Bioanalytical Strategies for Therapeutic Drugs and Their Conjugates (ADCs, Oligos, siRNA, ASOs and AOCs/ARCs) by QqQ/HRMS

Strategies and Case Studies

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AAPS Pharm Sci 360

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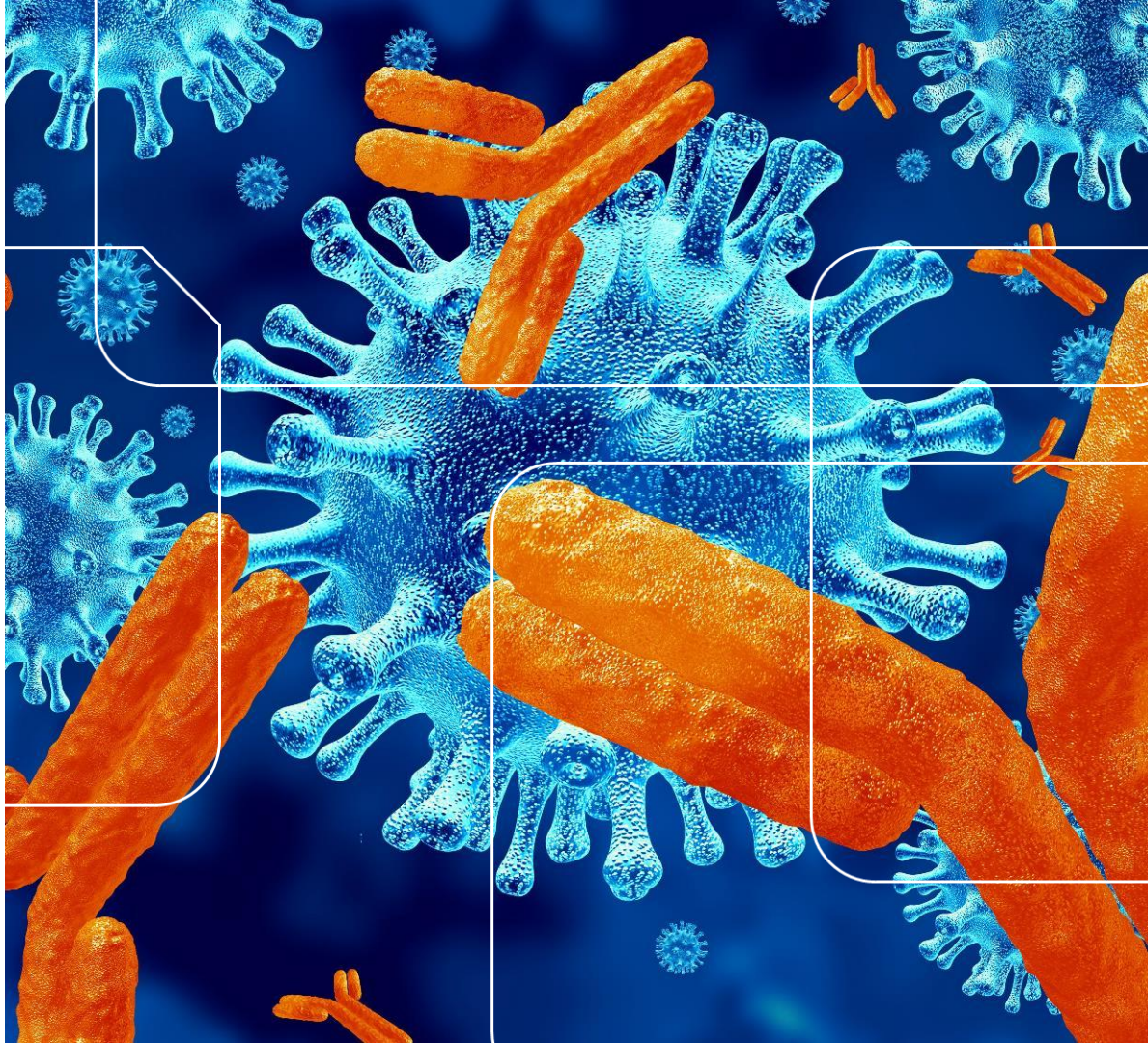
Kcas bio

SCIENCE ACCELERATED

CRO Capabilities Overview



Experience You can Trust

KCAS Bio is an established bioanalytical **CRO with over 45 years of experience** in biomarker and bioanalytical solutions.

Global Footprint

~400 experts dedicated to your bioanalytical & biomarker success operating in purpose-built facilities. We partner with our clients to help them with their drug development process around the globe.
We like solving problems **together.**

Method Development to Routine Testing

Discovery to Product Registration
Support all along the way

Matrices
Biofluids, Tissues, Cells

All Modalities
Large Molecules, Biologics, Small Molecules, Cell & Gene therapy, Vaccines

Non-Regulated / Regulated
GLP & GCLP Compliant

Core Scientific Expertise
KCAS Bio has decades of experience working with all key therapeutic areas and technologies

Services:
Full CRO Solution Suite

KCAS Bio brings full suite CRO solutions combining scientific expertise, technical innovation, and the highest standards of regulatory rigor.

- PK / TK Biopharmaceutical
- PK / TK Pharmaceutical
- Biomarkers – Soluble
- Biomarkers – Cellular
- Flow Cytometry Services
- Immunogenicity
- Biodistribution
- Dose Formulation Analysis
- Sample Kitting & Central Lab
- Molecular Services

Technology: KCAS Bio Leading the Way

Cutting-edge platforms and instrumentation that accelerate the discovery and development of life-changing drugs.

LBA

- ELISA
- MSD
- Luminex
- ELLA
- ELISPOT
- Lumipulse
- Simoa HD-X
- SMCxPro
- NULISA

LC / MS

- LC / MS / MS
- HPLC
- Hybrid MS / LBA
- LC / UV Fluorescence
- HRMS

Flow Cytometry

- Spectral Flow
- Conventional Flow
- Cell Sorting
- Validated Panels
- Backbone Panels

Molecular

- qPCR
- ddPCR
- DNA & RNA Assays
- Automated Extraction
- Automated Sample Prep





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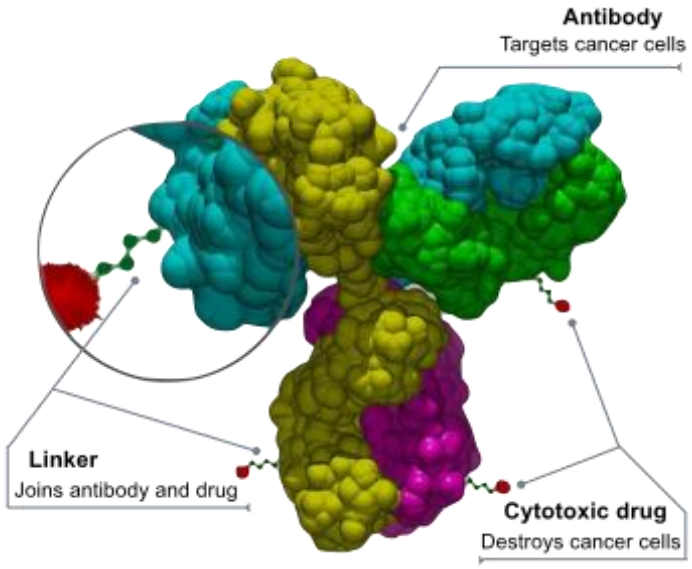
Antibody Drug Conjugates

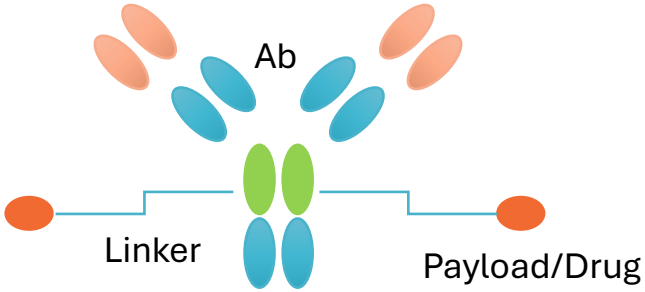
Background and Case Studies

Antibody Drug Conjugate



3 Main Parts of ADC

- Ab – directed against specific tumor antigen
- Drug
  - Payload or Cytotoxin
  - SiRNA
  - Other
- Linker – Cleavable or Uncleavable



Purpose: Develop various conjugated modalities to help aid in drug delivery, development, efficacy etc.

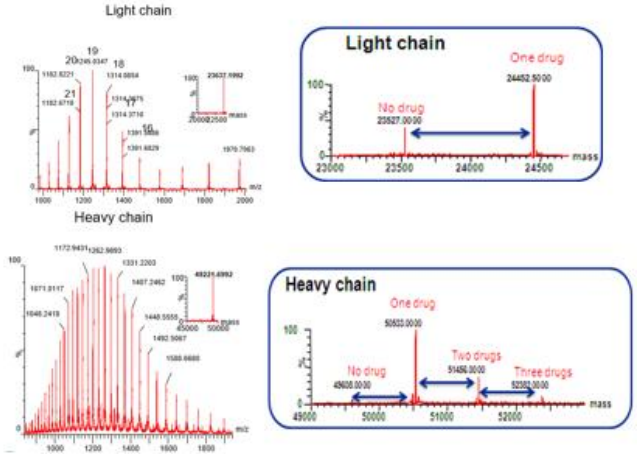
- Adding large molecules to increase half-life
- Adding toxins to targeted delivery (ADC) to minimize systemic exposure to toxic materials (Chemo)
- Adding caps/Abs to help protect stability etc (ARCs)

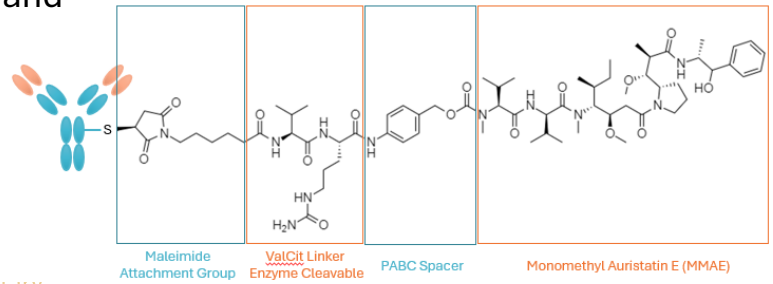
Types of ADCs

- Toxin
- siRNA/Oligo/ASO
- Peptide
- Radio isotope
- Degraders

Lexicon and Definitions

- **Total Ab** (TAb) – Antibody with or without any payloads LCMS or LBA
- Naked Ab – Ab with DAR = 0 (no Payload)
- **ADC** – Antibody Drug Conjugate – Ab with DAR >0
  - Conjugated payload – DAR sensitive (released payload) LCMS
  - Conjugated antibody – DAR insensitive (IP Payload, detect Ab) LCMS or LBA
- Payload – microtublin inhibitors (MMAE, mertansine-DM1), DNA Binders (calicheamicin, SN-38, Exatecan, camptothecin)
  - **Free Payload** LCMS
  - Total Payload
- DAR – Drug to Antibody Ratio – how many drugs are on the Ab (indicates level of drug loaded)
  - HRMS – characterization
- Linker – attaches payload to Ab (should be stable in circulation and only release payload at target)
  - Cleavable – Cathepsin, papain – Release Free Payload
  - Non-cleavable – less BioA options, more stability?
    - Release active payload-linker-amino acid moieties
    - May need Biotransformation to ID metabolites

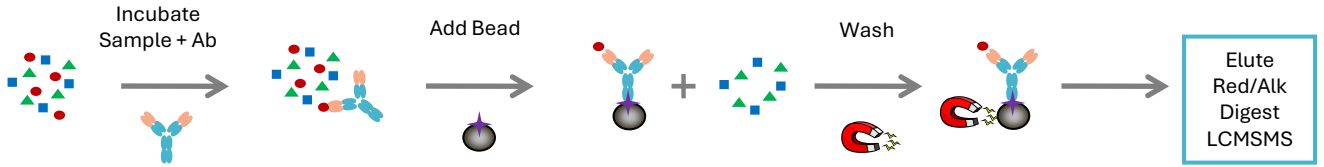




Principles of Hybrid LC-MS

- Definition: Hybrid LCMS is a technique which combines an enrichment step (typically an antibody enrichment on beads or columns) with the selectivity and sensitivity provided by LC-MS/MS
- Various surrogate peptides can be chosen for detection depending on needs

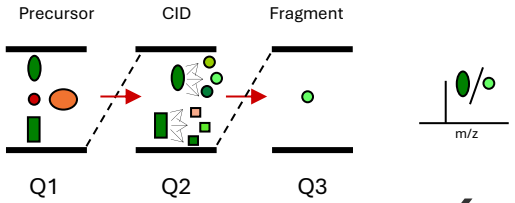
Capture 1.



Detector 2.

- MS detector allows one to "dial" in selectivity
- Only need 1 Ab/capture reagent

Multiple Reaction Monitoring



ADC – Strategies

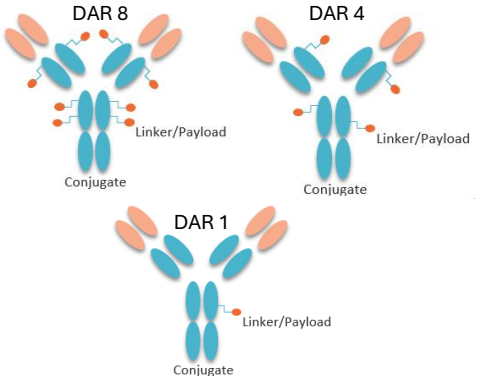
Hybrid LCMS

Total or Naked Ab
ADC
Free Payload
“Released” Payload for cleavable linkers

- Strategy is determined by conjugation and linker chemistry as well as what information is needed and available reagents

Strategy 1

- IP with Anti-Payload
- Measure IgG

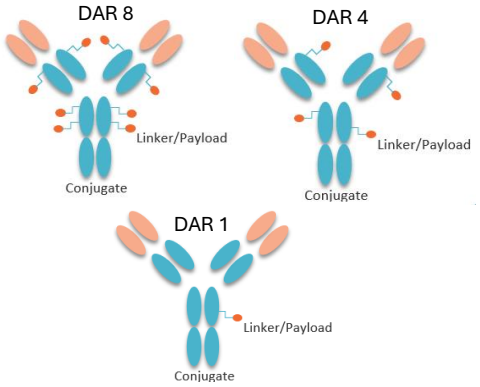


- DAR insensitive > 0
- Same data for all 3

Generic

Strategy 2

- IP with Anti-Ab
- Cleave Linker

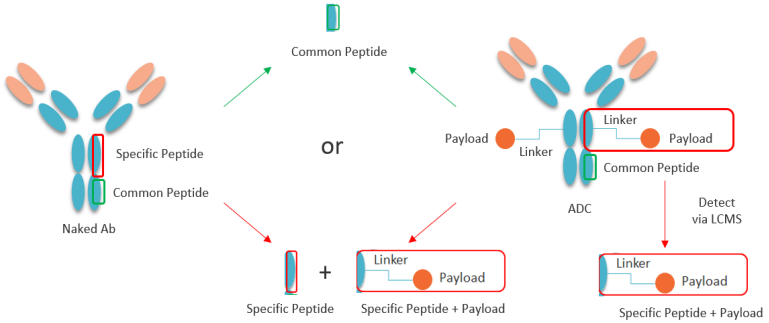


- DAR sensitive – Avg DAR
- Avg DAR changes with # payloads
- Possible to combine with Total Ab
- 1.5 plex or 2 plex

Val/Cit

Strategy 3

- IP with Anti-Ab
- Detect peptide +/- payload



- 1 Assay can detect Total Ab and ADC
- Limited shots on goal

Site Specific Conjugation

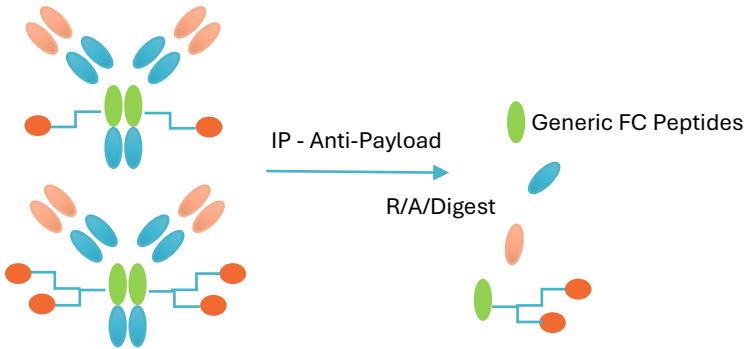
Case Study - “Generic” Assay for ADC

Non-clinical Assay – LCMS for ADC

- Goal: To develop a PK assay for a Human ADC in preclinical species for *in-vitro* and *in-vivo* studies – Various DAR’s with same payload (MMAE)
- Approach – Anti-MMAE IP followed by digestion and LCMSMS
  - +/- Reduction/Alkylation
  - Common Fc peptides
  - Several to choose from (Trp sites)
  - Must have Antibody to payload (MMAE or other payload with Ab available)
  - DAR insensitive – Will capture any DAR > 0
  - 20 different ADC’s – different DARs/conjugations but all had MMAE payload

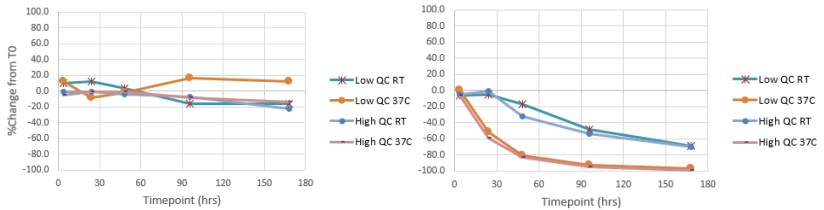
ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVP
SSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTC
VVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALP
APIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSD
GSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK

- Can “IP” or capture with reagent specific to Payload – Detect specific sequence by LCMS (generic or specific peptides)
  - FNW, GPS, VVSV, etc



- Step 1 – In-vitro stability to identify most stable ADCs
- Step 2 – In-vivo PK for top 3 ADCs

Example 2 ADC’s



Stable

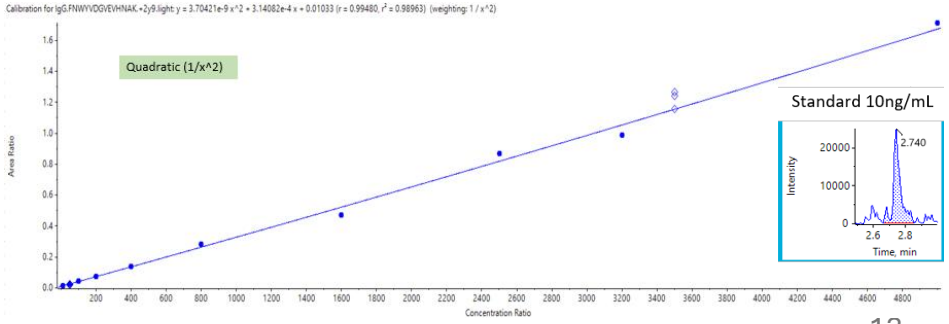
Unstable at both RT and 37C

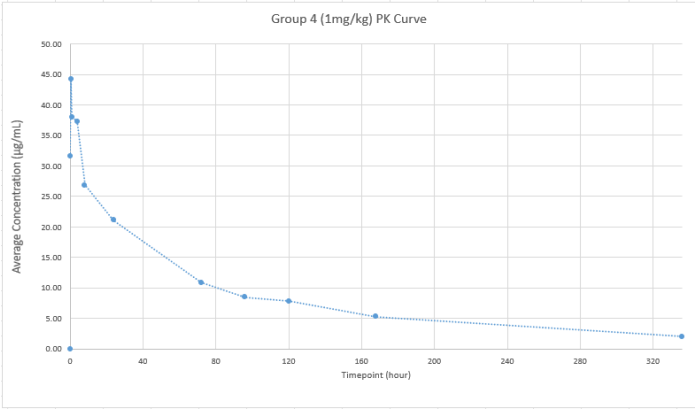
Case Study – Generic IgG - ADC

Example - STD and QC and PK Curve

Component Name	Actual Concentration (ng/mL)	Num. Values	Mean (ng/mL)	Average Accuracy	Value #1 (ng/mL)
IgG.FNWYVDGVEVHNAK.+2y9.light	10	1 of 1	10.3	**102.8**	10.3
IgG.FNWYVDGVEVHNAK.+2y9.light	50	1 of 1	39.2	**78.4**	39.2
IgG.FNWYVDGVEVHNAK.+2y9.light	100	1 of 1	112	**112.5**	112
IgG.FNWYVDGVEVHNAK.+2y9.light	200	1 of 1	207	**103.4**	207
IgG.FNWYVDGVEVHNAK.+2y9.light	400	1 of 1	411	**102.9**	411
IgG.FNWYVDGVEVHNAK.+2y9.light	800	1 of 1	858	**107.2**	858
IgG.FNWYVDGVEVHNAK.+2y9.light	1600	1 of 1	1452	**90.7**	1452
IgG.FNWYVDGVEVHNAK.+2y9.light	2500	1 of 1	2648	**105.9**	2648
IgG.FNWYVDGVEVHNAK.+2y9.light	3200	1 of 1	3006	**93.9**	3006
IgG.FNWYVDGVEVHNAK.+2y9.light	5000	1 of 1	5113	**102.3**	5113

Component Name	Actual Concentration (ng/mL)	Num. Values	Mean (ng/mL)	Standard Deviation	Percent CV	Average Accuracy Across Replicates	Value #1 (ng/mL)	Value #2 (ng/mL)	Value #3 (ng/mL)
IgG.FNWYVDGVEVHNAK.+2y9.light	0	0 of 3	N/A	N/A	N/A	N/A	0	0	0
IgG.FNWYVDGVEVHNAK.+2y9.light	50	3 of 3	43.7	7.8	17.88	87.5	34.7	47.9	48.6
IgG.FNWYVDGVEVHNAK.+2y9.light	3500	3 of 3	3694.3	166.3	4.50	105.6	3507	3752	3824





- 2 Day MD – verify peptides and P&A
- Typical range 25-5000 ng/mL
- Surrogate Matrix STD's (0.1% BSA) and Matrix QC's
- Several Fc peptides (FNW, VVSV, GPS, etc)

Case Study ADC and Total Ab – 1.5 Plex + Free Payload

Surrogate Peptide + Cleavable Payload – Split Sample – 3 Analytes by LC-MS/MS

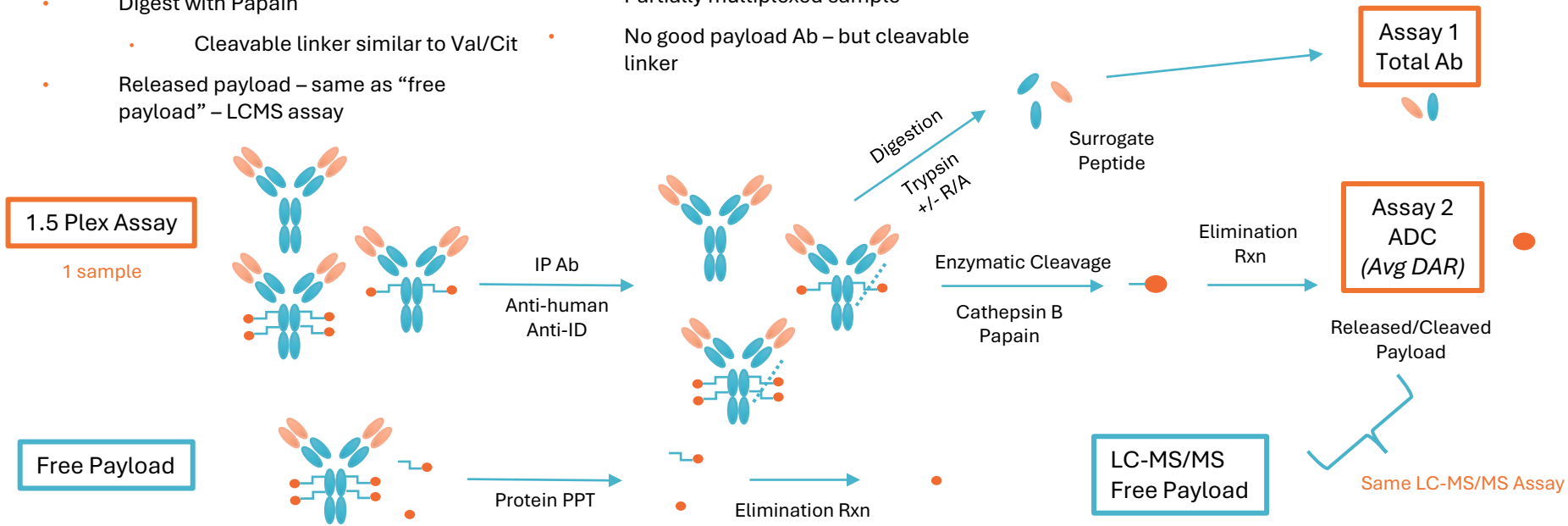
- Goal: To develop a bioanalytical assay for an ADC and Total Ab in Cyno Plasma from single sample

Approach

- IP with Anti-human
- Digest with Papain
  - Cleavable linker similar to Val/Cit
- Released payload – same as "free payload" – LCMS assay

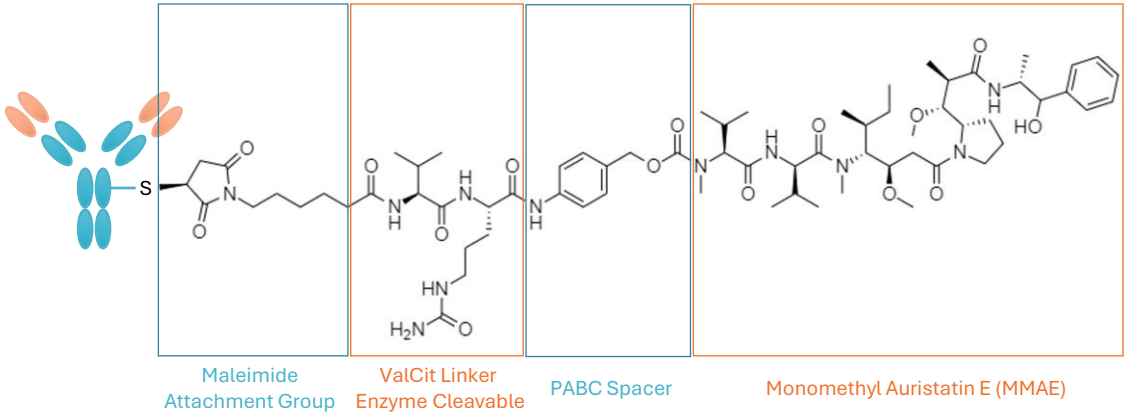
Challenges

- Single Sample – for both assays
- Partially multiplexed sample
- No good payload Ab – but cleavable linker

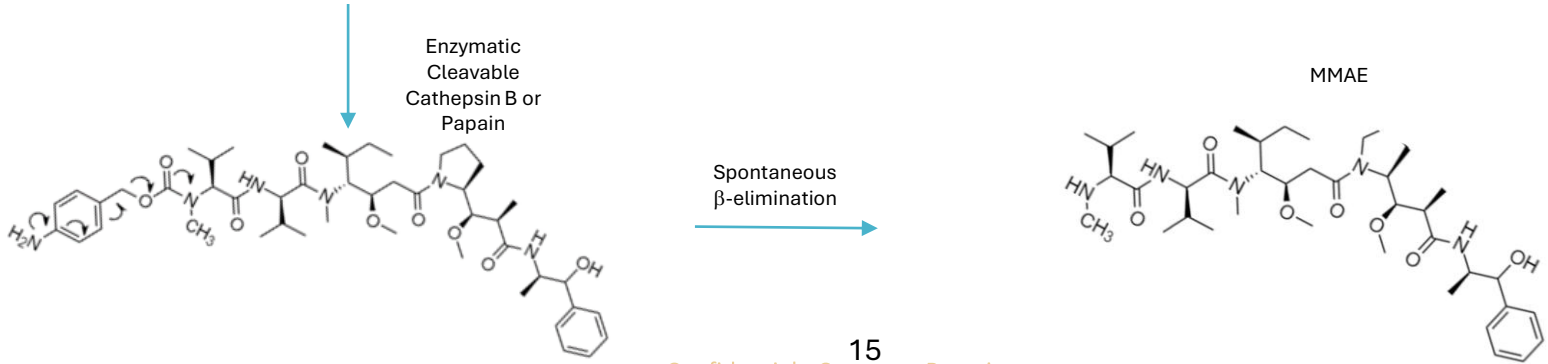


Case Study – ADC

Released Payload – Cleavable Linker Chemistry



- Example Chemistry for Cleavable linker
- Val Cit Linker
- 1, 6 Elimination to release “free” MMAE as measure of ADC (released)

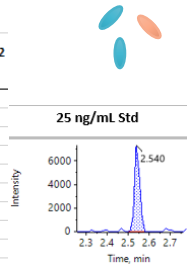


Case Study ADC and Total Ab – 1.5 Plex

Surrogate Peptide + Cleavable Payload – Split Sample

Total Ab Assay – Surrogate Peptide

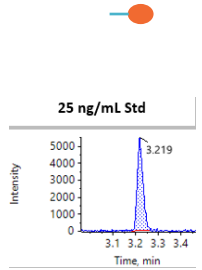
Component Name	Theoretical Concentration (ng/mL)	Num. Values	Mean	Standard Deviation	Percent CV	Average Accuracy (%)	Replicate #1 (ng/mL)	Replicate #2 (ng/mL)
IgG.FNWYVDGVEVHNAK.+2y10.light	25	2 of 2	25.7	4.60	17.9	102.9	22.5	29.0
IgG.FNWYVDGVEVHNAK.+2y10.light	50	2 of 2	47.1	9.10	19.4	94.1	40.6	53.5
IgG.FNWYVDGVEVHNAK.+2y10.light	100	2 of 2	102.0	13.60	13.3	102.0	92.4	111.6
IgG.FNWYVDGVEVHNAK.+2y10.light	200	2 of 2	189.5	2.50	1.3	94.7	191.2	187.7
IgG.FNWYVDGVEVHNAK.+2y10.light	400	2 of 2	390.8	7.2	1.8	97.7	385.7	395.9
IgG.FNWYVDGVEVHNAK.+2y10.light	800	2 of 2	908.2	52.50	5.8	113.5	945.4	871.1
IgG.FNWYVDGVEVHNAK.+2y10.light	1600	2 of 2	1568.6	54.4	3.5	98.0	1530.1	1607.1
IgG.FNWYVDGVEVHNAK.+2y10.light	3200	2 of 2	3106.1	445.3	14.3	97.1	2791.2	3421.0
IgG.FNWYVDGVEVHNAK.+2y10.light	4000	2 of 2	3840.8	146.6	3.8	96.0	3737.1	3944.4
IgG.FNWYVDGVEVHNAK.+2y10.light	5000	2 of 2	5189.0	368.1	7.1	103.8	4928.7	5449.2



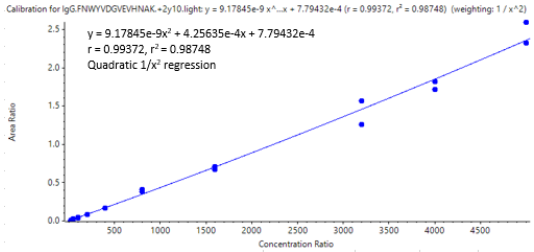
QC Level	Component Name	Dilution	Theoretical Concentration (ng/mL)	Num. Values	Mean (ng/mL)	Standard Deviation	Percent CV	Average Accuracy (%)	Replicate #1 (ng/mL)	Replicate #2 (ng/mL)	Replicate #3 (ng/mL)
LLOQ	IgG.FNWYVDGVEVHNAK.+2y10.light	1	25	3 of 3	26.2	3.50	13.3	104.8	29.50	22.60	26.50
Low	IgG.FNWYVDGVEVHNAK.+2y10.light	1	75	2 of 3	72.1	0.10	0.2	96.2	~~99.4~~	72.0	72.2
Medium	IgG.FNWYVDGVEVHNAK.+2y10.light	1	350	3 of 3	370	43.90	11.9	105.7	337.7	352.3	420.1
High	IgG.FNWYVDGVEVHNAK.+2y10.light	1	3750	3 of 3	3762.4	185.1	4.9	100.3	3549.2	3882.6	3855.4
100X Dilution QC	IgG.FNWYVDGVEVHNAK.+2y10.light	100	1,000	3 of 3	913.5	61.7	6.8	91.4	861.1	981.4	897.9

ADC Assay – Released Payload

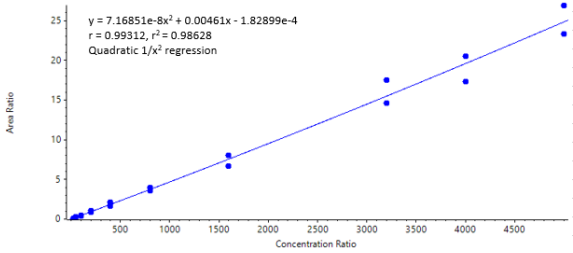
Component Name	Theoretical Concentration (ng/mL)	Num. Values	Mean	Standard Deviation	Percent CV	Average Accuracy (%)	Replicate #1 (ng/mL)	Replicate #2 (ng/mL)
Payload	25	2 of 2	25.2	3.80	15.0	100.8	22.5	27.9
Payload	50	2 of 2	49.2	9.40	19.2	98.3	42.5	55.8
Payload	100	2 of 2	99.3	14.40	14.5	99.3	89.1	109.5
Payload	200	2 of 2	203.0	29.70	14.6	101.5	182.0	224.0
Payload	400	2 of 2	403.1	64.8	16.1	100.8	357.2	448.9
Payload	800	2 of 2	804.8	59.20	7.4	100.6	762.9	846.6
Payload	1600	2 of 2	1560.1	204.9	13.1	97.5	1415.2	1705.0
Payload	3200	2 of 2	3305.7	407.4	12.3	103.3	3017.6	3593.8
Payload	4000	2 of 2	3874.0	440.6	11.4	96.9	3562.5	4185.6
Payload	5000	2 of 2	5045.1	479.0	9.5	100.9	4706.4	5383.8



QC Level	Component Name	Dilution	Theoretical Concentration (ng/mL)	Num. Values	Mean (ng/mL)	Standard Deviation	Percent CV	Average Accuracy (%)	Replicate #1 (ng/mL)	Replicate #2 (ng/mL)	Replicate #3 (ng/mL)
LLOQ	Payload	1	25	3 of 3	25.9	1.90	7.4	103.5	28.00	24.30	25.30
Low	Payload	1	75	2 of 3	74.6	0.70	1.0	99.4	75.1	74.0	~~58.2~~
Medium	Payload	1	350	3 of 3	364.3	15.80	4.3	104.1	350.7	360.7	381.6
High	Payload	1	3750	3 of 3	1070.6	58.4	5.5	107.1	1009.8	1126.3	1075.6
100X Dilution QC	Payload	100	1,000	3 of 3	3903.8	288.7	7.4	104.1	3591.5	4160.8	3959.0



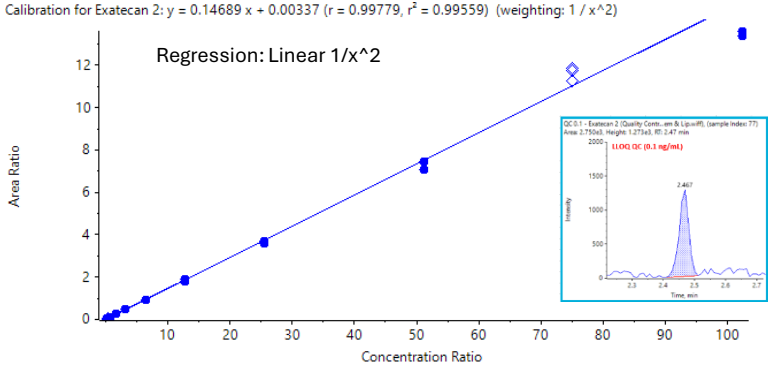
- Prevalidation Data – Both Assays
- Accuracy and Precision < 20%
- ADC signal is after IP enrichment and then cleavage of payload (Avg DAR)
- Surrogate peptide chosen – FNW – FC peptide since Cyno Matrix



Case Study – Free Payload

Free Payload Assay (Exatecan)

Human Plasma Standards								
Standard	Component Name	Actual Concentration (ng/mL)	Num. Values	Replicate 1	Replicate 2	Mean Concentration (ng/mL)	% CV	% Accuracy
1	Exatecan	0.1	2 of 2	0.09	0.09	0.09	0.7	94.3
2	Exatecan	0.2	2 of 2	0.21	0.22	0.21	4.0	106.5
3	Exatecan	0.4	2 of 2	0.42	0.44	0.43	3.0	106.9
4	Exatecan	0.8	2 of 2	0.81	0.83	0.82	1.4	102.3
5	Exatecan	1.6	2 of 2	1.69	1.71	1.70	0.9	106.4
6	Exatecan	3.2	2 of 2	3.25	3.38	3.32	2.8	103.7
7	Exatecan	6.4	2 of 2	6.34	6.39	6.36	0.6	99.4
8	Exatecan	12.8	2 of 2	12.15	12.85	12.50	3.9	97.7
9	Exatecan	25.6	2 of 2	24.40	25.11	24.75	2.0	96.7
10	Exatecan	51.2	2 of 2	50.85	47.98	49.41	4.1	96.5
11	Exatecan	102.4	2 of 2	92.59	91.15	91.87	1.1	89.7



QC	Component Name	Actual Concentration (ng/mL)	Num. Values	Replicate 1	Replicate 2	Replicate 3	Mean Concentration (ng/mL)	% CV	% Accuracy
1	Exatecan	0.1	3 of 3	0.079	0.085	0.089	0.08	6.0	84.5
2	Exatecan	0.3	3 of 3	0.254	0.274	0.266	0.26	3.9	88.2
3	Exatecan	3	3 of 3	2.65	2.90	2.87	2.80	4.9	93.4
4	Exatecan	75	3 of 3	71.64	68.75	72.17	70.85	2.6	94.5

Lot	Component Name	Actual Concentration (ng/mL)	Num. Values	% Accuracy	Value #1
Lot 1	Exatecan 2	0.3	1 of 1	100.03	0.30
Lot 2	Exatecan 2	0.3	1 of 1	99.36	0.30
Lot 3	Exatecan 2	0.3	1 of 1	107.93	0.32
Lot 4	Exatecan 2	0.3	1 of 1	102.59	0.31
Lot 5	Exatecan 2	0.3	1 of 1	100.24	0.30
Lot 6	Exatecan 2	0.3	1 of 1	92.01	0.28

Lot	Component Name	Actual Concentration (ng/mL)	Num. Values	% Accuracy	Value #1
Lot 1	Exatecan 2	75	1 of 1	110.1	82.58
Lot 2	Exatecan 2	75	1 of 1	102.1	76.57
Lot 3	Exatecan 2	75	1 of 1	111.54	83.65
Lot 4	Exatecan 2	75	1 of 1	109.68	82.26
Lot 5	Exatecan 2	75	1 of 1	109.18	81.88
Lot 6	Exatecan 2	75	1 of 1	103.29	77.46

- Pre-validation Data – Accuracy and Precision
  - Curves and QCs all pass acceptance (15/20%)
  - Matrix effect passes at low and high QC
- Curve regression – linear $1/x^2$
  - Quadratic – potentially better fit on high end
- Back end LC-MS/MS method same as ADC (conjugated drug)

Case Study ADC and Total Ab – 2 Plex

Surrogate Peptide + Acid Cleavable Payload

- Goal: To develop a bioanalytical assay for an ADC and Total Ab in Human Plasma from single sample

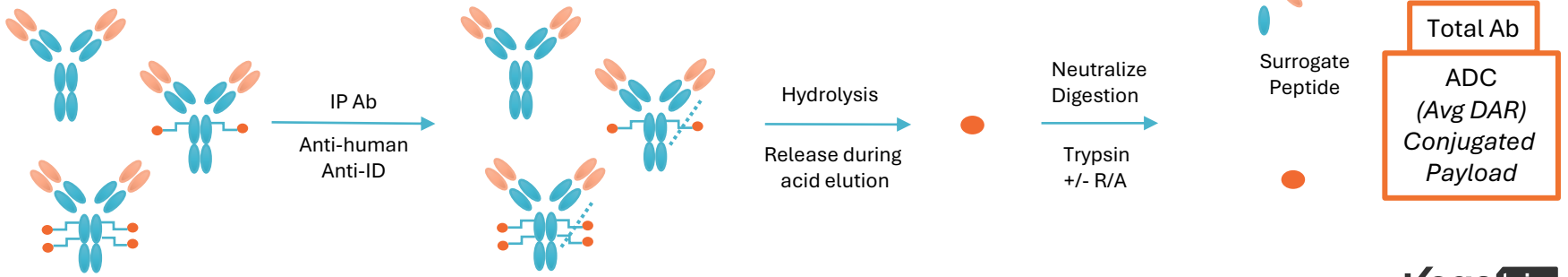
Acid cleavable ADC linkers are designed to release the drug payload under acidic conditions, which are characteristic of tumor cells. These linkers, such as hydrazone bonds, remain stable in the neutral pH of blood but cleave in the acidic environment of lysosomes or endosomes, enabling precise drug release at the target site

Approach

- IP with Anti-ID
- Hydrolyze payload during Ab elution
  - Acid cleavable
- Released payload – same as "free payload" – LCMS assay

Challenges

- Single Sample – for both assays
- Multiplexed sample
- No good payload Ab or stability concerns – but cleavable linker



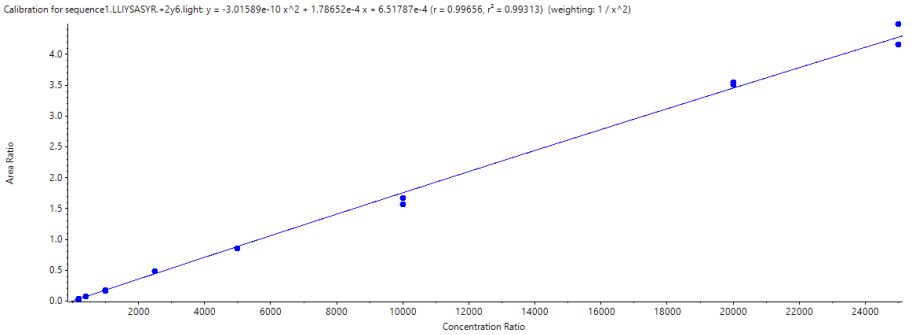
P&A Total Ab (TAb)

200-25,000 ng/mL ADC – Quantitation Surrogate Peptide

25-08-21 LC62 P&A-MMM
Standard Curve in Pooled HuPl

Sample ID	Actual Concentration (ng/mL)	Num. Values	Mean	Standard Deviation	Percent CV	Average Accuracy across Replicates	Value #1	Value #2
Std 1	200	2 of 2	200.224	36.137	18.05	100.11	225.776	174.671
Std 2	400	2 of 2	396.991	7.099	1.79	99.25	391.971	402.011
Std 3	1000	2 of 2	988.608	43.142	4.36	98.86	958.102	1019.115
Std 4	2500	2 of 2	2732.512	14.247	0.52	109.3	2742.586	2722.437
Std 5	5000	2 of 2	4839.442	11.192	0.23	96.79	4847.357	4831.528
Std 6	10000	2 of 2	9229.105	440.597	4.77	92.29	9540.654	8917.556
Std 7	20000	2 of 2	20456.26	119.829	0.59	102.28	20540.99	20371.52
Std 8	25000	2 of 2	25288.78	1434.712	5.67	101.16	26303.27	24274.28

Regression = Quadratic 1/x2



25-08-21 LC62 P&A-MMM
Quality Controls in Pooled HuPl

Sample ID	Actual Concentration (ng/mL)	Num. Values	Mean	Standard Deviation	Percent CV	Average Accuracy across Replicates	Value #1	Value #2	Value #3	Value #4	Value #5	Value #6
LLOQ QC	200	6 of 6	182.859	10.531	5.76	91.43	199.291	177.792	172.473	184.065	190.344	173.191
LQC	600	6 of 6	529.641	28.422	5.37	88.27	521.104	502.297	531.915	544.388	501.535	576.609
MQC	12500	6 of 6	11138.28	747.763	6.71	89.11	11674.15	10592.91	9979.098	11661.87	11021.93	11899.71
HQC	18750	6 of 6	18598.35	895.063	4.81	99.19	17822.23	18691.37	18001.24	19169.35	17843.77	20062.15

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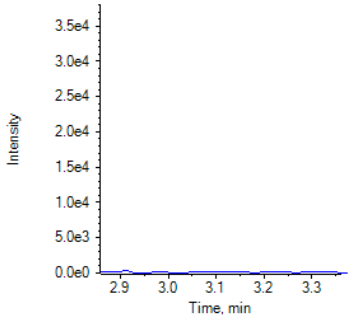
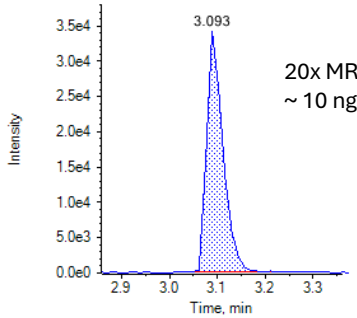


LLOQ at 200 ng/mL



20x MRD
~ 10 ng/mL

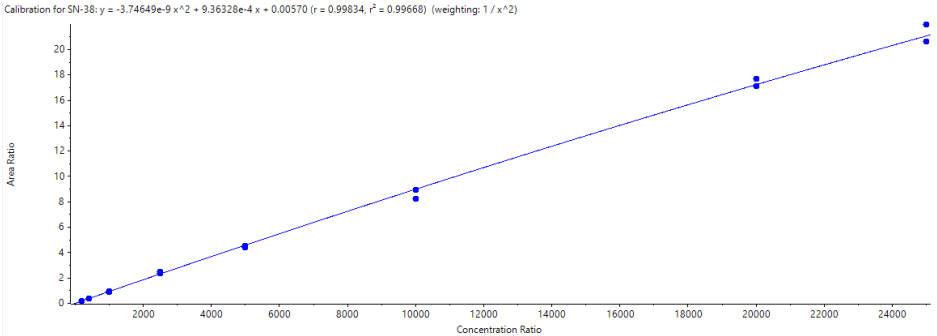
P&A Conjugated Drug (ADC)

200-25,000 ng/mL ADC – Quantitation Drug

25-08-21 LC62 P&A-MMM
Standard Curve in Pooled HuPl

Sample ID	Actual Concentration (ng/mL)	Num. Values	Mean	Standard Deviation	Percent CV	Average Accuracy across Replicates	Value #1	Value #2
Std 1	200	2 of 2	197.634	19.908	10.07	98.82	211.711	183.557
Std 2	400	2 of 2	409.093	5.103	1.25	102.27	405.485	412.702
Std 3	1000	2 of 2	993.858	68.161	6.86	99.39	945.661	1042.056
Std 4	2500	2 of 2	2606.779	125.613	4.82	104.27	2695.601	2517.957
Std 5	5000	2 of 2	4869.338	142.314	2.92	97.39	4768.707	4969.97
Std 6	10000	2 of 2	9564.001	572.429	5.99	95.64	9968.769	9159.232
Std 7	20000	2 of 2	20224.18	511.269	2.53	101.12	19862.66	20585.7
Std 8	25000	2 of 2	25298.78	1289.224	5.1	101.2	26210.4	24387.16

Regression = Quadratic 1/x2



25-08-21 LC62 P&A-MMM
Quality Controls in Pooled HuPl

Sample ID	Actual Concentration (ng/mL)	Num. Values	Mean	Standard Deviation	Percent CV	Average Accuracy across Replicates	Value #1	Value #2	Value #3	Value #4	Value #5	Value #6
LLOQ QC	200	6 of 6	180.404	10.538	5.84	90.2	198.775	181.21	167.804	176.581	183.493	174.561
LQC	600	6 of 6	537.377	23.32	4.34	89.56	513.367	519.87	548.649	556.179	517.703	568.497
MQC	12500	6 of 6	11368.81	741.94	6.53	90.95	12359.38	10545.82	10512.43	11426.23	11403.61	11965.41
HQC	18750	6 of 6	18639.77	1082.668	5.81	99.41	17363.69	18931.72	18042.94	19316.77	17882.59	20300.9

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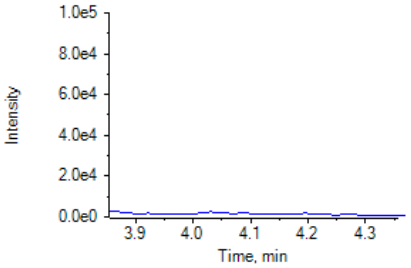
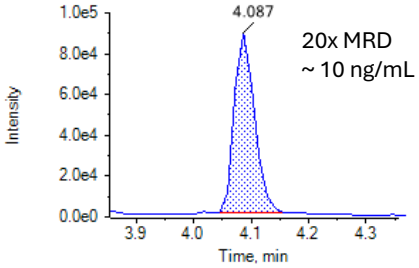


LLOQ at 200 ng/mL



Case Study ADC and Total Ab – 2 Plex

Why LCMS over LBA – Generic MAb +…

No reagents, multiplex

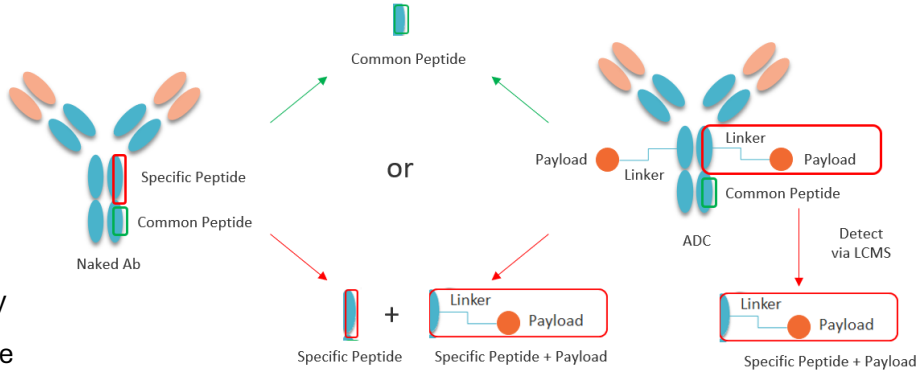
- Goal: To develop a bioanalytical assay for site-specific ADC and Total Ab in Cyno Plasma
- Affinity Capture ADC/Total Ab with Receptor that binds both

Challenges

- No Ab to Payload available nor planned
- Pab to CDR started
- Mab > 6 months away
- Receptor available

Approach

- Hybrid LC-MS/MS chosen as best choice due to limited reagent availability
- Both assays could be done by LCMS



ADC

- Non-cleavable linker
- Site-Specific – DAR 2
- Since no Ab MUST find peptide + payload – 1 option

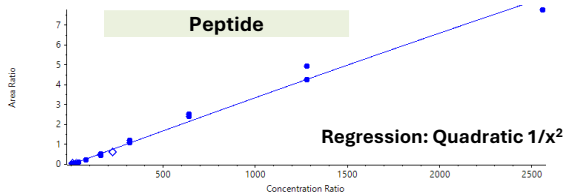
Total Ab

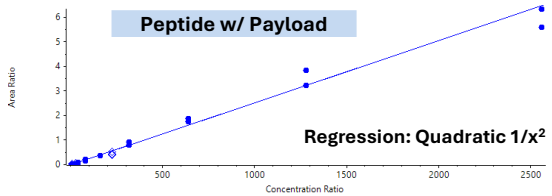
- Any peptide unique to Ab
  - Generic/common peptide to both
    - Specificity from IP or sequence
  - Peptide +/- payload summed

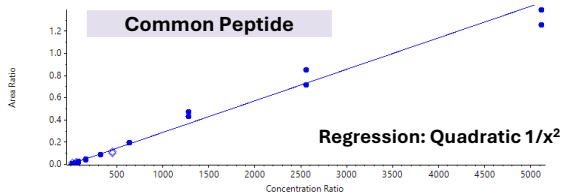
Case Study ADC and Total Ab – 2 Plex

Hybrid LCMS with Receptor IP

		Peptide			Peptide w/ Payload			Peptide + Peptide w/ Payload			Common Peptide		
Sample Name	Actual Concentration (ng/mL)	Calculated Concentration (ng/mL)	% CV	% Accuracy	Calculated Concentration (ng/mL)	% CV	% Accuracy	Total Antibody Calculated Concentration (ng/mL)	Sample Name	Actual Concentration (ng/mL)	Calculated Concentration (ng/mL)	% CV	% Accuracy
Mixed_Standard_10	10	10.5	7.1	104.9	9.74	9.1	97.4	20.2	Mixed_Standard_10	20	21.4	10.6	107.0
Mixed_Standard_20	20	19.2	11.4	96.2	22.7	6.0	113.5	41.9	Mixed_Standard_20	40	37.6	6.6	93.9
Mixed_Standard_40	40	36.5	29.2	91.3	36.1	13.9	90.2	72.6	Mixed_Standard_40	80	69.9	14.1	87.4
Mixed_Standard_80	80	74.9	19.2	93.6	70.2	7.7	87.7	145	Mixed_Standard_80	160	150	12.9	93.7
Mixed_Standard_160	160	148	0.2	92.8	146	8.9	91.2	294	Mixed_Standard_160	320	288	0.2	90.1
Mixed_Standard_320	320	342	9.9	106.9	337	6.1	105.5	680	Mixed_Standard_320	640	684	0.3	106.8
Mixed_Standard_640	640	723	5.4	113.0	731	3.3	114.3	1454	Mixed_Standard_640	1280	1576	6.0	123.1
Mixed_Standard_1280	1280	1400	11.9	109.4	1381	10.6	107.9	2781	Mixed_Standard_1280	2560	2745	12.7	107.2
Mixed_Standard_2560	2560	2357	8.8	92.1	2366	0.2	92.4	4723	Mixed_Standard_2560	5120	4647	7.4	90.8





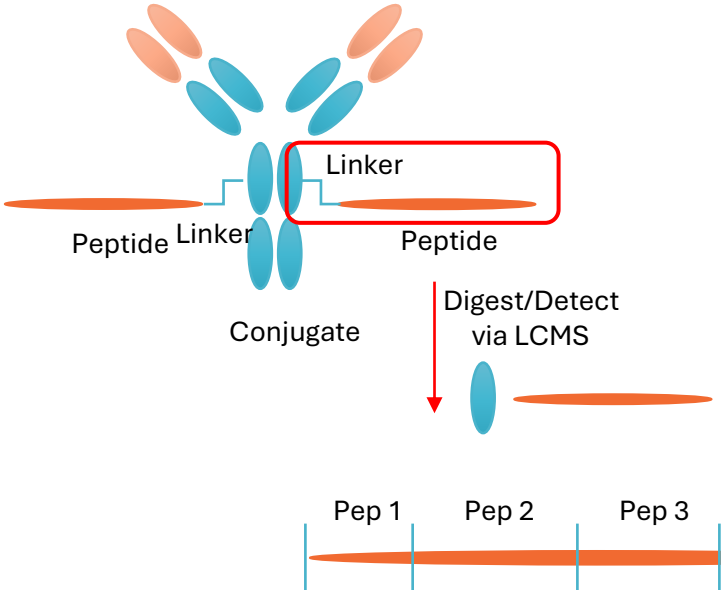


- Total Ab quantified by either common peptide or summing various forms

Peptide ADC - 2 Plex

Hybrid LCMS with Anti-human IP

- Affinity Capture Total Ab with Antihuman Ig…. Then detect
  - Generic peptides from Fc region of Ab (FNW, GPS etc)
  - Peptide – peptide released during digestion



- Can digest with various enzymes to give good coverage of Ab and Peptide (conjugate)
  - Ab
    - FC region – FNW, GPS, etc
  - Peptide
    - Cleave various parts of peptide

Peptide ADC

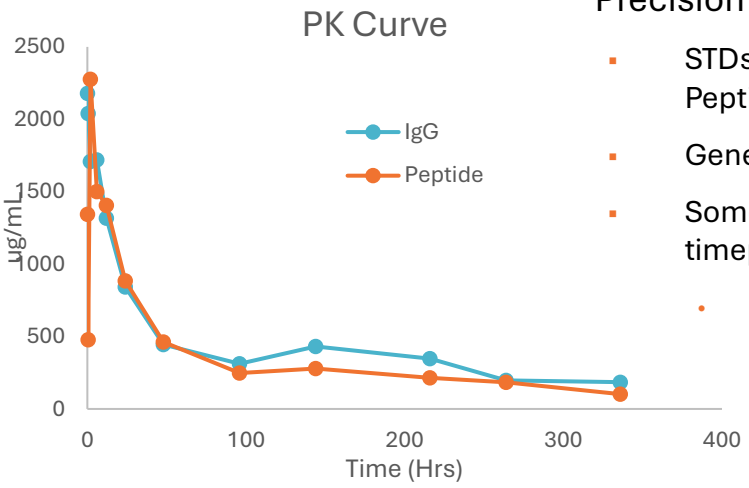
Hybrid LCMS with Anti-human IP

Row	Component Name	Theoretical Concentration (ng/mL)	Replicates Used	Mean Concentration (ng/mL)	% CV	% Accuracy
1	GPS-IgG	10	2 of 2	10.525	7.2	105.2
2	GPS-IgG	20	2 of 2	17.544	16.8	87.7
3	GPS-IgG	40	2 of 2	40.938	17.9	102.4
4	GPS-IgG	80	2 of 2	77.924	8.8	97.4
5	GPS-IgG	160	2 of 2	169.057	2.3	105.7
6	GPS-IgG	320	2 of 2	344.231	8.6	107.6
7	GPS-IgG	640	2 of 2	675.342	4.5	105.5
8	GPS-IgG	1280	1 of 2	1053.414	0.0	82.3
9	GPS-IgG	2560	2 of 2	2842.382	12.0	111.0
10	GPS-IgG	5120	2 of 2	4420.698	15.2	86.3

Row	Component Name	Theoretical Concentration (ng/mL)	Replicates Used	Mean Concentration (ng/mL)	% CV	% Accuracy
1	Peptide	10	2 of 2	10.552	15.5	105.5
2	Peptide	20	2 of 2	17.686	12.0	88.4
3	Peptide	40	2 of 2	41.199	11.4	103.0
4	Peptide	80	2 of 2	79.019	12.9	98.8
5	Peptide	160	2 of 2	144.396	24.4	90.2
6	Peptide	320	2 of 2	307.544	6.3	96.1
7	Peptide	640	2 of 2	788.759	13.0	123.2
8	Peptide	1280	2 of 2	1398.516	6.4	109.3
9	Peptide	2560	2 of 2	2492.635	19.5	97.4
10	Peptide	5120	2 of 2	4508.361	15.0	88.0

Component Name	Theory Conc (ng/mL)	Replicates Used	Conc (ng/mL)	% CV	% Accuracy
GPS-IgG	10	3 of 3	12.548	1.8	125.5
GPS-IgG	30	3 of 3	31.433	9.4	104.8
GPS-IgG	250	3 of 3	287.978	18.5	115.2
GPS-IgG	4000	3 of 3	4030.38	4.5	100.8

Component Name	Theory Conc (ng/mL)	Replicates Used	Conc (ng/mL)	% CV	% Accuracy
Peptide	10	2 of 3	8.67	13.7	86.7
Peptide	30	3 of 3	31.379	17.4	104.6
Peptide	250	3 of 3	295.459	7.5	118.2
Peptide	4000	3 of 3	4259.131	7.4	106.5



- Good Accuracy and Precision
  - STDs and QCs – IgG and Peptide
  - General overlap
  - Some deviation at later timepoints
- Stability?

Oligonucleotides

siRNA, ASO, ARCs

Background and Case Studies

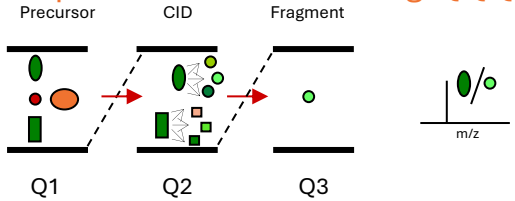
QqQ and HRMS

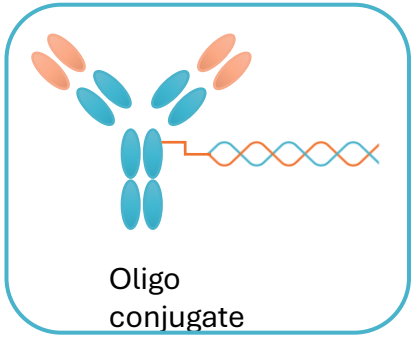
Oligonucleotide Bioanalysis – ARCs/siRNA

KCAS Assay approaches – Experience/Strategies

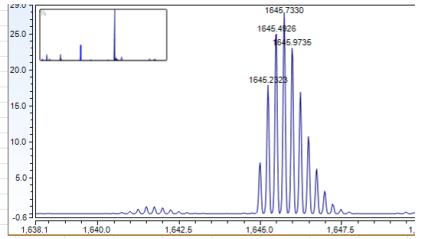
- Most recent Experience – siRNA, Antisense and ARCs – Hybrid or "traditional" LC-MS/MS on QqQ or HRMS
  - Early Discovery before Tox (HRMS – quick MD, sense and ASO info, some metabolite)
  - Tox/GLP – typically either HRMS or QqQ
- LC-MS/MS (QqQ 5 -50ng/mL or HRMS 1-20ng/mL)
  - Good when don't have any reagents
  - Also provides metabolite information
  - Sensitivity – ng/mL
  - Advantages/disadvantages for QqQ vs HRMS
- Hybridization ELISA – 100s pg/mL
  - Need specific probe (long if need Capture and Detector)
  - Sensitivity – can be 0.1 ng/mL but limited dynamic range (20-50x)
  - Typically cannot differentiate N-1 and N-2 from parent
- PCR – 10's pg/mL
  - Need specific probes
  - Good sensitivity
- Hybridization – LC-MS/MS (PNA, LNA) – 100's pg/mL – low ng/mL
  - Good specificity and good sensitivity
  - Need specific probe – but only for capture
  - Common sequences (generic approach)?
- UPLC-FD - low ng/mL
  - Good sensitivity
  - Needs fluorescent tag/probe
  - Common method

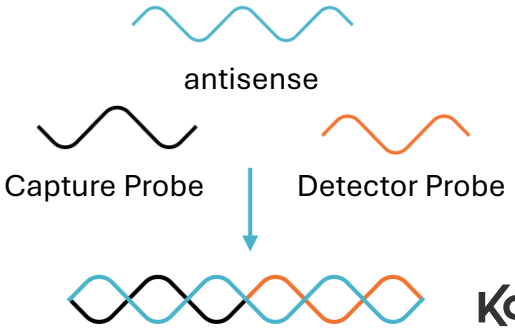
Multiple Reaction Monitoring/QQQ





HRMS – ASO 4⁻

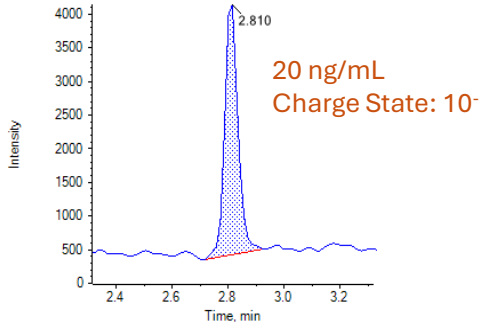


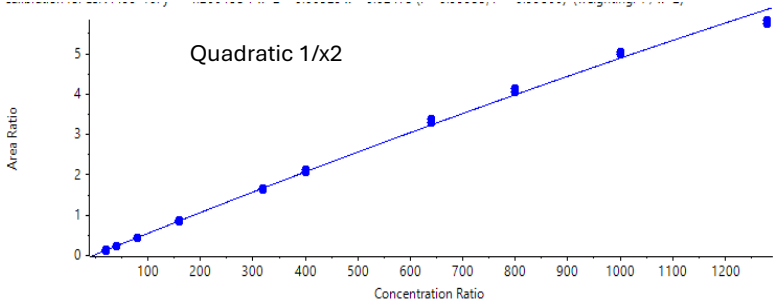


Assay Optimization – QqQ – 2 plex assay

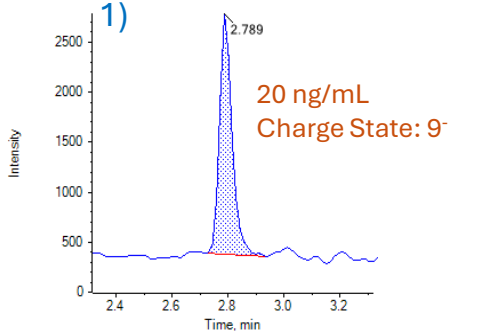
Analyte and Metabolite quant in 1 assay

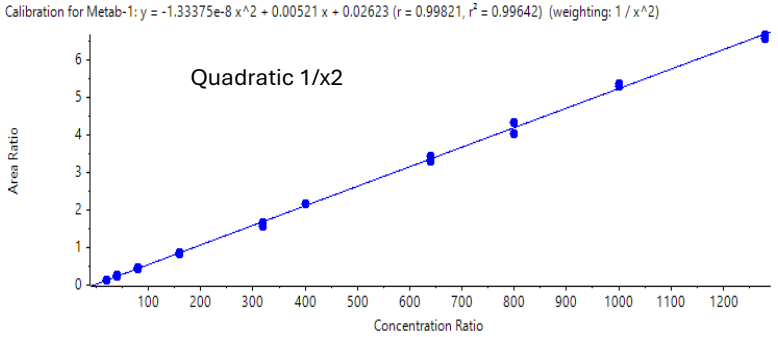
Parent





Metabolite (3' n-1)

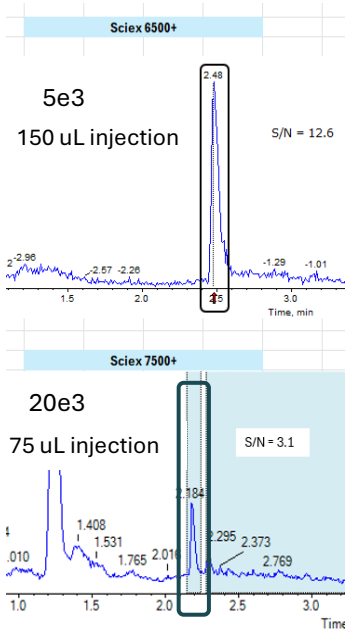




- ARC associated antisense assay
- Assay developed on SCIEX 7500 system
- 2-plex for LLOQ at 20 ng/mL in Cyno Plasma (Parent and Metabolite)
- Metabolite conjugate 3' n-1 of parent oligo (Antibody unchanged)
- IP Assay optimized with higher amounts of capture to allow for simultaneous quantitation of parent oligo conjugate and metabolite conjugate
- Metabolite elutes slightly before parent (no contribution observed)
- Curve Range 20-1,280 ng/mL
- 2-plex method fully validated with GLP sample analysis

Achieving the desired LLOQ

Instrument choice - Oligo dependent



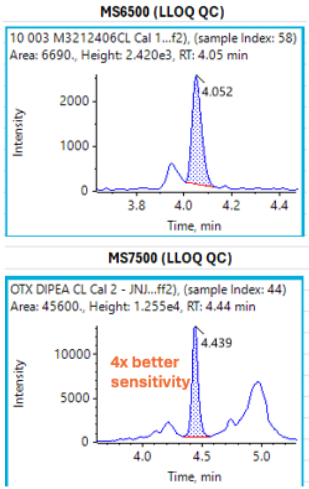
Instrument: SCIEX Triple Quad 6500+ system & SCIEX 7500 system

25 ng/mL

Molecule: T-AS (ARC dosed)

- See 2x worse sensitivity with using SCIEX 7500 system

Sample Name	Sciex7500 %Accuracy	Sciex6500 %Accuracy
LLOQ QC	74.4	134.1
LLOQ QC	104.9	96.2
LLOQ QC	102.2	97.8
LLOQ QC	109.9	94.6
LLOQ QC	106.4	97.2
LLOQ QC	105.1	87.2
Low QC	96.1	99.2
Low QC	98.3	99.4
Low QC	95.1	95.4
Low QC	87.6	94.1
Low QC	111.2	98.7
Low QC	92.8	101.4
Mid QC	95.2	99.9
Mid QC	109.2	101.6
Mid QC	78.9	100.3
Mid QC	88.1	103.7
Mid QC	97.4	101.6
Mid QC	119.5	98.4
High QC	84.4	94.5
High QC	97.0	103.5
High QC	88.9	100.2
High QC	87.6	110.4
High QC	105.8	109.9
High QC	109.1	101.8

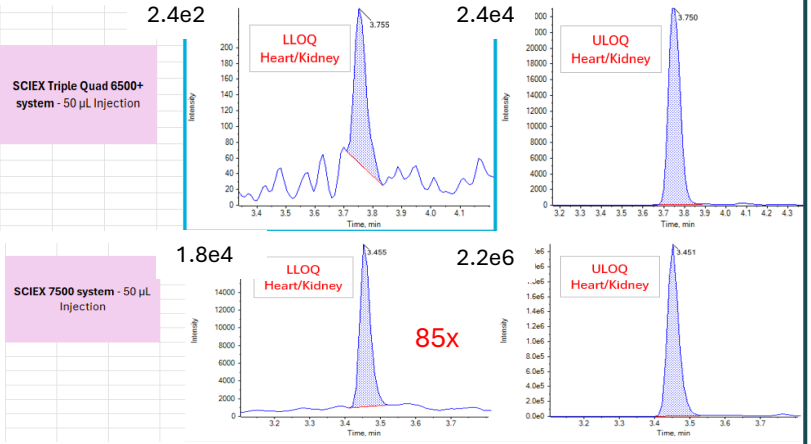


Instrument: SCIEX Triple Quad 6500+ system & SCIEX 7500 system

Range: 4-4000 ng/mL

Molecule: Duplex (~31Mer)

- See 4x better sensitivity with using SCIEX 7500 system and DIPEA



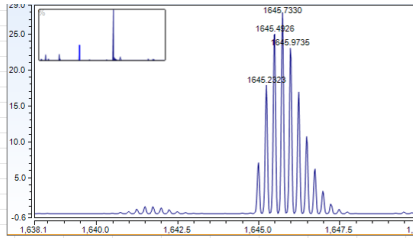
Sample: **5 ng/mL (LLOQ) and 1280 ng/mL ULOQ extracted ARC sample**

- SCIEX 7500 system gave ~ 85x higher sensitivity compared to SCIEX Triple Quad 6500+ system in Cyno heart/kidney homogenate samples
- Ion spray Voltage played a crucial role in sensitivity gain – 4x from 1700 to 4500

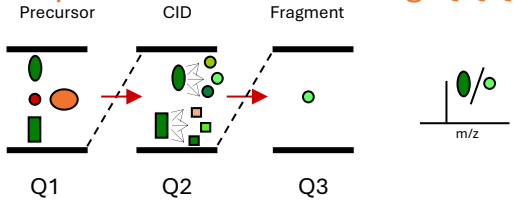
QQQ vs HRMS

Adv and Disadvantages

HRMS – ASO 4-



Multiple Reaction Monitoring/QQQ



- HRMS
  - Limited MD
  - Sense and Antisense info – Full scan
  - Good overall sensitivity 1-20 ng/mL
  - Clear advantages when have interferences in specific matrices
  - Works well when oligo doesn't fragment well
  - More complicated data analysis (slower)
  - Bigger file size – Need to have data storage plan
  - Better SLIS – differentiation – due to lower charge state (less cross talk/interference
- QqQ
  - MRM – specific MD
    - Varies per matrix – need to chromatographically separate interferences not typical seen in HRMS
  - Decent but variable sensitivity
  - Easy data processing
  - Good for regulated studies
  - SLIS can contribute to cross talk – interferences

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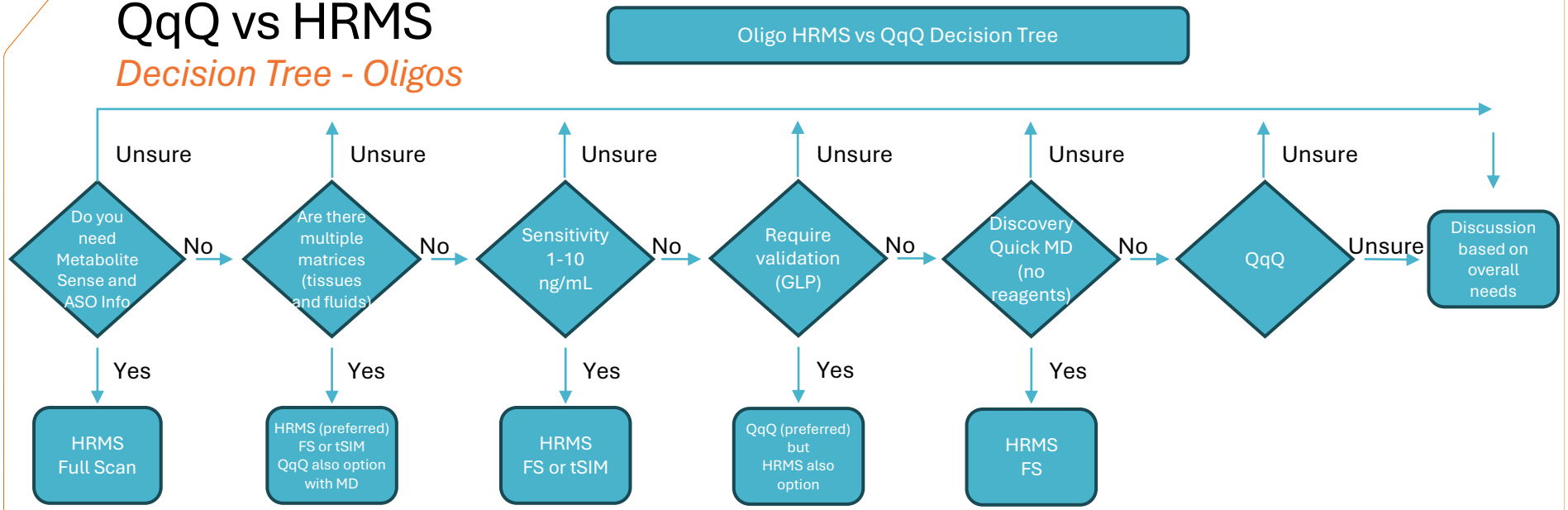
QqQ vs HRMS

Decision Tree - Oligos



HRMS vs QqQ

- QqQ is the workhorse for targeted quant
  - high throughput
  - quick data processing
  - small file size

BUT

- HRMS has a lot of power and can be more sensitive with less specific method development

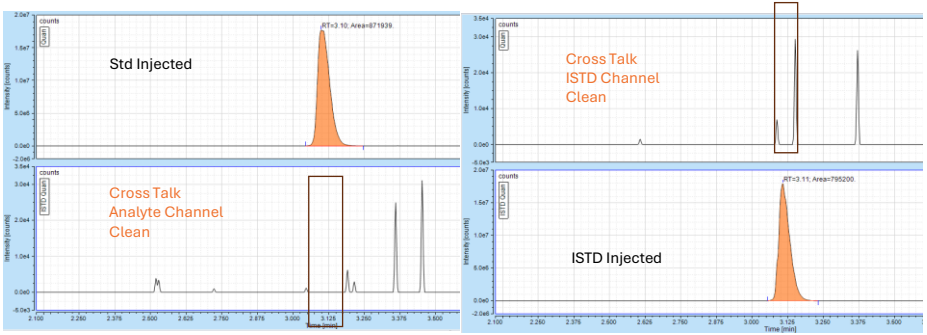
Philosophy Points to consider

- What sensitivity is needed
  - GLP or NonGLP
- Metabolite Info or Sense and ASO
  - Multiple matrices
- Quick MD - Full Scan data
  - Does Oligo Fragment
  - SLIS
- Is File Size Important

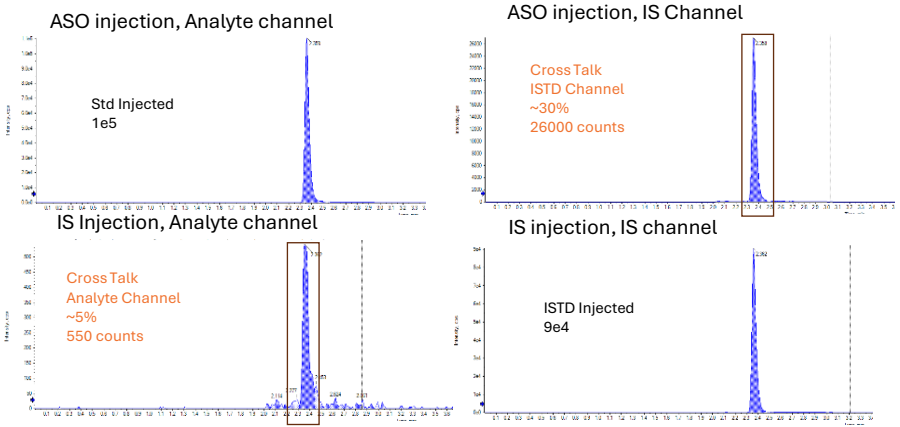
QqQ vs HRMS

SLIS Cross Talk

HRMS 4⁻ m/z



QqQ 9⁻ m/z

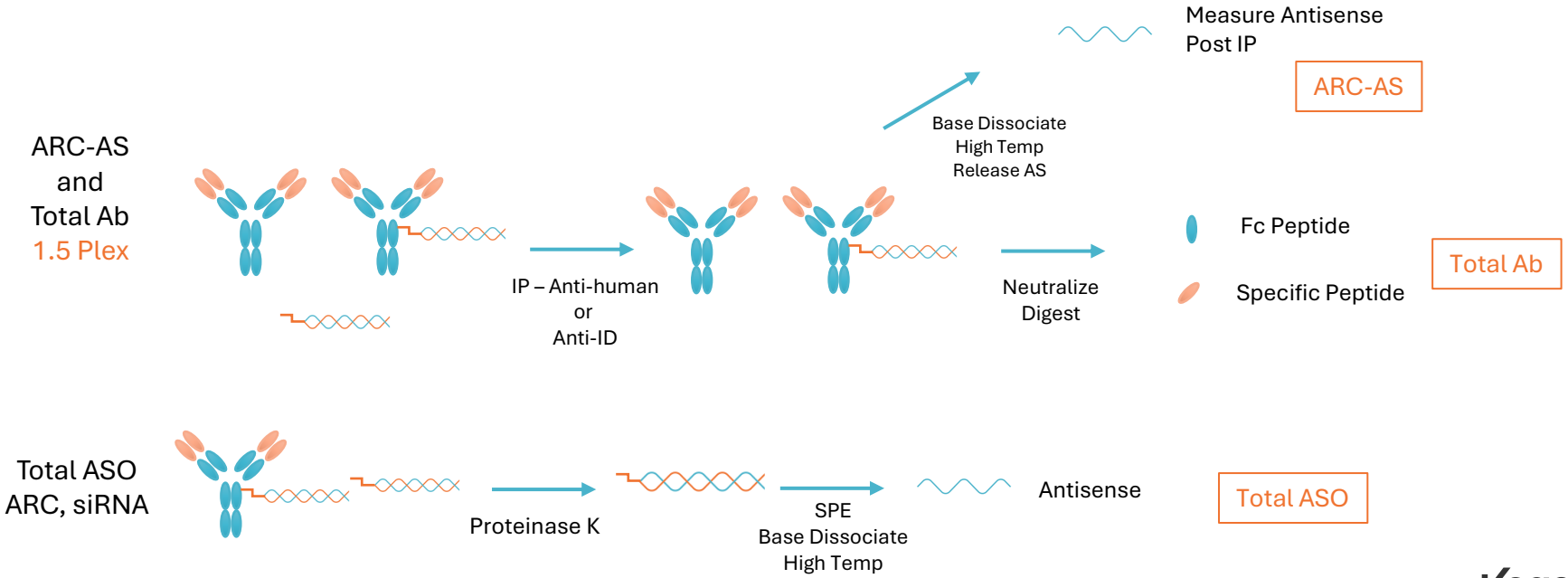


- Stable labeled internal standard – 27 Dalton delta
  - QqQ – 9 charge state – only 3 dalton difference = cross talk
  - HRMS – 4 charge state - ~7 dalton difference and HRMS = NO cross talk

Case Study – Antibody RNA Conjugate and siRNA - QqQ

ARC, Total Antisense and Total Ab (1.5 plex for ARC-AS and Total Ab)

- Goal: To develop a PK assay for an ARC (Antibody-siRNA-Conjugate) for ARC, total antisense and total IgG in various preclinical matrices



Case Study ARC and Total Ab – 1.5 Plex - QqQ

ARC associated AS and CDR Peptide – Split Sample

Total Ab Assay – CDR Peptide

Row	Component	Actual Conc (ng/mL)	No	Mean	STDev	% CV	% Accuracy	Value #1	Value #2
1	CDR Peptide	100	2 of 2	105.84	13.44	12.7	105.8	96.34	115.34
2	CDR Peptide	200	2 of 2	190.82	9.89	5.2	95.4	197.81	183.82
3	CDR Peptide	400	2 of 2	357.24	39.01	10.9	89.3	329.66	384.82
4	CDR Peptide	800	2 of 2	761.72	11.64	1.5	95.2	769.95	753.49
5	CDR Peptide	1600	2 of 2	1469.68	20.54	1.4	91.9	1484.20	1455.16
6	CDR Peptide	3200	2 of 2	3345.21	231.12	6.9	104.5	3181.78	3508.64
7	CDR Peptide	6400	2 of 2	6447.62	186.70	2.9	100.7	6315.61	6579.64
8	CDR Peptide	12800	2 of 2	13572.45	492.55	3.6	106.0	13224.17	13920.74
9	CDR Peptide	25600	2 of 2	27871.40	793.17	2.9	108.9	27310.55	28432.25
10	CDR Peptide	51200	2 of 2	52218.09	5386.08	10.3	102.0	48409.56	56026.63
11	CDR Peptide	102400	2 of 2	99842.96	8334.18	8.4	97.5	105736.11	93949.80

QC	Component	Actual Conc (ng/mL)	No	Mean	STDev	% CV	% Accuracy	Value #1	Value #2	Value #3
LLOQ	CDR Peptide	100	3 of 3	98.6	3.6	3.6	98.6	102.6	96.0	97.0
Low	CDR Peptide	600	3 of 3	549.4	34.5	6.3	91.6	522.9	536.9	588.4
Med	CDR Peptide	37500	3 of 3	39137.8	3150.2	8.1	104.4	35738.6	39715.9	41958.9
High	CDR Peptide	75000	3 of 3	65737.4	3722.5	5.7	87.7	62965.7	64278.1	69968.5

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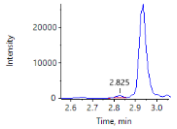
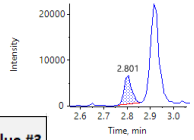


LLOQ – 100 ng/mL



ARC-AS Assay – antisense

Row	Component	Actual Conc (ng/mL)	No	Mean	STDev	% CV	% Accuracy	Value #1	Value #2
1	Antisense Strand	34.6	2 of 2	36.5	2.9	7.9	105.3	34.4	38.5
2	Antisense Strand	69.3	2 of 2	62.1	3.8	6.2	89.6	59.4	64.8
3	Antisense Strand	138.6	2 of 2	138.7	0.0	0.0	100.1	138.6	138.7
4	Antisense Strand	277.1	2 of 2	267.8	6.0	2.2	96.7	263.6	272.0
5	Antisense Strand	554.2	2 of 2	564.6	39.4	7.0	101.9	536.7	592.4
6	Antisense Strand	1108.4	2 of 2	1142.6	37.0	3.2	103.1	1168.7	1116.4
7	Antisense Strand	2216.8	2 of 2	2313.8	49.8	2.2	104.4	2278.6	2349.1
8	Antisense Strand	4433.5	2 of 2	4448.7	465.0	10.5	100.3	4119.9	4777.5
9	Antisense Strand	8867.0	2 of 2	8740.8	313.1	3.6	98.6	8962.2	8519.4

QC	Component	Actual Conc (ng/mL)	No	Mean	STDev	% CV	% Accuracy	Value #1	Value #2	Value #3
Low	Antisense Strand	52.0	3 of 3	53.7	3.9	7.3	103.3	49.7	53.8	57.5
Med	Antisense Strand	3247.2	3 of 3	3184.1	203.1	6.4	98.1	2949.6	3295.5	3307.1
High	Antisense Strand	6494.4	3 of 3	5944.9	642.4	10.8	91.5	5636.0	5515.4	6683.4

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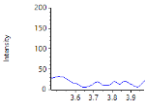
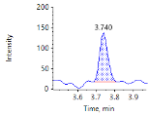
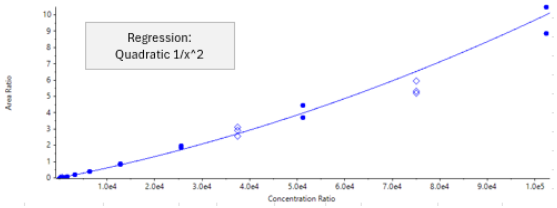


LLOQ – 35 ng/mL

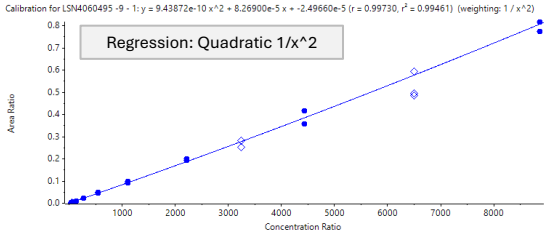


SCIEX 7500 System – Monkey Plasma



- Prevalidation Data – Both Assays
- Accuracy and Precision < 20%
- ARC signal is after IP enrichment and then release of antisense (ASO)
- Surrogate peptide chosen – CDR – Peptide (more specific)

SCIEX Triple Quad 6500+ system – Monkey Plasma



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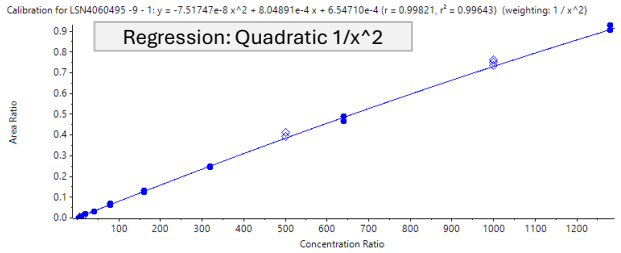
Case Study – Total Antisense - QqQ

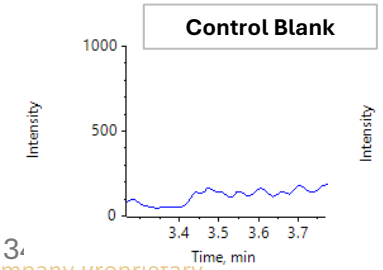
Example – Calibration Standards & Quality Controls

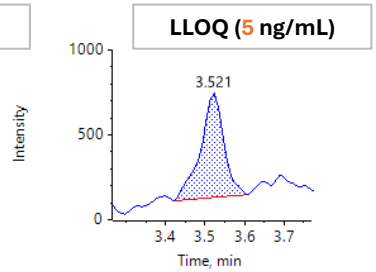
Row	Component	Actual Conc (ng/mL)	No	Mean	STDev	% CV	% Accuracy	Value #1	Value #2
1	Antisense Strand	5.0	2 of 2	5.0	0.3	6.7	99.3	5.2	4.7
2	Antisense Strand	10.0	2 of 2	9.9	1.1	10.6	98.9	9.2	10.6
3	Antisense Strand	20.0	2 of 2	21.4	0.1	0.3	107.0	21.4	21.3
4	Antisense Strand	40.0	2 of 2	38.2	0.4	1.1	95.5	38.5	37.9
5	Antisense Strand	80.0	2 of 2	80.4	5.7	7.1	100.5	84.5	76.4
6	Antisense Strand	160.0	2 of 2	160.6	8.9	5.5	100.4	154.3	166.9
7	Antisense Strand	320.0	2 of 2	315.7	3.7	1.2	98.7	313.1	318.3
8	Antisense Strand	640.0	2 of 2	631.2	24.3	3.9	98.6	648.3	614.0
9	Antisense Strand	1280.0	2 of 2	1295.1	28.8	2.2	101.2	1274.8	1315.5

QC	Component	Actual Conc (ng/mL)	No	Mean	STDev	% CV	% Accuracy	Value #1	Value #2	Value #3
LLOQ	Antisense Strand	5.0	3 of 3	4.9	0.6	11.8	98.1	5.5	5.0	4.3
Low	Antisense Strand	15.0	2 of 2	15.4	0.2	1.5	102.5	15.2	15.5	N/A
Med	Antisense Strand	500.0	3 of 3	520.0	15.2	2.9	104.0	510.4	512.0	537.5
High	Antisense Strand	1000.0	3 of 3	1030.1	21.4	2.1	103.0	1051.9	1009.1	1029.2

- Method Development
  - Total Antisense in Antibody-siRNA-Conjugate (ARC)
- Range: 5 ng/mL – 1280 ng/mL
- Matrix: Cynomolgus Heart/ Kidney Homogenate
- Detector: SCIEX Triple Quad 6500+ system
- Mobile Phases:
  - Mobile Phase A: 1% HFIP, 0.2% TEA in Deionized Water
  - Mobile Phase B: 1% HFIP, 0.2% TEA in Methanol
  - Rinse Solution: 1% HFIP, 0.2% TEA in Methanol
- Analytical Column: Phenomenex bioZen 1.7 µm Oligo 50 X 2.1 mm
- Run Time – 8.5 min







Case Study – siRNA QqQ

Good sensitivity by QqQ – various species/matrices

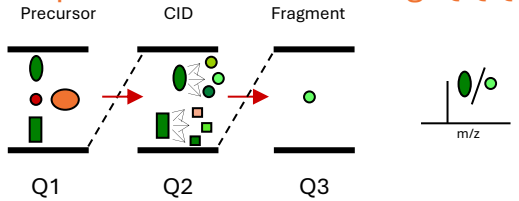
- Goal: To develop a PK assay for a total antisense (siRNA) via ASO in various rat and cyno tissues in a triple quad

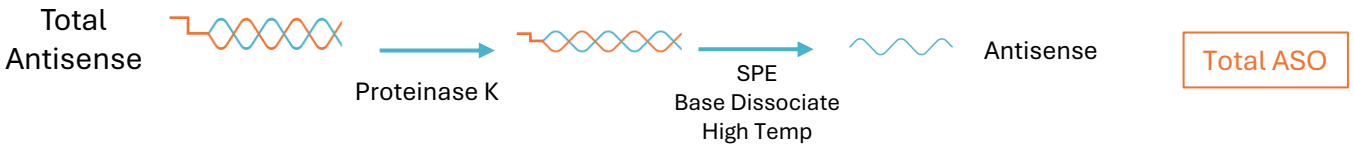
Assay needs to be sensitive and developed across 2 species (Rat and Cyno) across several tissue matrices

- Rat
  - Brain, Liver, Kidney
- Cyno
  - Brain Liver, Kidney, Gastro

Each matrix will need to be optimized for interferences as necessary

Multiple Reaction Monitoring/QQQ





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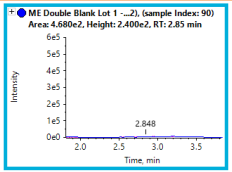
Case Study – siRNA QqQ

Cyno Tissues

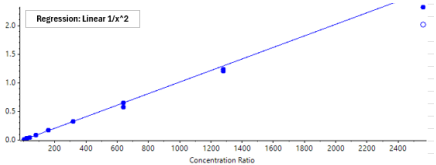


- Assay Range 5-2560 ng/mL
- Linear – all tissues
- All passing PA 15/20%

Brain

Actual Conc	Num. Value	Mean	StDev	% CV	% Acc
5	2 of 2	4.8	1.2	24.0	96.6
10	2 of 2	10.1	0.0	0.4	100.7
20	2 of 2	21.8	1.2	5.7	109.2
40	2 of 2	42.1	0.7	1.7	105.3
80	2 of 2	80.6	1.6	2.0	100.7
160	2 of 2	169.1	1.1	0.7	105.7
320	2 of 2	315.0	2.8	0.9	98.5
640	2 of 2	603.2	49.8	8.3	94.3
1280	2 of 2	1206.8	20.4	1.7	94.3
2560	1 of 2	2296.3	N/A	N/A	89.7

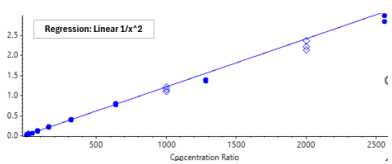
Actual Conc	Num. Value	Mean	StDev	% CV	% Acc
15	5 of 5	12.9	1.2	9.2	86.0
1000	5 of 5	925.2	15.1	1.6	92.5
2000	5 of 5	1779.3	44.8	2.5	89.0



Kidney

Actual Conc	Num. Value	Mean	StDev	% CV	% Acc
5	2 of 2	4.861	0.15	3.09	97.22
10	2 of 2	10.56	0.382	3.62	105.6
20	2 of 2	19.96	0.729	3.66	99.78
40	2 of 2	39.22	0.512	1.31	98.05
80	1 of 2	77.74	N/A	N/A	97.17
160	2 of 2	177.9	0.076	0.04	111.2
320	2 of 2	332	8.704	2.62	103.8
640	2 of 2	640.1	22.16	3.46	100
1280	2 of 2	1158	9.831	0.85	90.49
2560	1 of 2	2320	N/A	N/A	90.61

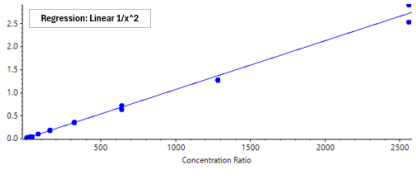
Actual Conc	Num. Value	Mean	StDev	% CV	% Acc
15	5 of 5	12.35	0.877	7.1	82.34
1000	5 of 5	937.8	14.47	1.54	93.78
2000	5 of 5	1777	65.45	3.68	88.85



Liver

Actual Conc	Num. Value	Mean	StDev	% CV	% Acc
5	2 of 2	4.853	1.023	21.09	97.07
10	1 of 2	11.19	N/A	N/A	111.9
20	1 of 2	17.71	N/A	N/A	88.53
40	2 of 2	43.63	2.032	4.66	109.1
80	2 of 2	82.18	2.5	3.04	102.7
160	2 of 2	167.3	6.124	3.66	104.6
320	2 of 2	324.6	18.12	5.58	101.4
640	2 of 2	611.2	11.84	1.94	95.5
1280	2 of 2	1239	4.888	0.39	96.79
2560	1 of 2	2185	N/A	N/A	85.35

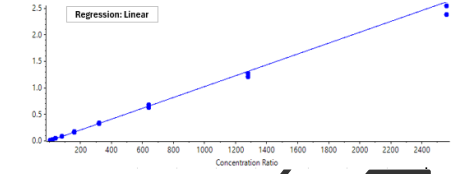
Actual Conc	Num. Value	Mean	StDev	% CV	% Acc
15	4 of 5	12.74	1.203	9.44	84.91
1000	5 of 5	926.9	26.72	2.88	92.69
2000	5 of 5	1803	57.94	3.21	90.14



Gastro

Actual Conc	Num. Value	Mean	StDev	% CV	% Acc
5	2 of 2	4.858	0.087	1.79	97.17
10	2 of 2	10.25	0.224	2.19	102.5
20	2 of 2	20.7	0.381	1.84	103.5
40	2 of 2	41.89	0.281	0.67	104.7
80	2 of 2	80.86	1.401	1.73	101.1
160	2 of 2	163	4.939	3.03	101.9
320	2 of 2	322.9	10.7	3.31	100.9
640	2 of 2	638.1	33.44	5.24	99.7
1280	2 of 2	1211	50.18	4.14	94.62
2560	2 of 2	2403	117	4.87	93.87

Actual Conc	Num. Value	Mean	StDev	% CV	% Acc
15	6 of 6	14.15	0.374	2.65	94.33
1000	6 of 6	934.5	30.47	3.26	93.45
2000	6 of 6	1848	48.55	2.63	92.42



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Case Study – siRNA QqQ

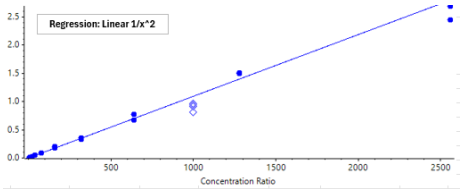
Rat Tissues

- Assay Range 5-2560 ng/mL
- Linear – all tissues
- PA 15/20% (1 out at 10 in Liver)

Brain

Actual Conc	Num. Value	Mean	StDev	%CV	%Acc
5	1 of 2	4.575	N/A	N/A	91.51
10	2 of 2	10.41	0.075	0.72	104.1
20	2 of 2	20.92	2.216	10.59	104.6
40	2 of 2	42.42	1.233	2.91	106.1
80	2 of 2	82.76	3.638	4.4	103.5
160	2 of 2	166.3	0.075	0.04	103.9
320	2 of 2	312.9	20.23	6.47	97.77
640	2 of 2	639.2	38.34	6	99.88
1280	2 of 2	1231	3.569	0.29	96.2
2560	2 of 2	2258	52.51	2.33	88.19

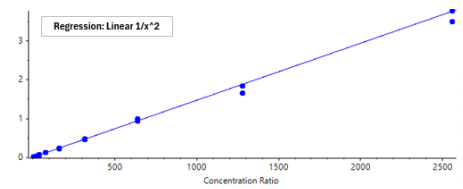
Actual Conc	Num. Value	Mean	StDev	%CV	%Acc
15	3 of 5	12.74	0.671	5.27	84.92
1000	5 of 5	907.4	22.99	2.53	90.74
2000	5 of 5	1892	100.9	5.34	94.58



Kidney

Actual Conc	Num. Value	Mean	StDev	%CV	%Acc
5	2 of 2	4.841	0.909	18.78	96.83
10	2 of 2	9.967	0.978	9.81	99.67
20	2 of 2	21.13	1.042	4.93	105.7
40	2 of 2	45.23	3.332	7.37	113.1
80	2 of 2	82.68	0.857	1.04	103.4
160	2 of 2	165.6	3.814	2.3	103.5
320	2 of 2	332.2	16.43	4.95	103.8
640	2 of 2	612.6	27.54	4.5	95.72
1280	2 of 2	1197	71.1	5.94	93.49
2560	2 of 2	2174	48.92	2.25	84.94

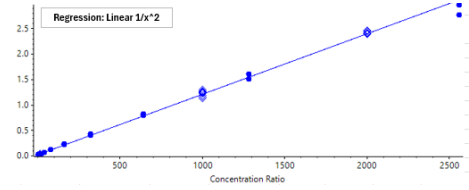
Actual Conc	Num. Value	Mean	StDev	%CV	%Acc
15	5 of 5	13.46	1.067	7.92	89.74
1000	5 of 5	941.9	35.15	3.73	94.19
2000	5 of 5	1862	51.74	2.78	93.09

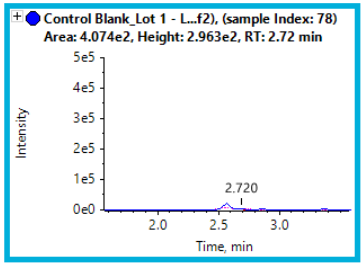


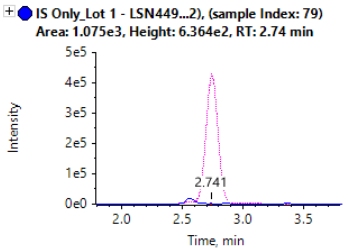
Liver

Actual Conc	Num. Value	Mean	StDev	%CV	%Acc
5	1 of 2	5.676	N/A	N/A	113.5
10	1 of 2	8.165	N/A	N/A	81.65
20	2 of 2	17.92	1.187	6.62	89.62
40	2 of 2	39.11	0.732	1.87	97.78
80	2 of 2	85.47	3.921	4.59	106.8
160	2 of 2	170	7.667	4.51	106.2
320	2 of 2	331.2	14.95	4.51	103.5
640	2 of 2	664	19.62	2.96	103.7
1280	2 of 2	1294	55.5	4.29	101.1
2560	2 of 2	2396	115	4.8	93.58

Actual Conc	Num. Value	Mean	StDev	%CV	%Acc
15	3 of 5	14.89	2.391	16.06	99.24
1000	5 of 5	982.8	37.08	3.77	98.28
2000	5 of 5	2023	26.54	1.31	101.2



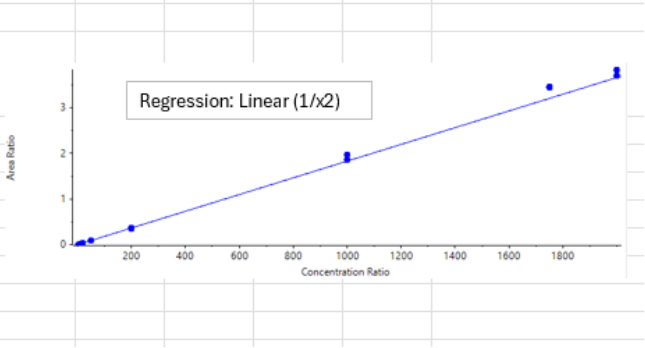


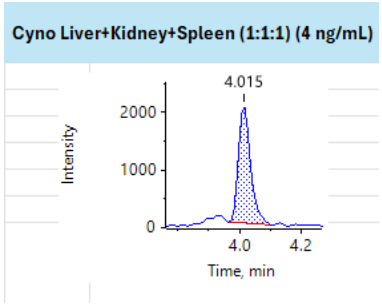


Surrogate Matrix Choices

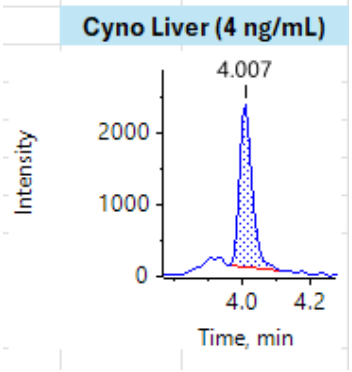
P&A Data for Cyno Kidney, Liver and Spleen

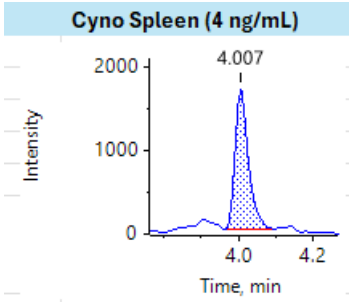
Curve in Cyno Liver+Kidney+Spleen Homogenate Mixture (1:1:1)					
Actual Concentration (ng/mL)	Num. Values	Mean	Standard Deviation	Percent CV	% Accuracy
4	2 of 2	4.15	0.105	2.5	103.8
8	2 of 2	7.72	0.399	5.2	96.6
20	2 of 2	18.4	0.588	3.2	92.2
50	2 of 2	46.6	0.787	1.7	93.2
200	2 of 2	198	4.77	2.4	98.9
1000	2 of 2	1050	43.7	4.2	104.6
1750	2 of 2	1890	8.25	0.4	107.8
2000	2 of 2	2060	50.7	2.5	102.8

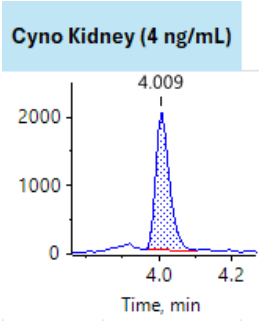




Cyno Kidney QCs					
Actual Concentration (ng/mL)	Num. Values	Mean	Standard Deviation	Percent CV	% Accuracy
4	6 of 6	4.21	0.446	10.6	105.2
12	6 of 6	12.2	0.641	5.3	101.6
400	6 of 6	416	15.5	3.7	104.0
1500	6 of 6	1590	96.6	6.1	106.1
Cyno Spleen QCs					
Actual Concentration (ng/mL)	Num. Values	Mean	Standard Deviation	Percent CV	% Accuracy
4	6 of 6	3.31	0.198	6.0	82.7
12	6 of 6	9.62	0.418	4.3	80.2
400	5 of 5	387	14.2	3.7	96.7
1500	6 of 6	1450	86	5.9	96.5
Cyno Liver QCs					
Actual Concentration (ng/mL)	Num. Values	Mean	Standard Deviation	Percent CV	% Accuracy
4	6 of 6	4.52	0.354	7.8	113.0
12	6 of 6	11.2	0.486	4.3	93.2
500	6 of 6	471	14.7	3.1	94.2
1500	6 of 6	1500	28.2	1.9	100.1



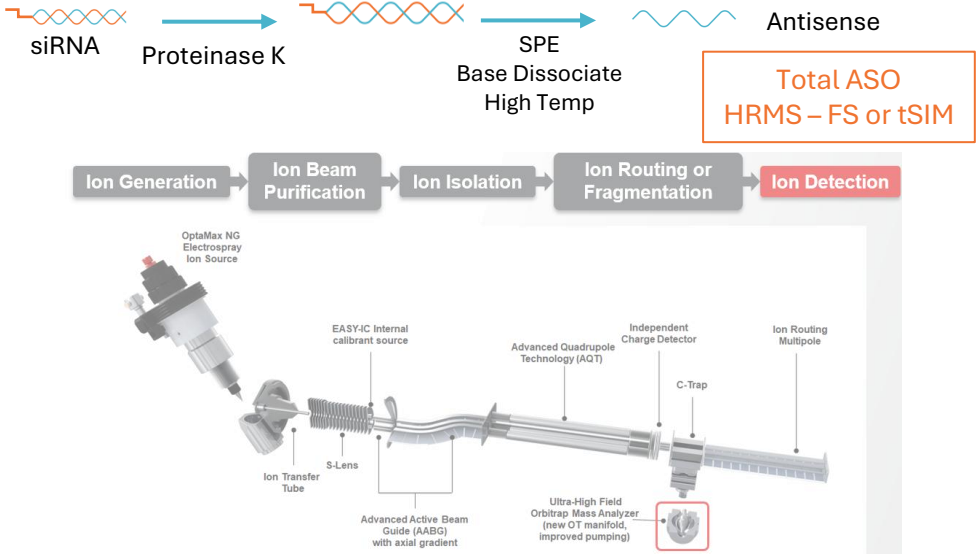


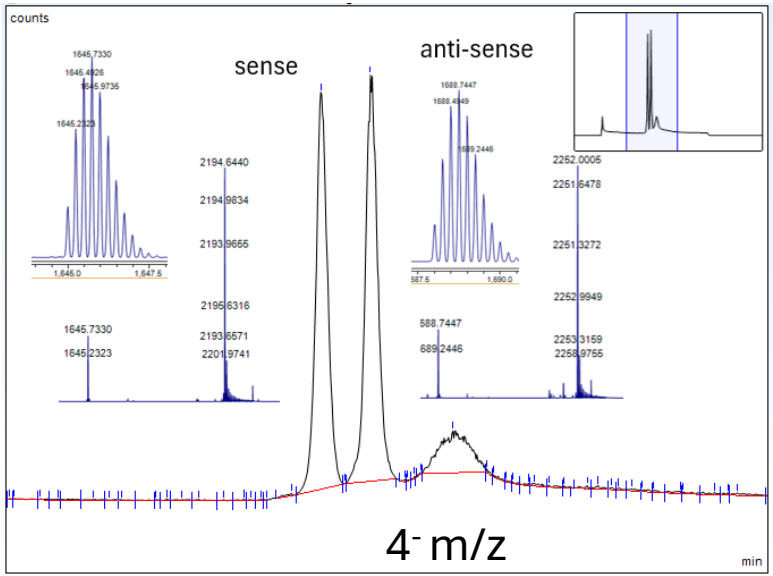


Case Study – siRNA – HRMS

Advantages of HRMS for "Discovery"

- Goal – to quickly be able to develop a "discovery" level method for various siRNA molecules on HRMS to determine an overall LLOQ and general method to be used across many species/matrix/oligos





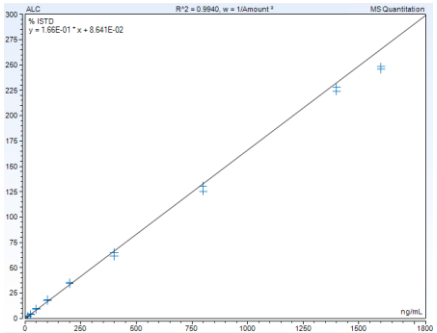
- Full scan data – in more discovery/non-GLP setting to get most information at quickest pace
- Get anti-sense and sense data
- Typically follow 3 or 4 charge state – using similar gradient with Oligo column and ion pairing agent
- Important – to use high purity solvents to minimize adducts etc.

Case Study – siRNA – HRMS

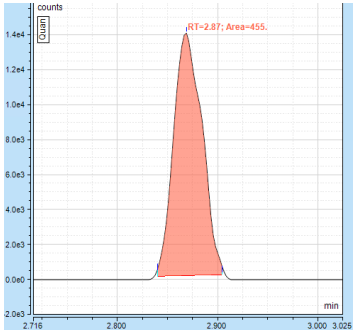
Rat Plasma – siRNA 1 - Precision and Accuracy

Standards							
Name	Theo. Amt ng/mL	Amount ng/mL	%Accuracy	Count	Average Amount	Average Accuracy	%CV
STD 12.5	12.5	12.6	98.83				
STD 12.5	12.5	11.6	107.1	2	12.13	**97.0**	**6.02**
STD 25	25	25.9	96.52				
STD 25	25	23.4	106.32	2	24.65	**98.6**	**7.03**
STD 50	50	53.8	92.34				
STD 50	50	57.3	85.48	2	55.54	**111.1**	**4.36**
STD 100	100	107.1	92.86				
STD 100	100	107	92.99	2	107.08	**107.1**	**0.09**
STD 200	200	206	96.98				
STD 200	200	213.4	93.3	2	209.72	**104.9**	**2.48**
STD 400	400	371.4	107.15				
STD 400	400	391.6	102.1	2	381.5	**95.4**	**3.74**
STD 800	800.00	757.2	105.35				
STD 800	800.00	780.2	102.47	2	768.7	**96.1**	**2.12**
STD 1400	1,400.00	1374.4	101.83				
STD 1400	1,400.00	1336.8	104.52	2	1355.56	**96.8**	**1.96**
STD 1600	1,600.00	1483	107.32				
STD 1600	1,600.00	1495.2	106.55	2	1489.07	**93.1**	**0.58**

Quality Controls							
Name	Theo. Amt ng/mL	Amount ng/mL	% Accuracy	Count	Average Amount	Average Accuracy	%CV
QC 12.5	12.5	12.4	101.02				
QC 12.5	12.5	12.5	100.19				
QC 12.5	12.5	10.4	116.86				
QC 12.5	12.5	12.7	98.3	4	12.0	**96.0**	**7.8**
QC 37.5	37.5	40.7	91.44				
QC 37.5	37.5	41.9	88.32				
QC 37.5	37.5	40	93.44				
QC 37.5	37.5	41.1	90.46	4	40.9	**109.1**	**1.7**
QC 600	600	661.7	89.72				
QC 600	600	678.6	86.91				
QC 600	600	656.3	90.62				
QC 600	600	655.9	90.69	4	663.1	**110.5**	**1.4**
QC 1200	1200	1357.5	86.87				
QC 1200	1200	1372	85.67				
QC 1200	1200	1289.6	92.53				
QC 1200	1200	1263.7	94.69	4	1320.7	**110.1**	**3.4**



Plasma 12.5 ng/mL



- PA in Rat Plasma
- Range 12.5-1600 ng/mL
- Limited MD, Full Scan
- Sense and Antisense data

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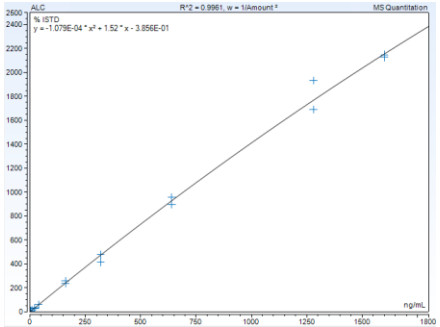
Case Study – siRNA – HRMS

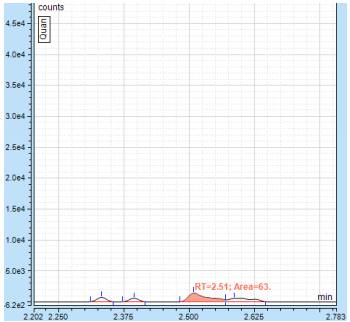
Rat Liver Homogenate– siRNA 1 - Precision and Accuracy

Standards							
Name	Theo. Amt ng/mL	Amount ng/mL	%Accuracy	Count	Average Amount	Average Accuracy	%CV
Std 2	2	2.00801	100.40058				
Std 2	2	1.92926	96.462959	2	1.968635415	**98.4**	**2.8**
Std 4	4	4.11562	102.89048				
Std 4	4	4.01507	100.37668	2	4.065343053	**101.6**	**1.7**
Std 10	10	10.0344	100.34394				
Std 10	10	10.6959	106.9588	2	10.36513713	**103.7**	**4.5**
Std 20	20	19.002	95.009803				
Std 20	20	21.766	108.83017	2	20.38399706	**101.9**	**9.6**
Std 40	40	38.4945	96.236125				
Std 40	40	38.8612	97.152954	2	38.6778158	**96.7**	**0.7**
Std 160	160	156.265	97.665401				
Std 160	160	172.246	107.65374	2	164.2553129	**102.7**	**6.9**
Std 320	320	277.636	86.761298				
Std 320	320	320.574	100.17936	2	299.1050544	**93.5**	**10.2**
Std 640	640	660.043	103.13171				
Std 640	640	616.536	96.333777	2	638.289543	**99.7**	**4.8**
Std 1280	1,280	1216.84	95.065483				
Std 1280	1,280	1414.58	110.51418	2	1315.709814	**102.8**	**10.6**
Std 1600	1600	1594.81	99.675385				
Std 1600	1600	1578.18	98.636293	2	1586.493428	**99.2**	**0.7**

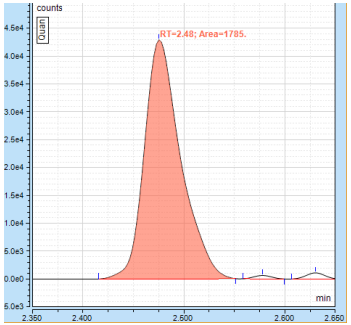
Quality Controls							
Name	Theo. Amt ng/mL	Amount ng/mL	% Accuracy	Count	Average Amount	Average Accuracy	%CV
Liver QC 2	2	1.69	84.75				
Liver QC 2	2	1.88	94.05				
Liver QC 2	2	2.24	111.86	3	1.9	**96.9**	**14.2**
Liver QC 6	6	5.85	97.55				
Liver QC 6	6	7.04	117.33				
Liver QC 6	6	6.87	114.51	3	6.6	**109.8**	**9.7**
Liver QC 600	600	625.53	104.25				
Liver QC 600	600	672.01	112.00				
Liver QC 600	600	641.59	106.93	3	646.4	**107.7**	**3.7**
Liver QC 1200	1200	1219.89	101.66				
Liver QC 1200	1200	1145.42	95.45				
Liver QC 1200	1200	1289.39	107.45	3	1218.2	**101.5**	**5.9**



Liver Zero



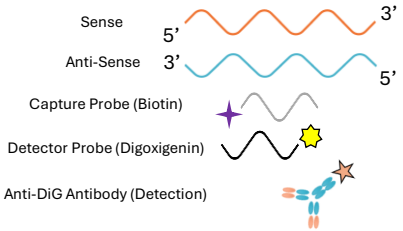
Liver 2 ng/mL



- PA in Rat Liver Homogenate
- Range 2-1600 ng/mL
- Limited MD, Full Scan
- Sense and Antisense data

Case Study – siRNA – hELISA

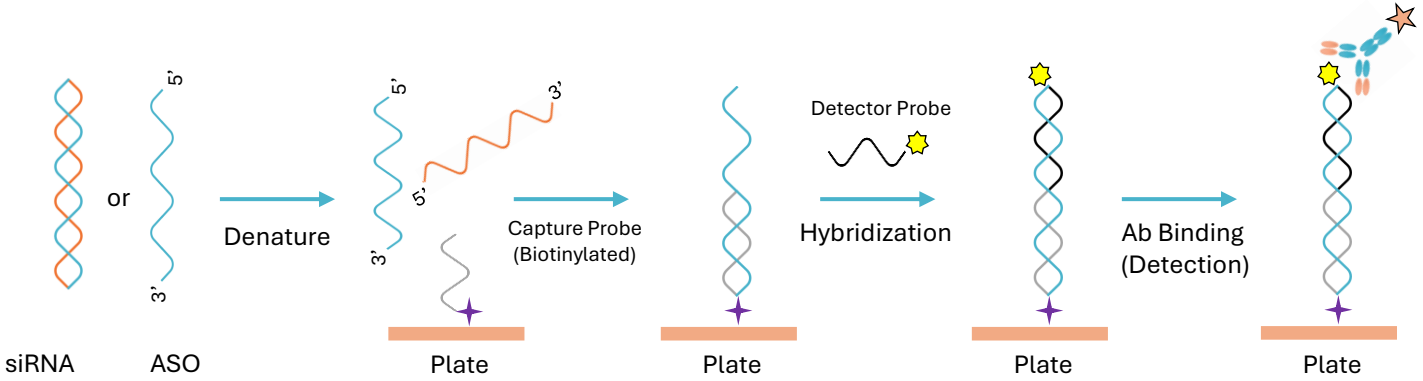
Hybridization ELISA for siRNA or Anti-sense Oligo



- Goal – To develop a hybridization ELISA in NHP Plasma for PK measurement of a gene therapy drug

- Background
  - SiRNA
  - Previous method (colorimetric) had some high background interference

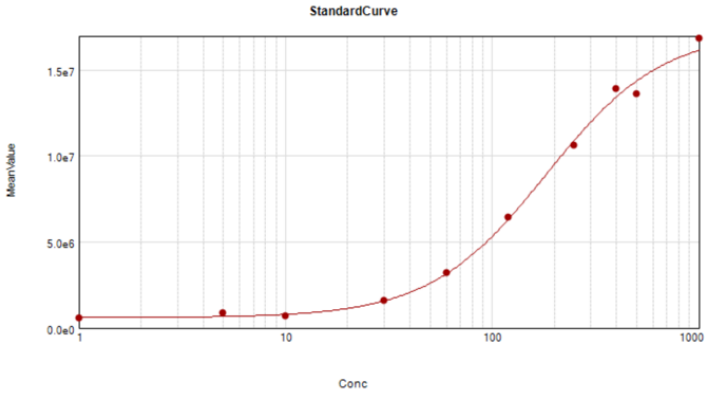
- Approach
  - Dual Hybridization Assay with 1 capture oligo and 1 detector oligo
    - Capture probe – Biotinylated on SA Plate
    - Detector Probe (labeled with Dig)
  - Optimize Assay
    - Anti-Dig Ab
    - Fluorescent detection, screen substrates



Case Study – siRNA – hELISA

Oligo PK for siRNA - Data in NHP plasma

- **Challenge:** Client had developed an internal method with a high background.
- **Solution:** Performed troubleshooting (plates, buffers, and detection labels) approaches.
- **Outcome:**
  - Identified plate type, optimal buffer, optimal DIG labeled detector, and fluorescence substrate.
  - Plasma method can be adopted to tissues as needed.
  - Fit-For Purpose non-regulated performance characteristics below
  - Tested pre-clinical samples for support of NHP Study



Performance Charateristics	Result (ng/mL)
Range of quantification	1-500 (%RE Range 5.7% to 10.5%)
HQC	370 (%RE Range 3.0% to 14.1%)
MQL	50 (%RE Range 1.0% to 14.7%)
LQC	15 (%RE Range 1.0% to 11.1%)
A/P	Passed (3 A/P runs performed)
Selectivity HQC	370 (%RE 0.4 to 18.8)
Selectivity LQC	15 (%RE Range 0.6% to 17.7%)

- Conclusion
  - Research Grade (FFP) method developed to measure non-GLP samples
  - LLOQ – 1 ng/mL

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Summary/Conclusions

ADCs, ARCs/AOCs, Oligos etc

- Pharmaceutical and Biotech companies continue to develop complicated, multifaceted drugs. This continues to push the Bioanalysis community into constantly re-evaluating and driving the science to support these new modalities
- As challenging modalities continue to emerge (ADCs, ARCs, bi/tri specifics etc), LC-MS/MS and/or Hybrid LC-MS/MS is becoming increasingly utilized for bioanalytical assays in drug development
  - Additional selectivity/specificity or information
  - Ability to multiplex and translate assays between species and matrices
- ADCs
  - Many types of ADCs in development – requiring several assays to properly "characterize" the therapeutic
  - Can require several bioanalytical strategies/techniques depending on the need
- Oligos – Conjugates or stand alone (siRNA, ASO, etc)
  - Many different complimentary techniques for the analysis of oligonucleotides
    - Each have advantages and disadvantages (LCMS, LBA, Hybrid, PCR, HPLC, etc)
  - Oligo analysis (ARCs or ASO) - LCMS
    - Choices
      - "Traditional" vs "Hybridization" techniques
      - QqQ vs HRMS
    - Dedicated instruments – ion pairing – chromatography – painful for flexibility at CRO
- Recommendation to choose a CRO that has ability to support these complicated therapeutics with several bioanalytical techniques/strategies

Thank you

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