qPCR assay for ASO quantification under GLP: out of ICH M10 scope

Evotec SE, Elena Vicentini, 18th EBF OS, 18-20 November 2025

Agenda

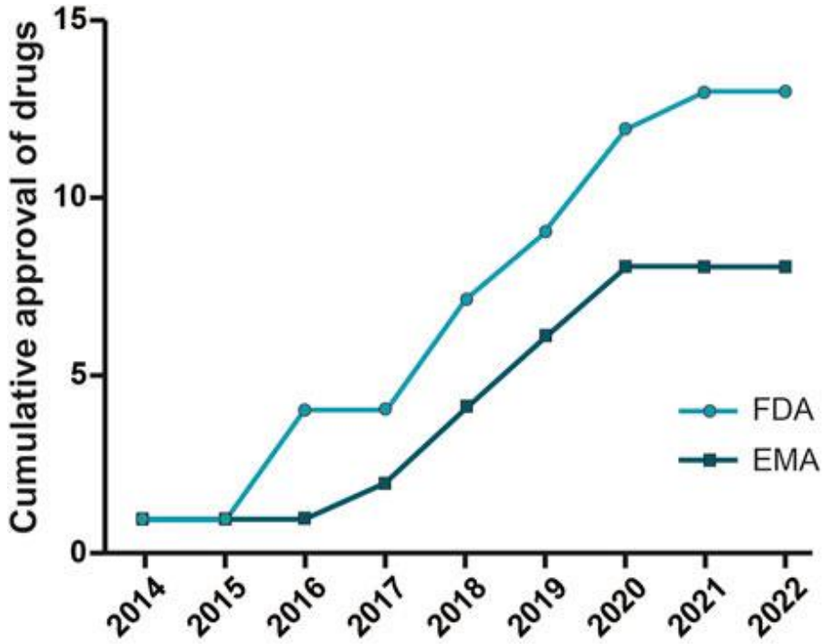
1. ASOs introduction
2. Approaches for ASOs quantification
3. SMCxPRO® Hybridization Assay
4. SplintR® qPCR
5. Technology Comparison in Sample Analysis
6. Validation Approach SplintR® qPCR Method
7. Reflection and Questions

ASOs Introduction

Challenges in Bioanalysis

Growing Class of therapeutic agents modulating gene expression

10 ASOs EMA/FDA approved since ’90s



Oligonucleotide drugs approved since 2013 by FDA and EMA for different therapeutic areas (neurological and neuromuscular, metabolic and endocrine, and infection diseases)

Challenges in bioanalysis

- **Structural Complexity and Diversity**
  ASOs diverse chemical modifications require tailored bioanalytical methods for quantification.
- **Sensitivity Challenges**
  Low-doses ASOs administration demands highly sensitive methods to quantify them in biological matrices.
- **Specificity Challenges**
  Overlap with homologous sequences in mRNA and discrimination of metabolites (N-1, N-2 shorter…)
- **Matrix Interference Issues**
  Biological sample variability requires robust assay validation to ensure reliable ASO quantification.
- **Standardization and Validation**
  Each bioanalytical approach offers both positive results and related challenges.

Front. Pharmacol. 14:1304342. doi: 10.3389/fphar.2023.1304342

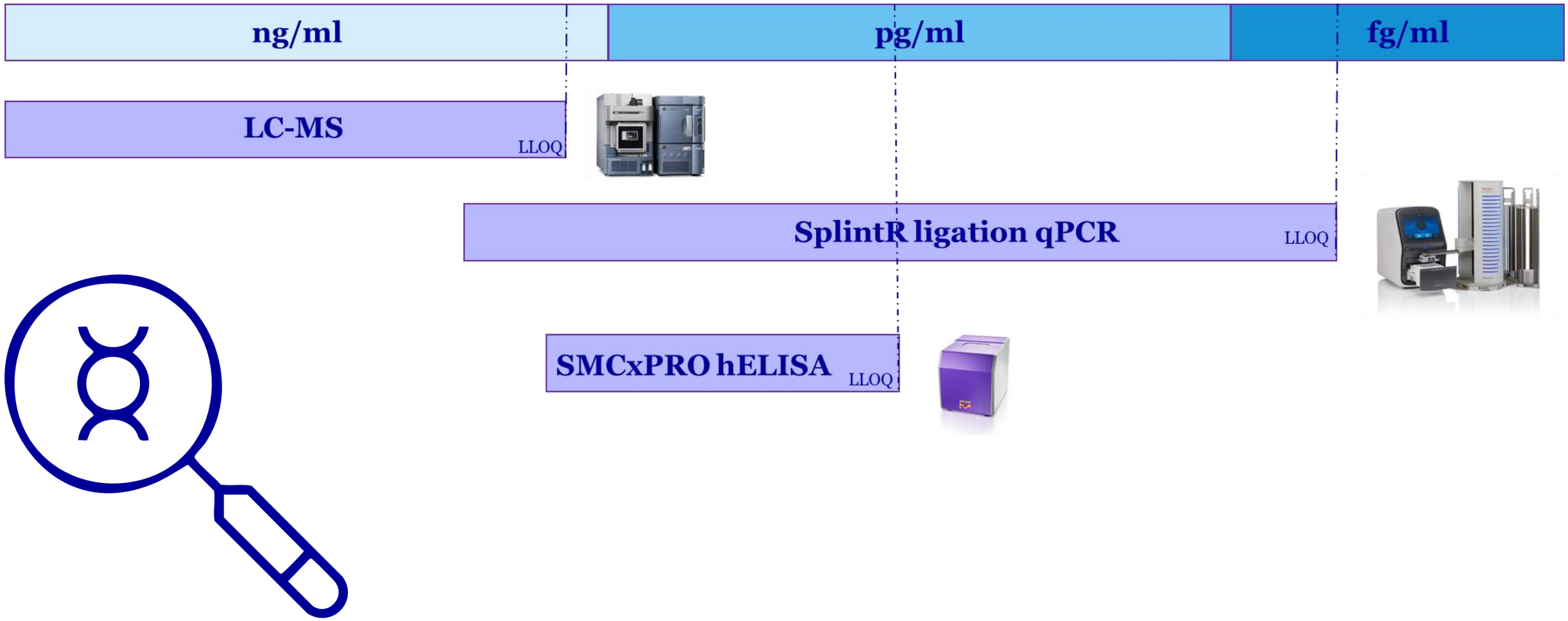
ASO quantification: different approaches

Technology comparison

Parameter	LC-MS	MSD/SMCxPRO® Platforms	SplintR® qPCR
Sensitivity	Matrix dependent. Down to 5-10 ng/mL for plasma and CSF. About 50-100 ng/g in tissue.	Probe and matrix dependent. About 40-80 pg/mL in plasma and CSF (SMC). About 1000 pg/g in solid tissues (MSD).	Probe and matrix dependent. About 750 fg/mL in plasma and CSF. About 100 pg/g in solid tissues.
Specificity	High selectivity: possibility to distinguish parent and metabolites	Dependent on the design of the probes, may recognize the parent and some metabolites (e.g. n-1, n-2)	Dependent on the design of the probes, may recognize the parent and some metabolites (e.g. n-1, n-2)
Dynamic range	Wide (up to 3 orders of magnitude)	Extensive (up to 4-5 orders of magnitude)	Extensive (5-6 orders of magnitude)
Applicability	Better with ASOs < 40 nt Applicable to siRNA	ASOs shorter than 16 nt → potential difficulties for optimal binding. Applicable to other nucleic acids (to be tested case-by-case).	ASOs shorter than 16 nt → potential difficulties for optimal binding. Applicable to other nucleic acids (to be tested case-by-case).
Cost	Low	Medium	Medium

ASO quantification: different approaches

Sensitivity & Dynamic Range

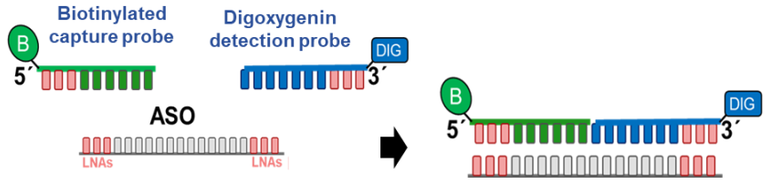


Hybridization ELISA Methods

Principles

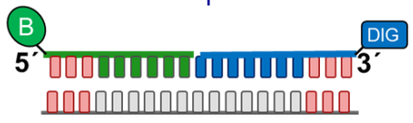
Principle of ASO immunodetection assay on MesoScale Discovery® and SMCxPRO® platforms

Hybridization reaction

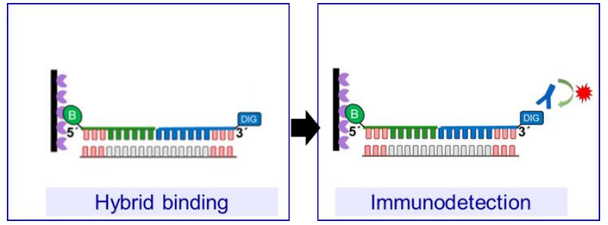


Simultaneous hybridization of probes to ASO target under denaturing conditions (on PCR cycler)

ASO-probes hybrid

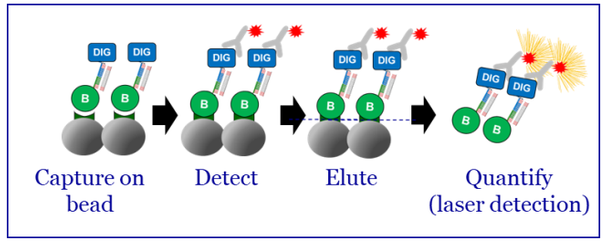


Immunoassay-based detection



MSD S600 ®

- High throughput and sensitivity
- ELISA-like, well customizable
- Very robust



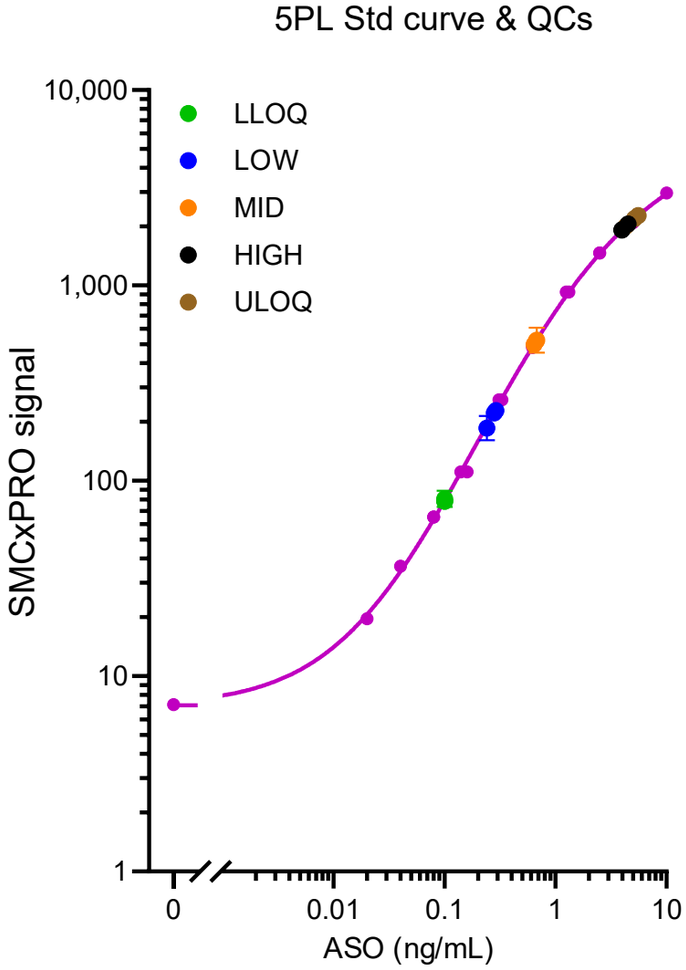
SMCxPRO ®

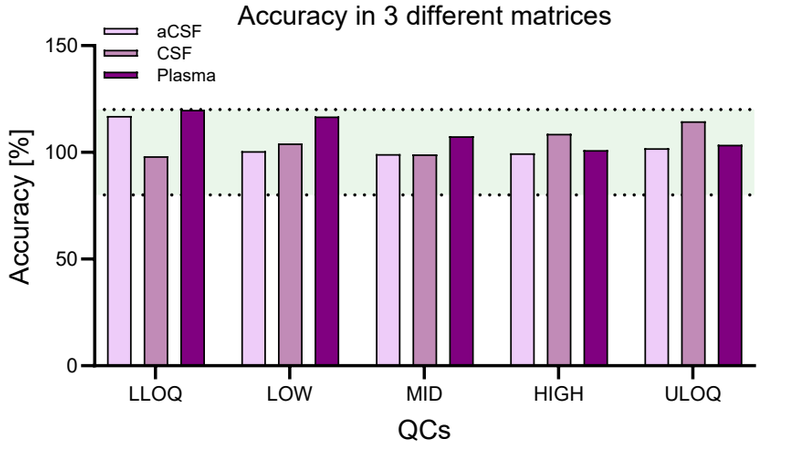
- Ultra-high sensitivity
- Single molecule counting
- Reliable and suitable for clinically relevant matrices

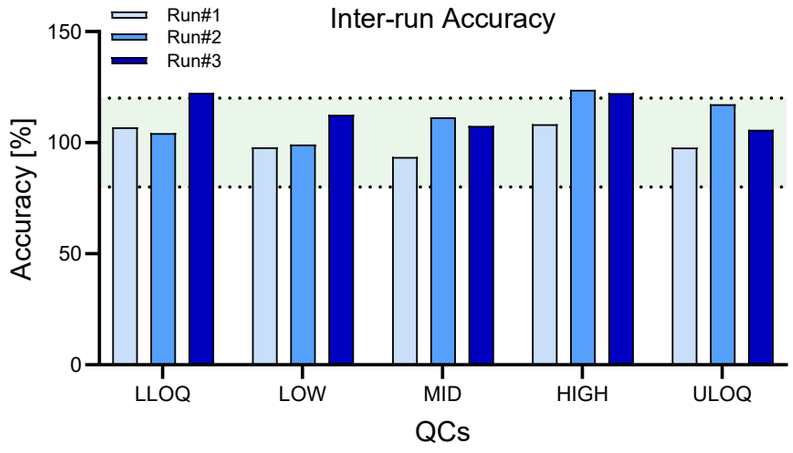
1 Thayer et al., POE Immunoassay: Plate based oligonucleotide electrochemiluminescent immunoassay for the quantification of nucleic acids in biological matrices. Scientific Reports | (2020) 10:10425.

SMCxPRO® Method Qualification

Representative Calibration Standards & QCs Performance







- LLOQ 0.08 ng/mL
- ULOQ 5.00 ng/mL

SplintR® qPCR method

Principles & Process

Development

Design and probes selection

- Design of 8-10 sets of probes
- Test of specificity and standard curve range definition (8-point curve)

Evaluate matrix effect

- Evaluate absence or extent of matrix effect
- Confirm 8-point standard curve range in matrix

Define standard curve and quality controls (QCs)

- Estimate limits of quantification (LLOQ and ULOQ)
- Define QCs levels

Qualification

Define sample dilutions

- Assess dilutional linearity
- Select appropriate sample dilutions

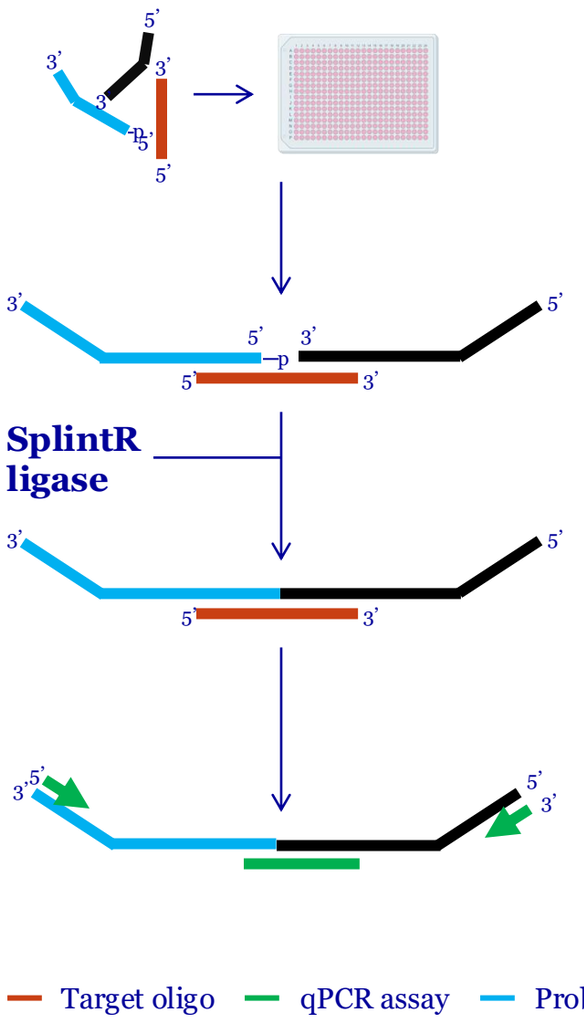
Evaluate method performance

- Accuracy and precision assessment with QC samples

In-study

Analysis of study samples (non-GLP)

- Assessment of study sample via standard curve absolute quantification method
- Standard curve and QCs acceptance criteria analysis for results evaluation

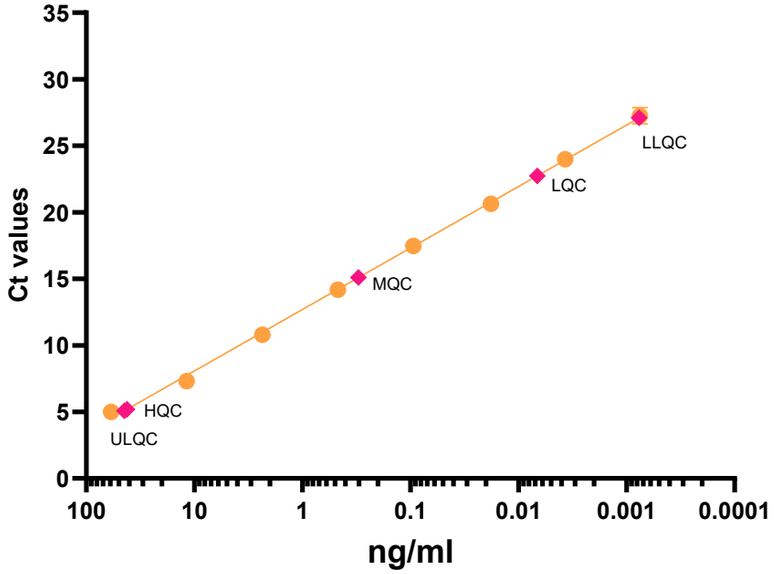


Quantification of Antisense Oligonucleotides by Splint Ligation and Quantitative Polymerase Chain Reaction. Shin M. et al., NAR, 2022

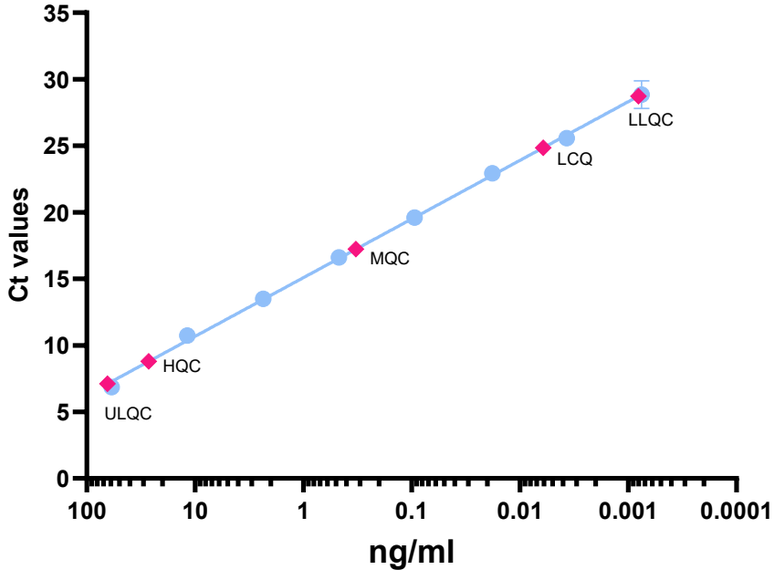
SplintR® qPCR method

Representative Calibration Standards & QCs Performance

Plasma quantification range standard curve (1:50 matrix dilution)



CSF quantification range standard curve (1:20 matrix dilution)



	Matrix dilution	R2	Linear range (ng/mL)
Water	/	>0.99	58 – 0.00006
Plasma	50	>0.99	58 – 0.00075
aCSF	20	>0.99	58 – 0.00075

CSF vs aCSF STD curve demonstrated

- LLOQ 0.0037 ng/mL
- ULOQ 58.0 ng/mL

Methods Performance Comparison

Qualification for CSF/plasma sample analysis vs Acceptance criteria

Qualification parameter	LC-MS/MS	SMCxPRO® Platform	SplintR® qPCR
Calibration model	Linear fitting	5 parameter logistic (5PL) model	Linear fitting
Qualified quantification range	5 to 2500 ng/mL	0.08 to 5 ng/mL	0.0037 to 58.0 ng/mL
Precision within run	≤15%	≤ 20/25% (in aCSF)	≤25/35%
Precision between runs	Not tested	≤ 20/25% (in aCSF)	Not tested
Accuracy within run	≤15%	75/80% ≤ Accuracy ≤ 120/ 125% (in aCSF)	≤25% (LLOQ/LQC/MQC) - 50% HQC/ULOQ (plasma) ≤25/35% (all levels in CSF)
Accuracy between runs	Not tested	75/80% ≤ Accuracy ≤ 120/ 125% (in aCSF)	Not tested
Prozone/hook Effect	------	Not observed	Not tested
Stability in CSF	Not tested	Not tested	Not tested
Stability in plasma	Not tested	Not tested	Not tested
Dilution Integrity/Linearity (CSF)	Up to 50-fold	Tested up to 1:254 in two individual control CSF samples	Linear range 58 – 0.00075, MRD 1:20
Dilution Integrity/Linearity (Plasma)	Up to 50-fold	Tested up to 1:1000 in two individual control plasma samples	Linear range 58 – 0.00075, MRD 1:50

ASO quantification results in non-GLP DRF study

Technology comparison

Dose Group	Time	Animal ID	ASO SplintR® qPCR	ASO SMCxPRO®	ASO LC-MS
0	Day 1 – 0h	1	**NQ**	**NQ**	**NQ**
GP1	Day 1 – 0h	1	**NQ**	**NQ**	**NQ**
GP2	Day 1 – 0h	1	**NQ**	**NQ**	**NQ**
GP1	Day 29 – 0h	1	Q	Q	**NQ**
		2	Q	Q	**NQ**
		3	Q	Q	**NQ**
GP2	Day 29 – 0h	1	Q	Q	**NQ**
		2	Q	Q	**NQ**
		3	Q	Q	Q
GP1	Day 43 – 0h	1	Q	Q	Q
		2	Q	Q	**NQ**
		3	Q	Q	Q
GP2	Day 43 – 0h	1	Q	Q	Q
		2	Q	Q	Q
		3	Q	Q	Q

Analysis of DRF Cyno CSF samples at longer timepoint(s) upon intrathecal administration

- **Method for comparison**
  - LC-MS/MS using a Turbo Spray IonDrive™ interface with negative ion multiple reaction monitoring was qualified over the range 5 to 2500 ng/mL
- **CSF Ratio Splint and SMC vs LC/MS overall near 1**
  - Range SMC/LC-MS: 0.7 – 1.3
  - Range Splint/LC-MS: 0.7 – 1.1
- **Next Step**
  - SplintR® qPCR potential method validation request to support regulated upcoming studies

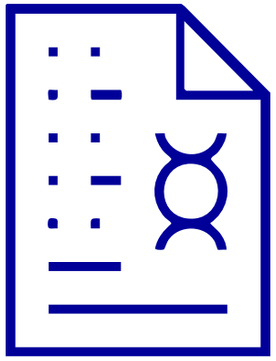
SplintR® qPCR Method Validation

Based on ICHM10 rules for LBA

1.1. Objective

This guideline is intended to provide recommendations for the validation of bioanalytical methods for chemical and biological drug quantification and their application in the analysis of study samples. Adherence to the principles presented in this guideline will ensure the quality and consistency of the bioanalytical data in support of the development and market approval of both chemical and biological drugs.

The objective of the validation of a bioanalytical method is to demonstrate that it is suitable for its intended purpose. Changes from the recommendations in this guideline may be acceptable if appropriate scientific justification is provided. Applicants are encouraged to consult the regulatory authority(ies) regarding significant changes in method validation approaches when an alternate approach is proposed or taken.



1.3. Scope

The information in this guideline applies to the quantitative analysis by ligand binding assays (LBAs) and chromatographic methods such as liquid chromatography (LC) or gas chromatography (GC), which are typically used in combination with mass spectrometry (MS) detection.

Validation parameter	LBA	SplintR® qPCR
Calibration model	4/5 –PL	Linear fitting
Precision within run	20%-25% at extremes	25%-35% at extremes
Precision between runs	20%-25% at extremes	25%-35% at extremes
Accuracy within run	20%-25% at extremes	25%-35% at extremes
Accuracy between runs	20%-25% at extremes	25%-35% at extremes
Prozone/hook Effect	To be tested	To be tested
Dilution Linearity	Accuracy and precision ≤20%	Accuracy and precision ≤ 25% (high dilution range needed)
Specificity	Unspiked <LLOQ; Accuracy ±25% (variants/structurally related)	Unspiked <LLOQ; Accuracy ±30% (Using n-1/n-2 sequences)
Selectivity	Unspiked <LLOQ/ Spiked 20-25% (LLOQ-HQC) incl hemolyzed matrix	Unspiked <LLOQ/ Spiked 25-35% (LLOQ-HQC) incl. hemolyzed matrix
Stabilities (short term, F/T, Long Term)	Accuracy and precision ≤20%	Accuracy and precision ≤25%
Stability/Lot to lot variation of critical reagents	Required for labelled reagent	Required for probes/enzymes (lot to lot variability)

SplintR® qPCR Method Validation

Points to be considered

Based on previous experience in validating assays using qPCR to support biodistribution studies in regulated environment, all ICH M10 required assessments can be performed for TK/PK application.

Based on qPCR assay amplification nature, higher variability is observed at the extremes in terms of accuracy and precision. Careful evaluation of the quantification range (LLOQ and ULOQ) should be done based on study requirements of sensitivity and dynamic range.

Required Dynamic Range should imply huge sample dilutions at timepoints near Cmax (based on LC-MS dynamic range) and extensive Dilution Linearity assessment must be performed.

Ideally same analytical approach to be used within the same study/project to compare results.

Different analytical approaches can be introduced but “cross validation” among techniques should be considered to demonstrate equal concentrations are obtained resulting in equal exposure results.
e.g. QC samples prepared spanning the quantification range and real samples to be assessed with both technologies.

Reflections and Questions

- Need for more sensitive assay(s) due to route of administration (e.g. it, icv), low circulating levels and potential inter- subject variability for low doses administered
- Relevant matrices with low concentration to be quantified depending on route of administration (e.g. CSF)
- Hybridization based methods impacted by specificity issues (e.g. metabolites, impurities….)
- SplintR® qPCR method could replace LC-MS methods or could complement LC-MS for quantification at longer timepoints, in CSF/plasma matrices based on strong result correlation to be assessed during method validation
- Clinical relevance: bioanalytical assays suitable for clinical monitoring
- Any regulatory feedback on the use of alternative bioanalytical platforms such as qPCR outside ICH M10?

Acknowledgements

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