

Quantitation of siRNA and Metabolites in Plasma, Excreta, and Tissues by LC-QToF-MS in Regulated Studies

Daniel Schulz-Jander, Ph.D.



siRNA Quantitation by LC-QToF-MS

IND/CTA-ENABLING STUDIES FOR OLIGONUCLEOTIDES: ALL NEED BIOANALYTICAL DATA

▶ *In vitro*

- ◆ Metabolism
 - *Metabolic Stability / Clearance*
 - *Metabolite Profiling / Identification*
 - *Reaction Phenotyping*
- ◆ Plasma Protein Binding
- ◆ Drug-Drug Interactions
 - *Up / down regulation of drug metabolizing enzymes; Cellular Uptake/Distribution*
 - *CYP450 inhibition*
 - *CYP450 induction*
 - *Drug Transporters*

▶ *In vivo*

- ◆ PK / PD / Biodistribution Studies
 - *Rat Single and/or Multiple Dose*
 - *NHP Single and/or Multiple Dose*
- ◆ General Toxicity Studies
 - *Rat DRF & TK*
 - *Rat Definitive Toxicity & TK (4 weeks or 13 weeks)*
 - *NHP DRF & TK*
 - *NHP Definitive Toxicity & TK (4 weeks or 13 weeks)*
- ◆ Genetic Tox / Safety Pharma Studies
 - *Rat Micronucleus & TK*
 - *NHP CV & TK*

▶ Radiolabel ADME Studies

- ◆ Mass Balance / Excretion
- ◆ QWBA
- ◆ Metabolite Profiling/ID (plasma/tissue)



siRNA Quantitation by LC-QToF-MS

IND/CTA-ENABLING STUDIES FOR OLIGONUCLEOTIDES: ALL NEED BIOANALYTICAL DATA

▶ Bioanalysis

- ◆ Modifications (thiophosphorolate, PMO, cholesterol, GalNAc, lipids, ...)
- ◆ Species
 - Mice, rat, NHP, mini-pig, rabbit, human
- ◆ Matrices
 - Hepatocytes, cellular fractions
 - Plasma
 - Excreta (urine, cage wash, bile, feces)
 - Tissues (liver, kidney, adrenal, thymus, thyroid, brain, CSF, lung, heart, intestine, pancreas, spleen, testes, ovaries, ...)

▶ Immunogenicity / ADA

▶ Criteria

- ◆ GLP: plasma and 'critical' tissues
- ◆ Non-GLP: urine for excretion; tissues for biodistribution

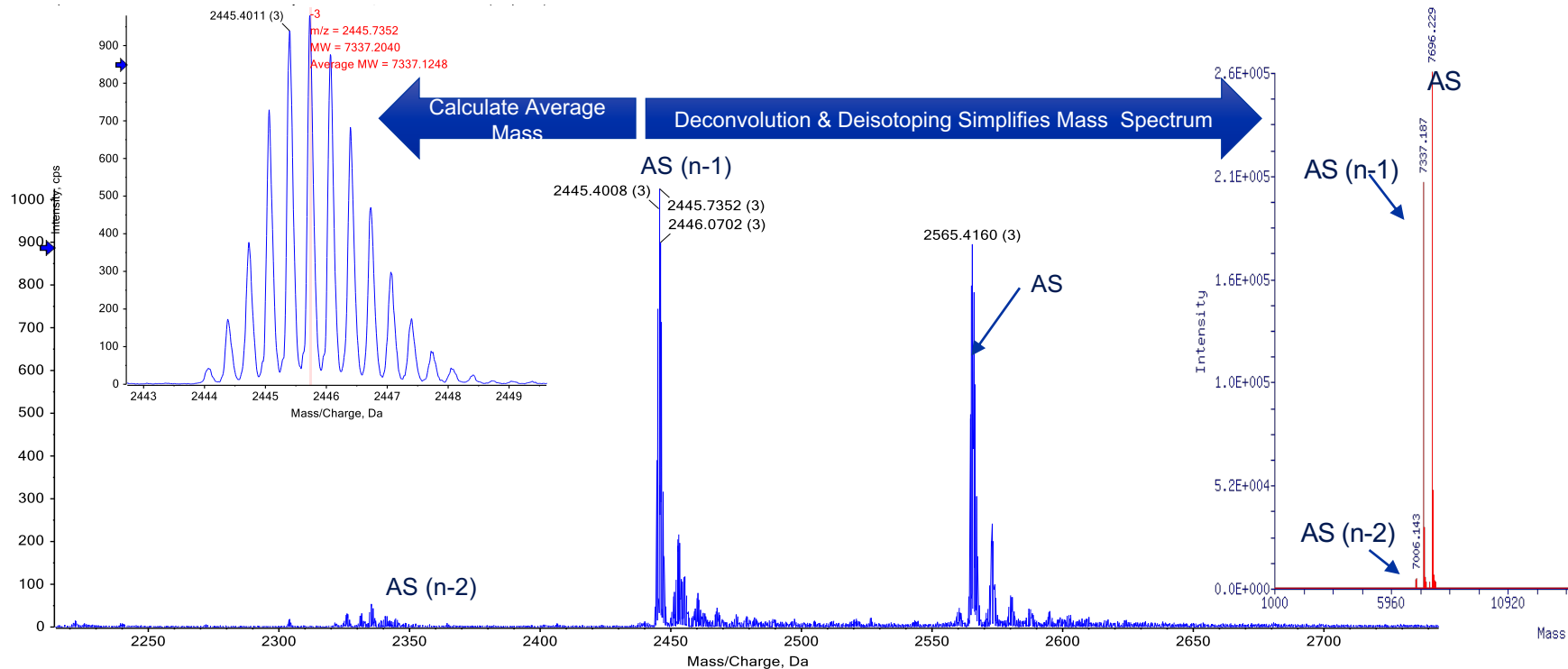
▶ Chromatography vs. Ligand Binding

- ◆ Perception
 - Hybridization ELISA more "sensitive"
 - hLC-Fluorescence or hLC-Mass Spec more "sensitive"
 - LC-Mass Spec more "specific"
- ◆ Reality
 - Mostly based on historical data & 'comfort'
 - Whatever works, driven by
 - Sensitivity
 - Tissue concentration usually high
 - Transferability from plasma to excreta and tissues
 - Metabolism
 - Nucleases (exo/endo) vs. oxidative deamination vs. glycosidases



siRNA Quantitation by LC-QToF-MS

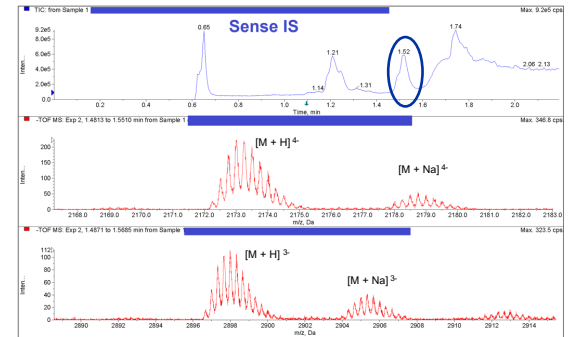
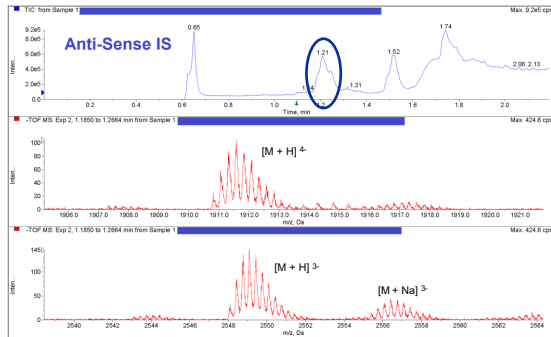
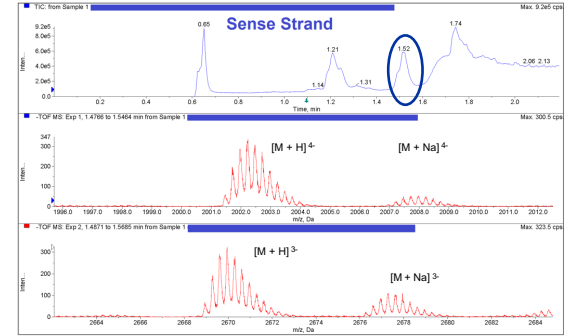
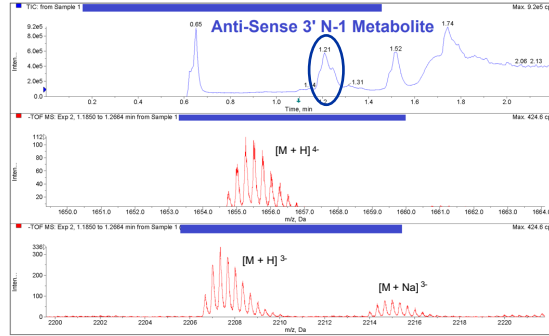
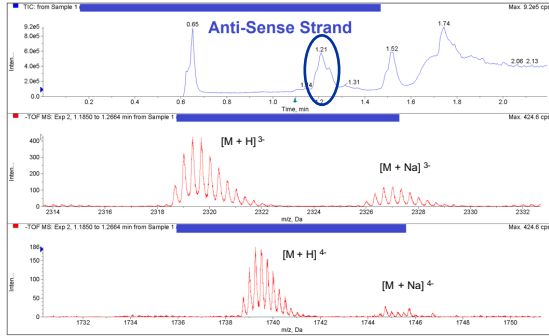
EXAMPLE: OLIGONUCLEOTIDES NEGATIVE ESI MASS SPECTRUM SHOWING AS³⁻ (PARENT, N-1, N-2)





siRNA Quantitation by LC-QToF-MS

EXAMPLE: TIC AND CORRESPONDING MASS SPECTRA OF ANTI-SENSE, AS (N-1), AND SENSE STRANDS IN HUMAN PLASMA





siRNA Quantitation by LC-QToF-MS

EXAMPLE: BACK-CALCULATED CONCENTRATIONS (NG/ML) OF ANTI-SENSE AND SENSE STRANDS IN NHP PLASMA

Run Date	Run ID	15.000	20.000	60.000	150.000	500.000	2000.000	6000.000	8000.000	Run ID	15.000	20.000	60.000	150.000	500.000	2000.000	6000.000	8000.000
xx-xxx-xxxx	1	13.190	21.499	63.400	162.819	494.371	1833.130	6051.825	8840.723	1	14.100	22.447	63.072	158.597	515.777	1926.201	6467.072	8219.630
		16.010	19.189	62.924	142.573	479.980	1929.086	5949.792	*EQB		14.391	19.804	61.203	139.332	494.451	1819.142	5422.217	8398.151
xx-xxx-xxxx	4	13.706	18.009	55.136	150.596	472.211	1920.926	5714.063	7891.184	4	14.454	21.150	*49.996	132.914	439.058	2275.491	5394.285	8041.336
		16.098	22.196	65.317	157.323	*597.066	2050.698	6467.582	*10881.741		*19.614	*25.661	*73.939	157.589	539.660	2013.296	6036.099	8261.483
xx-xxx-xxxx	5	13.823	20.158	53.143	162.026	465.587	1870.904	6246.336	7660.460	5	14.997	20.360	57.735	145.970	487.065	1808.463	5627.720	8861.397
		14.721	22.161	68.506	145.238	519.646	1917.488	5877.656	8632.701		14.626	19.940	64.921	159.280	512.670	2015.147	6036.734	7914.181
xx-xxx-xxxx	7	15.430	19.035	65.516	137.301	456.705	2084.381	5924.925	7514.660	7	15.344	18.897	66.989	142.975	453.329	*2366.524	6244.893	8087.479
		*21.413	19.645	59.398	165.348	525.127	1827.802	6149.786	8585.295		*20.058	19.864	*48.842	152.278	498.068	2128.488	5689.786	7896.502
xx-xxx-xxxx	8	15.440	18.312	*78.675	155.560	536.446	1859.304	5538.565	9072.964	8	15.790	18.902	62.930	154.305	505.504	2021.011	5457.217	7520.344
		15.279	19.867	62.363	162.480	453.524	1907.866	5638.443	8699.376		14.890	*24.656	53.405	166.449	443.645	2088.555	6265.643	8717.702
xx-xxx-xxxx	9	16.157	17.538	54.585	159.073	494.371	2147.200	5341.302	8927.331	9	16.263	22.920	65.339	149.972	447.172	1877.093	6254.040	8797.669
		15.023	21.083	57.331	148.016	533.255	1987.162	6059.684	7566.178		13.005	18.013	59.131	143.718	544.729	2100.018	5569.221	7427.324
xx-xxx-xxxx	10	12.658	20.738	54.048	164.472	455.721	1825.448	6277.228	8215.063	10	12.079	21.808	52.386	169.905	471.981	2073.515	5944.341	7916.064
		17.210	19.881	57.843	168.642	506.175	2073.168	5793.520	7861.672		17.236	20.653	51.027	168.150	470.315	2095.471	5893.342	8110.671
xx-xxx-xxxx	11	16.196	18.231	58.156	150.714	518.161	1985.653	5656.058	9197.344	11	13.895	22.000	54.774	135.517	516.381	2220.126	5981.032	7889.852
		15.499	19.076	56.852	160.686	502.269	2024.471	5954.051	7328.612		14.675	21.733	56.600	145.558	536.565	1978.200	6264.309	7558.646
xx-xxx-xxxx	12	13.988	18.684	60.059	143.666	517.027	2007.383	5837.624	8196.975	12	16.236	20.380	60.833	155.256	499.268	2032.258	5832.451	8196.927
		15.665	21.344	67.271	142.948	496.115	1916.690	5759.748	8425.254		14.216	18.772	60.431	139.397	492.449	2118.246	5839.825	7987.168
xx-xxx-xxxx	13	17.167	19.789	58.797	163.116	539.502	1957.882	6288.826	8040.715	13	14.409	19.211	64.773	164.305	531.033	1803.114	5757.264	8461.214
		14.751	17.100	53.156	172.247	451.351	1864.424	5939.785	*EQB		16.051	*15.287	51.654	155.523	*409.497	1825.336	6290.744	8356.597
xx-xxx-xxxx	14	15.702	20.743	59.252	153.623	517.606	1956.345	5689.468	7477.982	14	16.906	19.410	57.339	141.287	508.919	2086.446	5329.365	*6337.454
		14.887	18.259	*79.850	145.662	516.911	1940.035	6343.861	8567.735		15.074	17.573	*88.193	140.441	553.429	2111.285	6295.350	8155.387
Mean		15.171	19.661	59.653	155.188	497.717	1949.429	5931.824	8247.485		14.932	20.202	59.141	150.851	498.165	2019.852	5904.225	8132.177
S.D.		1.196	1.466	4.684	9.707	29.120	88.661	285.679	582.288		1.255	1.506	5.058	10.920	34.081	134.233	341.472	388.348
%CV		7.9	7.5	7.9	6.3	5.9	4.5	4.8	7.1		8.4	7.5	8.6	7.2	6.8	6.6	5.8	4.8
%RE		1.1	-1.7	-0.6	3.5	-0.5	-2.5	-1.1	3.1		-0.5	1.0	-1.4	0.6	-0.4	1.0	-1.6	1.7
n		21	22	20	22	21	22	22	19		20	19	18	22	21	21	22	21

EQB: Exceeds Quadratic Bounds

* Deactivated according to SOP QPS-BA-001



siRNA Quantitation by LC-QToF-MS

EXAMPLE: STANDARD CURVES OF ANTI-SENSE, AS (N-1), AS (N-2), AND SENSE STRANDS IN HUMAN PLASMA (LLOQ @ 5 ng/mL AND 1 ng/mL)

Calibration Curve Parameters for Anti-Sense Strand Calibration Standards in Human Plasma by LC-TOF-MS

Run Date	Run ID	A	B	C	R-Squared	Regression Footnote(s)
Day 1	36	-0.00000006	0.003819670	-0.011560698	0.9951	1
Day 2	30	-0.00000012	0.004052494	-0.009053454	0.9966	1
Day 3	34	-0.00000014	0.003581962	-0.002954172	0.9969	1
Day 4	37	-0.00000010	0.004465860	0.000321158	0.9924	1
Day 5	38	-0.00000016	0.003670701	-0.004392901	0.9926	1

Quadratic Regression with $1/x^2$ Weighting

Regression Footnote(s):

1) Resp. = A * (Conc.**2) + B * Conc. + C

Calibration Curve Parameters for Anti-Sense 3' N-1 Metabolite Calibration Standards in Human Plasma by LC-TOF-MS

Run Date	Run ID	A	B	C	R-Squared	Regression Footnote(s)
Day 1	36	-0.00000104	0.010317655	-0.005277962	0.9922	1
Day 2	30	-0.00000044	0.009893031	-0.002362463	0.9963	1
Day 3	34	-0.00000208	0.010450719	-0.002749375	0.9956	1
Day 4	37	-0.00000165	0.007991487	0.004228573	0.9935	1
Day 5	38	-0.00000313	0.012478697	-0.002909850	0.9922	1

Quadratic Regression with $1/x^2$ Weighting

Regression Footnote(s):

1) Resp. = A * (Conc.**2) + B * Conc. + C

Calibration Curve Parameters for Anti-Sense 3' N-2 Metabolite Calibration Standards in Human Plasma by LC-TOF-MS

Run Date	Run ID	A	B	C	R-Squared	Regression Footnote(s)
Day 1	36	-0.00000157	0.012459182	-0.007114476	0.9933	1
Day 2	30	-0.00000157	0.011743405	-0.004888467	0.9912	1
Day 3	34	-0.00000395	0.012705437	-0.002720330	0.9959	1
Day 4	37	-0.00000329	0.011105167	-0.002518838	0.9942	1
Day 5	38	-0.00000415	0.014417882	-0.002621136	0.9948	1

Quadratic Regression with $1/x^2$ Weighting

Regression Footnote(s):

1) Resp. = A * (Conc.**2) + B * Conc. + C

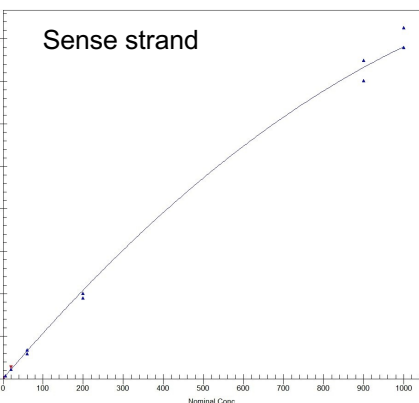
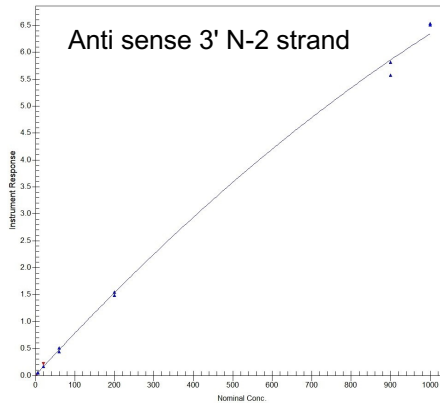
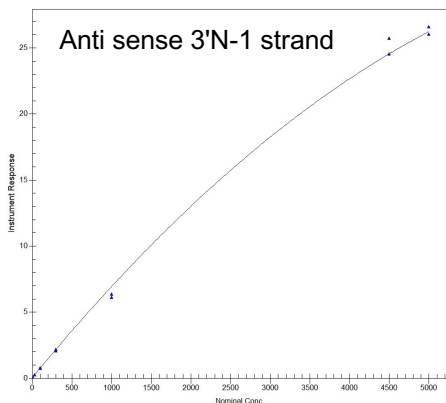
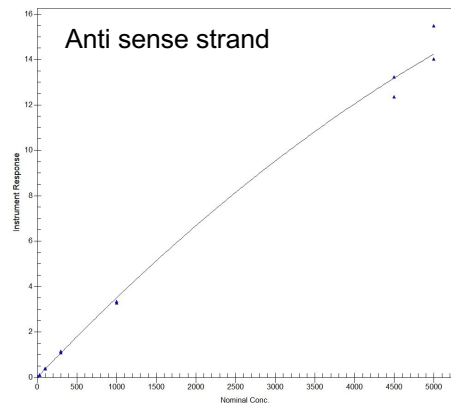
Calibration Curve Parameters for Sense Strand Calibration Standards in Human Plasma by LC-TOF-MS

Run Date	Run ID	A	B	C	R-Squared	Regression Footnote(s)
Day 1	36	-0.00000014	0.004089592	-0.007317355	0.9919	1
Day 2	30	-0.00000025	0.004474451	-0.003289805	0.9950	1
Day 3	34	-0.00000025	0.003762272	-0.003002574	0.9934	1
Day 4	37	-0.00000042	0.007331004	-0.002179958	0.9923	1
Day 5	38	-0.00000159	0.004356572	-0.004784018	0.9964	1

Quadratic Regression with $1/x^2$ Weighting

Regression Footnote(s):

1) Resp. = A * (Conc.**2) + B * Conc. + C





siRNA Quantitation by LC-QToF-MS

EXAMPLE: INTRADAY PRECISIONS & ACCURACY (ng/mL) OF ANTI-SENSE AND SENSE STRANDS IN HUMAN URINE

Run Date	Run No.	10.000 (ng/mL)	30.000 (ng/mL)	400.000 (ng/mL)	4000.000 (ng/mL)	8000.000 (ng/mL)
xx-xxx-xxxx	4	9.628	32.665	410.473	3500.527	8372.543
		8.904	#36.848	422.344	3895.790	8335.535
		10.737	31.481	420.543	3753.631	8679.257
		10.252	33.428	437.848	3643.109	8528.072
		9.227	33.474	437.373	3664.978	8026.947
		11.604	32.129	415.376	3762.311	7704.733
Intra-run Mean		10.059	33.338	423.993	3703.391	8274.515
S.D.		1.010	1.882	11.337	133.624	354.226
%CV		10.0	5.6	2.7	3.6	4.3
%RE		0.6	11.1	6.0	-7.4	3.4
n		6	6	6	6	6
xx-xxx-xxxx	2	9.415	#34.570	421.081	3727.745	7699.368
		9.195	33.034	#466.776	3630.197	8393.761
		9.564	34.089	435.561	3427.571	7518.592
		11.760	32.161	442.462	3816.636	7965.332
		10.497	32.815	438.999	3768.821	7941.758
		9.689	31.698	425.768	3711.253	8569.326
Intra-run Mean		10.020	33.061	438.441	3680.371	8014.690
S.D.		0.961	1.101	16.060	138.575	401.151
%CV		9.6	3.3	3.7	3.8	5.0
%RE		0.2	10.2	9.6	-8.0	0.2
n		6	6	6	6	6
xx-xxx-xxxx	6	10.304	32.292	428.389	3963.266	8739.975
		9.773	32.704	435.890	3978.822	8514.264
		10.900	#34.861	437.923	3651.129	9175.286
		9.908	29.756	424.322	3775.331	8701.066
		10.709	31.647	435.732	3573.929	#9314.492
		9.965	30.356	412.558	3772.263	8467.749
Intra-run Mean		10.260	31.936	429.136	3785.790	8188.805
S.D.		0.461	1.822	9.643	162.524	348.955
%CV		4.5	5.7	2.2	4.3	4.0
%RE		2.6	6.5	7.3	-5.4	10.2
n		6	6	6	6	6
Inter-run Mean		10.113	32.778	430.523	3723.184	8369.336
Inter-run S.D.		0.804	1.663	13.374	144.350	488.790
Inter-run %CV		8.0	5.1	3.1	3.9	5.8
Inter-run %RE		1.1	9.3	7.6	-6.9	4.6
n		18	18	18	18	18

[%RE] > 15% from Nominal

Run Date	Run No.	10.000 (ng/mL)	30.000 (ng/mL)	400.000 (ng/mL)	4000.000 (ng/mL)	8000.000 (ng/mL)
xx-xxx-xxxx	4	10.981	33.472	416.956	3533.206	8459.925
		11.775	##37.236	412.114	3718.112	8072.350
		11.285	31.996	420.292	3630.524	8249.649
		11.270	34.147	416.033	3467.150	7254.214
		11.981	33.626	424.252	3690.902	8049.408
		#12.257	32.436	427.404	3679.823	8728.610
Intra-run Mean		11.592	33.819	419.509	3619.953	8135.693
S.D.		0.490	1.853	5.634	99.236	501.622
%CV		4.2	5.5	1.3	2.7	6.2
%RE		15.9	12.7	4.9	-9.5	1.7
n		6	6	6	6	6
xx-xxx-xxxx	2	11.206	32.061	##463.803	3750.329	8557.748
		#12.083	34.292	458.008	3759.381	7255.836
		11.265	32.841	438.566	3970.782	8544.897
		11.856	32.200	450.040	4150.629	8697.330
		10.280	34.496	435.734	3844.580	##9475.690
		9.922	33.504	438.152	3718.987	7494.992
Intra-run Mean		11.102	33.232	447.384	3865.781	8337.749
S.D.		0.853	1.038	11.732	166.620	824.171
%CV		7.7	3.1	2.6	4.3	9.9
%RE		11.0	10.8	11.8	-3.4	4.2
n		6	6	6	6	6
xx-xxx-xxxx	6	11.553	31.286	421.248	3677.049	7974.568
		10.625	32.978	421.181	3725.791	7763.235
		10.573	33.677	431.090	3631.529	8636.794
		10.953	30.137	417.097	3685.080	8234.166
		9.790	32.248	416.578	3805.417	9076.507
		11.317	33.085	412.676	3910.882	7477.794
Intra-run Mean		10.802	32.235	419.978	3739.291	8193.844
S.D.		0.626	1.316	6.320	102.395	587.053
%CV		5.8	4.1	1.5	2.7	7.2
%RE		8.0	7.5	5.0	-6.5	2.4
n		6	6	6	6	6
Inter-run Mean		11.165	33.095	428.957	3741.675	8222.429
Inter-run S.D.		0.715	1.513	15.536	157.521	618.699
Inter-run %CV		6.4	4.6	3.6	4.2	7.5
Inter-run %RE		11.7	10.3	7.2	-6.5	2.8
n		18	18	18	18	18

[%RE] > 20% from Nominal

[%RE] > 15% from Nominal



siRNA Quantitation by LC-QToF-MS

PROCEDURE: LC-MS (HRMS OR MS/MS) METHOD

▶ Calibration Curve

- ◆ Depending the purpose and matrix availability
- ◆ Standalone curve – plasma (exposure), urine (total renal excretion), other matrices (potential safety concern)
- ◆ ‘Surrogate’ curve – rare matrices from ‘higher’ species
 - *Plasma curve as surrogate matrix, if possible*
 - CSF
 - Tissues for biodistribution studies
 - Specific mouse strain [C57BL6 vs. CByB6F1-Tg(HRAS)2Jic] for carcinogenicity studies
- ◆ Part of method development process; whatever matrix that provide the best surrogate curve

▶ Sample Preparation

- ◆ Feces and tissues need homogenization followed by lysis
 - *Very laborious involving two people in parallel*
- ◆ Extraction
 - *Sample volume: 25µL to 100µL recon into 100µL or 200µL*
 - *Phenomenex Clarity OTX SPE 100 mg/96-well plate (mixed mode SAX)*
 - *Modified protocol, e.g., no NaN₃, ..., ...*
 - *Automated, still very laborious 2.5 – 3 hours procedures with multiple conditioning, wash, and elution steps*
 - *Recovery: ≥70% ↔ ≤95%*



siRNA Quantitation by LC-QToF-MS

PROCEDURE: LC-MS (HRMS OR MS/MS) METHOD

▶ Chromatography

- ♦ Run-time usually ≤ 5 min injection-to-injection
- ♦ Column temperature $\geq 70^\circ\text{C}$
 - *ds 'Oligos' denature on column to Anti-Sense and Sense strands ($r_t \text{ AS} < r_t \text{ S}$)*
- ♦ Column
 - *C18 2.1 mm x 50 mm 1.7 μm 130Å fully porous*
 - *Column life: 300 – 400 injections*
 - *300Å for specific applications, e.g., AOC / POC*
- ♦ Ion Pairing Reverse Phase Chromatography
 - *Initial @ ~10% MeOH to final ~90% MeOH depending on the Sense strands*
 - *Ion Pairing reagent: DIPA, HFIP*
- ♦ Injection Volume: 3-10 μL

▶ Mass Spectrometry

- ♦ IS: Analogue or Stable Isotopically Labeled
 - *Analyte AS & S & analogue AS & S*
 - *Cation adducts:*
 - $\text{H}_n\text{Na}_0\text{K}_0 \rightarrow \text{H}_{n-1}\text{Na}_1\text{K}_0; \text{H}_{n-1}\text{Na}_0\text{K}_1 \rightarrow \text{H}_{n-2}\text{Na}_2\text{K}_0; \text{H}_{n-2}\text{Na}_1\text{K}_1; \text{H}_{n-2}\text{Na}_0\text{K}_2 \rightarrow \dots$
 - *Analogue IS: Potential overlapping isotopic mass*
 - *SIL-IS: expensive, control isotopic overlap?*
- ♦ Mass Resolution (Theoretic vs. Operating)
 - *Triple Quad (unit resolution)*
 - *QTOF (uniform resolution; ~40K) vs. Ion trap (mass dependent; $\geq 120\text{K}$ @ 200 amu)*
 - *UPLC peak width @ 3-6 seconds; with 12+ data-point operating resolution @ ~35K*



siRNA Quantitation by LC-QToF-MS

PROCEDURE: LC-MS (HRMS OR MS/MS) METHOD

▶ Tripe Quadrupoles

- ◆ -ve ion mode
- ◆ LLOQ: ≤ 2 ng/mL
 - *100 μ L sample volume, 100 μ L recon, 3 μ L – 5 μ L injection*
 - *Sub-1 ng/mL (if higher sample volume, lower recon, higher volume injection)*
- ◆ Assay range: 3 orders of magnitude
- ◆ LC-MS/MS
 - *Q1 @ higher charge envelope, e.g., M^9*
 - *Q3 @ lower charge envelope (similar to peptides MS/MS), mostly <400 m/z*
- ◆ Data Intensity
 - *Two 96-well plate: 2MB*

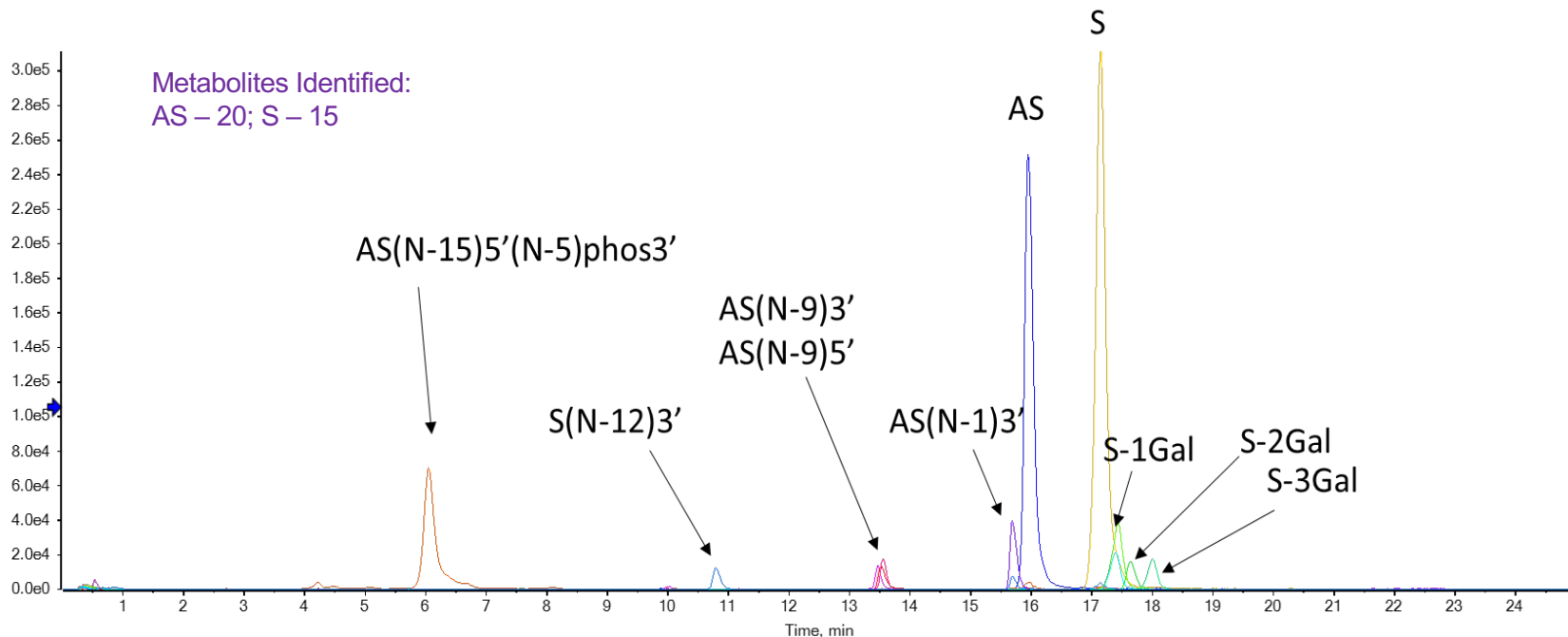
▶ QTOF

- ◆ -ve ion mode
- ◆ LLOQ: ≤ 5 ng/mL
 - *100 μ L sample volume, 100 μ L recon, 3 μ L – 5 μ L injection*
 - *Sub-1 ng/mL (if higher sample volume and lower recon, higher volume injection)*
- ◆ Assay range: 3 orders of magnitude
- ◆ Full scan LC-HRMS
 - *Quan and Qual*
 - *Sum multiple isotopic mass @ lower charge state, e.g., M^4 and M^3*
- ◆ Data Intensity
 - *Two 96-well plate: 3GB*



siRNA Quantitation by LC-QToF-MS: Power of LC-HRMS

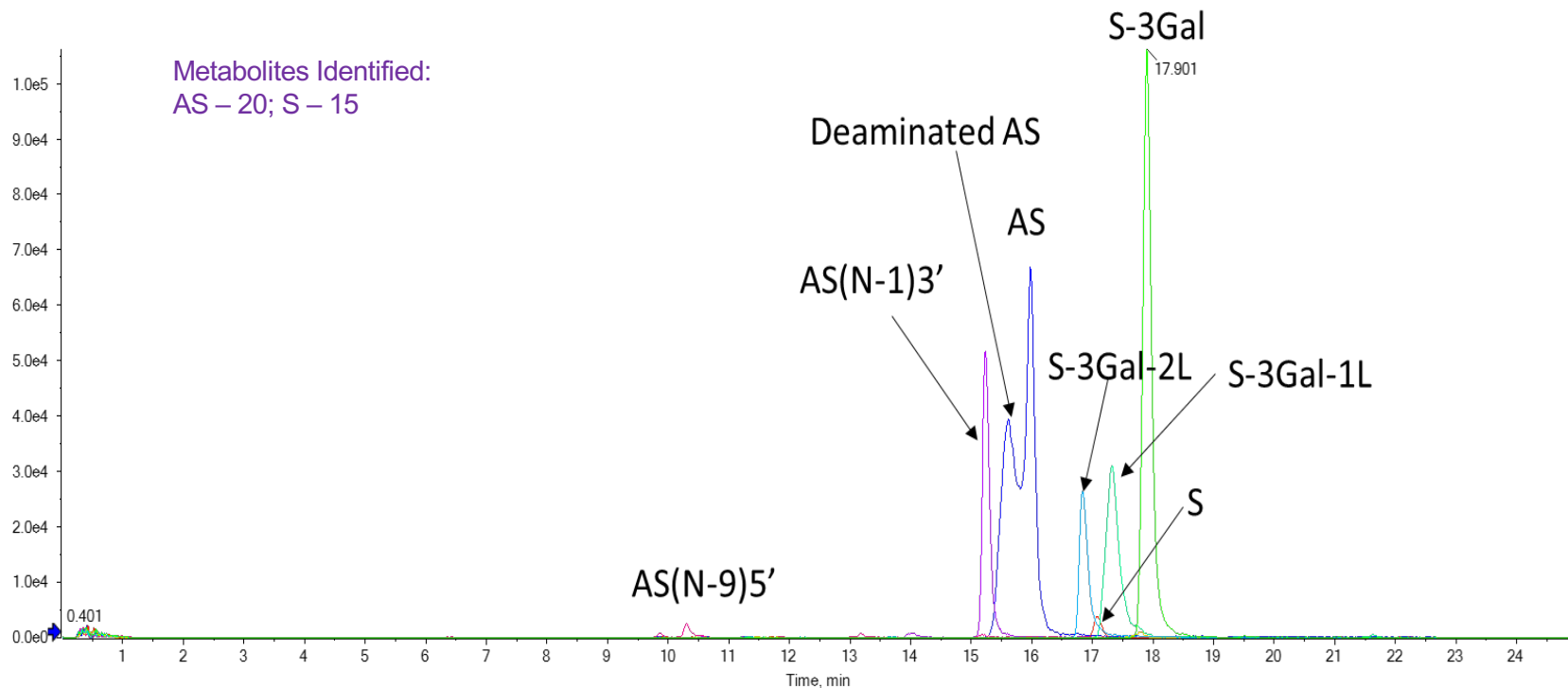
EXAMPLE: RECONSTRUCTED XIC OF siRNA METABOLISM VIA NUCLEASES & GLYCOSIDASES...





siRNA Quantitation by LC-QToF-MS: Power of LC-HRMS

EXAMPLE: RECONSTRUCTED XIC OF siRNA METABOLISM VIA OXIDATIVE DEAMINATION

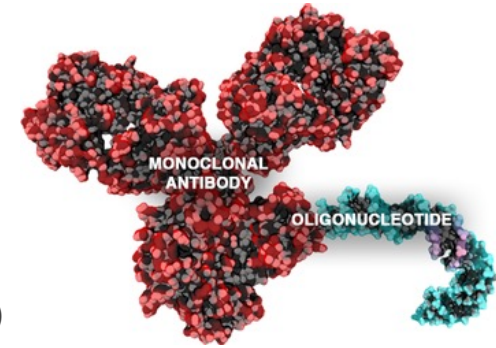




siRNA Quantitation by LC-QToF-MS

ANTIBODY/PROTEIN OLIGONUCLEOTIDE CONJUGATES

- ▶ Immuno pulldown (with antibody to protein/antibody)
 - ◆ Protease digestion
 - ◆ Quantitation of *signature peptide* reflective of the 'total' Protein/Antibody
 - Typical reverse phase workflow
 - ◆ Quantitation of *oligonucleotides* reflective of the original POC
 - Typical ion-exchange / ion-pairing workflow
- ▶ Immuno pulldown (with complementary strand/antibody to oligonucleotides)
 - ◆ Protease digestion
 - ◆ Quantitation of *oligonucleotides* reflective of the original POC and 'free' oligonucleotides
 - Typical ion-exchange / ion-pairing workflow



Coat

Capture

Digest

Liquid Chromatography

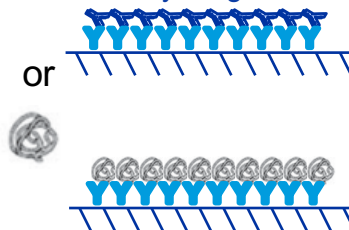
Mass Spectroscopy

Y

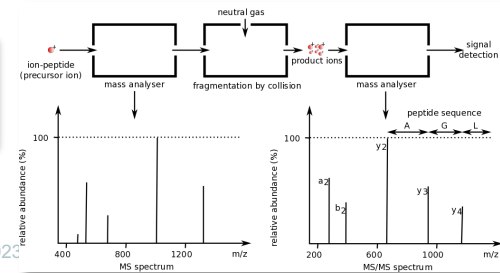
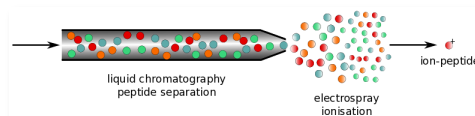


Magnetic beads are coated with antibody to antibody or protein target.

Y Antibody drug



digestion into peptides





siRNA Quantitation by LC-QToF-MS

ACKNOWLEDGEMENT

▶ QPS Delaware, USA

- ◆ Amelie Chen
- ◆ Diane Grotz
- ◆ Haotong Chen
- ◆ Jay Su
- ◆ Jiyi Wang
- ◆ Lan Li
- ◆ Lata Venkatarangan
- ◆ Rick Hamler
- ◆ Tim Snow
- ◆ Vicki North
- ◆ Yongdong Zhu
- ◆ Zamas Lam
- ◆ Zihua Yang

▶ QPS Netherlands

- ◆ Eric van de Horst
- ◆ Benjamin Steenge