qPCR Support of Cell and Gene Therapies – What to Measure and How

November 16th, 2023

EBF Open Symposium

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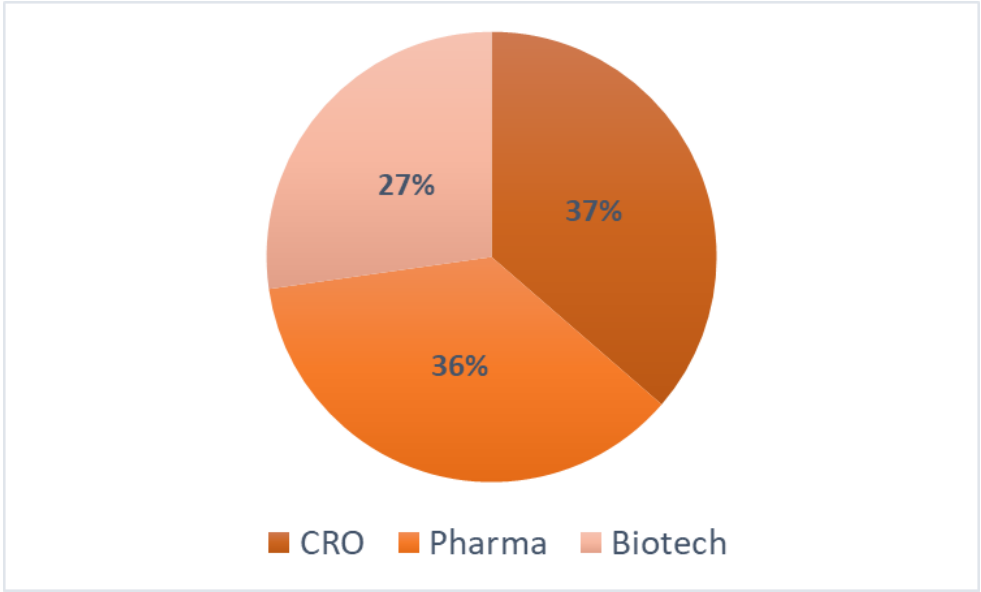
On Behalf of the AAPS Bioanalytical Community PCR Working Group

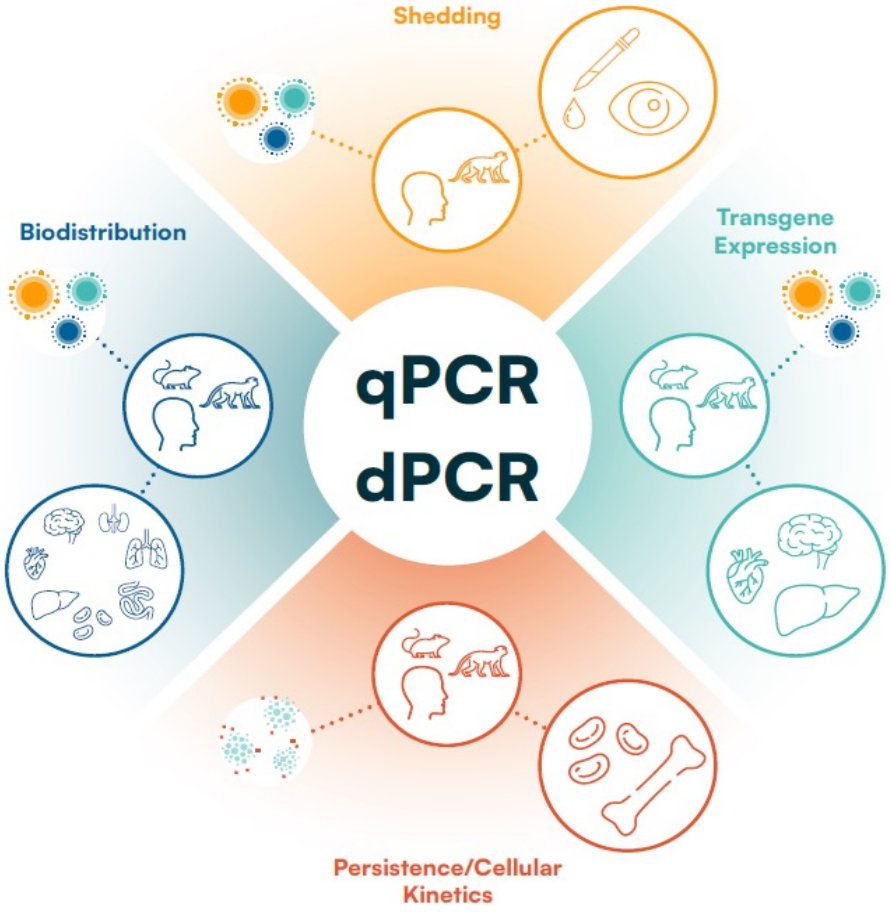
AAPS PCR Working Group

- Formed in December 2021 to address the lack of regulatory guidelines and harmonization
- 37 Industry Experts from 24 organizations
- Scope: Cell and Gene Therapies
  - Biodistribution
  - Transgene Expression
  - Viral Shedding
  - Persistence, Cellular Kinetics



AAPS PCR Working Group

- Divided group into 4 sub teams to enhance consensus building
  - Assay Design
  - Critical Reagents
  - Validation
  - Sample Analysis
- Manuscript submitted to AAPS Journal
  - Special topic issue “Emerging Trends in Preclinical and Clinical Development of Cell and Gene Therapies”
  - The views and conclusion presented in this paper are those of the authors and do not necessarily reflect the representative affiliations or company positions on the subject.



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What to Measure?

Cell and Gene Therapy

Biodistribution

Purpose	Measures the gene/cell therapy product in target and non-target tissues and biofluids.
Phase	Preclinical, Clinical
Matrices*	Injection site, gonads, adrenal gland, brain, spinal cord, liver, kidney, lung, heart, spleen, blood, etc.
Bioanalytical Assay Platforms	qPCR/dPCR, Flow Cytometry

*Dependent on the vector type, tissue tropism, route of administration, the expression product, animal, disease, and pathophysiology.

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What to Measure?

Purpose	Measures the gene/cell therapy product in target and non-target tissues and biofluids.	Measures the release of virus-based gene therapy products from the patient through excreta and secreta to understand exposure to the environment and transmission to other humans.
Phase	Preclinical, Clinical	Preclinical, Clinical
Matrices*	Injection site, gonads, adrenal gland, brain, spinal cord, liver, kidney, lung, heart, spleen, blood, etc.	Urine, saliva, tears, feces, nasopharyngeal fluids, wounds, sores, etc.
Bioanalytical Assay Platforms	qPCR/dPCR, Flow Cytometry	qPCR/dPCR, cell-based infectivity

*Dependent on the vector type, tissue tropism, route of administration, the expression product, animal, disease, and pathophysiology.

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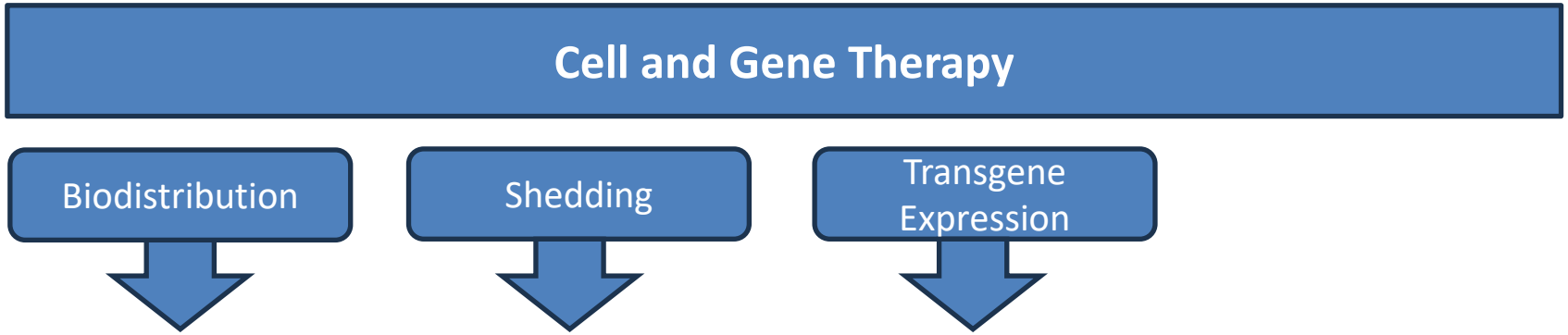
What to Measure?



Purpose	Measures the gene/cell therapy product in target and non-target tissues and biofluids.	Measures the release of virus-based gene therapy products from the patient through excreta and secreta to understand exposure to the environment and transmission to other humans.	Measures the expression of the transgene that is delivered by the gene/cell therapy product in target and non-target tissues and biofluids.
Phase	Preclinical, Clinical	Preclinical, Clinical	Preclinical, Clinical
Matrices*	Injection site, gonads, adrenal gland, brain, spinal cord, liver, kidney, lung, heart, spleen, blood, etc.	Urine, saliva, tears, feces, nasopharyngeal fluids, wounds, sores, etc.	Target tissues, gonads, adrenal gland, brain, spinal cord, liver, kidney, lung, heart, spleen, blood, etc.
Bioanalytical Assay Platforms	qPCR/dPCR, Flow Cytometry	qPCR/dPCR, cell-based infectivity	RT-qPCR/RT-dPCR

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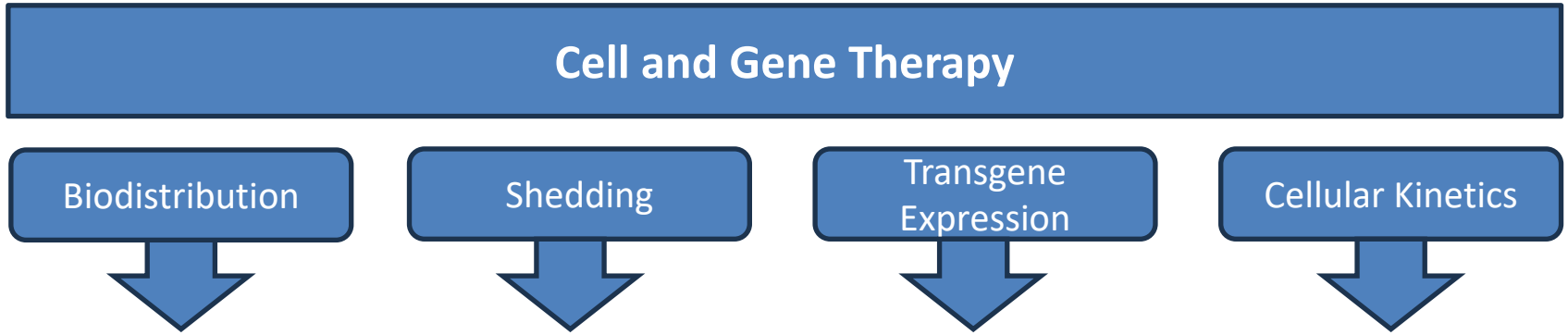
What to Measure?



Purpose	Measures the gene/cell therapy product in target and non-target tissues and biofluids.	Measures the release of virus-based gene therapy products from the patient through excreta and secreta to understand exposure to the environment and transmission to other humans.	Measures the expression of the transgene that is delivered by the gene/cell therapy product in target and non-target tissues and biofluids.	Measures the pharmacokinetics of the cell therapy after administration.
Phase	Preclinical, Clinical	Preclinical, Clinical	Preclinical, Clinical	Preclinical, Clinical
Matrices*	Injection site, gonads, adrenal gland, brain, spinal cord, liver, kidney, lung, heart, spleen, blood, etc.	Urine, saliva, tears, feces, nasopharyngeal fluids, wounds, sores, etc.	Target tissues, gonads, adrenal gland, brain, spinal cord, liver, kidney, lung, heart, spleen, blood, etc.	Blood, PBMCs, bone marrow aspirates, etc.
Bioanalytical Assay Platforms	qPCR/dPCR, Flow Cytometry	qPCR/dPCR, cell-based infectivity	RT-qPCR/RT-dPCR (mRNA level), various platforms (protein level)	qPCR/dPCR, Flow Cytometry

*Dependent on the vector type, tissue tropism, route of administration, the expression product, animal, disease, and pathophysiology.

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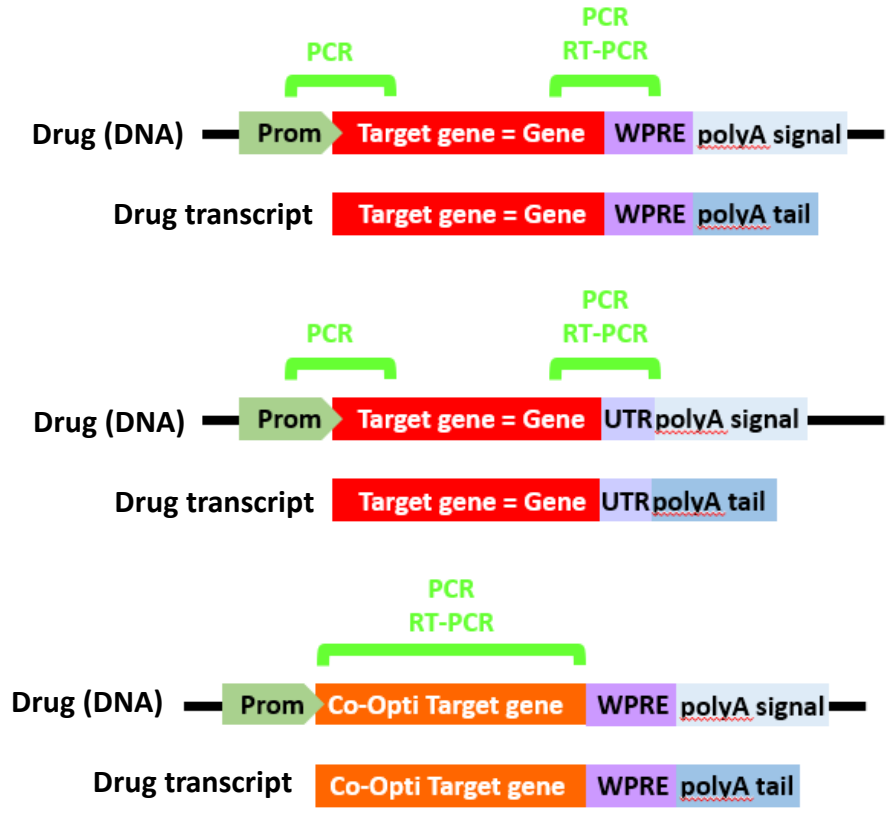
How to Measure?

- Assay Design:
  - Consider assay purpose and COU when designing primer/probe sets
  - Primer/Probe Design (qPCR vs. dPCR)
    - Set with suboptimal PCR efficiency but viable in dPCR
    - Positive/neg partitions are distinguishable
  - Multiplex consideration for CGT applications
    - RT-qPCR/RT-dPCR for gene expression
    - Cellular kinetics
    - Reportable result in copies/cell



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How to Measure?

- Extraction Considerations
  - First step in the PCR workflow
  - Considered part of the method
  - Development and validation
  - Changes in procedure can trigger qualification, partial validation activities

Proposed Minimum Evaluation Approach*	Full Validation	Partial Validation	Bridging Study
Change	• Extraction platform or kit change • PCR Platform • Primer/probe sequence change	• Single Reagent change within extraction kit initiated by manufacturer. • PCR Mastermix or RT Reagent Part Change	• Single Extraction Reagent • Provider Change • PCR Oligo Provider Change • TLDA card lot change • Calibrator/QC lot change • Surrogate matrix lot change • DNAse manufacturer or enzyme (i.e., benzonase or MNase) change

*The proposed approach should be on a case-by-case basis and determined based on the COU.

- Nucleic Acid Input and Assay Reportables
  - COU dictates reportables (e.g., copies/ug, relative expression or copies/volume)
  - Low DNA/RNA yield in certain shedding matrices (e.g., tears, saliva, urine)
    - Load equal undiluted volumes
    - Reported as copies per mL
  - For solid shedding matrices (e.g., feces)
    - Measure mass as extraction input
    - Reported as copies per mg

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How to Measure?

- qPCR versus dPCR Considerations
  - What are you measuring?
    - Target with low expression or low copy numbers
    - Need for higher precision for rare events
    - Matrix with PCR inhibitors
  - Expected copy numbers of target
    - Platform limitations
    - Setting dynamic range to limit sample dilutions
  - Considerations for phase in development process
    - Sample throughput
    - Reagent cost

What to Measure?

Method Development	Method Validation
Primer and probe set evaluation: • qPCR efficiency • dPCR pos/neg separation	Precision Accuracy
PCR optimization	PCR Efficiency
Calibration curve optimization (qPCR)	Dilutional Linearity (if applicable)
Extraction optimization	Co-linearity (if applicable)
Recovery Extraction Efficiency	Sensitivity: • LOB (if applicable) • LOD • LLOQ
Precision Accuracy	Specificity Selectivity
Specificity Selectivity	Robustness and Ruggedness
Sensitivity	Stability (if applicable): • Freeze/Thaw • Bench Top • Frozen
Other parameters as needed	Extraction Efficiency

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Extraction Efficiency

- The preanalytical procedure comprising the steps from sampling, to transport, storage have an impact on extraction recovery and contribute with more confounding variation than the actual analytical qPCR/dPCR procedure.

- **Design:**
  - Spike in drug product, or reference material
  - > 1 QC level
  - Matrices: biofluids, representative tissues
  - ≥ 3 extraction/lots per matrix type

- **Criteria:**
  - Results are reported and generally acceptable with a spike recovery between >20% and <120%.
  - Consider the COU if the performance exceeds this acceptance criteria.

Precision and Accuracy

- **Design:**
  - Assessed over ≥6 runs, ≥2 analysts, ≥2 days
  - ULOQ, HQC, MQC, LQC, and LLOQ
    3 determinations per level per run
  - Positive control template spiked into relevant nucleic acid, or full process
  - Drug product or surrogate reference material
  - Representative matrix or pool (±20 %RE of comparator matrix)
- **Criteria:** Based on demonstrated performance and COU

Suggested:

- Intra- and Inter-assay Precision:
  - CV ≤30 % for QCs
  - CV ≤50 %CV for LOQs for interpolated qPCR or absolute dPCR copy number
- Intra- and Inter-assay Accuracy
  - qPCR: -50 to 100 %RE on interpolated copies (1 cycle is ½ or 2x conc.)
  - dPCR: absolute copies measured should be %RE ≤ 30 for QCs, and ≤ 50 for LOQs

PCR Efficiency and Linearity

- **Purpose:**
  - To assess the amplification performance of the assay, matrix interference and linearity of test sample dilutions
  - Inherent to qPCR
    - calculated from slope of the linear regression
    - theoretical amplification efficiency should be 100%, resulting in a doubling of template with every amplification cycle
  - dPCR does not require a calibrator curve and interpolation
    - Recommended that linearity still be assessed
    - Demonstrates linear dynamic range of the assay

- **Design:**
  - qPCR: Calibrator curve
  - dPCR: Dilution series or QCs including LOQs
  - Assess in ≥6 runs, ≥1 curve per run, ≥2 analysts, ≥2 days, (≥2 PCR instruments)

- **Suggested:**
  - All concentrations within the ROQ > LOD
  - $R^2 \geq 0.98$ and slope -3.1 and -3.6 (corresponding to 90-110% efficiency) within the ROQ
  - 95% confidence interval can also be reported
  - Efficiency not meeting these criteria may be acceptable depending on the assay purpose and should be justified

Sensitivity

Limit of Blank (LOB)

- Based on COU and Technology

Design

- NTC prepared with mock sample (water) with matrix
- 20 replicates in validation
- Empirical (19/20 Neg)
- Statistical (dPCR):

$$LOB = Mean_{NTC} + 1.645(SD_{NTC})$$

Criteria

- LOB is reported as determined
- LOB < LOD

Limit of Detection (LOD)

- Detection with 95% probability

Design

- ≥3 runs, ≥2 analysts, ≥2 days
- Reference template in extracted matrix or Full Process
- 1.5-2-fold dilutions from LLOQ level
- Empirical (≥20 replicates)
- Statistical method
  - Probit regression analysis
  - dPCR: LOD = LOB + 1.645(SD)

Criteria

- LOB < LOD ≤ LLOQ
- LOD reported with 95% confidence interval
- Results below LOD reported as Below the Limit of Detection (BLD)

Limit of Quantification (LLOQ)

- FDA ICH S12 no LLOQ requirement

Design

- Determined from P&A runs
- Included in the calibration curve, suggested QC for dPCR

Criteria

- LLOQ ≥ LOD
- Within P&A criteria

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Summary

- The purpose of the working group discussions and consensus publication is to improve consistency, clarity, and sound scientific validations for qPCR and dPCR assays that are used to support gene and cell therapies.

- The information presented in the publication is built on experience from molecular and bioanalytical scientists in the industry who have experience in developing and validating these assays for regulatory submissions.

- The recommendations are intended to present general considerations and examples of experimental design and suitable acceptance criteria for assays used to support cell and gene therapies by qPCR and dPCR, especially in the absence of regulatory guidance for analytical validation of these assays.

Acknowledgments

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Amanda Hays (BioAgilytix)
Wendy Adamowicz (PPD)
Mark Wissel (Eurofins Viracor)
Kelly Colletti (Beam Therapeutics)
Russell Soon (BioMarin)
Mitra Azadeh (Ultragenyx)
Justin Smith (Pfizer)
Rajitha Doddareddy (Merck)
Melanie Chalfant (GSK)
Swarna Suba Ramaswamy (Eli Lilly)
Sanjay L. Dholakiya (BMS)
Sebastian Guelman (Genentech)
Bryan Gullick (BioAgilytix)
Jennifer Durham (BioAgilytix)
Keith Rennier (Labcorp)
Pruthvi Nagilla (Asher Bio)
Anamica Muruganandham (BioAgilytix)
Manisha Diaz (Eurofins Viracor)
Cassandra Tierney (Pfizer)
Kaarthik John (Regenxbio)
Jenny Valentine (Regeneron)
Timothy Lockman (PPD)
Hsing-Yin Liu (Janssen)
Benjamin Moritz (CRL)
Jean Paul Ouedraogo (CRL)
Marie-Soleil Piche (CRL)
Muriel Smet (Sanofi)
Jacqueline Murphy (Janssen)
Kaylyn Koenig (Alta Sciences)
Agnes Zybura (Labcorp)
Carrie Vyhlidal (KCAS)
Jonathan Mercier (Flowmetric)
Niketa Jani (BioAgilytix)
Mikael Kubista (Institute of Biotechnology Czech Academy of Sciences)
Donald Birch (Alta Sciences)
Karlin Morse (Alta Sciences)
Oskar Johansson (TATAA)

Back-up Slides

PCR Efficiency and Linearity

- Dilutional Linearity – only performed in validation if high-concentration samples are expected to require dilution to yield signals within the assay's ROQ.
  - To determine if the dilution impacts reaction efficiencies and accuracy of interpolation
  - The shorter dynamic range of dPCR

- Co-Linearity – performed to demonstrate linearity between two reference materials to allow use of a surrogate reference material in the assay.
  - To demonstrate that a surrogate reference material (e.g., plasmid DNA) behaves equivalently in the assay
  - Co-linearity should be performed if valuable given the assay's COU

Stability Considerations

- Stability assessment
  - Assessed with QCs as a surrogate
    - Prepare a sufficient number of LQC and HQC aliquots to cover stability time-points
    - Short-term, room temperature
    - Freeze/thaw cycles
    - Long-term stability
  - Assessed with contrived samples
    - Surrogate, relevance to actual sample is unclear
    - Acceptance criteria based on the demonstrated extraction efficiency variability
  - Assessed with endogenous samples, if applicable, or study samples (incurred samples)