Recent Advances in Oligonucleotides & Peptides Bioanalysis by Triple Quad and HRMS

Regulated Bioanalysis Interest Group (RBIG)

5:45 – 7:00 pm, Wednesday, June 8th 2022

Presiding:
Jian Wang (Crinetics Pharmaceuticals), Wenkui Li (Novartis-NIBR), Dian Su (Mersana Therapeutics)

Panelists:
Kevin Guo, Ph.D. (Avice Laboratories), Nicole A. Schneck, Ph.D. (GlaxoSmithKline),
Ming Wang, M.A. (Merck), and Karan Agrawal, Ph.D. (Janssen Research & Development)

Introduction

Kevin Guo, Ph.D.

Kevin Kun Guo, Ph.D. had worked as a Senior Applications Scientist at Thermo Fisher Scientific for 9 years, with 18 years of experience running LC/MS, specializing in oligonucleotide analysis and other pharmaceutical and biopharmaceutical applications. Before joining Thermo Fisher he was a Mass Spectrometry Scientist at Immunogen, demo scientist at Bruker, and completed his Ph.D. at the University of Alberta. Kevin is currently the president of the Avice Laboratories, his own oligonucleotide LC/MS lab.

Nicole A. Schneck, Ph.D.

Investigator in the Bioanalysis, Immunogenicity and Biomarkers Department at GSK since 2019. Scientist in the Analytical Department within the Vaccine Research Center, National Institutes of Health (NIH) in Maryland USA (2016 – 2019). Ph.D. in Analytical Chemistry from the University of Maryland, College Park (2011 – 2016). Experience in both discovery and regulated bioanalysis in a pharmaceutical setting leading internal biomarker, PK, and pCQA projects of large biomolecules by LC-MS. Additional expertise in high-resolution LC-MS characterization of vaccine and biotherapeutics during biopharmaceutical process development. Member of ASMS since 2013

Ming Wang, M.A.

Principal Scientist in Regulated Bioanalytics at Merck & Co since 2004. Principal Scientist in DMPK at Purdue Pharm (2000-2004). MA in analytical chemistry from City College of New York (1995-1997). 25+ years of experience in discovery and regulated bioanalysis in pharmaceutical industry. Developing preclinical and clinical bioanalytical assays of both small and large molecules in LC/MS and ligand binding-LC/MS hybrid platforms. Managing internal projects of both small and large molecules to support TK and PK studies.

Karan Agrawal, Ph.D.

Scientist in Bioanalytical Discovery and Development Sciences at Janssen Research & Development since 2021. Staff Scientist in Bioanalytical Services at Labcorp Drug Development (2018-2021). Ph.D. in Pharmacology and Toxicology from University of California-Davis (2013-2017), M.Sc. in Forensic Science from King's College London (2012-2013), B.A. from Cornell University (2012). 4+ years of experience in discovery and regulated bioanalysis in pharmaceutical industry developing and validating methods for PK and biomarker analysis of small molecules, oligonucleotides and proteins/antibodies by LC-MS/MS, ligand binding and ligand binding-LC-MS/MS hybrid platforms.

Agenda

05:45-05:50 pm – Introduction

05:50-05:55 pm – ***Recent Advances in LC-HRAM Oligonucleotide Analysis***

-Kevin Guo, Ph.D. (Avice Laboratories)

05:55-06:05 pm – ***Overview: Analytical Strategies for Intact Peptide Bioanalysis by LC-MS***

-Nicole A. Schneck, Ph.D. (GlaxoSmithKline)

06:05-06:10 pm – ***Bioanalysis of Intact Peptide with LCMS***

-Ming Wang, M.A. (Merck)

06:10-06:15 pm – ***Hybrid LC-MS/MS Workflows for siRNA Analytes***

-Karan Agrawal, Ph.D. (Janssen Research & Development)

06:15-07:00 pm – Panel Discussions

Kevin Guo, Ph.D.

Kevin Kun Guo, Ph.D. had worked as a Senior Applications Scientist at Thermo Fisher Scientific for 9 years, with 18 years of experience running LC/MS, specializing in oligonucleotide analysis and other pharmaceutical and biopharmaceutical applications. Before joining Thermo Fisher he was a Mass Spectrometry Scientist at Immunogen, demo scientist at Bruker, and completed his Ph.D. at the University of Alberta. Kevin is currently the president of the Avice Laboratories, his own oligonucleotide LC/MS lab.

Recent Advances in LC-HRAM Oligonucleotide Analysis

Kevin Guo, Ph.D.

Kevin.guo@avicelabs.com
Avice Laboratories,
289 Great Road, Acton, MA

Recent Development: Chromatography Separation

- Liquid Chromatography
  - Reversed-phase Ion Pairing: much less ion pair and HFIP concentration is one of the trends with the new development of column chemistry

	Low Concentration Range	Common Concentration Range
	(with new column chemistry)	
TEA	2 - 7 mM	8 - 20 mM
DIPEA (or DIEA)	1 - 3 mM	4 -8 mM
HFIP	10 - 50 mM	70 - 400 mM

  - HILIC
    - Promising, however, some oligo won't work well in chromatography.
- CE and Zip-Chip
  - Short oligo works fine on ZipChip.

MS2, MSn, Pseudo-MS3, and Software Progress

- ddMS2 offers advantages over traditional MS1-based oligo characterization
  - Provides unambiguous sequence confirmation base-by-base and accurate localization of the modification (for short and medium oligo).
  - Enable identification and structural elucidation of unknown or unexpected impurities/metabolites.
  - ddMS2 is easy to execute without a need to build a target mass list.
  - Commercial software is available for ddMS2 data
- ddMS2 of digested mRNA
- UVPD, negative ETD, and other fragmentation techniques
- MS2 challenges, MS3, MSn and Pseudo-MS3 (on hybrid orbitrap and Qtof)
- Software progress

Oligo Quantitation: MS1 vs MS2

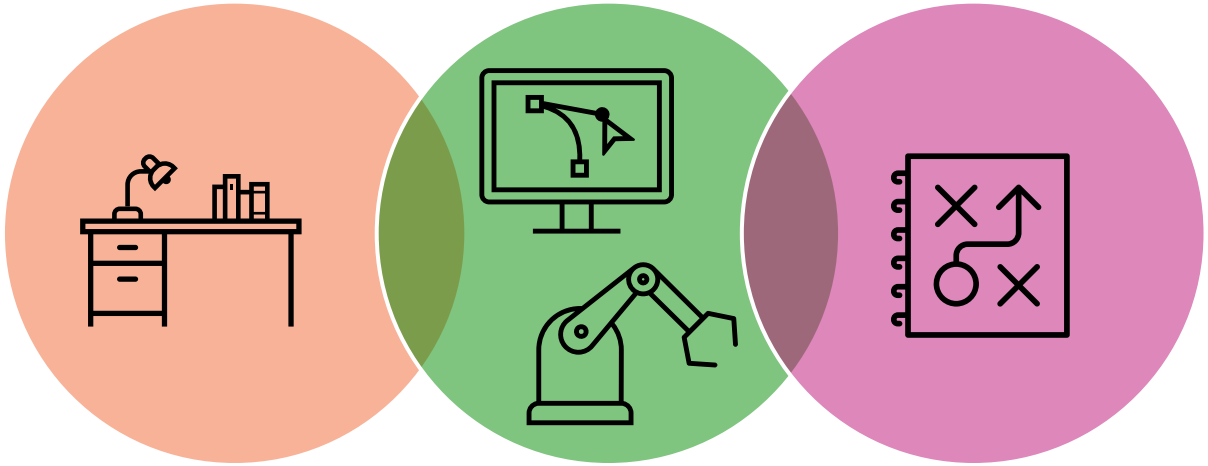
- MS1, preferably targeted HRAM MS1, is easy to execute
  - without the need for optimal collision energy
  - Sensitive, take advantage of summation of multiple charge states and isotopes (for high-resolution isotopic resolving spectra)
- MS2, preferably targeted HRAM MS2, (not ddMS2) offers advantages over commonly used (targeted) MS1-based quantitation
  - High specificity with HRAM MS2
  - Order of magnitude lower background than HRAM MS1.
  - The optimal collision energies are needed.
- Choices of internal standards in isotope dilution quantitation

Nicole A. Schneck, Ph.D.

Investigator in the Bioanalysis, Immunogenicity and Biomarkers Department at GSK since 2019. Scientist in the Analytical Department within the Vaccine Research Center, National Institutes of Health (NIH) in Maryland USA (2016 – 2019). Ph.D. in Analytical Chemistry from the University of Maryland, College Park (2011 – 2016). Experience in both discovery and regulated bioanalysis in a pharmaceutical setting leading internal biomarker, PK, and pCQA projects of large biomolecules by LC-MS. Additional expertise in high-resolution LC-MS characterization of vaccine and biotherapeutics during biopharmaceutical process development. Member of ASMS since 2013

Overview: Analytical Strategies for Intact Peptide Bioanalysis by LC-MS

Nicole A. Schneck, Ph.D.



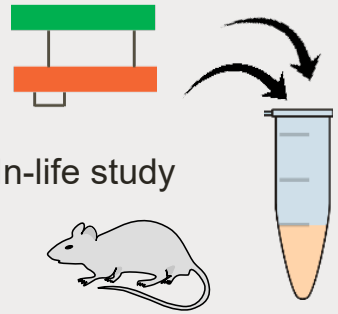
1.) Intact peptide quantification – **case study** with focus on method development

2.) Advancing method quality by **Design of Experiment** (DoE) + **Automation**

3.) LC-MS strategies for >10 kDa peptides

Method Development Strategy for ≈ 6.2 kDa Disulfide-Bonded Peptide Bioanalysis using LC-MS/MS (QqQ) – Intact Peptide Level

Case study



What: measure dosed peptide in rat plasma

How: targeted LC-MS/MS (via SRM / QqQ)

Why: determine PK

Challenges

Plasma composition

↓

Intact peptide + disulfide bonds

↓

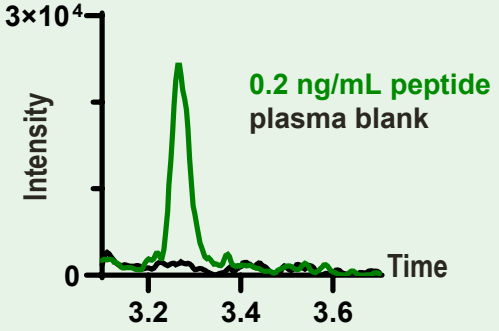
Sub-ng/mL LLOQ needed

Solutions to improve assay sensitivity

Peptides: Discovery → Development

- Extraction: PPT, SPE, IAP
- + I.S.
- RPLC
- MS/MS

Example XIC at LLOQ



2021 ASMS presentation (Schneck, NA., *et al.)*

PPT = protein precipitation
SPE = solid phase extraction
IAP = immunoaffinity purification
I.S.= internal standard

Integrating Design of Experiment (DoE) to Help Achieve Sensitive & High-Quality LC-MS/MS Assays for Challenging Large Peptides

Factor	# Levels	Levels
Pre-treatment / PPT	1	2% FA
Wash Comp.	1-2	MeOH vs. ACN
Wash % Organic	5-6	5% – 50%
Elution Comp.	2	MeOH vs. ACN
Elution % Organic	1	50%

1.) Wash
2.) Elution
3.) Pre-treatment
4.) Re-fine

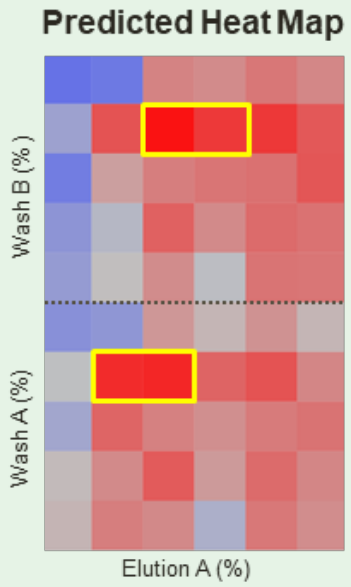
Current State

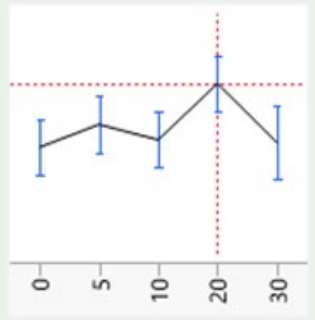
Desired Future State

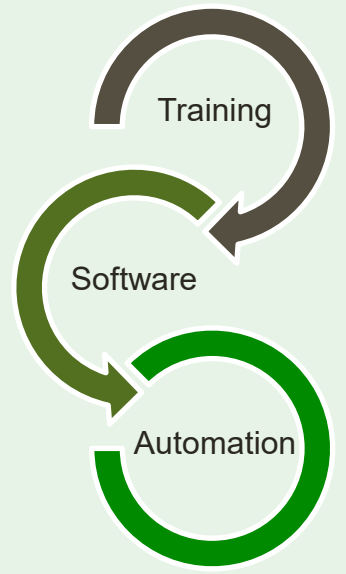
1.) Wash ↔ Elution ↔ Pre-treatment
2.) Re-fine

Rely on statistical analysis

Factor	# Levels
Pre-treatment / PPT	2
Wash Comp.	2
Wash % Organic	5
Elution Comp.	2
Elution % Organic	6





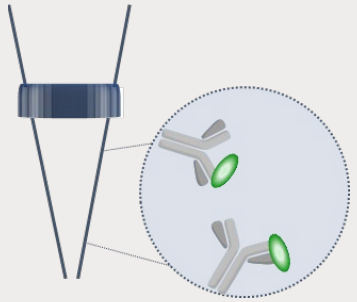


Design
Screen
Optimize

How to fill the gap? Advantages vs. Disadvantages

LC-MS Considerations for Peptides Larger than 10 kDa - HRMS

Case study

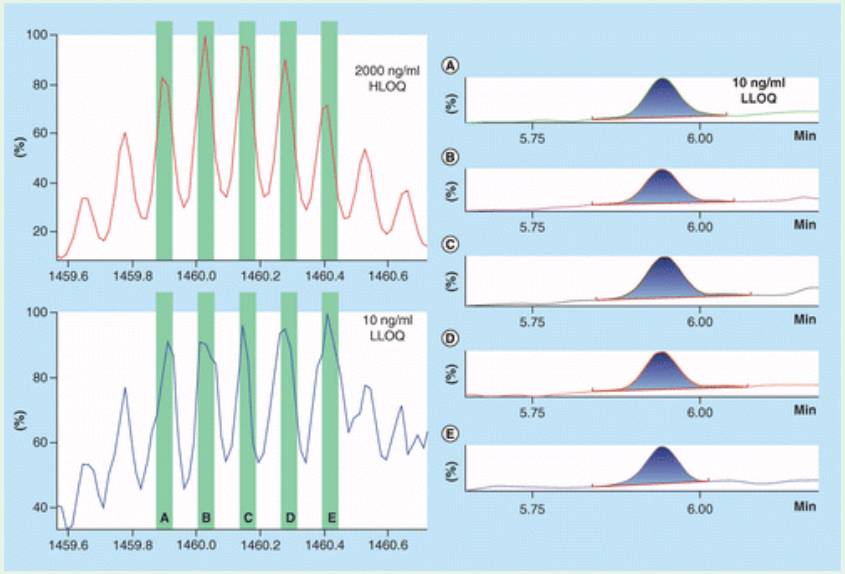


What: measure domain antibody (12 kDa) in rat or human plasma

How: immunocapture & HRMS (MS1 scan)

Why: establish intact protein quantification best practice

Example HRMS data from multiple isotope quantitation of a domain antibody



(**Left**): Isotopes for a single charge state (+8) shown with a 0.05 m/z XIC integration; (**Right**): XICs from each isotope within the charge state

Quantification Mode	Entire charge state	One isotope	Five isotopes
1 charge state	0.995	0.993	0.995
2 charge state	0.995	0.996	0.996
3 charge state	0.996	0.995	0.997
4 charge state	0.996	0.996	0.995
Deconvoluted mass	0.979	N/A	N/A

Charge state data demonstrated better linearity

Quantitation of 1 or a few charge states demonstrated good S/N while maintaining assay linearity

Kellie, JF., et al. Bioanalysis (2017), 9(23), 1883-1893

Acknowledgements

Statements

– All studies were conducted in accordance with the GSK Policy on the Care, Welfare and Treatment of Laboratory Animals and were reviewed the Institutional Animal Care and Use Committee either at GSK or by the ethical review process at the institution where the work was performed.

GSK
Protein Mass Spectrometry Group

John Mehl
Jack Kellie
Tom Angel
Hermes Licea-Perez
Ahmed Moghieb
Bo An
Lela Mortezavi
Zhuo Chen
Tim Sikorski
Huaping Tang

Ming Wang, M.A.

Principal Scientist in Regulated Bioanalytics at Merck & Co since 2004. Principal Scientist in DMPK at Purdue Pharm (2000-2004). MA in analytical chemistry from City College of New York (1995-1997). 25+ years of experience in discovery and regulated bioanalysis in pharmaceutical industry. Developing preclinical and clinical bioanalytical assays of both small and large molecules in LC/MS and ligand binding-LC/MS hybrid platforms. Managing internal projects of both small and large molecules to support TK and PK studies.

Bioanalysis of Intact Peptide with LCMS

Ming Wang

- Increased specificity
  - Multiple aspects - sample preparation, LC separation, MS/MS
  - Surrogate peptide analysis may not reflect the modifications in the molecule
- Ability to readily quantitate multiple analytes including metabolites in the same assay
- Help to evaluate in vivo structural modification, including deamidation, biotransformation, etc.
- Large dynamic range compared to LBA assay
- Improved reproducibility, precision, and accuracy
- **Currently, LCMS bioanalysis of intact peptide is our primary approach for TK and PK sample analysis**

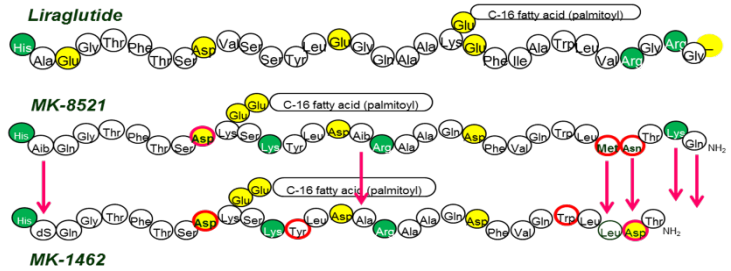
MERCK
INVENTING FOR LIFE

Bioanalysis of Intact Peptide with LCMS - Immunoaffinity Purification (IAP), Example 1

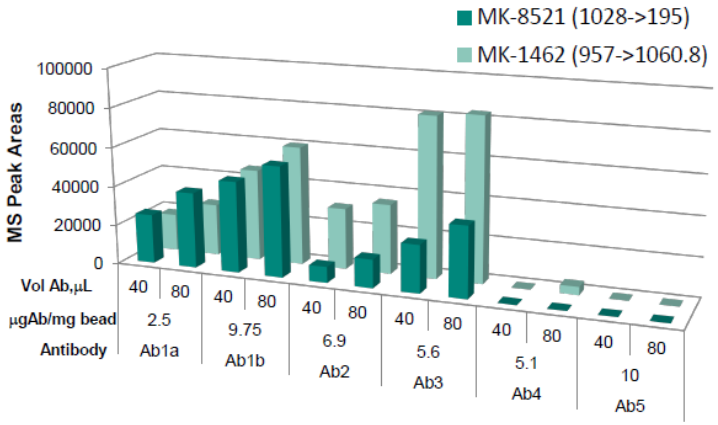
Considerations of IAP procedures

- Specific antibody *vs.* generic approach
- Supply resource and cost
- Ratio of antibody and magnetic beads
- Ratio of Ab-beads *vs.* sample volume
- Wash solvent composition and volume
- Dissociation solvent composition and volume

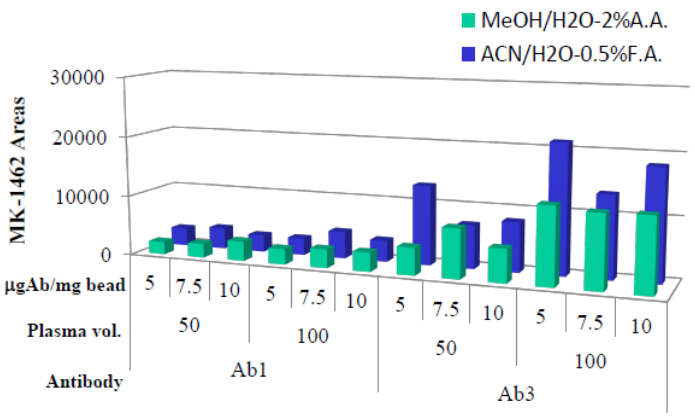
Glucagon-like peptide-1 receptor (GLP-1R) agonists



Selection and Optimization of Ab concentration and volume



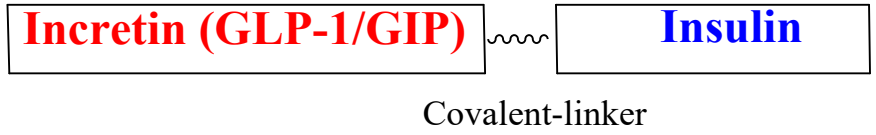
Optimization of dissociation solvent and sample volume



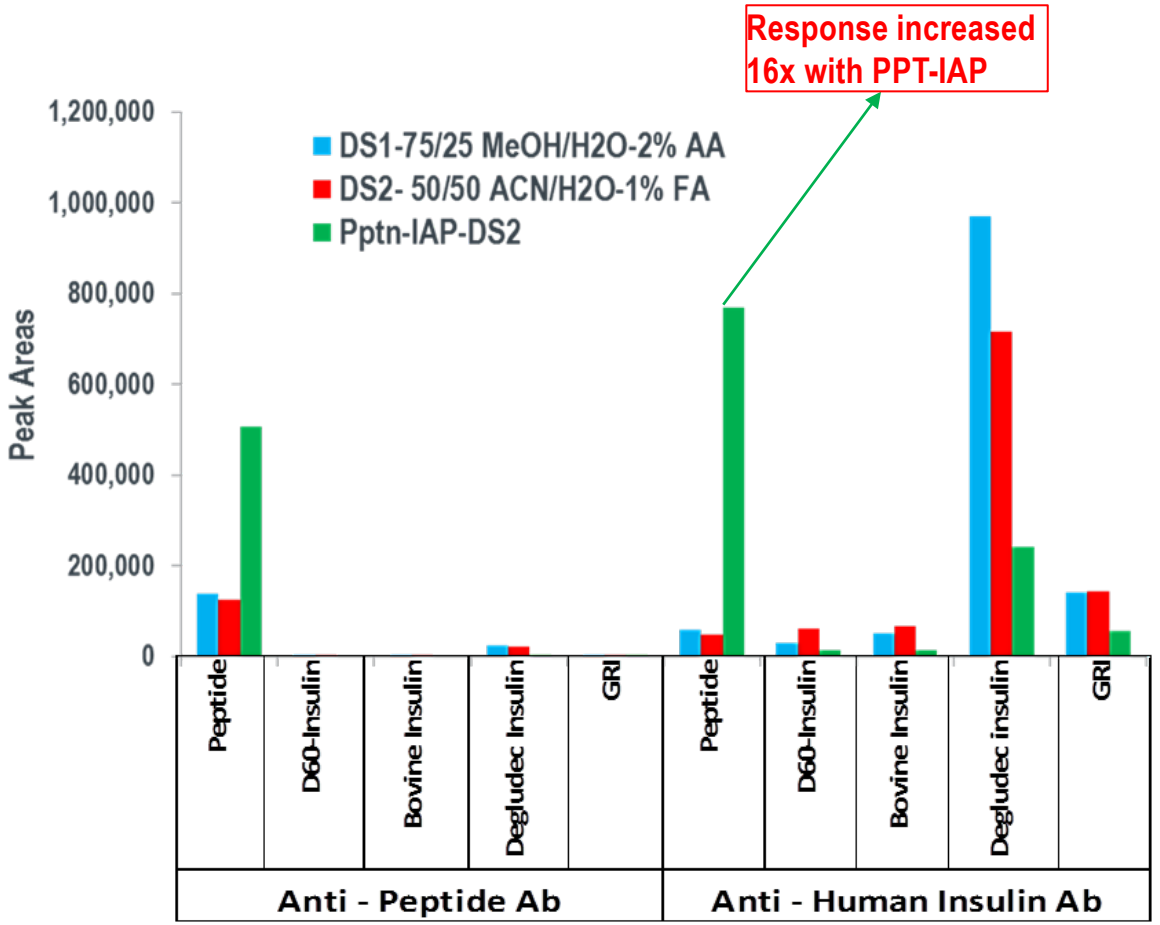
MERCK
INVENTING FOR LIFE

Bioanalysis of Intact Peptide with LCMS - Immunoaffinity Purification (IAP), Example 2

Incretin-insulin fusion drug, MW ~10500 Da



- 0.5 -200 ng/mL in rat and monkey plasma with 100 µL plasma
- Protein precipitation + IAP
- Optimization of Ab and internal standard
  - SIL-Peptide (D60)-failed specificity requirements
  - Insulin Analogs: D60 Insulin, Bovine Insulin, Insulin Degludec, Glargine

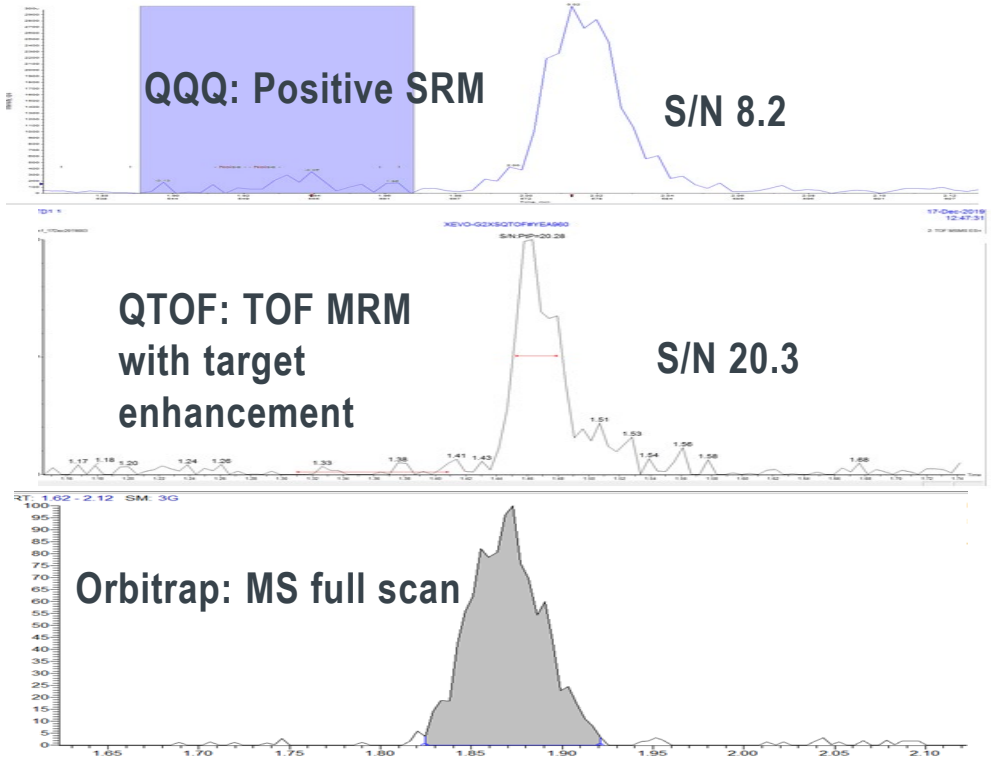


MERCK
INVENTING FOR LIFE

Bioanalysis of Intact Peptide with LCMS – QQQ vs. HRMS

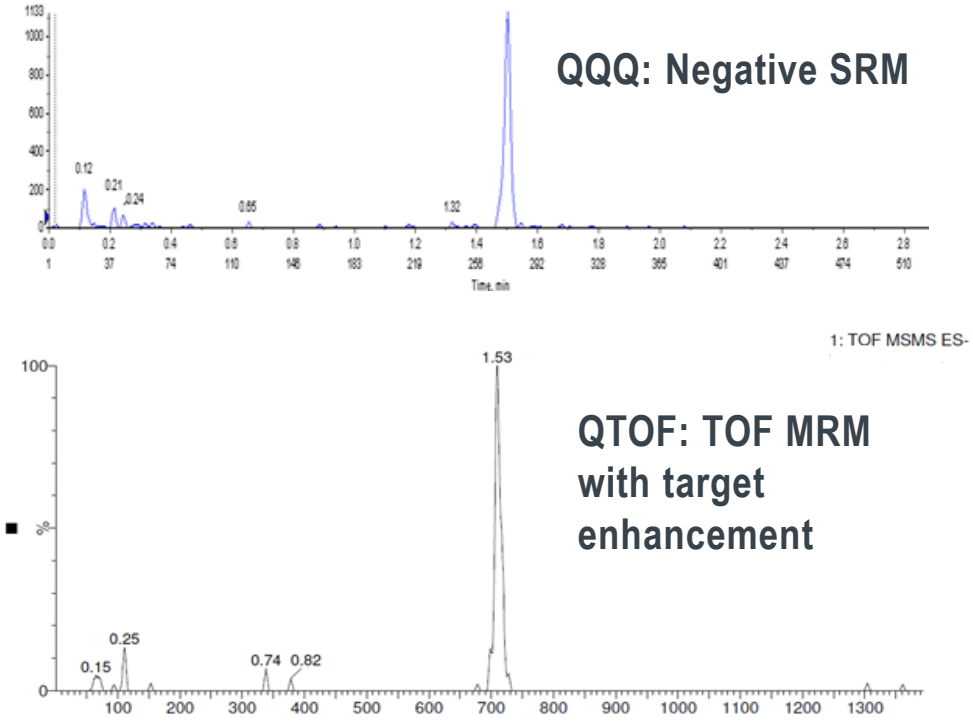
Cyclic peptide 1, MW: ~1600 Da

0.5 ng/mL extracted human plasma sample



Cyclic peptide 2, MW: ~2500 Da

2 ng/mL extracted monkey plasma sample

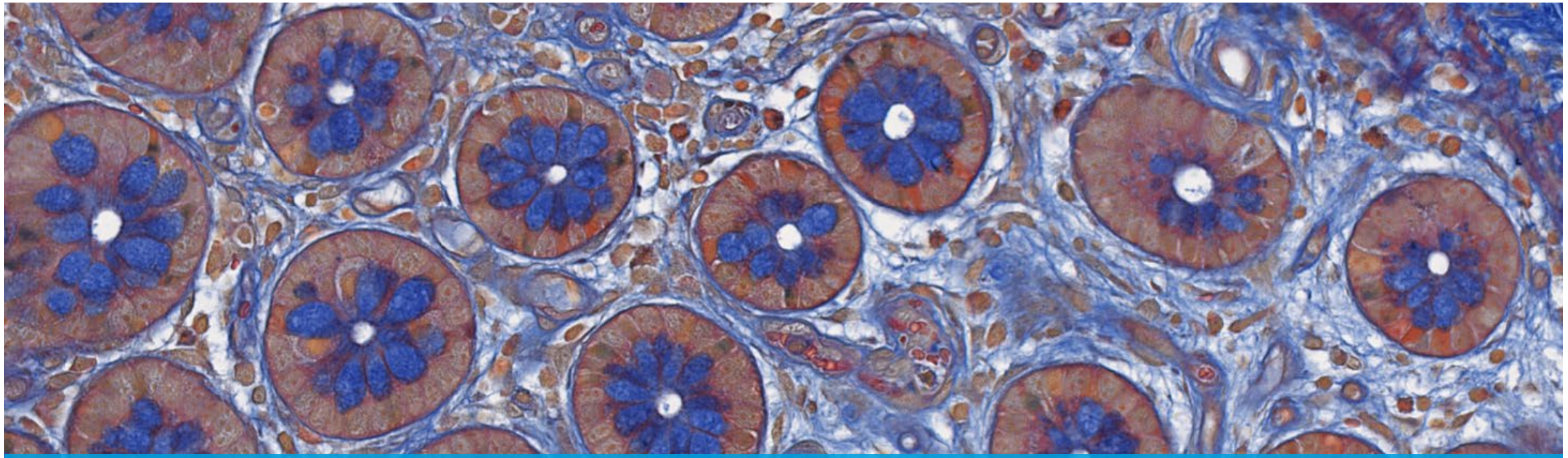


Assay accuracy, precision, selectivity, specificity and matrix effect were evaluated with QQQ and HRMS. The results were comparable between these two platforms.

MERCK
INVENTING FOR LIFE

Karan Agrawal, Ph.D.

Scientist in Bioanalytical Discovery and Development Sciences at Janssen Research & Development since 2021. Staff Scientist in Bioanalytical Services at Labcorp Drug Development (2018-2021). Ph.D. in Pharmacology and Toxicology from University of California-Davis (2013-2017), M.Sc. in Forensic Science from King's College London (2012-2013), B.A. from Cornell University (2012). 4+ years of experience in discovery and regulated bioanalysis in pharmaceutical industry developing and validating methods for PK and biomarker analysis of small molecules, oligonucleotides and proteins/antibodies by LC-MS/MS, ligand binding and ligand binding-LC-MS/MS hybrid platforms.



Hybrid LC-MS/MS Workflows for siRNA Analytes

Karan Agrawal
Scientist, BDDS
08-June-2022

Colonic mucosa highlighted by Picro-Mallory trichrome special stain, from an exploratory study for Pulmonary Arterial Hypertension (PAH)

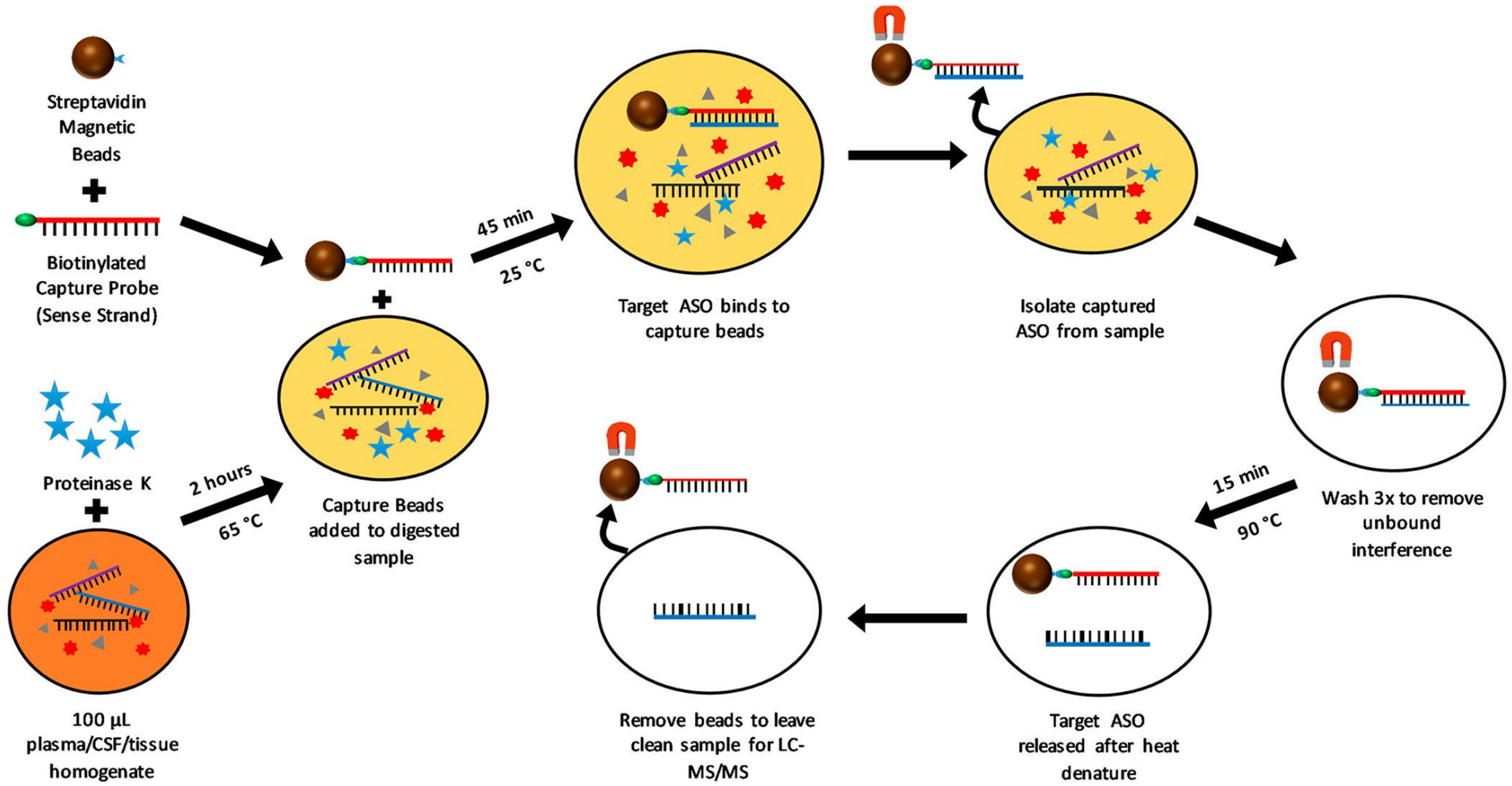
Credit: Vini Carreira, Pathology, Preclinical Sciences & Translational Safety

Published Hybrid LC-MS/MS Methods for Oligonucleotides

- **Li et al, Anal Chem 2020**
  - 17-mer ASO analysis using AB Sciex 6500+
  - Calibration range of 0.5 – 500 ng/mL using 100 µL of plasma
  - Recovery ~ 80-100%

- **Sips et al, Bioanalysis 2019**
  - 16-mer ASO analysis using AB Sciex 4000
  - Calibration range of 10 – 10,000 ng/mL using 20 µL of plasma
  - Recovery ~ 90-100%
  - SPE analysis of the same OGNs had similar sensitivity and recovery, but using 50 µL plasma

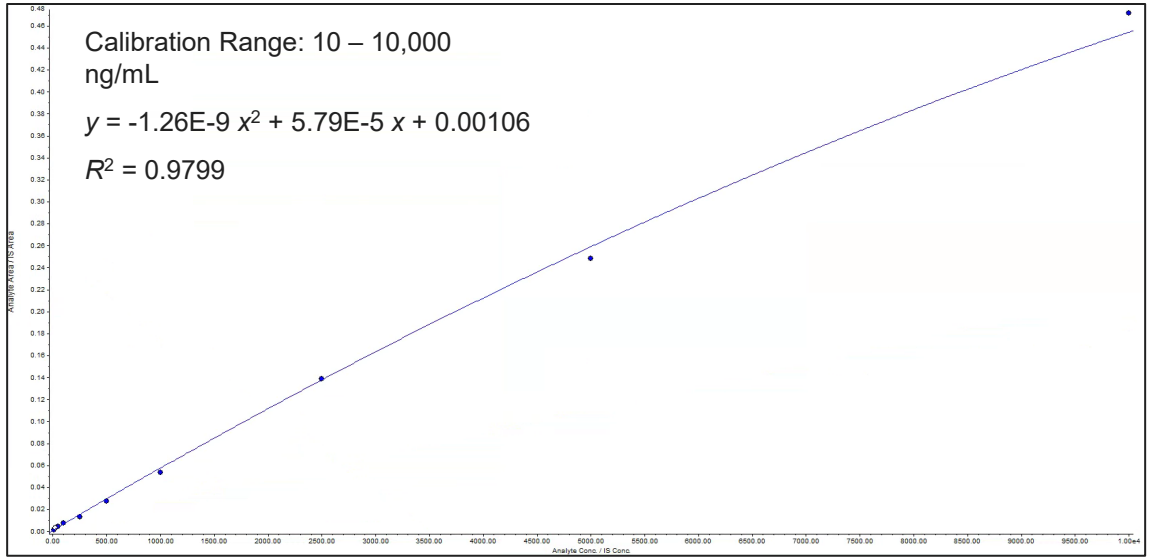
Hybrid LC-MS/MS for ASO Analytes

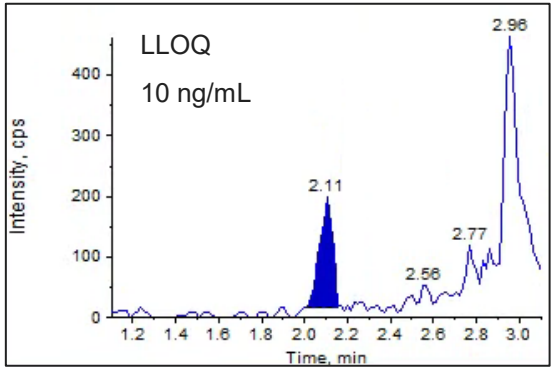


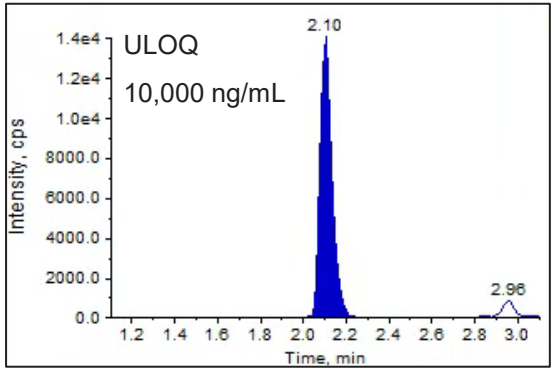
Pei et al, Anal Chem 2020

Hybrid LC-MS/MS for siRNA Analytes

- 20-mer cholesterol conjugated siRNA
- 10 µL plasma aliquot volume
- Capture using 15 µL streptavidin-coated beads conjugated with 90 pmol DNA probe with locked nucleic acids
- Heat to 95 C to un-anneal siRNA and anneal to probe by cooling to 22 C over 75 min
- Elute into 200 µL of starting mobile phase
- Analyze using Shimadzu 20 series autosampler coupled to AB Sciex API 5000 mass spectrometer







QC Concentration (ng/mL)	Accuracy (%)	%CV	Recovery (%)
8000	115	5.81	24.1
4000	94.7	3.67	23.6
400	97.7	14.8	~~82.1~~*
40	130	24.9	~~149~~*

* Impacted by non-specific binding of analyte in the recovery solution

Method Optimization Points for Oligonucleotide Hybrid LC-MS/MS Assays

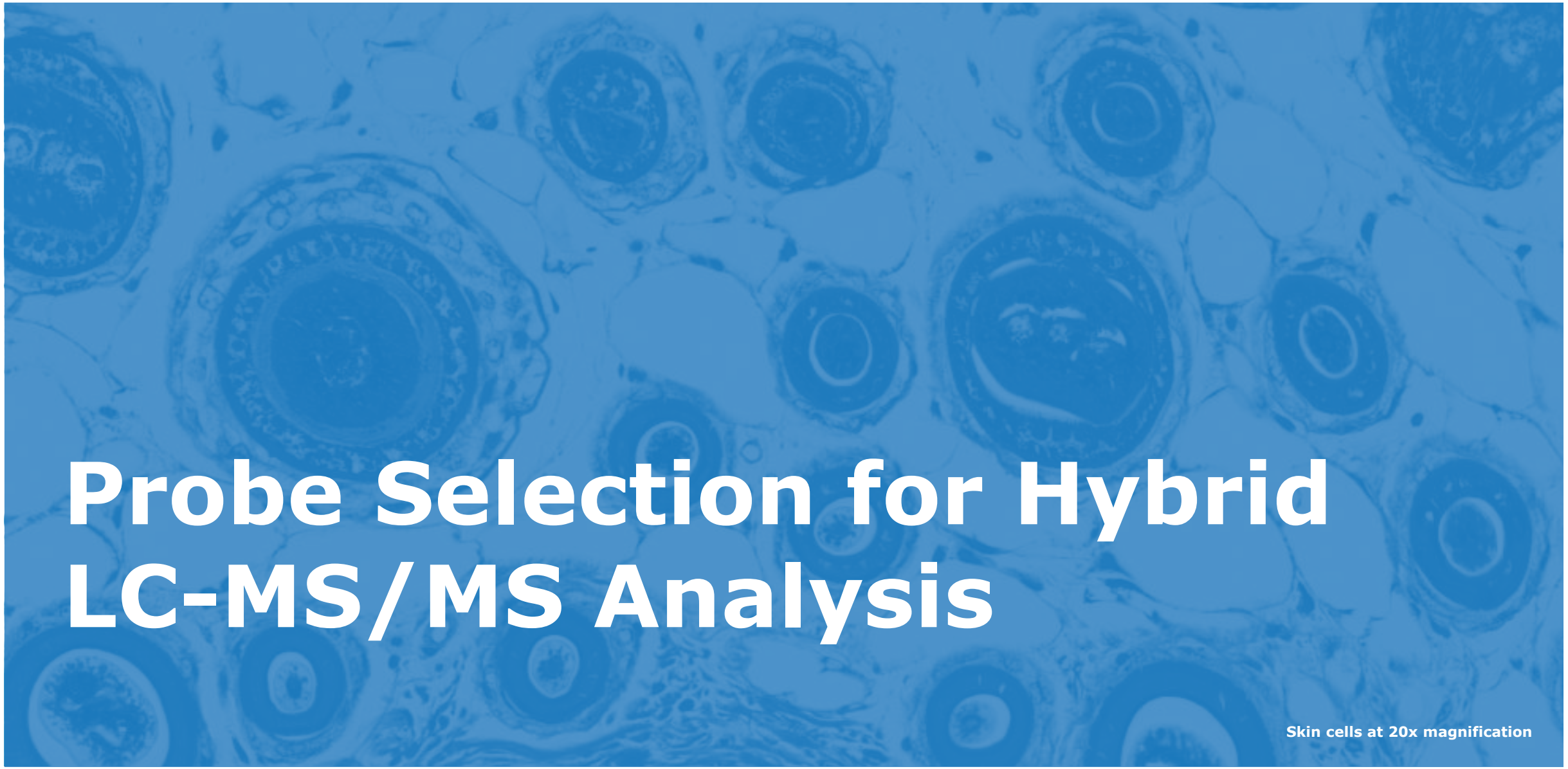
- **Capture probe type:** Complement Sequence v/s LNA v/s PNA
- **Ratio of matrix to capture probe:** Balancing aliquot volume with desired sensitivity
- **Protease K digestion:** Amount of Protease K and Digest Time
- **Un-annealing temperature:** Separating strands for siRNA analytes without compromising biotin-streptavidin integrity of the capture probe
- **Elution temperature:** Eluting only the analyte without separating the probe from the streptavidin beads

Backup for Q/A

Q/A 1.1

- ***Role of Design of Experiments (DoE)?***
  - ***To achieve low quantification levels for an intact peptide***
  - ***Should (DoE) be utilized more <u>regularly</u> to develop LC-MS assays, especially in cases where dual enrichment strategies are employed?***
  - ***What are some <u>bottlenecks</u> to applying DoE and statistical analysis for efficient and high-quality method development outcomes?***
  - ***What types of automation are being utilized to help with assay development?***

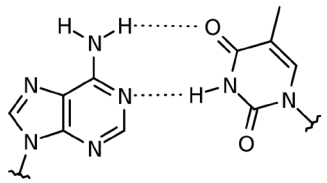
Probe Selection for Hybrid LC-MS/MS Analysis



Skin cells at 20x magnification

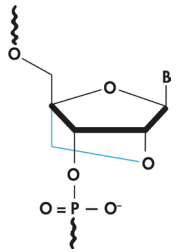
Probe Options for Hybrid LC-MS/MS Analysis

Complement Strand Probe



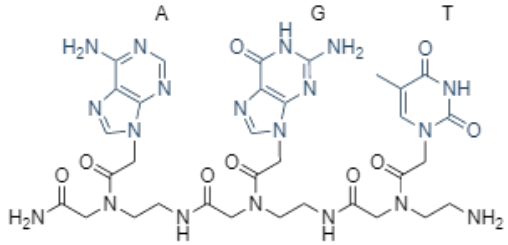
- Identical to sense strand in siRNA without additional modifications
- Lowest thermal stability
- Potential for competition with sense strand during annealing process
- Lowest probability of self-hybridization

Locked Nucleic Acid (LNA) Probe



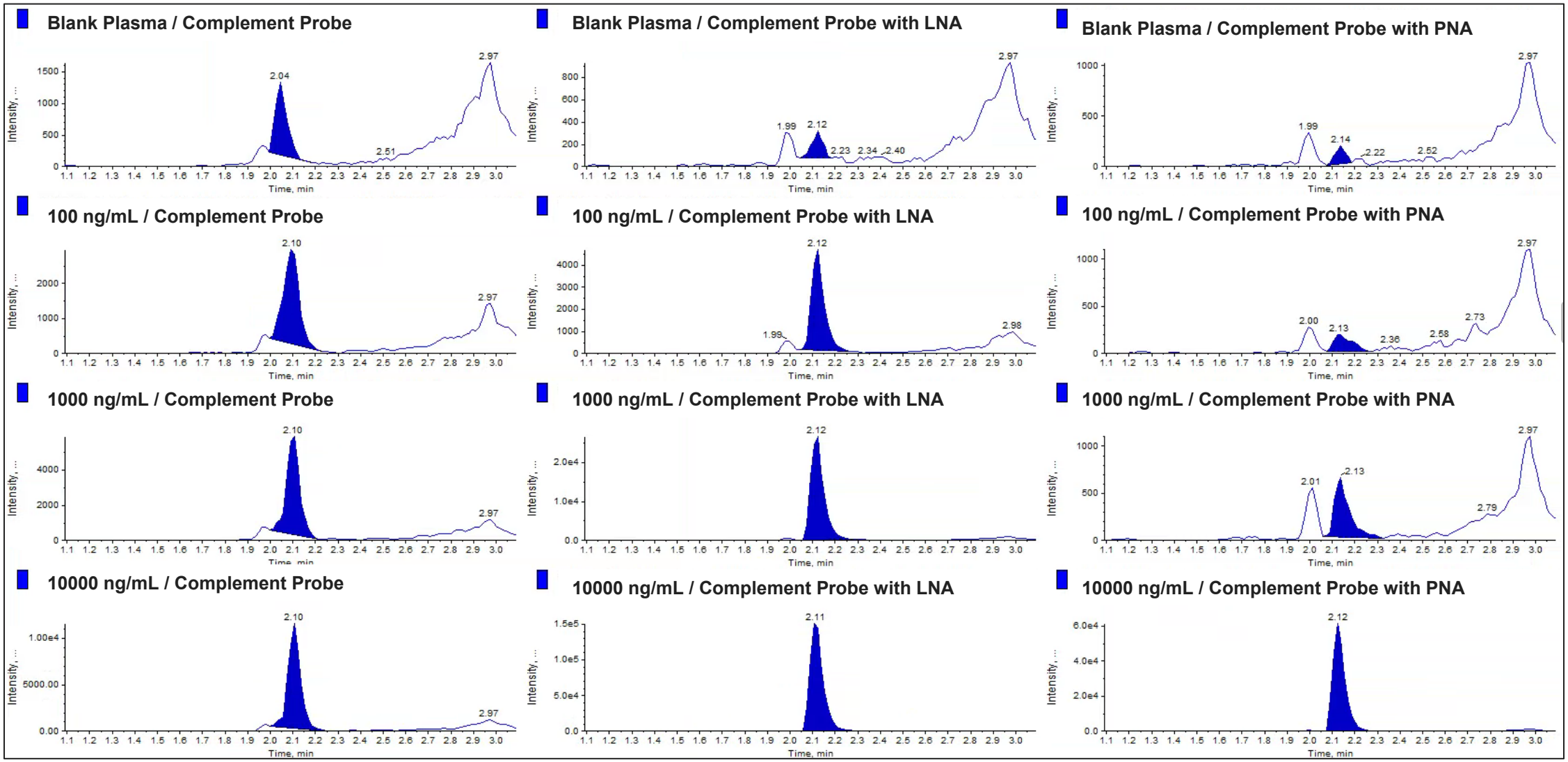
- Ribose ring is "locked" in the ideal conformation for Watson-Crick binding
- Higher thermal stability for LNA-DNA interaction compared to DNA-DNA
- Ideally want self-hybridization score < 30
- Generally, 5-7 LNAs in the probe are sufficient

Peptide Nucleic Acid (PNA) Probe



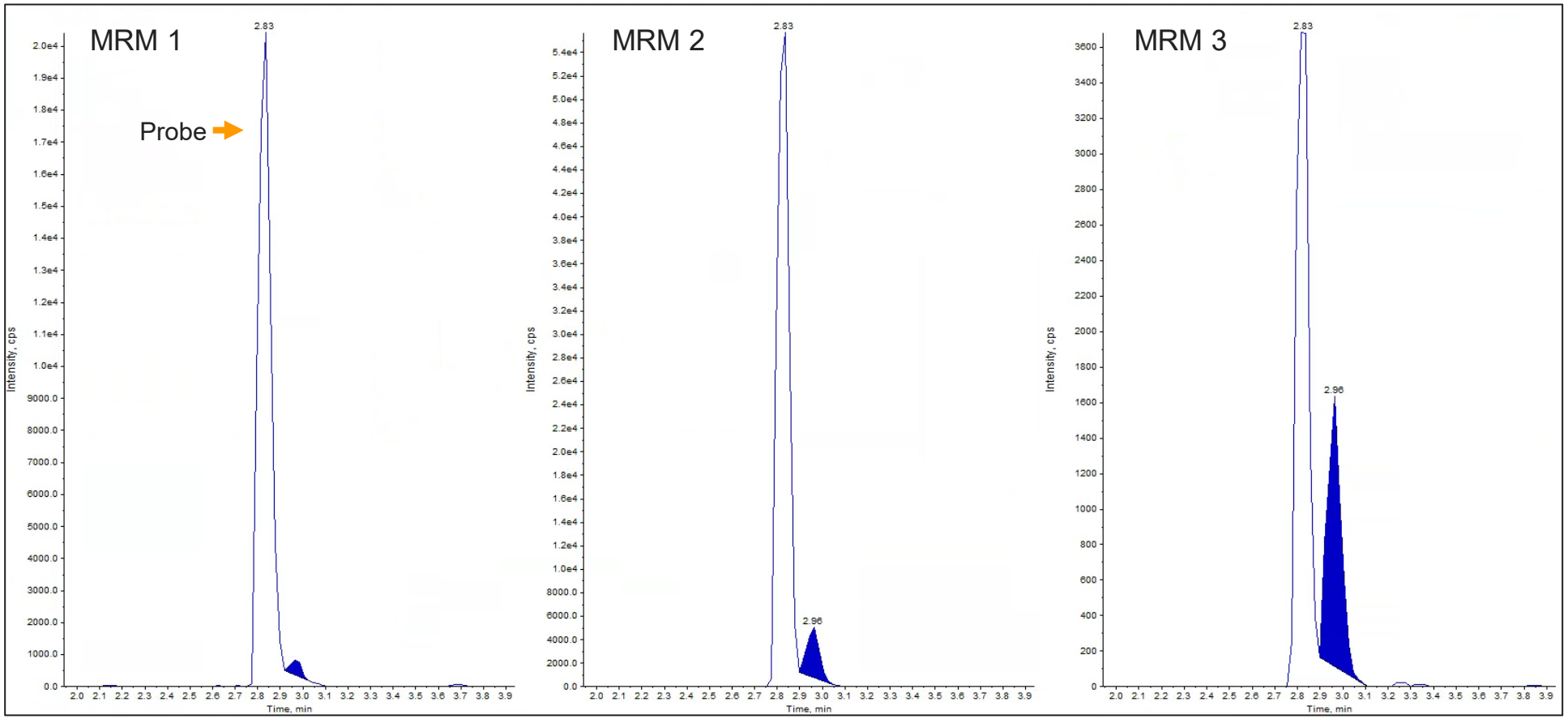
- Sugar-phosphate backbone replaced by pseudopeptides
- Thermal stability of PNA-DNA interaction higher than DNA-DNA
- PNA-PNA interactions are strongly preferred to PNA-DNA interactions
  - Avoid sequences with potential for self-hybridization
- Recommend to avoid purine-rich sequences due to solubility concerns

Impact of Probe Type on siRNA Recovery

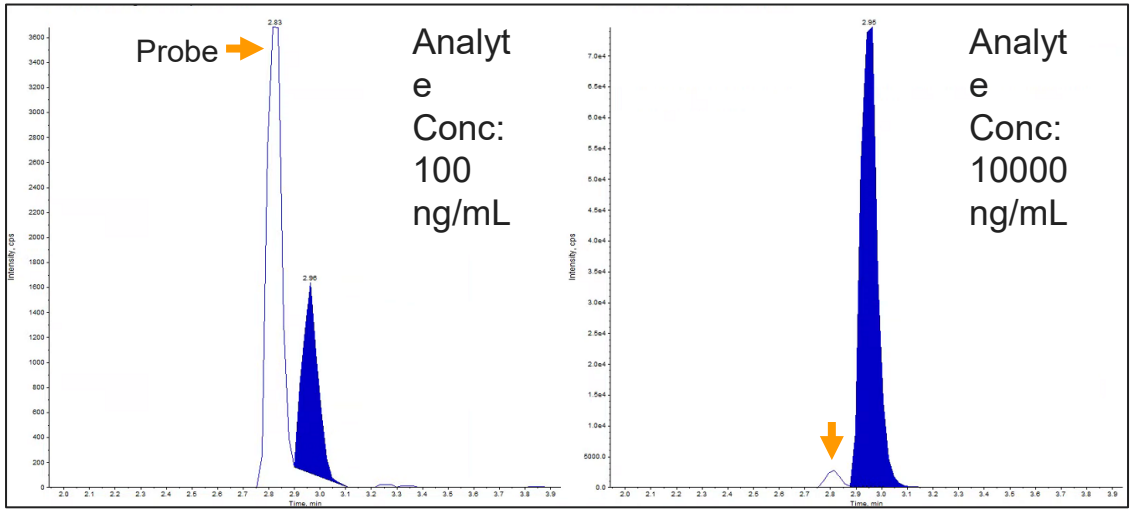


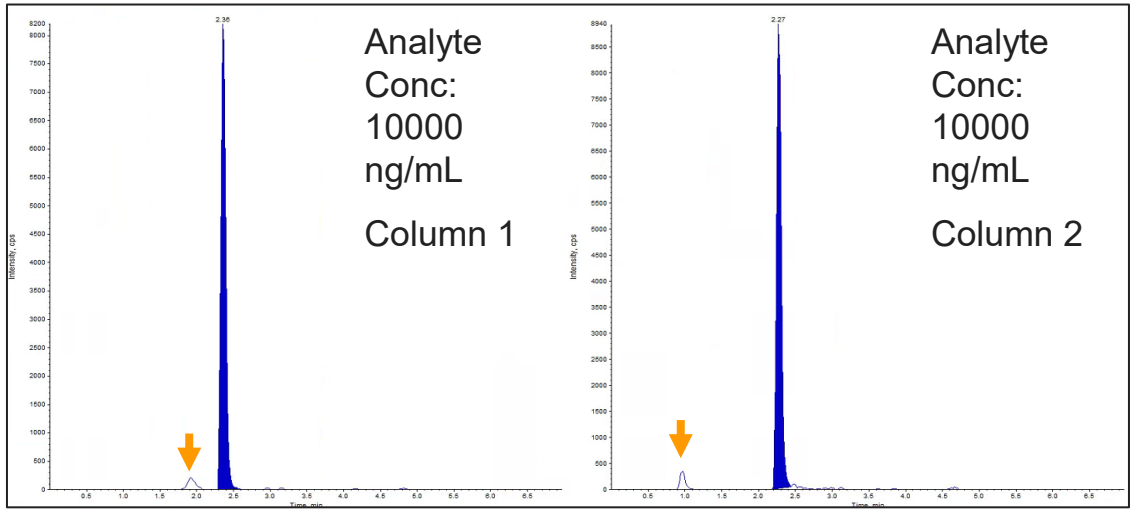
Probes Can Demonstrate Interfering Peaks That Could Impact Sensitivity

Analyte Conc:
100 ng/mL



Chromatography is One Alternative to Solving Interferences from the Probe

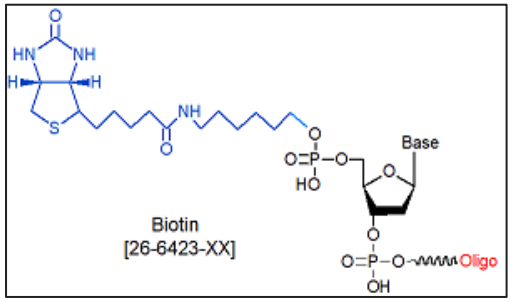


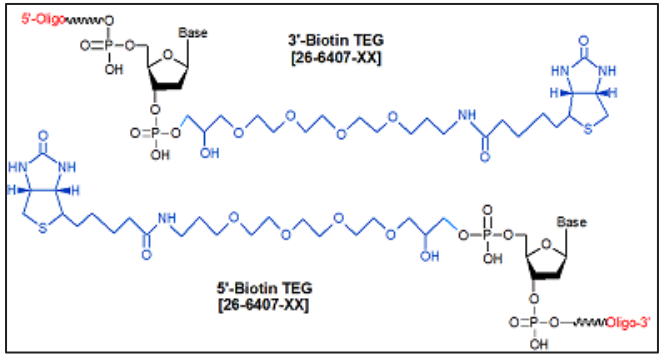


Time (min)	%B
0.00	5
0.75	5
4.00	50
4.20	95
6.00	95
6.20	5
9.00	5

Time (min)	%B
0.00	15
0.75	15
4.00	50
4.20	95
6.00	95
6.20	15
9.00	15

Use of Alternative Spacers and/or Additional Bases





genelink.com

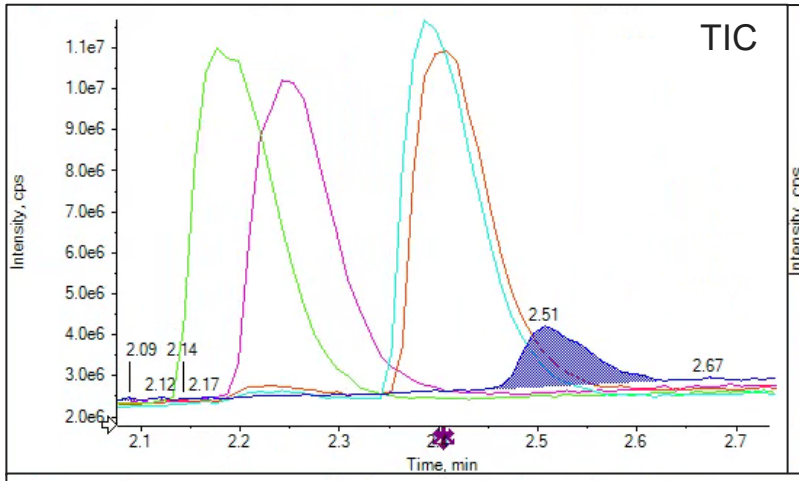
Analyte

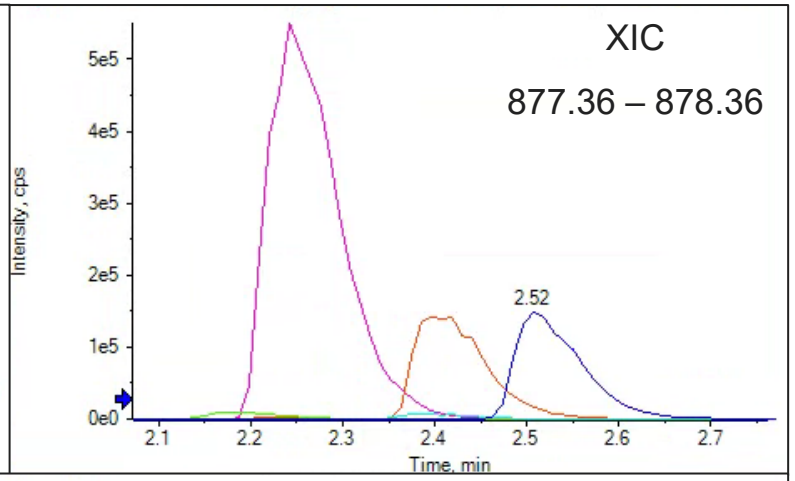
Complement LNA Probe with C6 Spacer

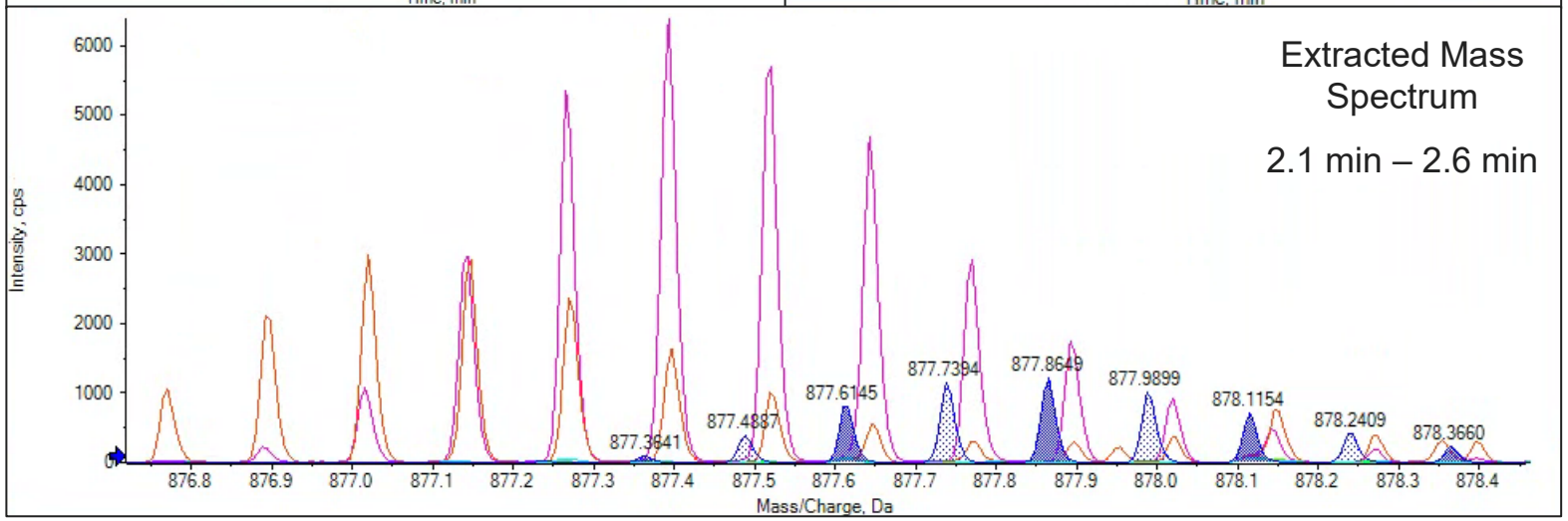
Complement LNA Probe with TEG Spacer

Complement LNA Probe with Addl Base and C6 Spacer

Complement LNA Probe with Addl Base and TEG Spacer







Questions to Consider

- Is a complement strand probe sufficient, or would an LNA or PNA probe be better?
- If interfering peaks are observed, is it better to resolve the issue by chromatography, or add an additional base to ensure no mass overlap?
- Is a C6-biotin spacer sufficient or would a TEG-biotin spacer be better to avoid steric hindrance when binding to streptavidin beads
- 3’ biotinylation v/s 5’ biotinylation

Q/A 1.3

- **Technical Details for Ion Pairing-RP LC-MS**
  - How to effectively clean ion-pairing reagents and HFIP from HPLC (and mass spec)?
  - How to minimize the carry-over issue in IP-RP MS analysis?
  - How much concentration of EDTA is appropriated, and how to make EDTA stock solution for IP-RP MS oligo analysis?
  - What are the best practices to minimize the salt adducts

Q/A 1.4

- **How to mitigate the impact of non-specific binding?**
  - Maximize peptide solubility
  - Determine the best solvent
  - Add organic (ACN, IPA, MeOH) / aqueous (water or buffer)
  - Use the drug product, if available, as a reference standard
  - Add carrier proteins (plasma, bovine serum albumin BSA) or surfactant (Tween-20, Triton X-100)
    - May cause chromatographic issues during working solution stability assessment
  - Spike high concentration stock into matrix and perform serial dilution in the matrix
  - Evaluate the choice of containers for automation, single use only

Q/A 2.1

- ***Assay development/enrichment***
  - ***Can more details be shared about assay development strategies or alternative approaches for enriching intact peptides from biomatrix?***

Q/A 2.2

- ***HRMS for Oligonucleotides/Peptides***
  - ***Is intact molecule HRMS being utilized for oligonucleotides and peptides, and what quantitation strategies are being applied?***

Q/A 2.3

- **How to overcome instability of peptide in matrix?**
  - Chemical degradation: deamidation, oxidation, reduction, etc.
  - Enzymatic activities in biological matrices
    - More frequently observed in rodents (rat, mouse)
  - Stabilization Strategies:
    - Sample storage temperature control (-70°C vs. -20°C)
    - Sample handling
    - Preparation on ice-water bath vs. the standard ambient temperature
    - Reduce/avoid multiple freeze-thaw cycles
    - Test protease and esterase inhibitors
    - Acidification to stabilize in plasma (formic, phosphoric acids)
  - Nonspecific binding or insolubility can be mistaken for instability

Method Validation for Oligonucleotide Hybrid LC-MS/MS Assays

Skin cells at 20x magnification

Questions to Consider

- Is a typical small molecule approach (based on FDA/EMA guidance) sufficient for validating oligonucleotide LC-MS/MS assays?
- What should acceptance criteria for accuracy and precision be?
- Do we need to demonstrate stability of reagents such as capture probe reference material and capture probe conjugated to magnetic beads?
- Should optimization of matrix:capture probe ratio be demonstrated during validation as it is for LBAs?
- What should be considered an acceptable recovery?

Q/A 3.3

- **What are the approaches to increase assay sensitivity for intact peptide bioanalysis?**
  - Low ionization efficiency comparing to small molecules. Peptides administrated by Sub-Q, PO and inhalation usually require a lower LLOQ assay.
  - MS strategies of increasing assay sensitivity:
    - Summing multiple MRM transitions
    - Opening resolution from “unit” to “Low” for Triple Quad instruments
      - Check for interference from matrix with extracted samples
    - Pseudo-MRM for peptide with poor fragmentation efficiency
  - LC strategies of increasing assay sensitivity
    - Column, organic, buffer and pH of mobile phase optimization
    - Mobile phase modifier, e.g., ammonium fluoride, DMSO
    - Column pre-treatment/condition to reduce peak tailing and lot-to-lot variability
    - Micro-LC
  - Sample preparation strategies of increasing assay sensitivity
    - Immunoaffinity enrichment

Workshop Summary

- There were four excellent (short) presentations from the experts in the field followed by panel discussions and Q/A on wide-range of important topics.
- We could proudly say that it was an “Oral Grade” but interactive session. It could easily go on at least another hour time permitted. We have suggested to ASMS office to explore the possibility to extend the time for the workshops where the presenters/panelists and the audiences can have more two-way meaningful conversations.
- There were 30-40 attendees this year. RBIG workshops used to draw 100-150 attendees. Unfortunately, it was low turnout all around this year due to various reasons. Per ASMS, there were more workshops including “independent” ones which could have diverted the flow as well.
- ASMS office encourages us to focus on what is being discussed and how involved the participants are.
- There were positive feedbacks from the audiences on the quality of the presentations and discussions in a well organized interest group workshop.