
# 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

# 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;<br>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

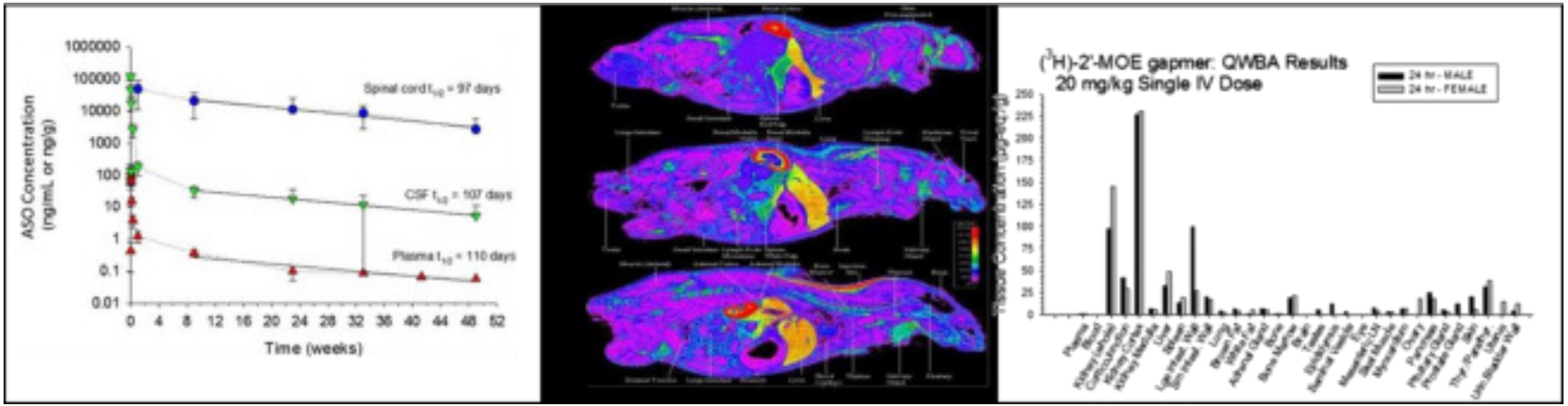
## FEATURES OF KEY BIOANALYTICAL PLATFORMS

| 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<br>Truncated product detection | Good specificity<br>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
  - High molecular weight
- 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*

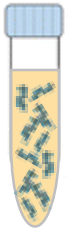
- PK/PD/tissue distribution studies
  - Single or multiple dose (rat, NHP)
- Toxicity studies
  - DRF and TK (rat, NHP)
  - Toxicity and TK (rat, NHP)
- Safety Pharma Studies
  - DRF and TK (rat, NHP)
  - Toxicity and TK (rat, NHP)
- Radiolabeled ADME Studies
  - Mass Balance
  - QWBA
- Metabolite ID/profiling

# 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

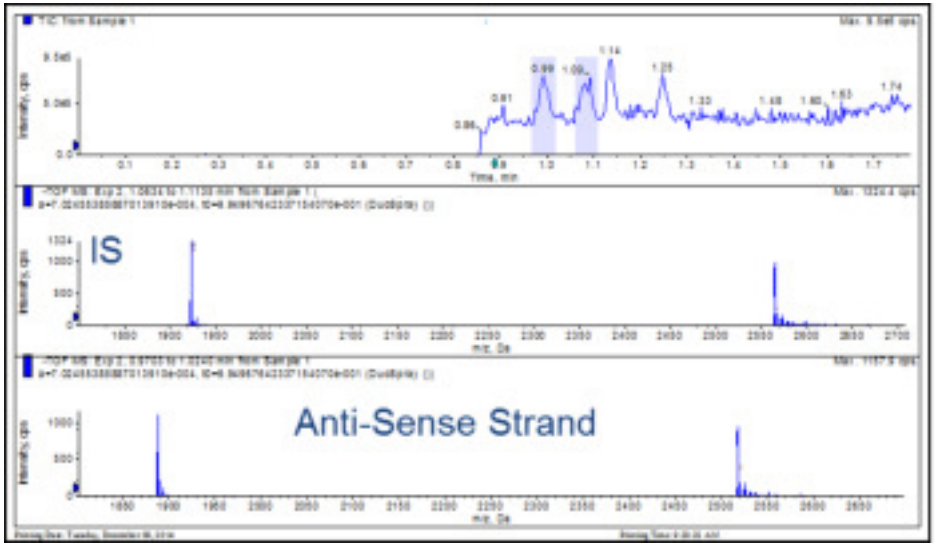
lot-to-lot difference in ion pairing reagents

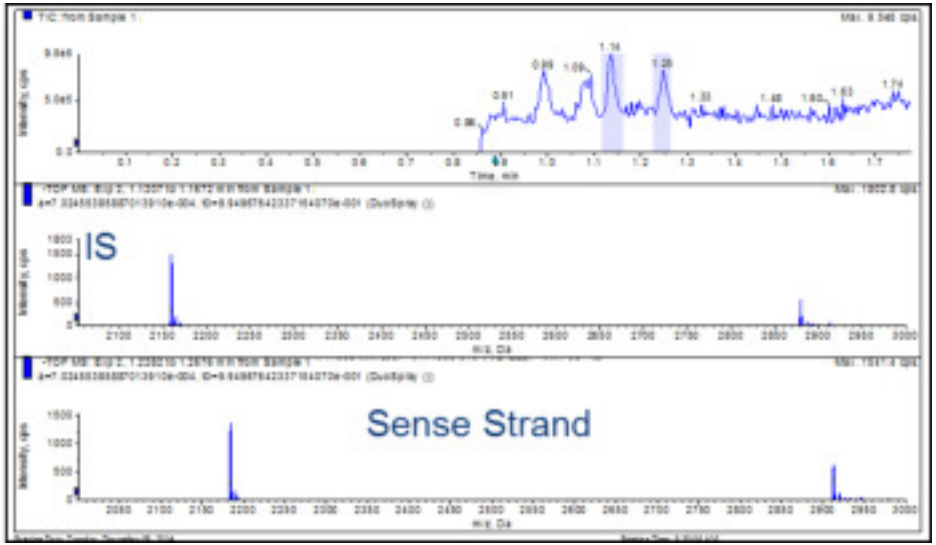
↑high mass ion transmission
↓multiple charge state

# “Small Oligos” – UPLC-ToF Quantitation

## DOUBLE STRANDED siRNA – CHROMATOGRAPHY (RAT PLASMA)

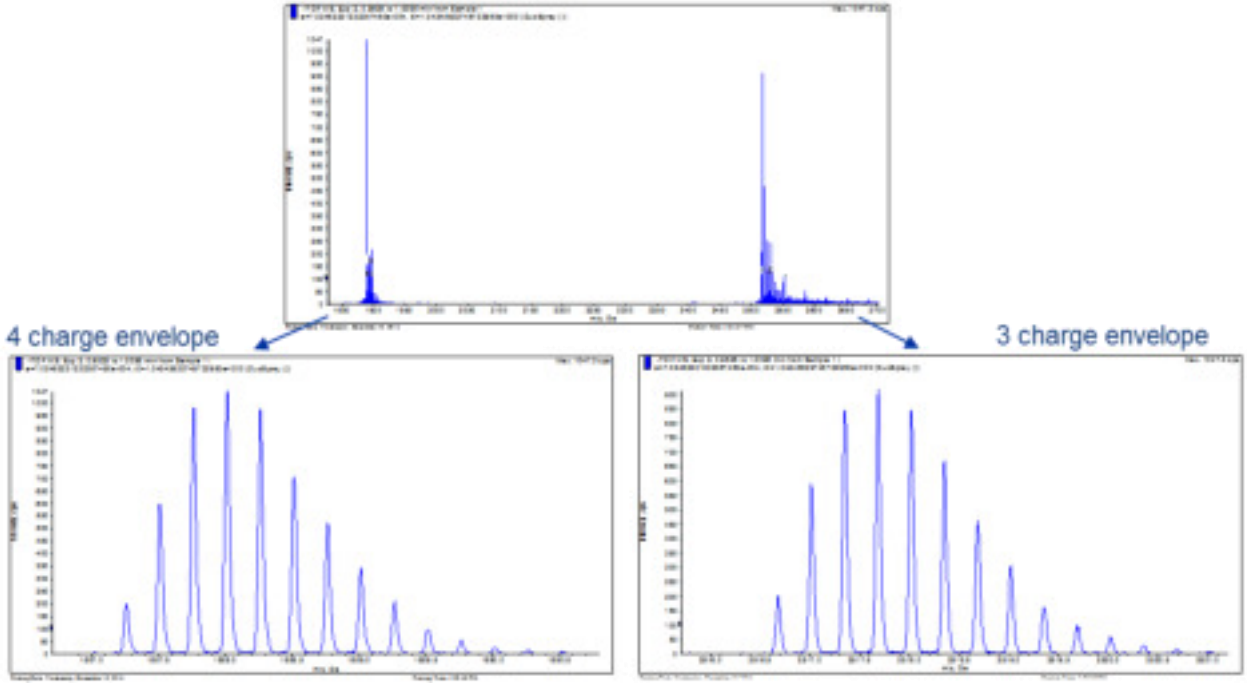
Monitoring multiple isotopic peaks of the different charge envelopes for anti-sense and sense strands



# “Small Oligos” – UPLC-ToF Quantitation

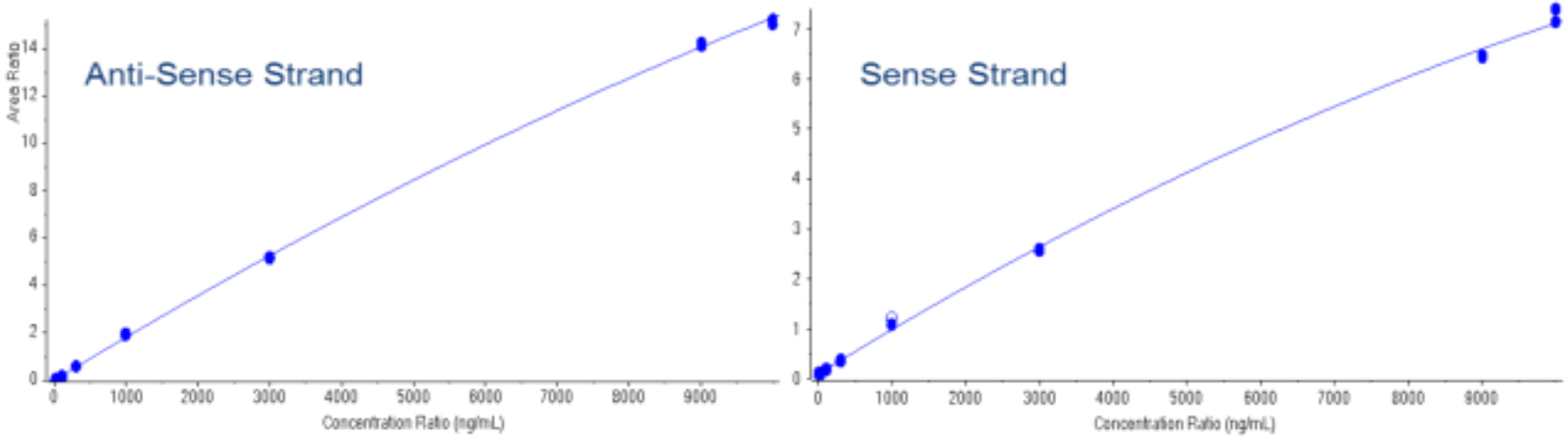
## DOUBLE STRANDED siRNA – MASS SPECTRA



# “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+)

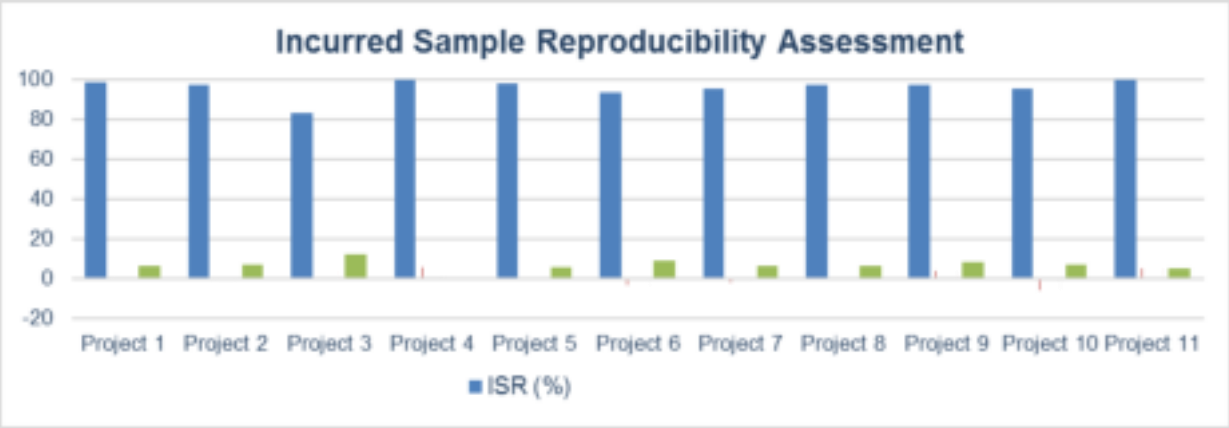



**Species** – mouse, rat, NHP, rabbit, and human
***In vitro*** – plasma, microsomes, S9, hepatocytes, lysosome (tritosomes), CYP450
***In vivo*** – plasma, urine, feces, tissues

# “Small Oligos” – UPLC-ToF Quantitation

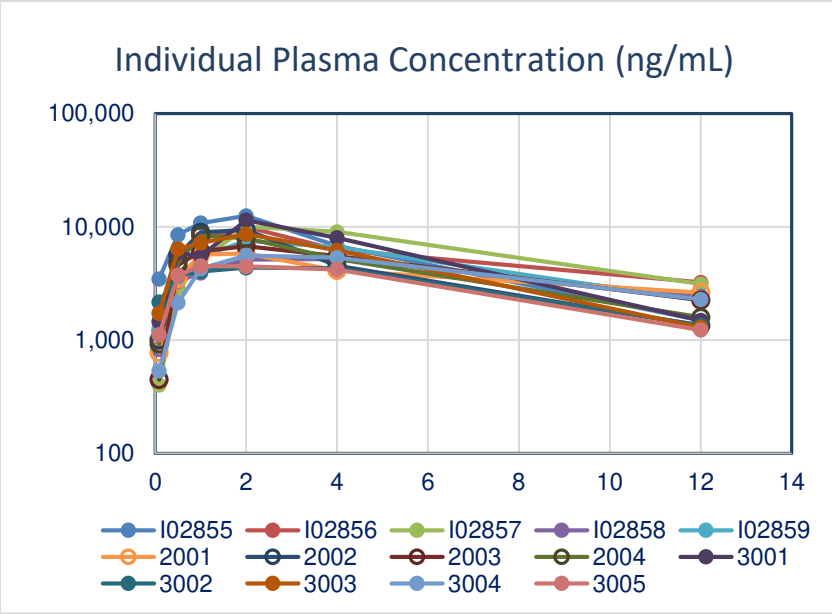
## METHOD VALIDATION

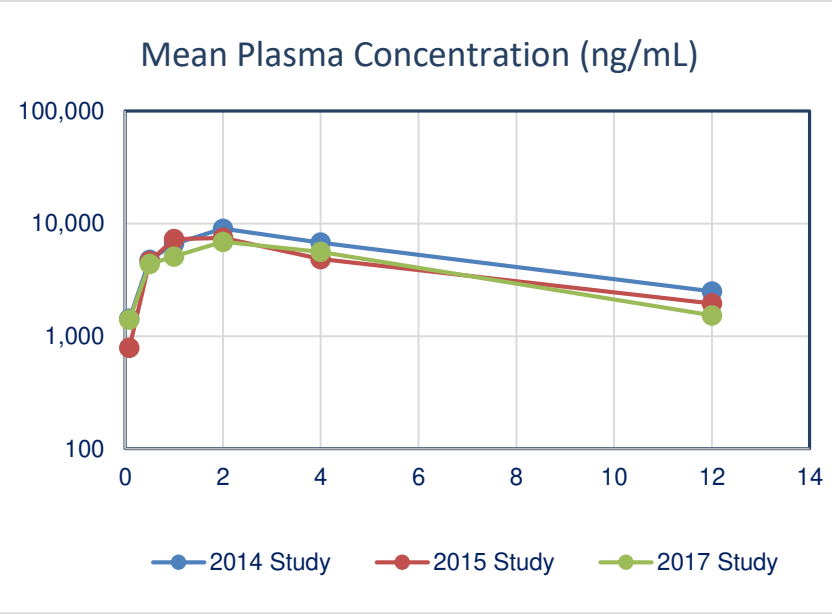
- Guidelines for chromatographic methods
- Small molecules acceptance criteria
- Plasma and Urine
- ISR
  - ±20.0% difference between the original result and the repeated analysis (2/3 of the ISR)



# “Small Oligos” – UPLC-ToF Quantitation

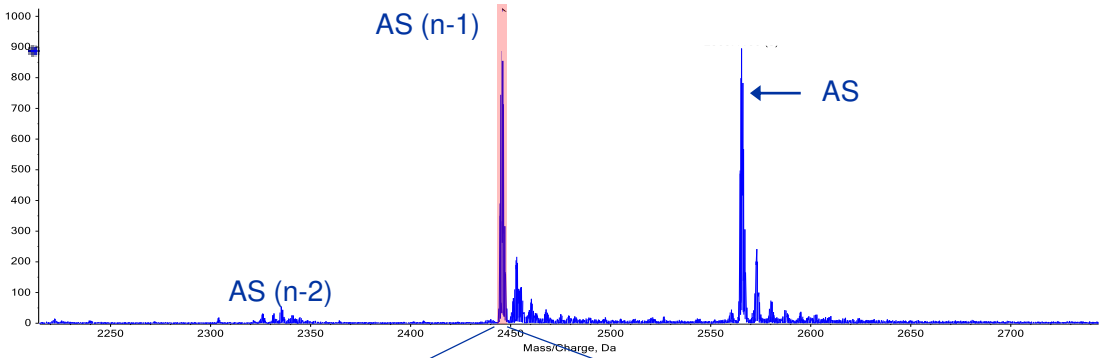
EXAMPLE OF SAMPLE ANALYSIS – 30 mg/Kg SC, DAY 1 PLASMA CONCENTRATIONS IN NHP

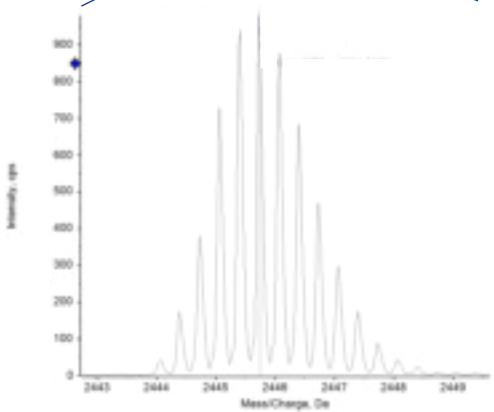


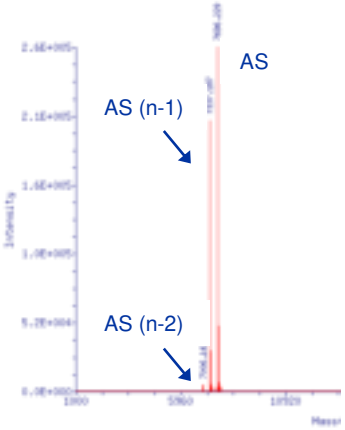
# Metabolite ID for Oligonucleotides by HRMS

## 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

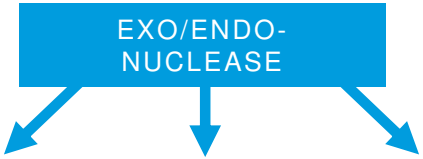
## COMPARE ALL MASS SPECTRA TO A METABOLITE DATA BASE CREATED FOR THE TEST COMPOUND

### 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)<br>AS(n-2)5’ (L, K) | AS(n-8)3’ (U)<br>AS(n-9)3’ (U)<br>AS(n-10)3’(U) | AS(n-1)3’ (P)<br>AS(n-2)3’ (P)<br>AS(n-3)3’ (P) |

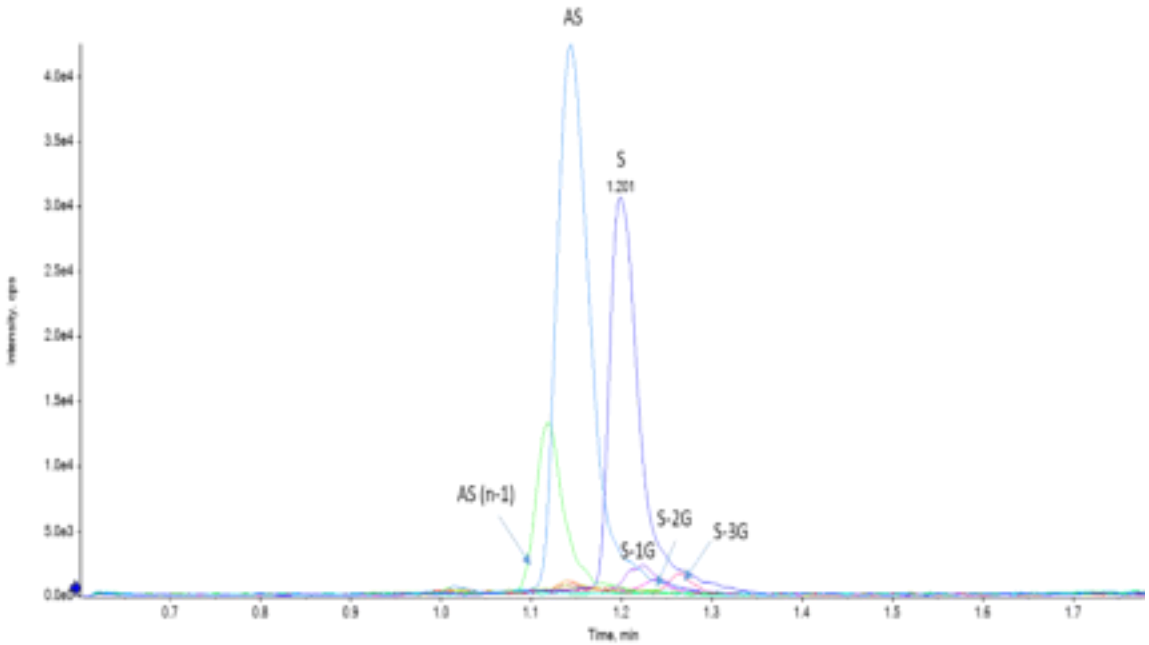
# Metabolite ID

## 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

## 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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