Bioanalytical monitoring of gene therapy trials: methodologies for PK-PD assessment and patient eligibility

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Therapeutic oligonucleotides

DNA and RNA (or analogue) fragments as treatment for genetic diseases

- MicroRNA (miRNA) and Small Interfering RNA (siRNA)
  - Interfere with gene expression by binding to mRNA
- RNA or ssDNA, Allele-Specific Oligonucleotides (ASO)
  - Block mRNA translation
  - Small (<25 bases)
- Aptamers
  - Act through their 3D-structure by specifically binding to a target
- Chemical modification are introduced to the backbone, base or termini (2'-OH)
  - Locked Nucleic Acid (LNA)
  - Peptide Nucleic Acid (PNA)
  - PEGylated oligonucleotides

Therapeutic oligonucleotides

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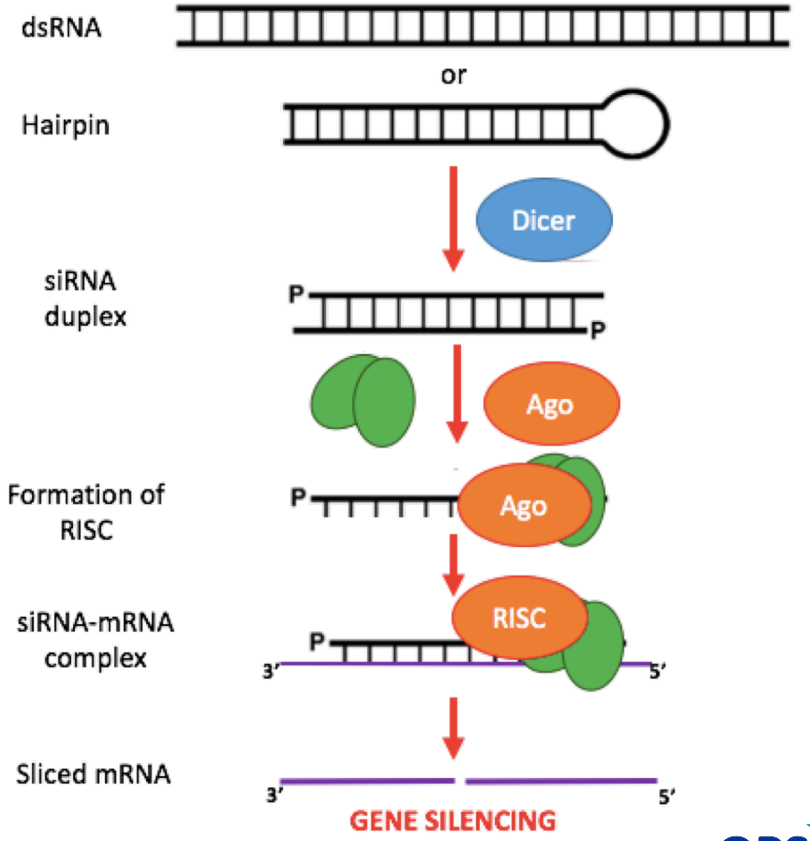


Nucleic Acid Therapy – Bioanalysis

FEATURES THAT INFLUENCE BIOANALYTICAL PLATFORM CHOICE

Antisense Oligonucleotides	siRNA	**Synthetic mRNA**
ssRNA	dsRNA	ssRNA
4,000-6,000 MW	13,000-16,000 MW	450,000-600,000 MW
14-20 nucleotides	two 22-27 nucleotide strands	1,500-2,000 nucleotides
Translation attenuation; RNase H based degradation	RISC based degradation	Gene expression
Often chemically modified	Also chemically modified	Typically un-modified

lots of phosphates

less Phospates
with phosphorodiamidate
morpholino oligomers

even more phosphates

Nucleic Acid Therapy – Bioanalysis

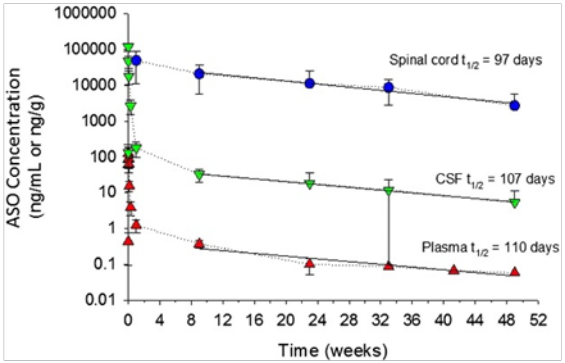
FEATURES OF KEY BIOANALYTICAL PLATFORMS

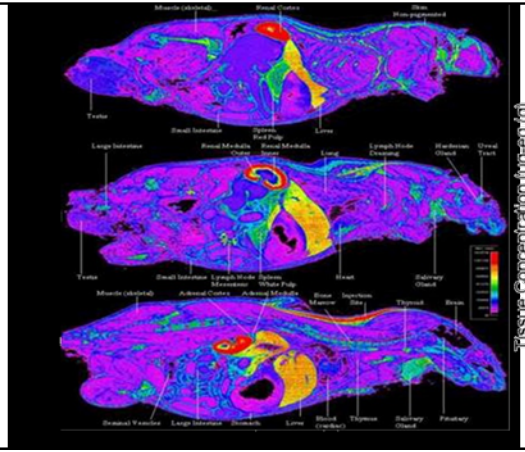
LC-MS	Hybridization ELISA or LC/FLD	qPCR
Mass	Specific hybridization target (<25 bases)	Specific hybridization target (>60 bases)
In matrix or SPE or LLE	In matrix or SPE	Extraction
Un-amplified	Enzymatic signal amplification (~106+)	Exponential signal amplification (~109+)
1 -10 ng/mL LLOQ	1 ng/mL LLOQ	50 copies LLOQ
ISR	ISR	-
Excellent specificity Truncated product detection	Good specificity Background possible	Excellent specificity

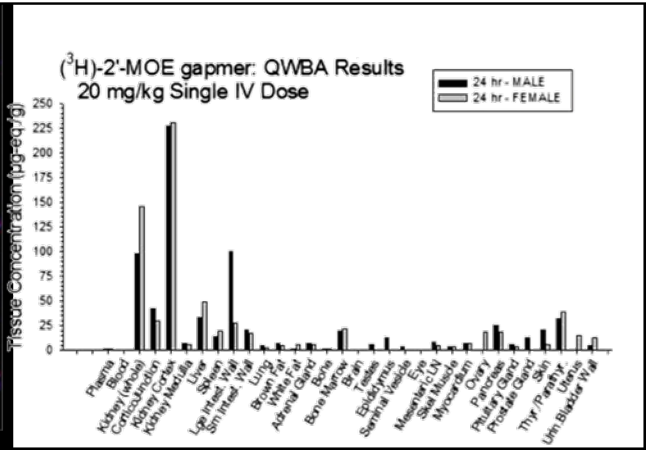
newest HRMS <1 ng/mL

Challenges

UNDERSTAND PHARMACOKINETIC AND PHARMACODYNAMIC







Geary *et al.* (2014), *Adv. Drug Delivery Reviews*, 87: 46-51

Bioanalytical methods for:

- PK
  - plasma
  - tissue distribution
  - potential renal excretion
- Biomarkers
  - endogenous small molecules in plasma and/or urine

Challenges

UNDERSTAND PHARMACOKINETIC AND PHARMACODYNAMIC

- Molecular challenges
  - Highly charged drug
  - Very polar biomarkers
- Describe plasma PK and tissue distribution
  - accurate and selective method: LC-MS for siRNA (GLP compliant HRMS quantitation)
  - a software solution to quantitate by summing multiple charge states and multiple isotopic forms
  - a robust ion-source to run thousands of samples for IND-/CTA-enabling studies without major cleaning
  - stable calibration for high sample throughput
  - metabolite identification
  - uniform resolution over a large mass range to accurately determine the mass of multiply charged parents and metabolites
- Biomarkers
  - accurate and selective bioanalytical platform (LC-MS or LBA)
  - exclusion/inclusion criteria (guidance and laboratory standards, CLIA)
  - primary end-point for decision making (validated methods, GLP)
  - fast turn-around

Bioanalytical strategy

APPROACHES TO NUCLEIC ACID QUANTITATION

Bioanalysis

- Plasma
- Tissues
  - liver, kidney, adrenal, thymus, brain, lung, heart, testis, jejunum, pancreas, spleen
  - urine and feces
- Immunogenity
  - Anti-drug Antibodies (ADA)

in vitro

- Metabolism
  - Metabolic stability
  - Reaction phenotyping
  - Profiling/identification
- Plasma
- Drug-Drug interaction (CYP450 up/down regulation)
- Cellular uptake/Distribution (Drug transport)

in-vivo

- PK/PD/tissue distribution studies
  - Single or multiple dose (rat, NHP)
- Toxicity studies
  - DRF and TK (rat, NHP)
  - Toxicity and TK (rat, NHP)
- Safety Pharma Studies
  - DRF and TK (rat, NHP)
  - Toxicity and TK (rat, NHP)
- Radiolabeled ADME Studies
  - Mass Balance
  - QWBA
- Metabolite ID/profiling

Oligonucleotides bioanalysis by HRMS

GENERAL EXPERIMENTAL SETUP

Workflow

Internal standard analogue

WAX SPE

NH_4OAc/AcN

NEXERA UHPLC

Acquity BEH C18, 2.1x50 mm 1.7 µm

(400 injections)

Ion pairing chromatography

H_2O/DIPA/HFIP and

H_2O/MeOH/DIPA/HFIP

(injection-to-injection 3-4 min)

TripleTOF 5600 / 6600

35,000 resolution

Full scan (10 ions x 2 charge envelopes)

sample stability
lysis buffer
homogenization

pH effect
drying

4-in-1 assay
↓adduct formation

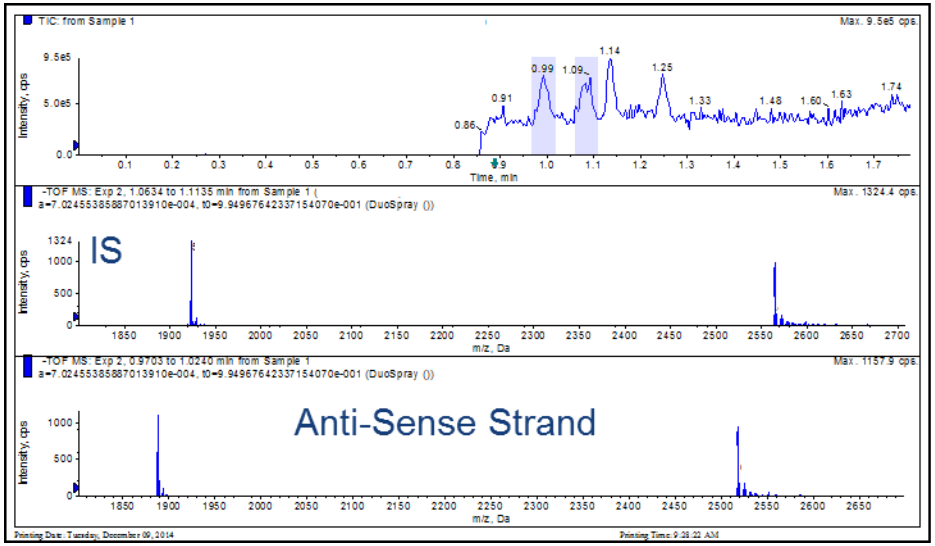
lot-to-lot difference in ion pairing reagents

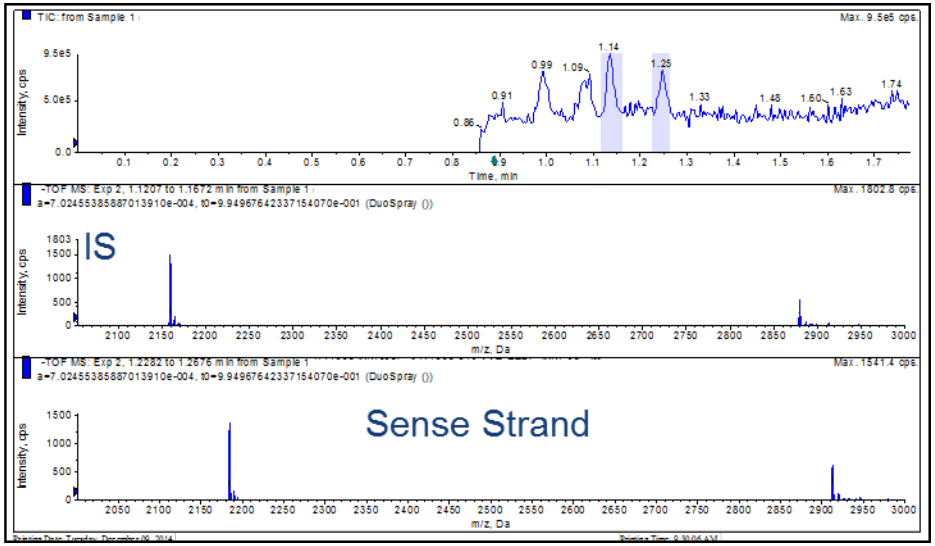
↑high mass ion transmission
↓multiple charge state

“Small Oligos” – UPLC-ToF Quantitation

DOUBLE STRANDED siRNA – CHROMATOGRAPHY (RAT PLASMA)

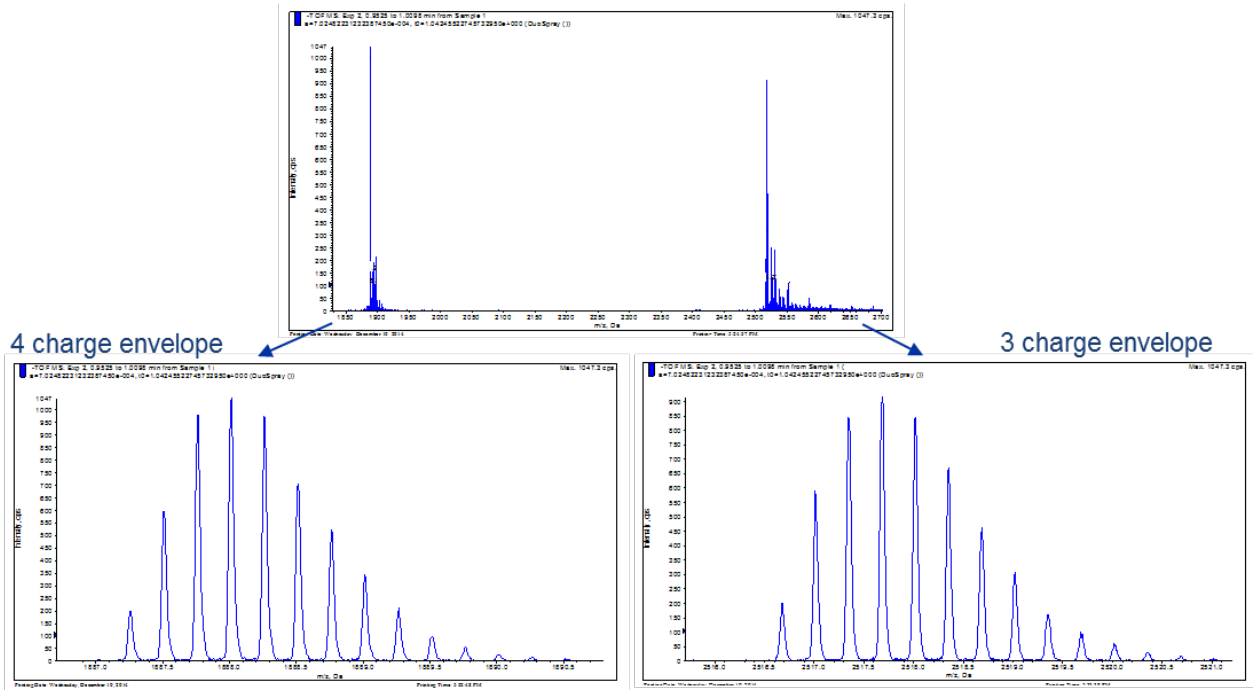
Monitoring multiple isotopic peaks of the different charge envelopes for anti-sense and sense strands





“Small Oligos” – UPLC-ToF Quantitation

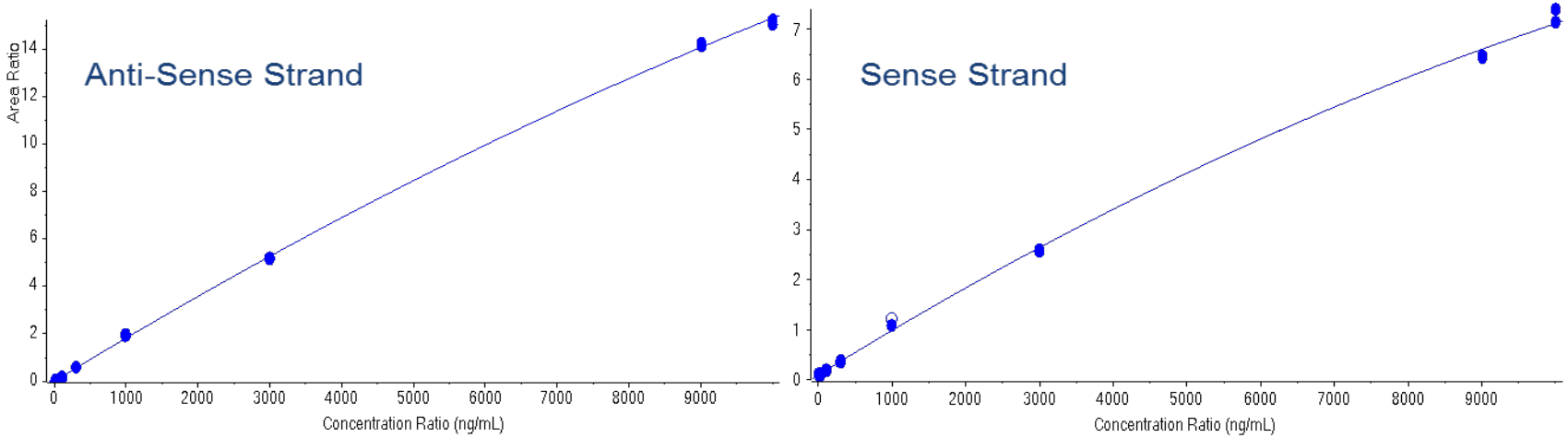
DOUBLE STRANDED siRNA – MASS SPECTRA



“Small Oligos” – UPLC-ToF Quantitation

CALIBRATION CURVES – Assay range 10-10,000 ng/mL

Assay Range ~ 10-10,000 ng/mL (5600), sub-1 ng/mL (6600+)

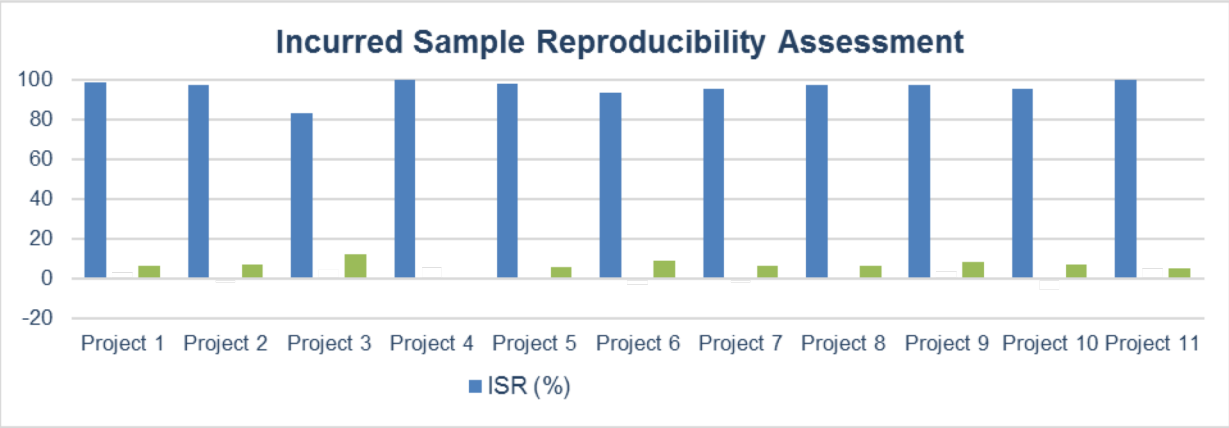


Species – mouse, rat, NHP, rabbit, and human
In vitro – plasma, microsomes, S9, hepatocytes, lysosome (tritosomes), CYP450
In vivo – plasma, urine, feces, tissues

“Small Oligos” – UPLC-ToF Quantitation

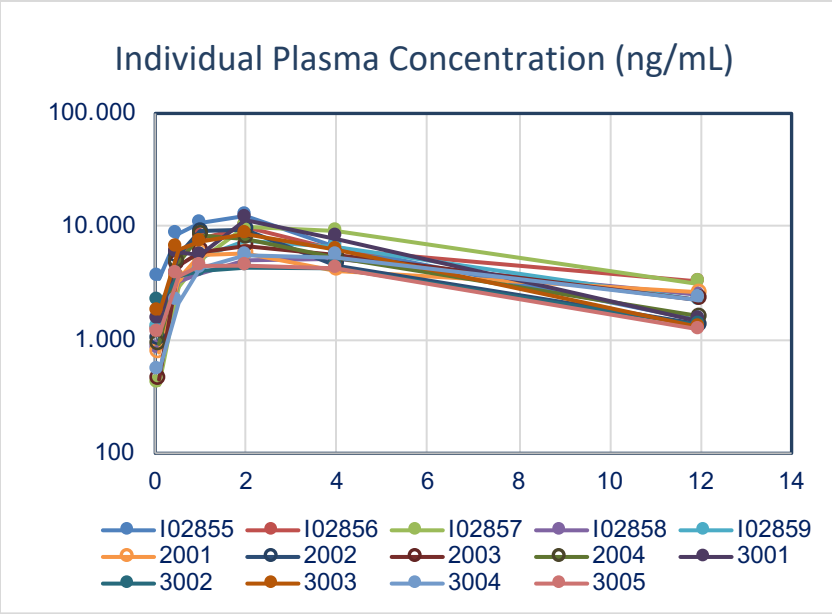
METHOD VALIDATION

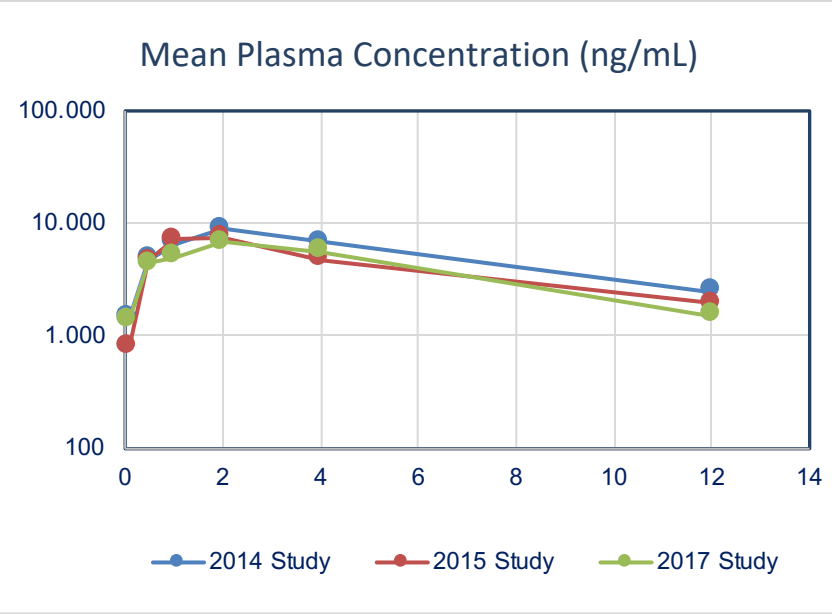
- Guidelines for chromatographic methods
- Small molecules acceptance criteria
- Plasma and Urine
- ISR
  - ±20.0% difference between the original result and the repeated analysis (2/3 of the ISR)



“Small Oligos” – UPLC-ToF Quantitation

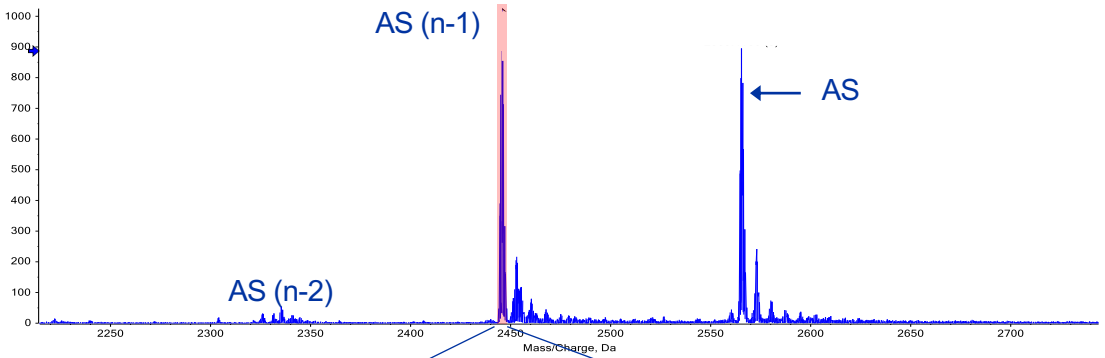
EXAMPLE OF SAMPLE ANALYSIS – 30 mg/Kg SC, DAY 1 PLASMA CONCENTRATIONS IN NHP

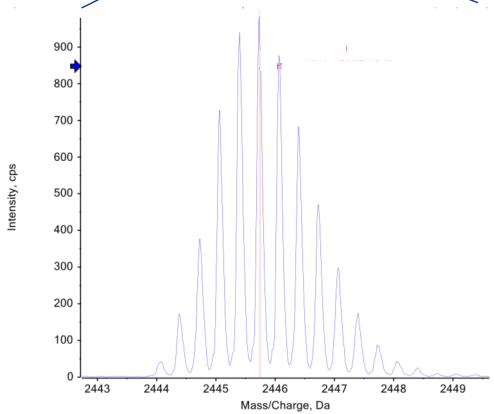




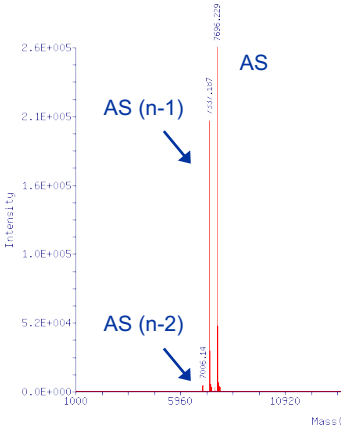
Metabolite ID for Oligonucleotides by HRMS

CONVERT ALL HIGH RESOLUTION SPECTRA TO AVERAGE OR MONOISOTOPIC MW





Deconvolution & Deisotoping
Simplifies Mass Spectrum



Deconvoluted mass spectra are match to a database with all possible metabolites

Identified metabolites are reviewed to ensure they make mechanistic sense and a metabolic pathway is proposed.

Metabolite ID for Oligonucleotides by HRMS

COMPARE ALL MASS SPECTRA TO A METABOLITE DATA BASE CREATED FOR THE TEST COMPOUND

Sequence Ladder Summary

RT (min)	Calculated Mass (Da)	Observed Mass (Da)	Intensity	Sequence
1.15	7368.9	7366.186	2.58E+004	AS(n-1)5’
1.06	5680.8	5677.963	7.84E+003	AS(n-6)5’
1.15	7009.6	7007.145	4.46E+003	AS(n-1)3’
1.26	1985.4	1982.432	8.99E+002	AS(n-17)3’
0.96	3973.6	3971.649	7.94E+002	S(n-9)3’+3'Phos
0.96	6092.0	6089.976	5.93E+002	AS(n-4)5’+5'Phos
0.96	5320.5	5317.884	4.26E+002	AS(n-7)3’
0.96	5760.8	5757.938	3.00E+002	AS(n-5)5’+5'Phos
0.96	6343.2	6345.612	2.96E+02	AS(n-4)5’

[^{14}C]Test Article Metabolic Pathway

5’- [Radio-labeled Anti-Sense Strand] -3’

(metabolites from the 5’ end)	(metabolites from somewhere in the middle)	(metabolites from the 3’ end)
AS(n-1)5’ (L, K) AS(n-2)5’ (L, K)	AS(n-8)3’ (U) AS(n-9)3’ (U) AS(n-10)3’(U)	AS(n-1)3’ (P) AS(n-2)3’ (P) AS(n-3)3’ (P)

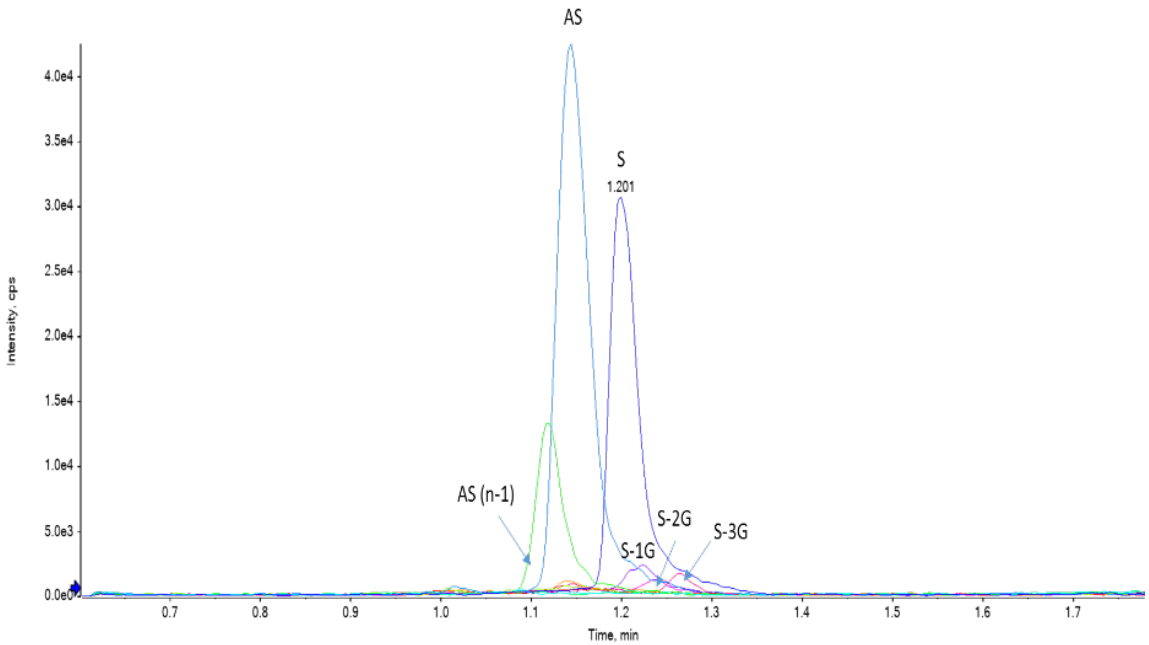
Metabolite ID

GENERATE XIC METABOLITE PROFILES AND QUANTITATION

XIC metabolic profiles are generated for each metabolite using ±0.7 da mass windows from the center of the most intense ion of the molecular ion cluster. This improves overall sensitivity.

Semi-Quantification can be performed for any metabolite, in any matrix, at any time point by radio-chromatography, or by comparison of relative ion count (MS response) to an authentic standard if available.

For precise “cold’ quantification, up to 10 of the most intense ions from the isotope clusters are integrated, across ±70 mDa windows to optimize signal-to-noise.



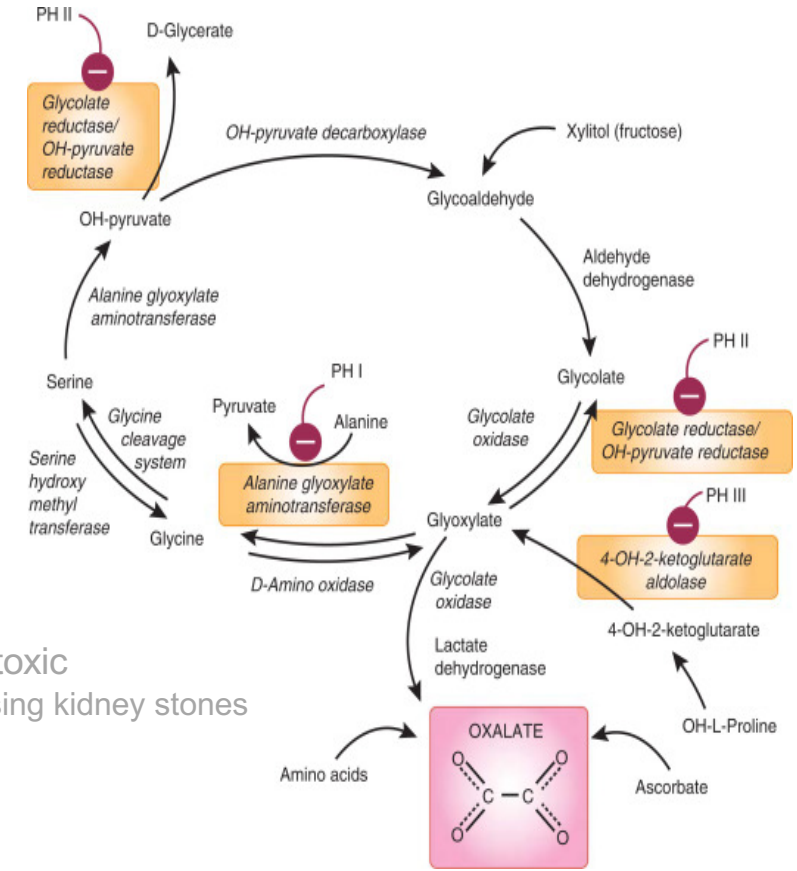
Biomarkers

DRUG EFFICACY AND PATIENT ELIGIBILITY

PRIMARY HYPEROXALURIA

Autosomal recessive disorder of glyoxylate metabolism

Excessive production of **glycolate** leading to urinary calcium oxalate (CaOx) supersaturation



High levels of **oxalate** are toxic

Accumulate in the kidney causing kidney stones

Biomarkers

DRUG EFFICACY AND PATIENT ELIGIBILITY

PRIMARY HYPEROXALURIA
Autosomal recessive disorder of glyoxylate metabolism

Excessive production of **glycolate** leading to urinary calcium oxalate (CaOx) supersaturation

PD (monitor drug efficacy)

- Patient eligibility
  - Inclusion exclusion criteria
  - Define early onset biomarkers to allow appropriate early treatment
  - Biomarkers for diagnose of disease state should be the same as the one used to demonstrate drug efficacy (primary endpoint)
- Typical LC-MS/MS to quantify metabolites (very small polar molecules)

Requirements and solutions

- Validated plasma, urine, and tissue assays
  - to selectively quantitate both the antisense and the sense strand
  - to understand metabolic clearance
- CLIA- and GLP-validated small molecule biomarker assays

- UPLC-HRMS workflow for siRNA quantitation and Met-ID
  - to support preclinical and clinical studies for the largest gene therapy trial to-date
- CLIA workflow for inclusion/exclusion criteria using LC-MS/MS biomarker monitoring that is more accurate, robust, and reliable than the current LBA assays used by physicians for patient inclusion/exclusion criteria

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