tissue, taking into account the size of the sample relative to the tissue you are testing.

2019 Survey – Inhibition Assessment



2019 Survey – Incurred Sample Reanalysis

Parameter	Cell Therapy	Gene Therapy	Oncolytic Virus	Gene expression
Yes, on the DNA/RNA stored after the first analysis	50%	29%	25%	33%
Yes, repeating the DNA/RNA extraction	17%	0%	25%	0%
Yes, following a HA request	17%	0%	0%	0%
No, but we never submitted the dataset to HA	33%	43%	50%	67%
No, and that was not an issue for submission	33%	43%	25%	33%

DISCUSSION
Sample processing: NA quantification and inhibition

- If we are expressing target DNA relative to total genomic DNA, how good are our total DNA/RNA assays?
- Are DNA/RNA assays validated? If not how are they controlled? QCs?
- Are extraction and total nucleic acid quantification kits critical reagents?
- How do we assess inhibition of the qPCR reaction?

Future directions

- Efficient data processing
- Direct PCR to eliminate sample extraction for some sample types
- Digital PCR
- CRISPR technology potential for qualitative testing
- Lateral flow strip, chip/capillary technologies, in combination with iPhone for sample screening or field testing

Acknowledgements

- Philip Timmerman and SC
- Rob Nelson – Covance
  - Milena Blaga, Charles River
  - Chris Cox, Psioxus
  - Philippe Ancian, Citoxlab
  - Manuela Braun, Bayer
  - Paul Bryne, Covance
  - Chiara Cazzin, Aptuit
  - Bing Hu, Merck
  - Lydia Michaut, BioAgilytix
  - Alexandra Rogue, Citoxlab
  - Veronica Ventura, Envigo
  - Tong-Yuan Yang, Janssen/J&J
  - Kelly Colletti, Charles River

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