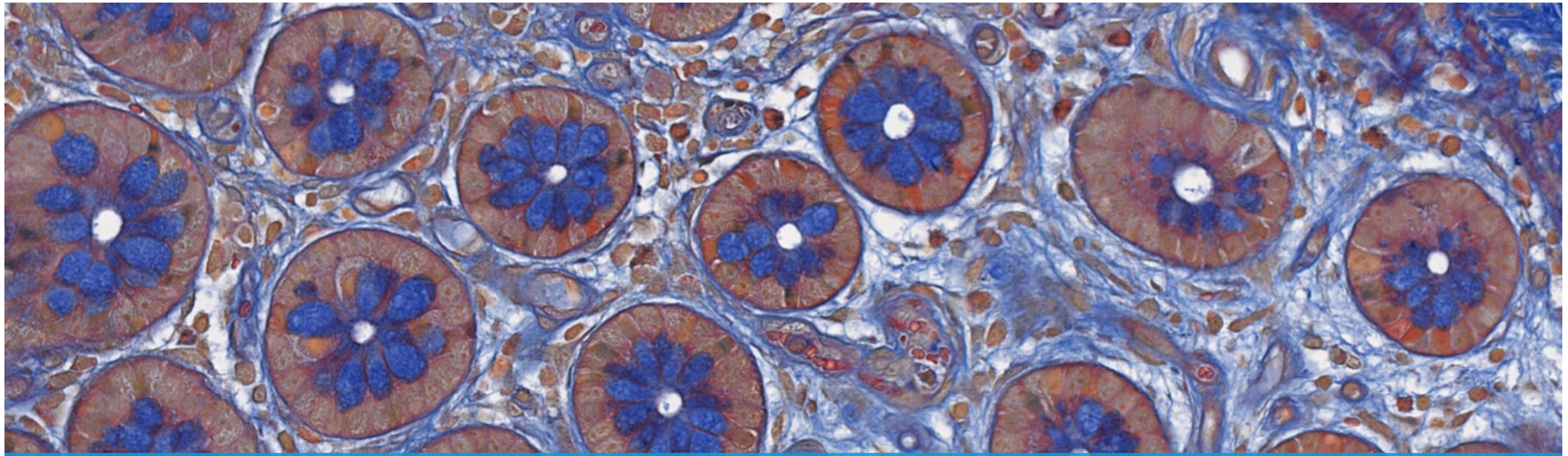


Bioanalytical challenges for analysis of oligonucleotides with LC-MS/MS

Emmanuel Njumbe Ediage, PhD
Development Bioanalysis, Janssen R&D, Beerse BELGIUM

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

Outline

1 Introduction

- Therapeutic oligonucleotide (OGN) landscape and available Bioanalytical platforms
- LC-MS/MS workflow for analysis of OGN

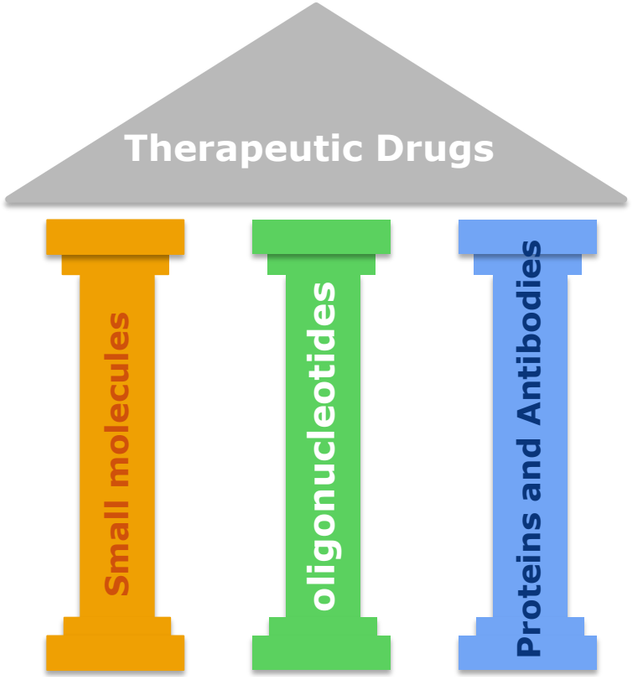
2 Bioanalytical challenges in OGN analysis

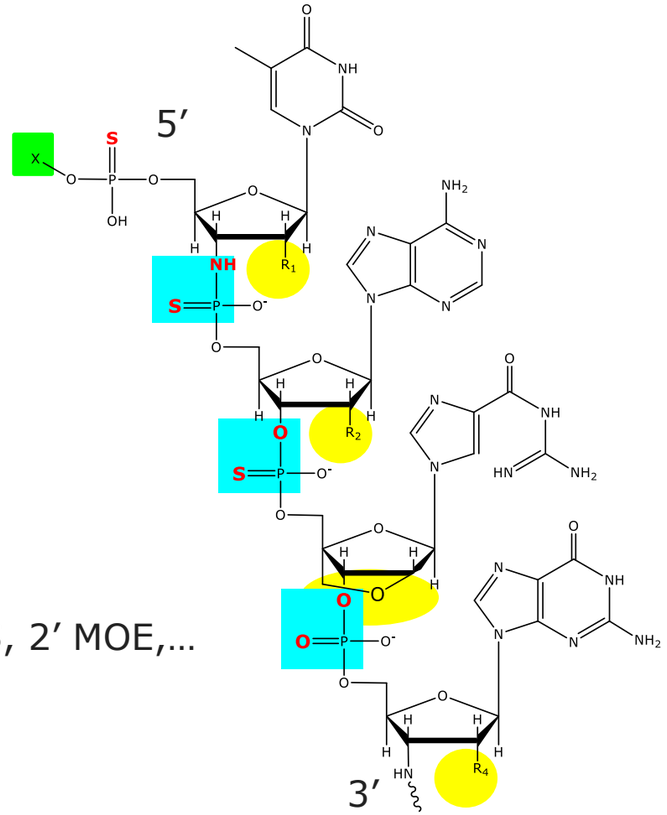
- Case study 1:Purity/interference/cross talk evaluation
- Case study 2:Matrix effect and choice of Internal standard
- Case study 3:Carryover mitigation strategy and system reproducibility

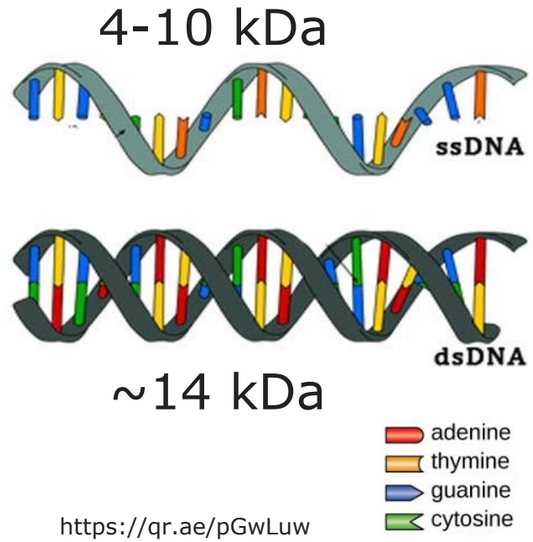
3 Extraction of OGN from biological samples using LNA Probes + LC-MS/MS analysis

4 Recommendation and conclusion

Therapeutic OGN landscape and available Bioanalytical platforms







https://qr.ae/pGwLuw

- **ASOs** (ssDNA/RNA, **14-25 nt**)
  - Structural design/modifications: gapmers, PMO, PS, 2’ MOE,…
  - Approved drugs: fomivirsen, nusinersen, ...
- **siRNAs** (dsRNA **2x 20-25 nt**)
  - antisense strand (AS) or guide strand
  - sense strand (SS) or passenger strand
  - Structural modifications: PS, 2’F/OMe, conjugation,…
  - Approved drugs: patisiran (2018), givosiran (2020), inclisiran (2021),….
- **Other oligo modalities:** example mRNA therapeutics

Crooke *et al., Cell Metabolism 27:714-739 (2018)*
Bennet and Swayze, *Annu Rev Pharmacol Toxicol 50:259-293 (2010)*

Bioanalytical Platforms for OGN quantification

	LC-MS	LC-Fluorescence	hELISA	PCR
Format	LC-HRMS LC-MS/MS	Hybridization Chromatography	Hybridization	Stem-loop qPCR
Sensitivity	0.5-1 ng/mL	0.1-1 ng/mL	High, 10 pg/mL	Highest, pg/mL
Specificity	High	Mid	Low	Low
Metabolite detection	Yes	Yes, but limited identification	No	No
Reagent	Internal standard (Analog)	Custom DNA/PNA	Custom probe	Custom Probe and Primer
Challenges	Chromatography, Ionization, Sensitivity, Ruggedness	Probe design, Specificity	Probe design, Specificity	Primer design, Specificity
Assay development	Fast, days-weeks	Slow, weeks-months	Slow, weeks-months	Medium, weeks
Regulatory BA	Yes (SMOL) Givosiran, Lumasiran, and Inclisiran	Yes (SMOL) Patisiran	Yes (LBA guidance)	Not routine

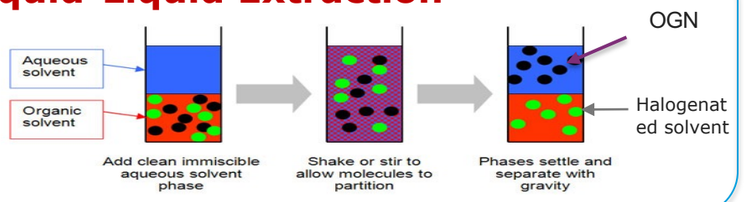
LC-MS/MS workflow for the analysis of OGNs

Skin cells at 20x magnification

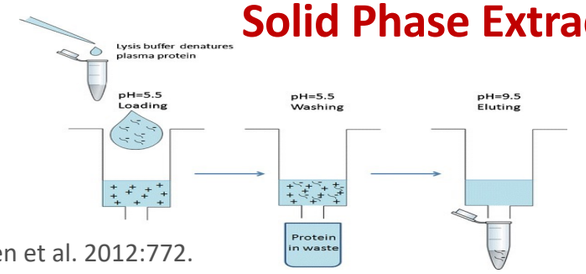
LC-MS Assay for Oligonucleotide Therapeutics

Sample Extraction

Liquid-Liquid Extraction

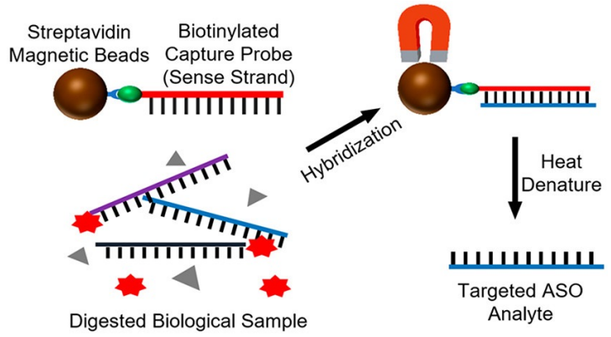


Solid Phase Extraction



Chen et al. 2012:772.

Hybridization

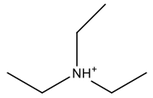


Li et al, Anal Chem. 2020: 10548

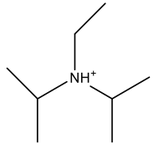
LC Separation

Ion-pair Reverse Phase LC

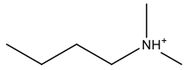
Alkyl amine



Triethylamine
Boiling point: 89°C

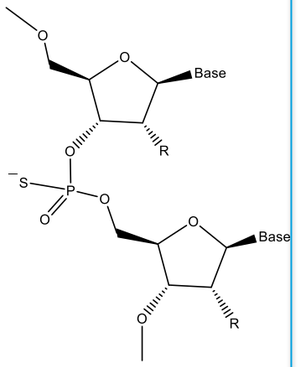


Diisopropylethylamine
Boiling point: 127°C

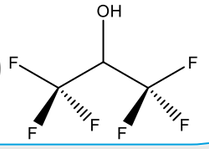


Dimethylbutylamine
Boiling point: 127°C

Oligonucleotide



Fluoroalcohol

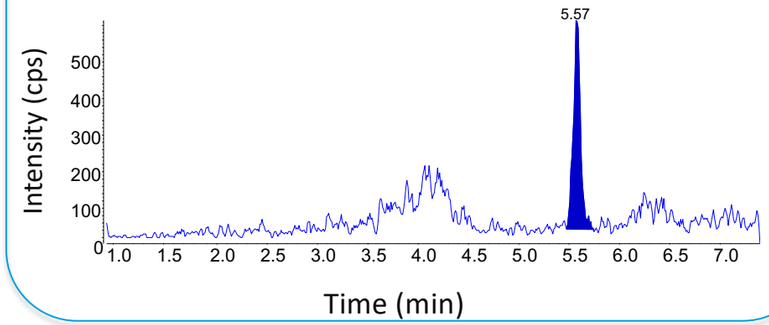


HFIP (Hexafluoroisopropanol)
Boiling point: 58.2°C

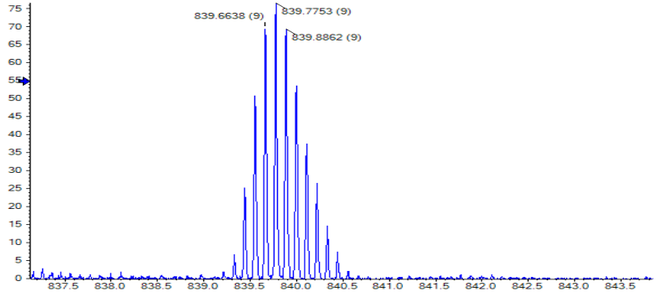
Hydrophilic Interaction Chromatography (HILIC)

Mass Spec Detection

LC-MS/MS (MRM)



LC-MS (HRMS)



- Sum of the isotope/charge state ions improves sensitivity
- Fragmentation independent
- Improves selectivity
- Metabolite ID
- Trouble shooting

janssen | PHARMACEUTICAL COMPANIES OF Johnson & Johnson

siRNA IP-RP chromatography

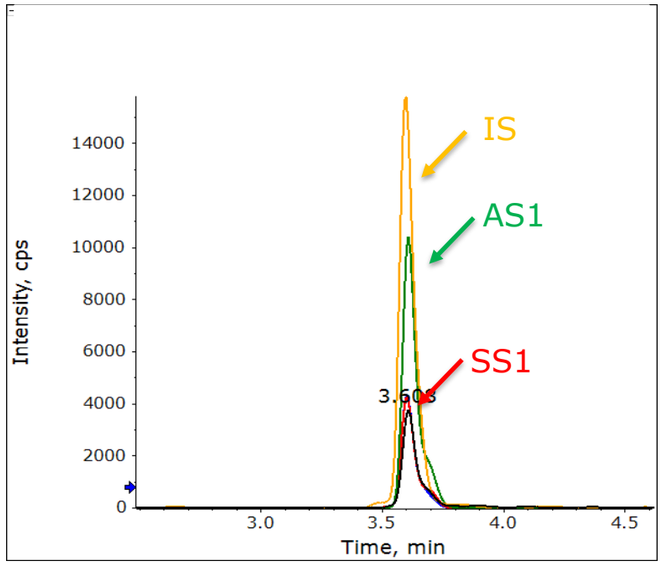
LC parameter	Shimadzu LC 20AD
Column	DNA Pac RP (50 x2.1 mm)
Flow rate	0.25 mL/min
MP A	47.3 mM HFIP(0.5%)/ 12.2 mM DMBA (0.2%)
MP B	CH_3CN/IPA (95/5)
gradient	2% ->50 %B in 2 min
Rinse solution	Water/MeOH/DMBA (93/5/2, V/V/V)

Time (min)	% A	% B
0.00	98	2
2.00	98	2
4.00	50	50
4.01	5	95
7.00	5	95
7.01	98	2

MS parameter	Sciex 6500+
CAD	8
CUR	30
GS1	80
GS2	50
DP and CE	Analyte/transition dependent

	MRM transitions
SS1	793.5 (11-) > 438; 793.5 > 358
AS1	685.8 (10-) > 334; 762.1 (9-) > 604
IS	634.1 (8-) > 617.1

Liver homogenate 5 µg/mL

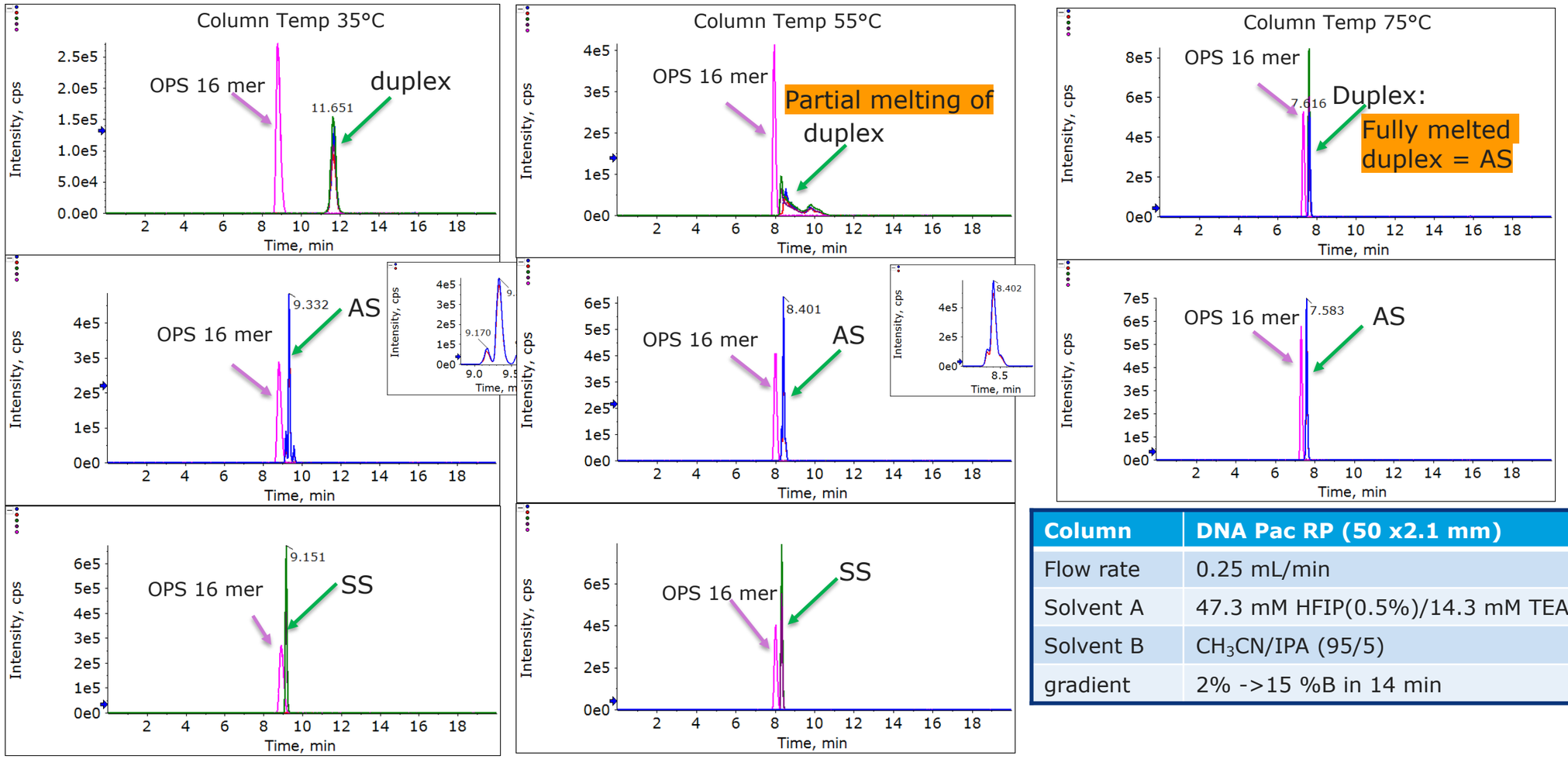


IS = internal standard

AS1: antisense strand 1

SS1: sense strand 1

IP-RP chromatography with an siRNA

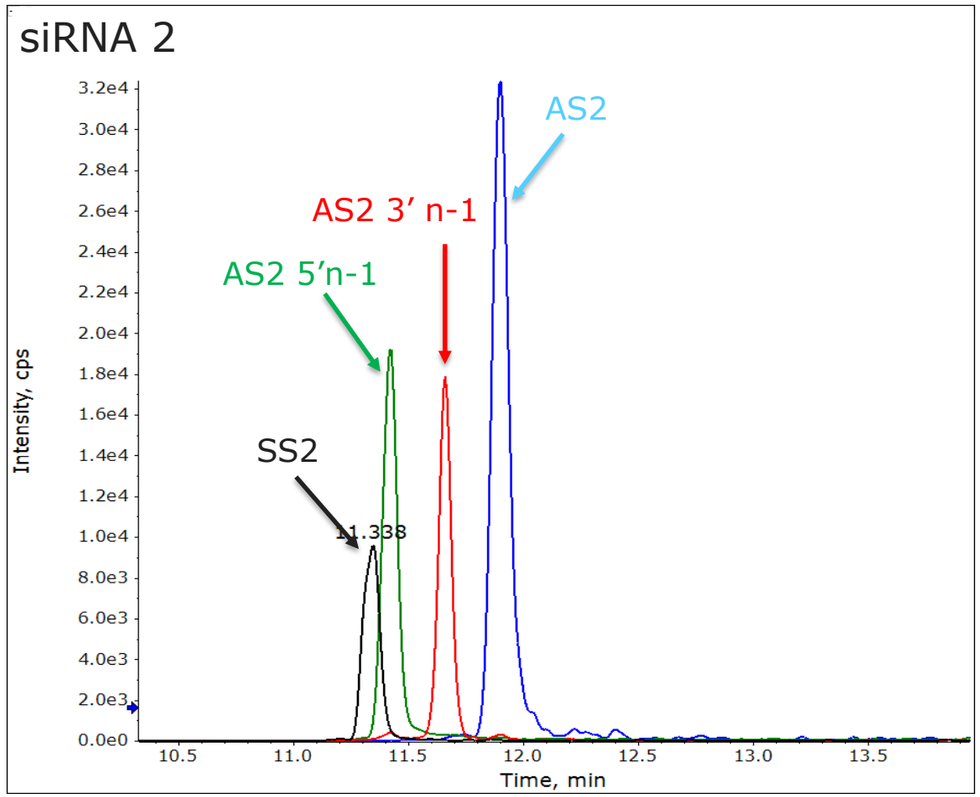


Column	DNA Pac RP (50 x2.1 mm)
Flow rate	0.25 mL/min
Solvent A	47.3 mM HFIP(0.5%)/14.3 mM TEA(0.2%)
Solvent B	CH_3CN/IPA (95/5)
gradient	2% ->15 %B in 14 min

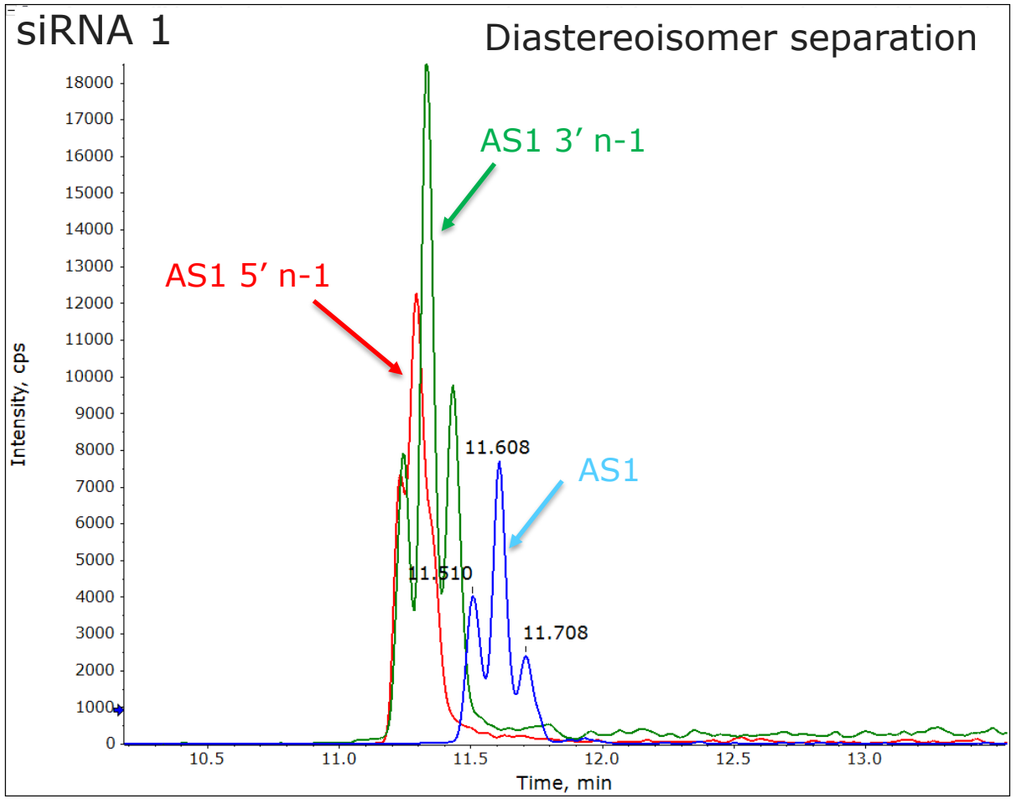
- Only the antisense strand is quantified, and concentration (ng/ml or ng/g) reported as **duplex equivalent**

LCMS separation of truncated metabolites of siRNAs

- Adapting chromatography: slow gradient 1-15% B over 20 min



- Both siRNA have same number of phosphorothioate (OPS) linkages



- Diastereomeric selectivity: C>G>A>T

Bioanalytical challenges in OGN analysis

Case study 1:

Purity/interference/cross talk evaluation

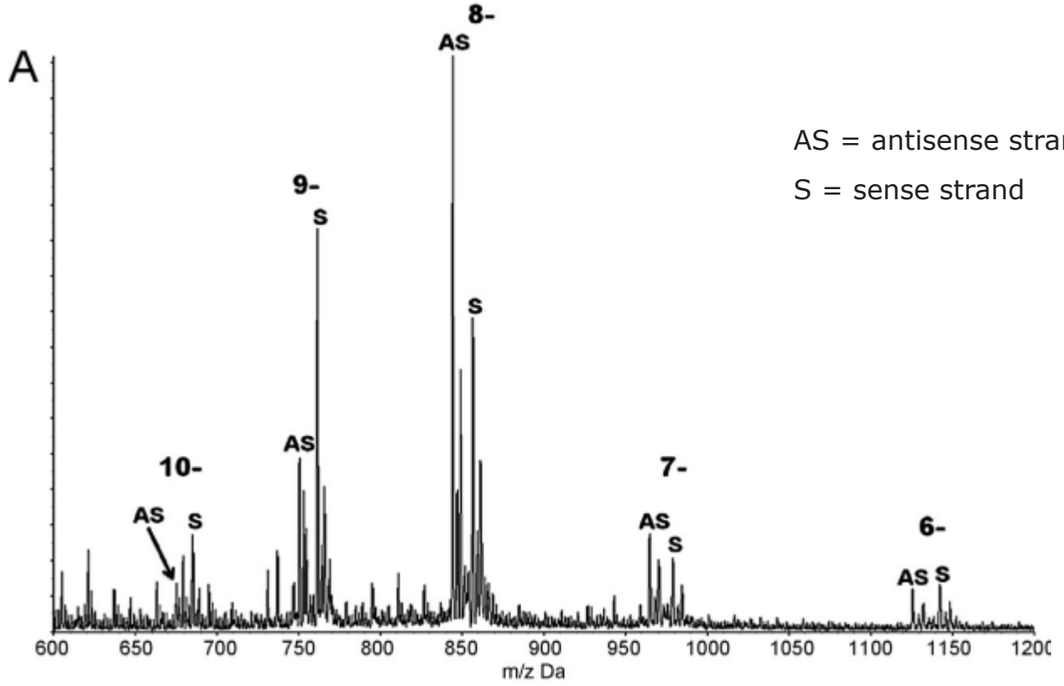
Skin cells at 20x magnification

Rationale for purity/interference evaluation

- **A MUST for MS/MS analysis with co-elution** of multiple OGN **species (drug + metabolites)** and impurity is expected
- OGNs show multiple charge states with Na/K adducts,
  - Similar m/z values can be expected

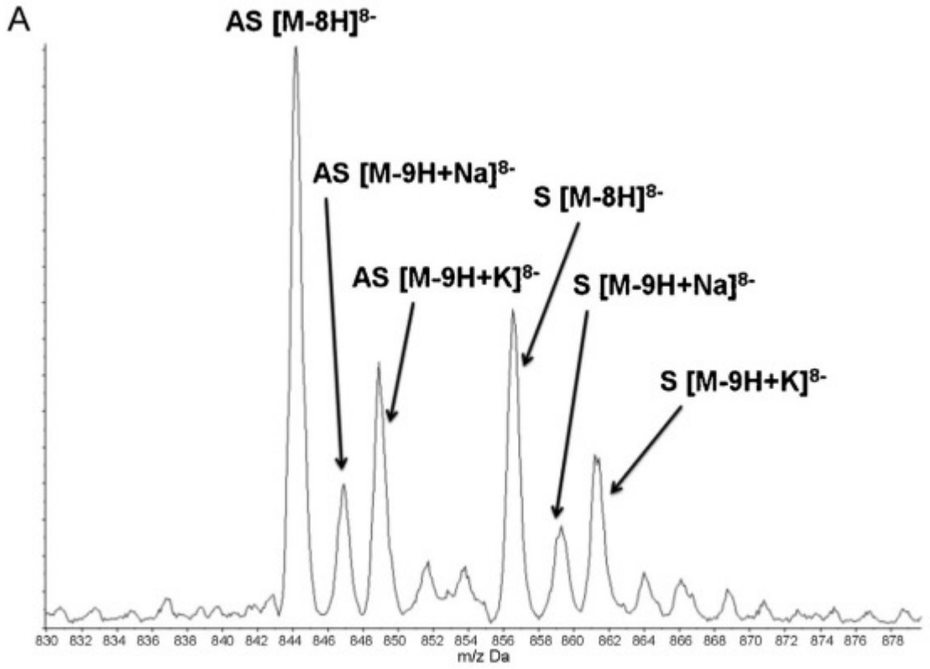
m/z of -11 is **697.0** for siRNA-1

m/z of -10 is **697.1** for siRNA-2



AS = antisense strand

S = sense strand



R. Wheller et al. / International Journal of Mass Spectrometry 345–347 (2013) 45–53

Rationale for purity/interference evaluation(2)

- The use of structural analogues (e.g siRNA or ASO) **as internal standard** in discovery phase projects
  - Check the contribution of the analogue **IS to the LLOQ of OGN unchanged drug**
  - Check the contribution of the OGN drug to the response of the analogue IS
- In multi-analyte analysis:
  - The possibility to combine 2 (or more) siRNA's/ASO in 1 calibration curve

Our internal Sequence of events (Internal process)

- **On-the-column Tuning** of the different OGNs (Q1) and select the 3-4 most intense charge states in Q1;
- Via infusion, optimize the other MS/MS parameters
- Of each charge state search for the most intense product ions
- Prepare standard solutions of the same concentration
- Inject the standard solutions separately and monitor the other transitions

Example Purity/interference check

- Acceptance criteria: % impurity should be < 2%
- Standards were injected at equimolar concentrations

			AS1	% impurity	AS1	% impurity	AS1	% impurity	AS1	% impurity
Monitored transitions			**(-10a)**		**(-10b)**		**(-10c)**		**(-9a)**	
Unchanged drug (siRNA)	**Duplex1**		7.96E+05		5E+05		4E+05		8E+05	
	Duplex1		7.54E+05		5E+05		4E+05		8E+05	
	Duplex1		7.74E+05		5E+05		4E+05		8E+05	
Injected										
Truncated forms and internal standards	As1 (n-1)'5		19668	**2.5**	18134	**3.4**	5799	**1.5**	17653	**2.2**
	As1 (n-1)'3		20949	**2.7**	20527	**3.9**	10834	**2.9**	7713	**0.9**
	As1 (n-2)'5		4838667	**624.8**	9E+05	**176.9**	1E+05	**39.1**	12216	**1.5**
			5092		1787					
	As1 (n-2)'3		133242	**17.2**	1E+05	**23.5**	76500	**20.4**	4133	**0.5**
			1061							
	16MER-OPS		720	**0.1**	2923	**0.5**		**0.0**		**0.0**

- Based on these data, -9a charge state was selected

Case study 2:

Matrix effect and choice of analogue internal standard in Discovery projects

Skin cells at 20x magnification

Performance of analogue ASO IS: incurred samples vs calibrators

- Choice is limited to analogue antisense OGN (ASO) and siRNA with 100% sequence dissimilarity compared to the analyte to be quantified
- @Janssen; historically, started with ASO 16mer OPS as IS. Used to support all projects
- We observed matrix-dependent IS response with brain and tissue homogenate. IS added prior to SPE extraction

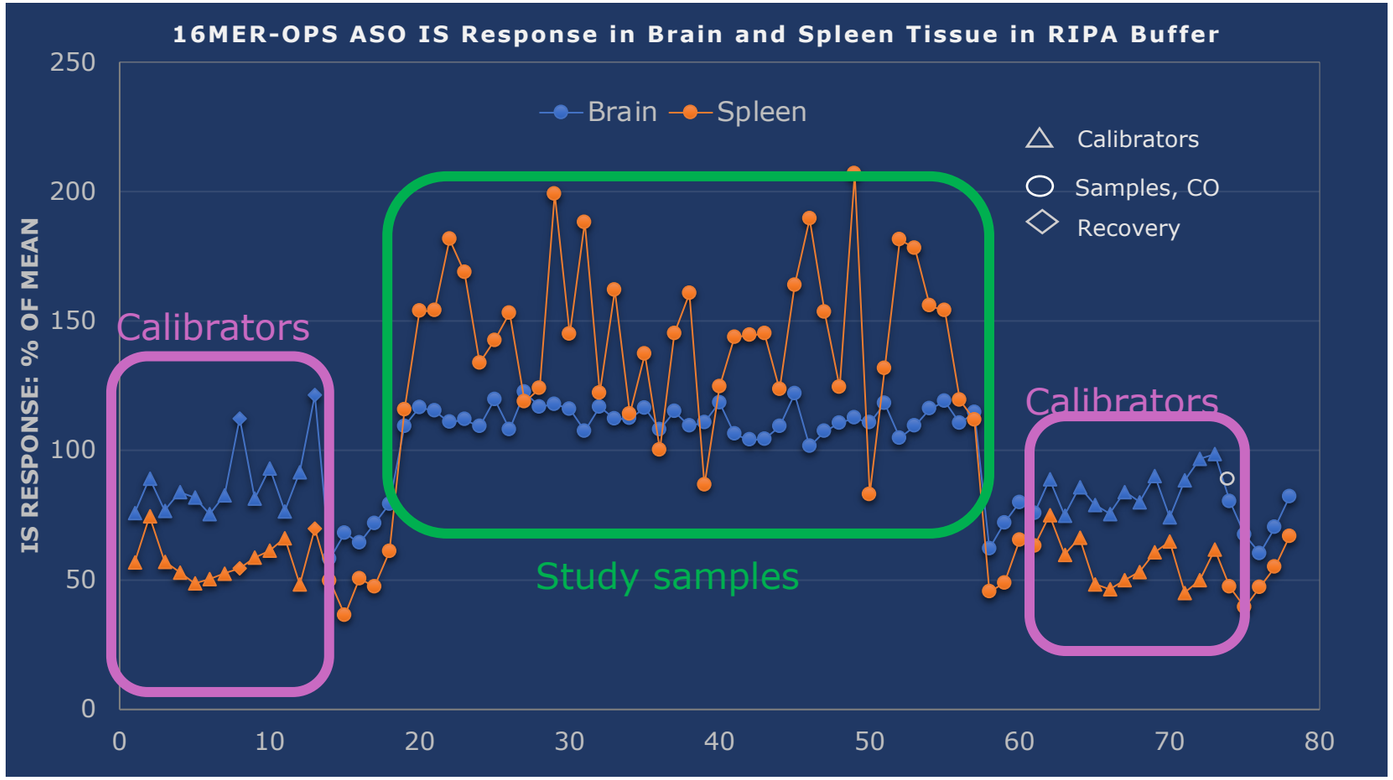


Fig .1 incurred samples and the calibrators were prepared with **separate protocols**

- **Sample clean-up with Clarity OTX SPE 96 well plate**
- Incurred samples and calibrators were prepared by different labs. **Sample extraction with Clarity OTX plate was performed by 1 lab**
- Different IS response between incurred samples and calibrators prepared in RIPA buffer
- IS response in incurred samples more variable with spleen than with Brain homogenates: **extraction efficiency?**
- Note: RIPA may contain SDS which might interfere with SPE extraction. **Recommended to use 10 mM EDTA as extraction buffer**

Performance of analogue lipid conjugated ASO vs siRNA

Fig .3a and b: 16MER-OPS ASO IS and siRNA IS in brain homogenates

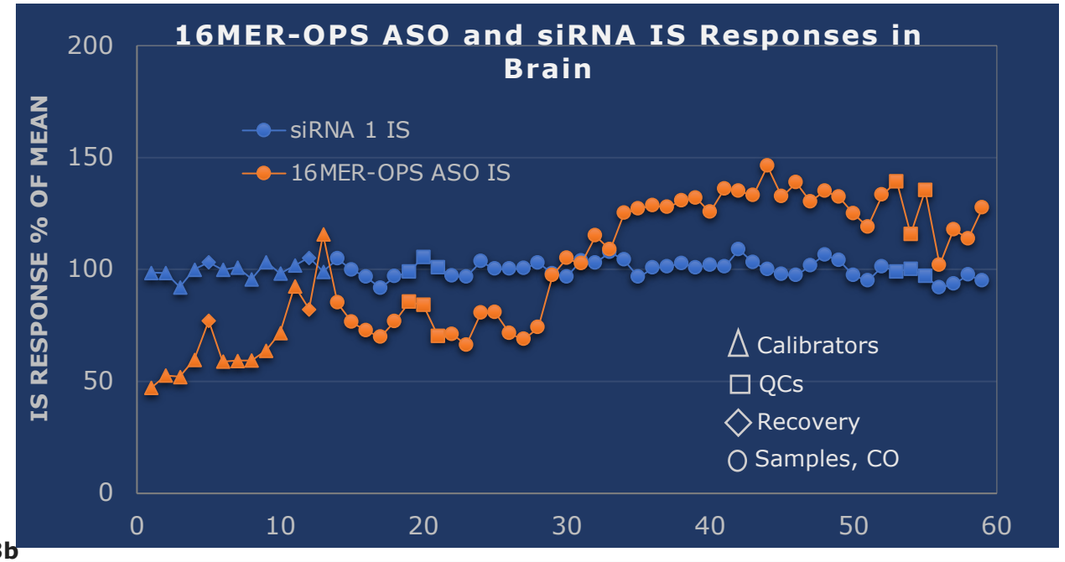


Fig .3b

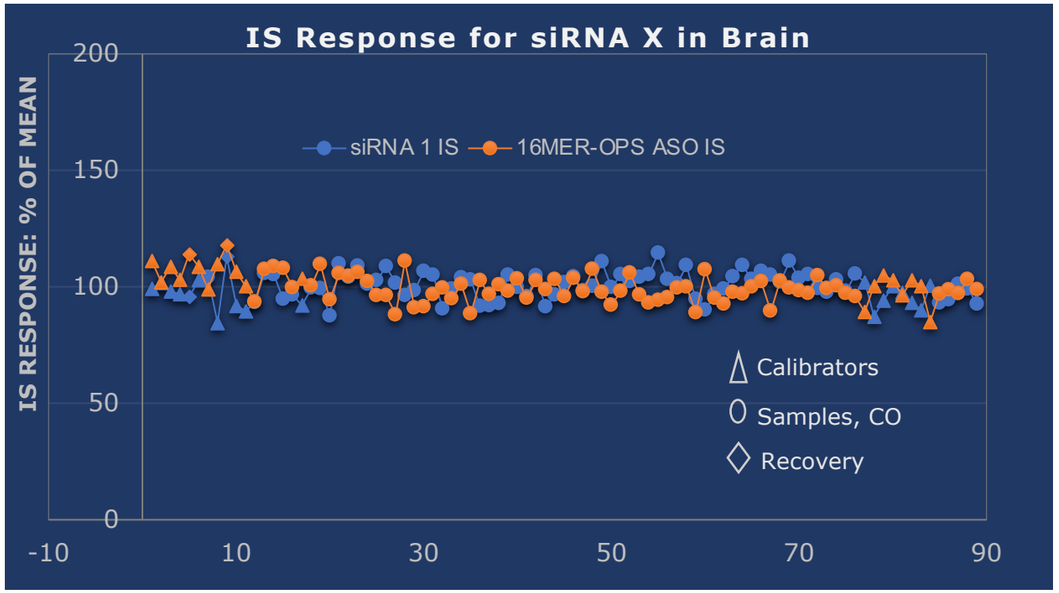


Fig .4 a and b 16MER-OPS ASO IS and siRNA IS in spleen homogenates

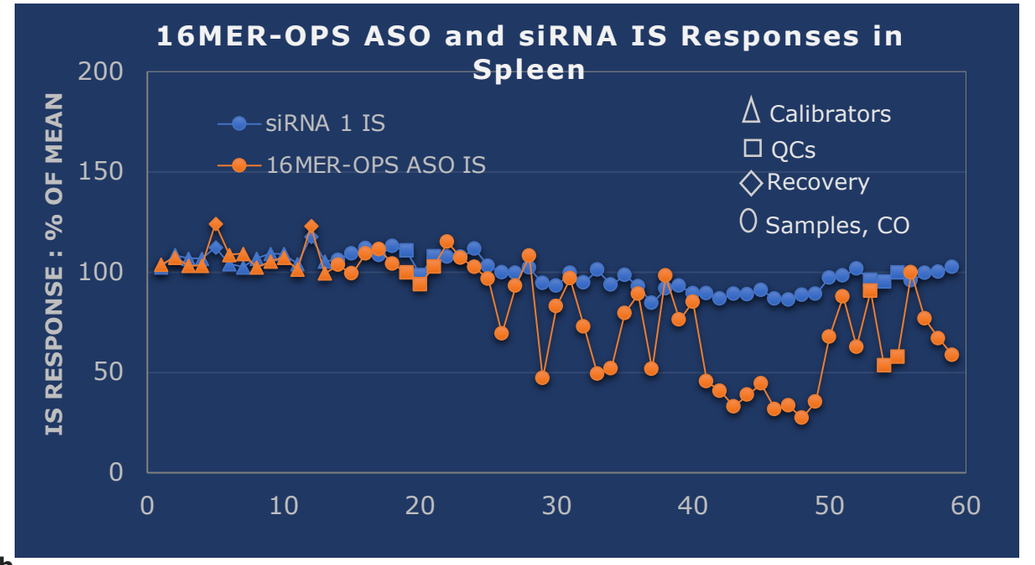
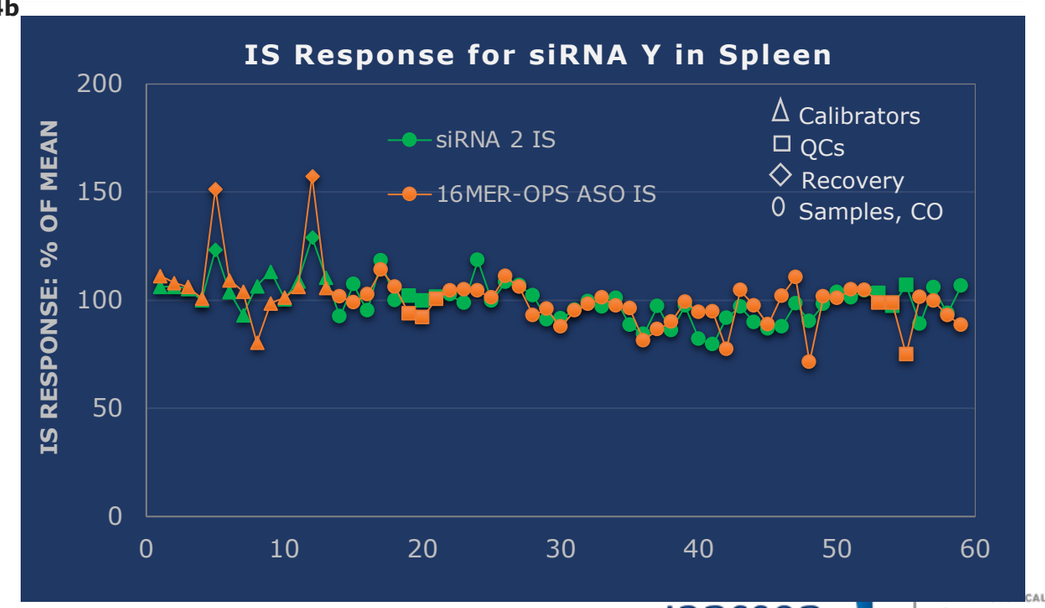


Fig .4b



➢ Batch to batch and matrix evaluation is required to guide decision

Case study 3:

Carryover mitigation strategy and system reproducibility

Skin cells at 20x magnification

Carryover Mitigation Strategy and System Reproducibility

Case study: Carryover observed during study sample analysis

Sample ID	Sample type	Calculated Conc (ng/ml)	% Acc	JNJ-1 (-9g) Peak Area	% CarryOver w.r.t ULOQ	% CarryOver w.r.t LLOQ
LLOQ	Standard	21.1	**105.5**	9783		
ULOQ	Standard	11300	**113.2**	3071974		
CO	Unknown	98		31096	1.01	317.9
CO	Unknown	40.9		14785	0.48	151.1
CO	Unknown	18.8		8521	0.28	87.1
CO	Unknown	14.4		7086	0.23	72.4
CO	Unknown	13.1		6671	0.22	68.2

- After days of troubleshooting (analytical column, tubing's, mobile phase) the CO issue was resolved

1. Carryover mitigation:

- ✓ Compound dependent but also different LC systems tend to have different CO --→ next slide
- ✓ **Use appropriate column fittings to avoid dead volumes**: pre-flushing is recommended
  - — NanoViper (Thermo Scientific) (pH limited recommended not to use Infront of the column)
  - — SecurityLINK (PEEKSIL) finger fitting (Phenomenex),
  - — PEEK tubing. New tubing gives distorted peaks. Equilibration with sample extract is needed

Carryover mitigation strategy and System reproducibility (2)

1. Carryover mitigation (continuation):

- ✓ Totally Inert UHPLC system. Is it needed for biological samples or Myth – Is stainless steel OK?
- ✓ Backflush the analytical column with Mobile phase B – prior to use
- ✓ BioInert analytical columns and frit
  - — **Acquity Premier BEH C18 (Waters),** pH 1-12
  - — **YMC Triart C18, 300Å (YMC)**: pH 1-12 + temperature recommendation
  - — **BioZen Oligo (Phenomenex),** core shell technology: pH 1-9
  - — DNAPac™ RP (Thermo Scientific) (Not Bio inert). Polymeric column, very stable over pH9

2. System reproducibility

- ✓ **Equilibrate** by injecting high calibrator standard (1/4 ULOQ) extract until a stable signal is reached. DNAPac columns may need more injections
- ✓ **Use a dedicated** LC-MS/MS system: keep it on constant low flow
- ✓ Mitigate drift in MS response by keep mobile phase(s) on ice during analytical run especially mobile phase containing HFIP and ion pairing reagent
- ✓ Use mobile phase solvent Debubbler if needed

Lessons learnt

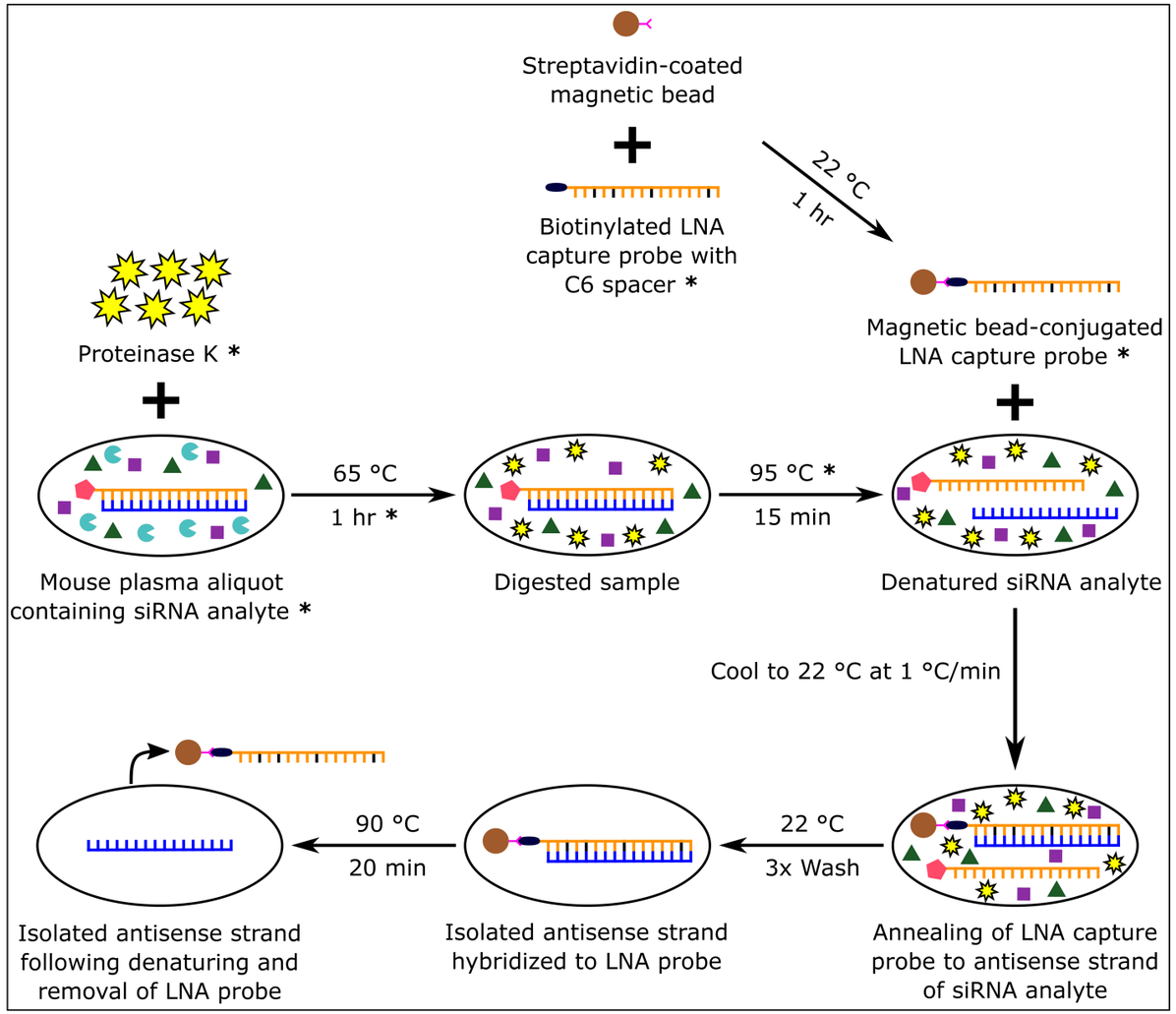
1. Interference evaluation is critical when setting up bioanalytical methods for multiple analytes
2. RIPA as **homogenization buffer** is not our preferred choice since it often result in poor MS response due to signal suppression
3. Combination of structural analogues IS provide more flexibility to select the best IS post analysis to mitigate matrix effect. But interference check must be performed
4. Analogue siRNA performed better than an analogue ASO. Most likely related to differences in ionisation efficiency between the ASO and siRNA. **Is it sequence dependent?**
5. Other analogues with close s**equence similarity** to the target analyte(s) would be preferred (if available)
6. Carryover is analyte dependent: phosphorothioate (OPS) modified and lipid conjugated OGN tend to show more carryover
7. Use of Bioinert UHPLC systems and columns can help mitigate carryover of OGN
8. Minimize dead volumes created by incompatible column fittings and tubing's
9. Carryover investigation is an art and should be approached systematically

Extraction of OGN using LNA probes

Skin cells at 20x magnification

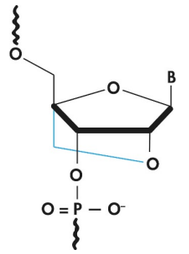
Agrawal et al. (2023) submitted to Bioanalysis journal

Hybrid siRNA LC-MS/MS Analysis Workflow



Agrawal et al. (2023) submitted to Bioanalysis journal

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

Method Optimization Points for Hybrid ASO Analysis

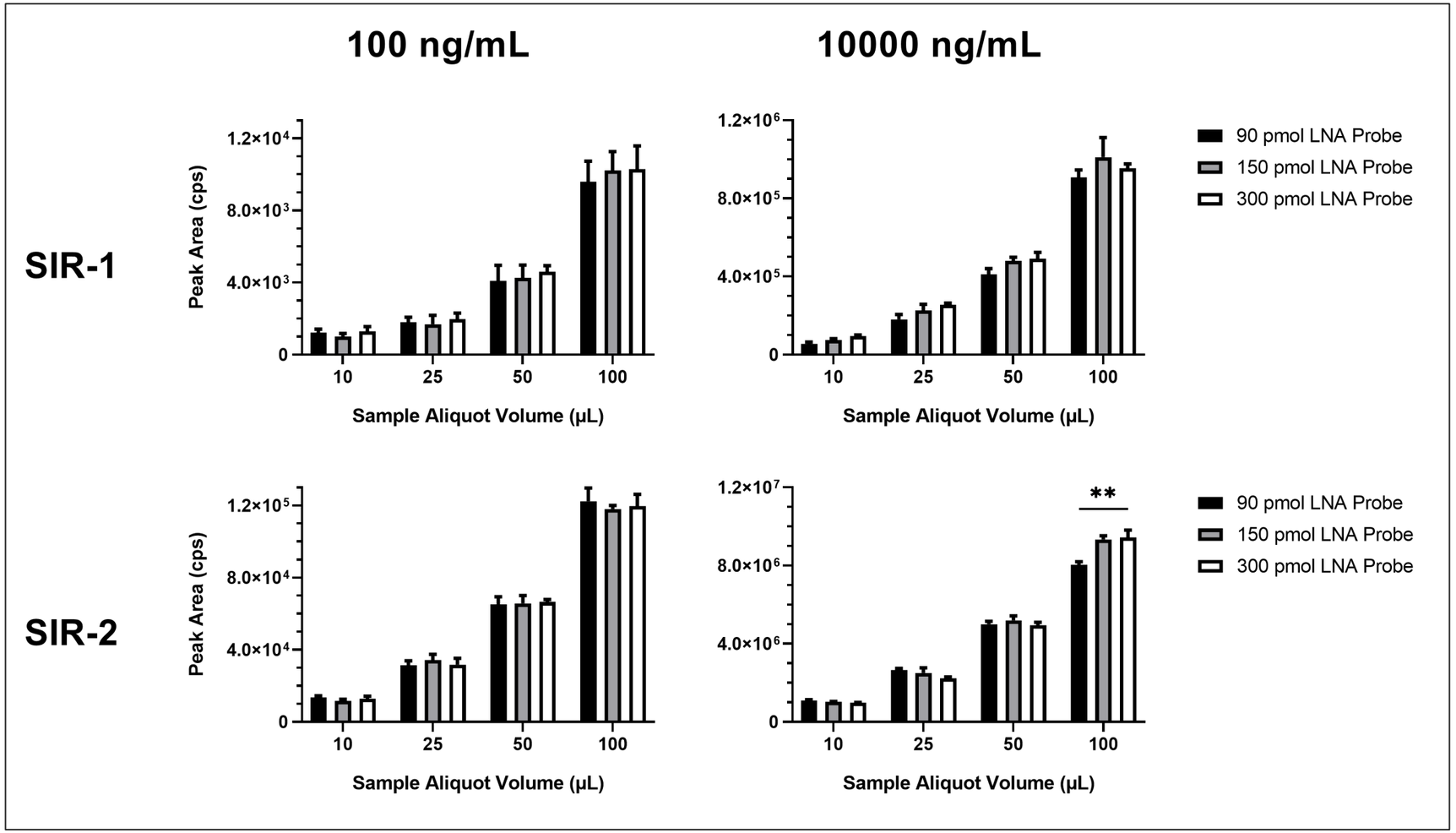
- Buffer for Proteinase K digestion and amount of Proteinase K
- Duration of Proteinase K digest
- Capture Probe TEG spacer position

Additional Challenges for siRNA

Challenges for siRNA	Approach
Competition from sense strand	Probe with higher affinity
Double stands need to be separated	Heat treatment to un-anneal the double strands
Self-hybridization of the probes	Optimize sample/probe ratio

Agrawal et al. (2023) Submitted for publication. Sips et al, Bioanalysis 2019 ; Li et al, Anal Chem 2020

Optimization of Probe Amount and Sample Volume

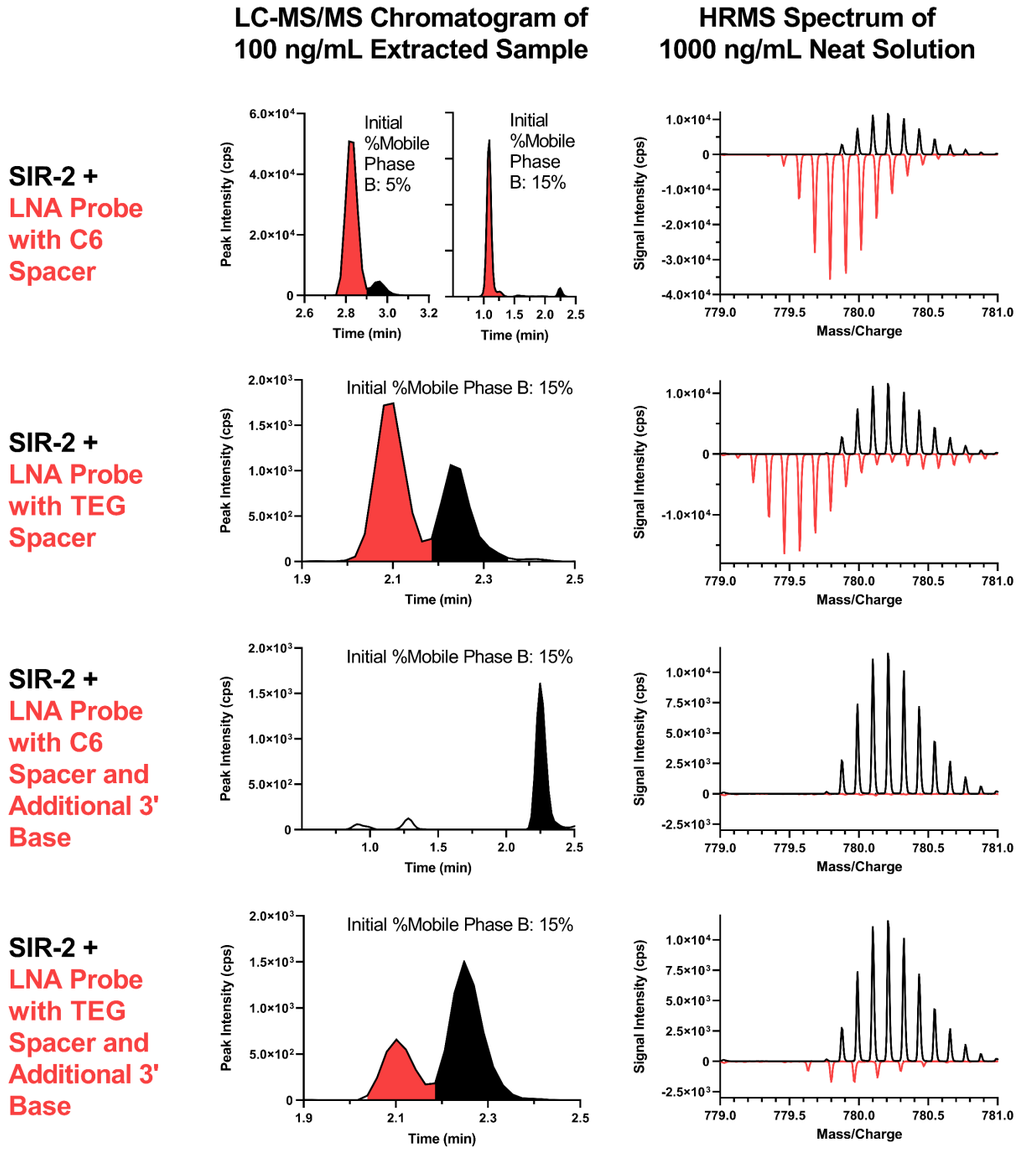


- Adding > 90 pmol probe/sample did not significantly increase siRNA recovery in general
- Using 90 pmol probe, SIR-1 response increases proportionally with sample aliquot volume from 25 µL to 100 µL at both 100 ng/mL and 10,000 ng/mL
- Using 90 pmol probe, SIR-2 response increases proportionally with sample aliquot volume from 10 µL to 50 µL at both 100 ng/mL and 10,000 ng/mL

Agrawal et al. (2023) submitted to Bioanalysis journal

Capture Probes can cause Interference Peaks for Analytes

- LNA probe that was direct complement to SIR-2 antisense strand with C6 spacer had interference peak for all MS/MS transitions evaluated
  - Peak resolvable from analyte peak by changing LC gradient, but concerns of inter-column variability in retention time
- Addition of mass to probe using TEG spacer (C15 v/s C6) caused a different interference peak to be observed
- Adding an additional nucleotide to the 3’ end and using C6 spacer shifted probe mass sufficiently to eliminate interference peak
- Adding an additional nucleotide to the 3’ end and using TEG spacer did not aid with removing the interference peak
- Interference peak not exactly the same Q1 mass as antisense strand (0.2 – 0.7 Da difference based on HRMS analysis)
  - Mass shift is easily resolved on HRMS system
  - Mass shift cannot be resolved on MS/MS system



Method Performance Assessment for FLP-1 (25 µL Aliquot / Sciex API 5000 MS)

QC Level	Nominal Conc (ng/mL)	Fresh Extract			7-day Reinjection Reproducibility
		%Bias (%CV)	*Recovery (%)*	*Matrix Factor*	*%Bias (%CV)*
LLOQ QC	25.0	7.13 (25.1)	N/A	N/A	N/A
LQC	75.0	17.1 (9.46)	34.9 ± 2.63	0.90 ± 0.09	-2.14 (12.4)
LMQC	400	22.1 (7.21)	N/A	N/A	23.8 (3.02)
MQC	4000	13.6 (10.4)	23.0 ± 1.44	1.02 ± 0.02	21.3 (9.21)
HQC	8000	12.4 (14.5)	22.7 ± 3.60	1.04 ± 0.03	19.4 (15.3)

Matrix Lot	LQC %Bias (%CV)
1	2.01 (21.2)
2	7.17 (16.3)
3	19.7 (1.95)
4	21.7 (10.2)
5	26.5 (12.8)
6	7.54 (13.5)

Conclusions: Use of LNA probes for sample extraction

1. First known assay for analyzing siRNA analytes using LNA probes for sample extraction/clean-up

2. The assay is a specific and sensitive hybrid LC-MS siRNA assay which uses less sample volume, making it highly suitable in preclinical analysis

3. LLOQ and linear range in mouse plasma were:

   - siRNA-1 from 25.0 – 10000 ng/mL using a 25 µL aliquot
   - siRNA-2 from 0.500 – 500 ng/mL using a 10 µL aliquot

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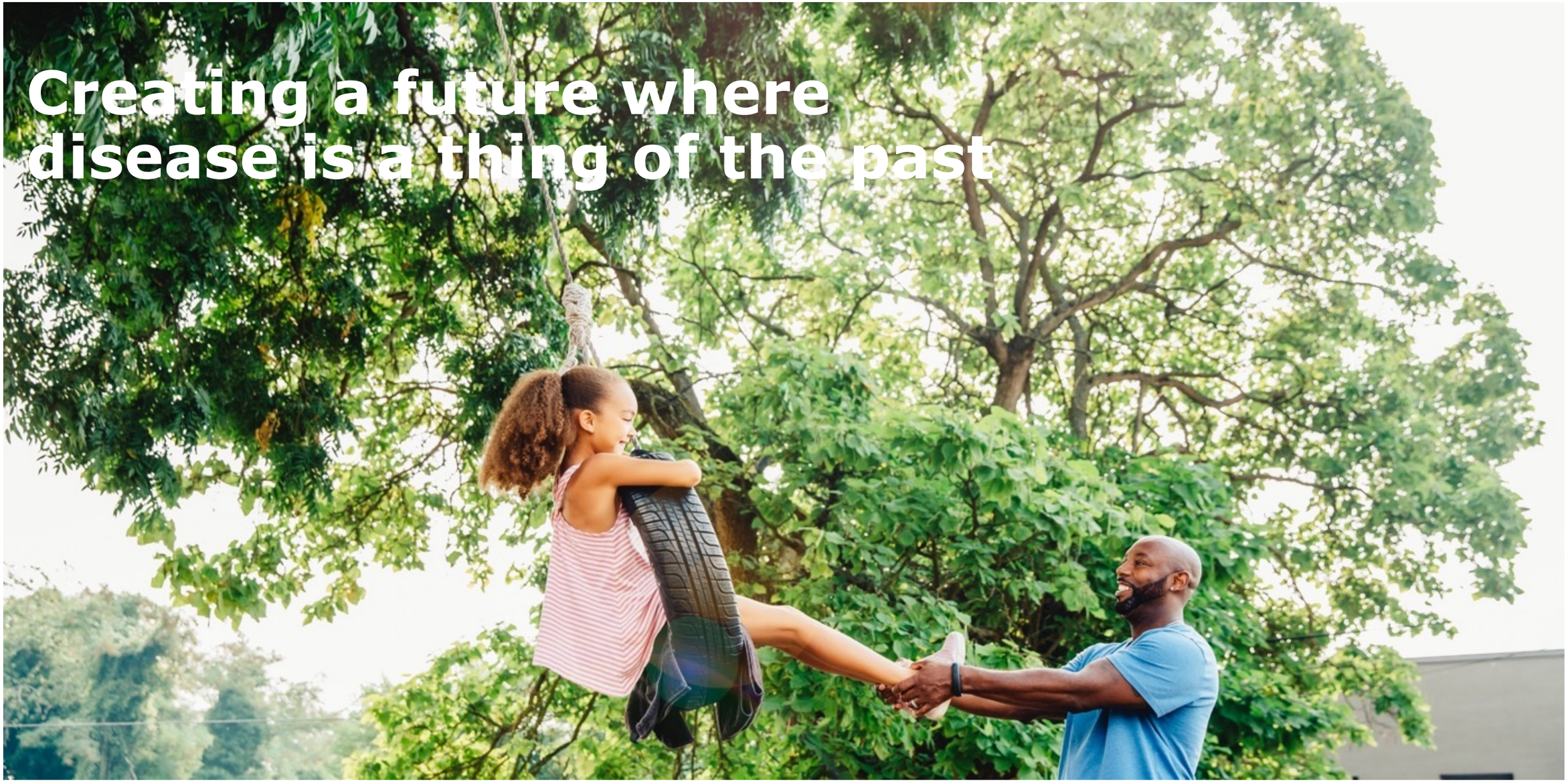
Creating a future where disease is a thing of the past

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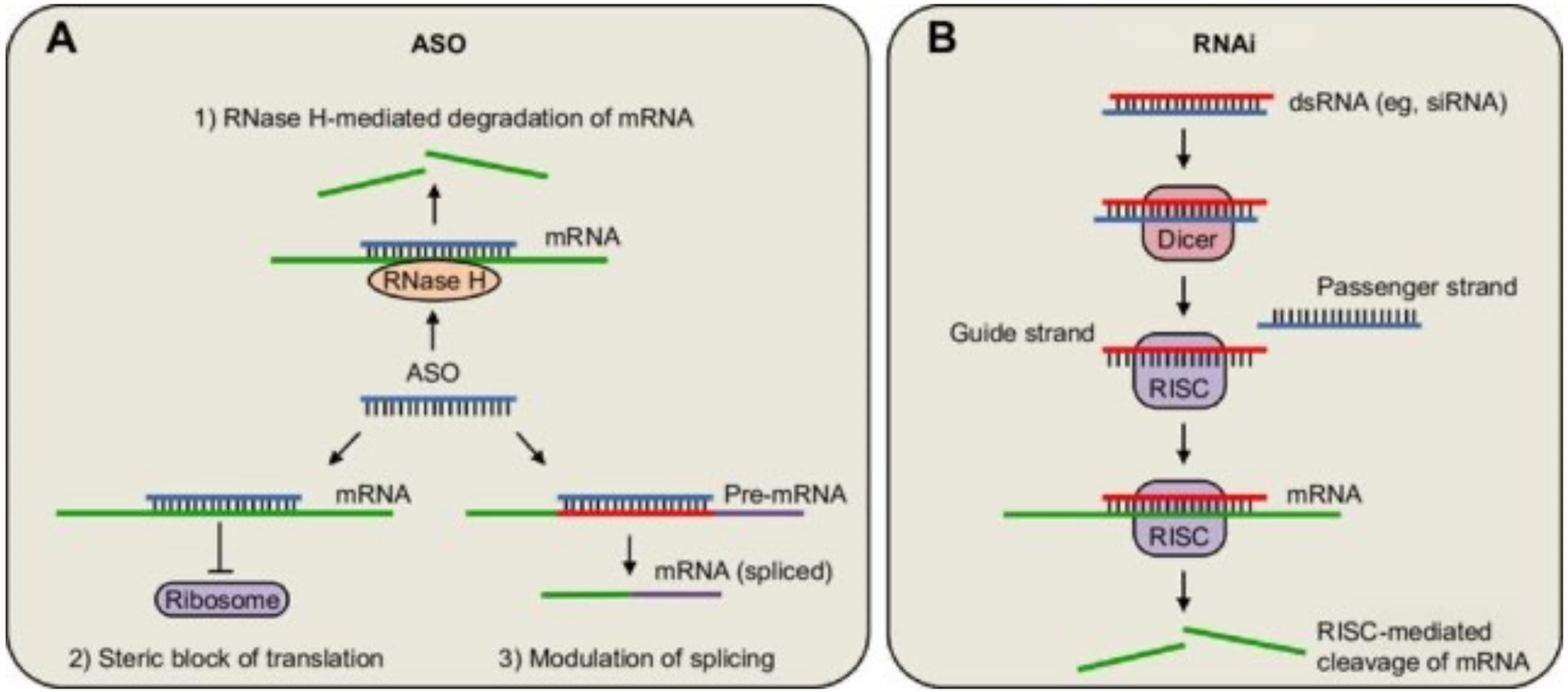
Johnson&Johnson

Gene silicing - mode of action



https://www.researchgate.net/publication/332881818/figure/fig1/AS:755726863593477@1557190733922/Mechanisms-of-oligonucleotides-Notes-A-ASOs-use-RNase-H-mediated-mRNA-degradation_W640.jpg
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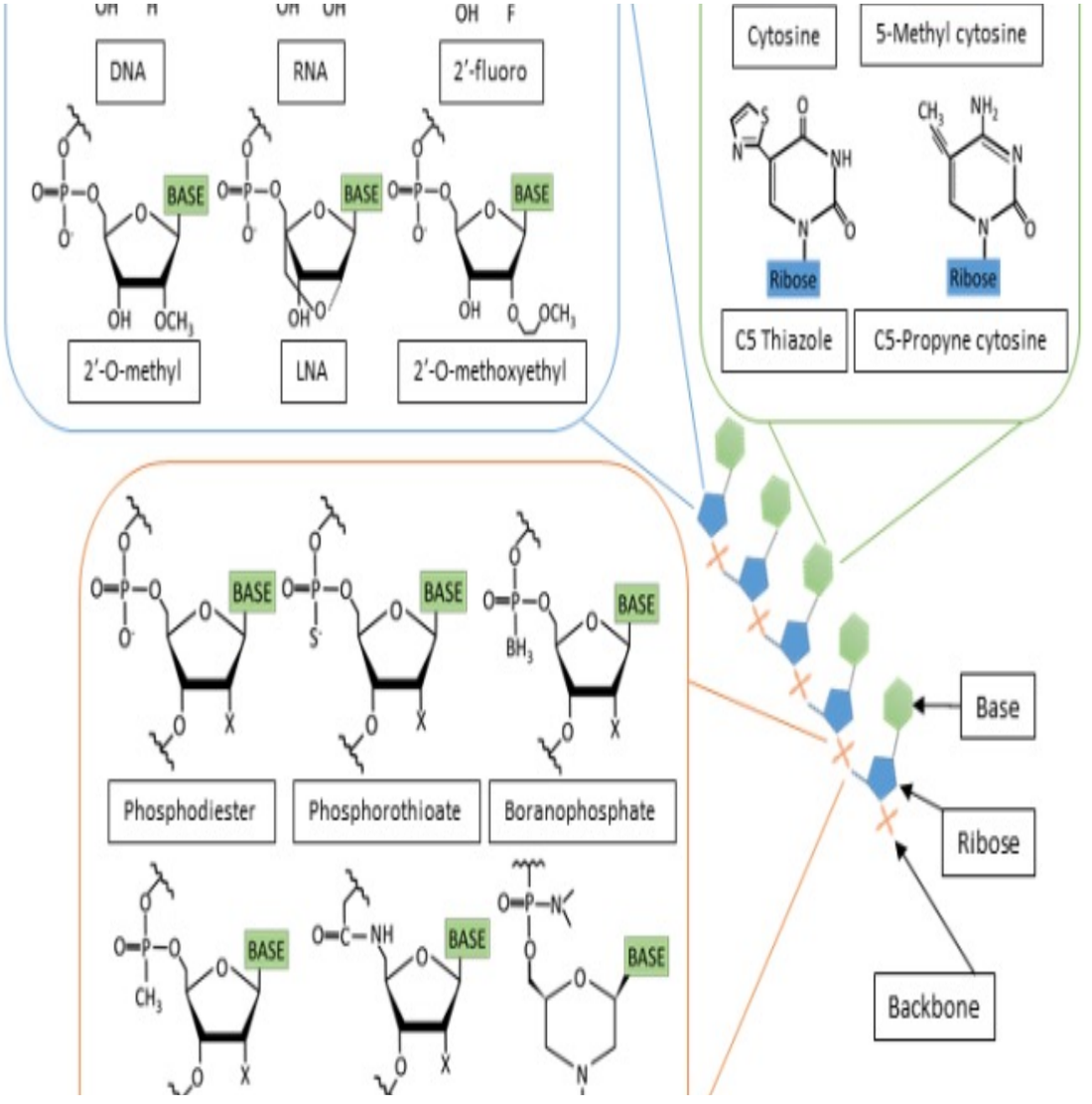
DNA
RNA
2′-fluoro
BASE
2′-O-methyl
LNA
2′-O-methoxyethyl
Cytosine
5-Methyl cytosine
Ribose
C5 Thiazole
C5-Propyne cytosine
Phosphodiester
Phosphorothioate
Boranophosphate
Base
Ribose
Backbone