Challenges with a LCMS method for quantification of an oligonucleotide

Pictured above: HIV absorption

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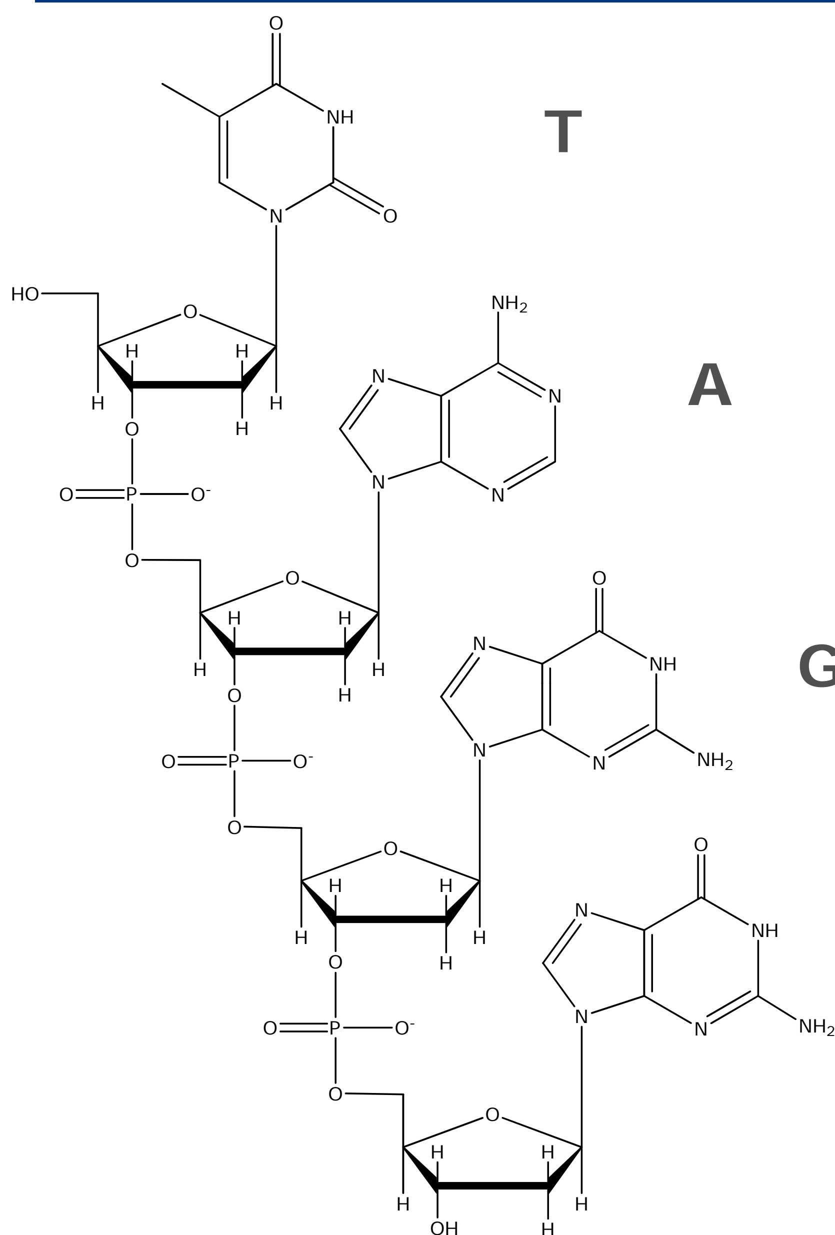
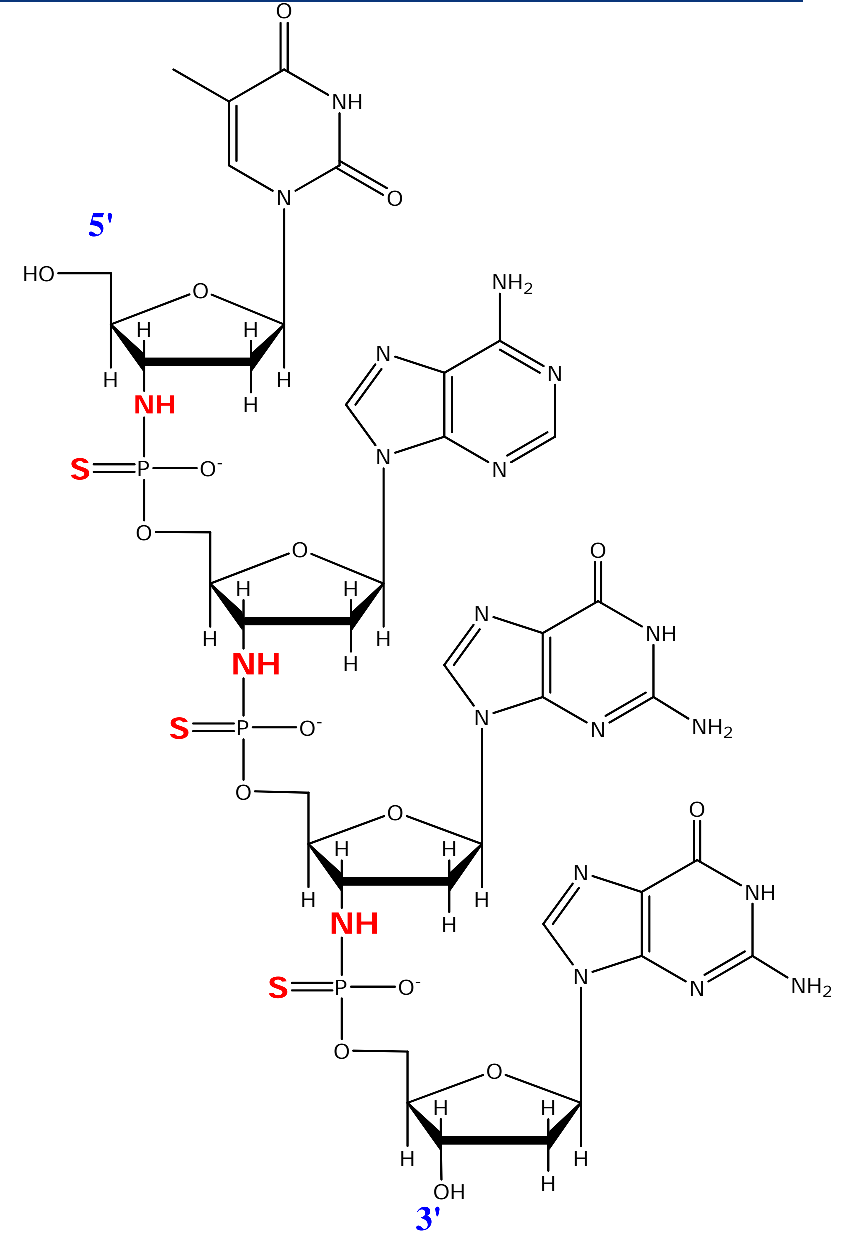
Outline

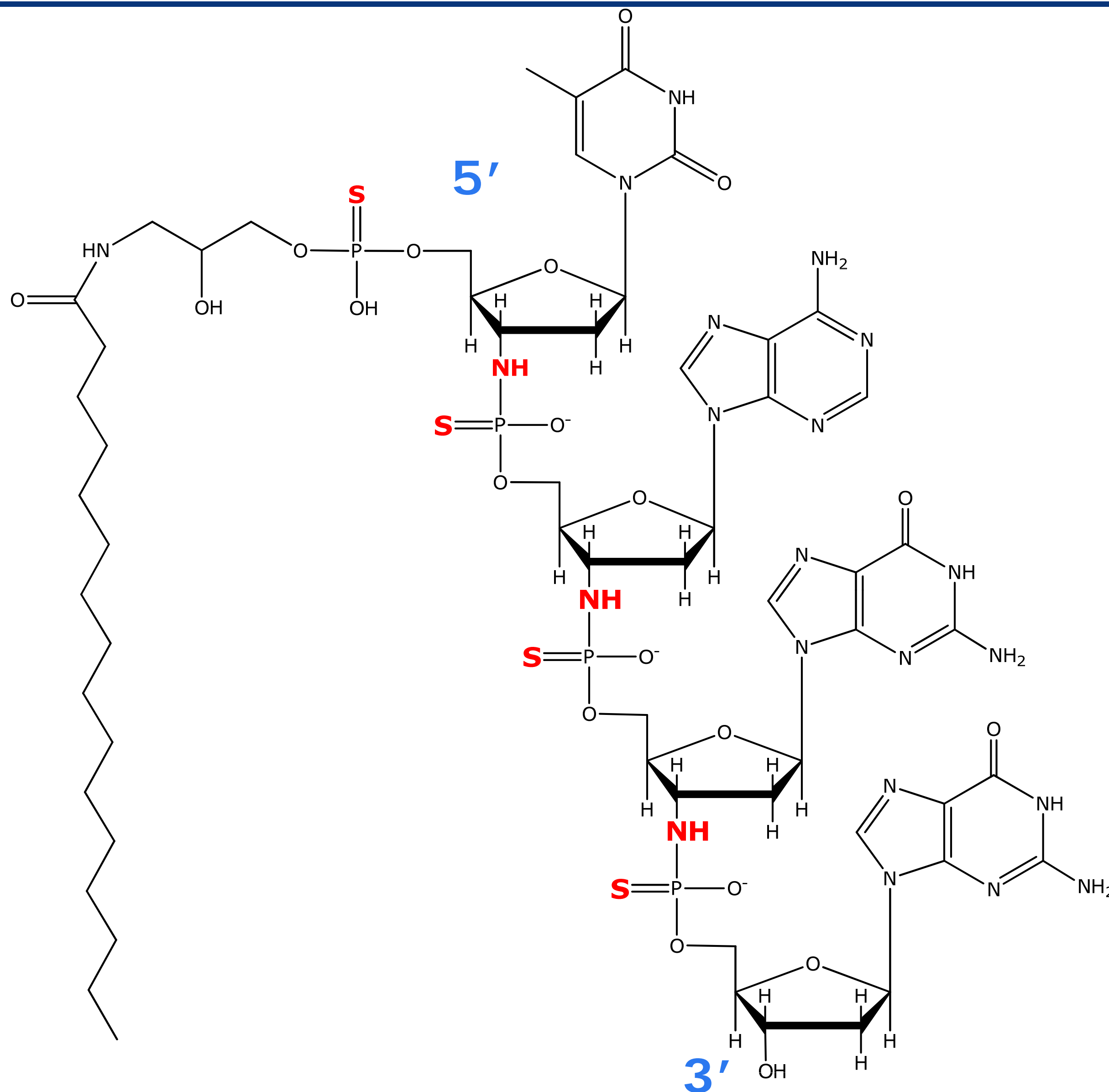
- Introduction to the compound
- Anticipated challenges
- LC-MS/MS method development
- Extraction from plasma

OGN – phosphorothioate linkage





OGN with Palmitoyl tail



Further background

- In licensed compound
- Oligonucleotide therapeutic – 13 mer – MW 4610
- ASO (antisense oligo) for cancer treatment
- API also referred to as "FLP-NPS" (Full Length Product-NPS). NPS indicates the bonding segment N-P=S

Quantitative assay

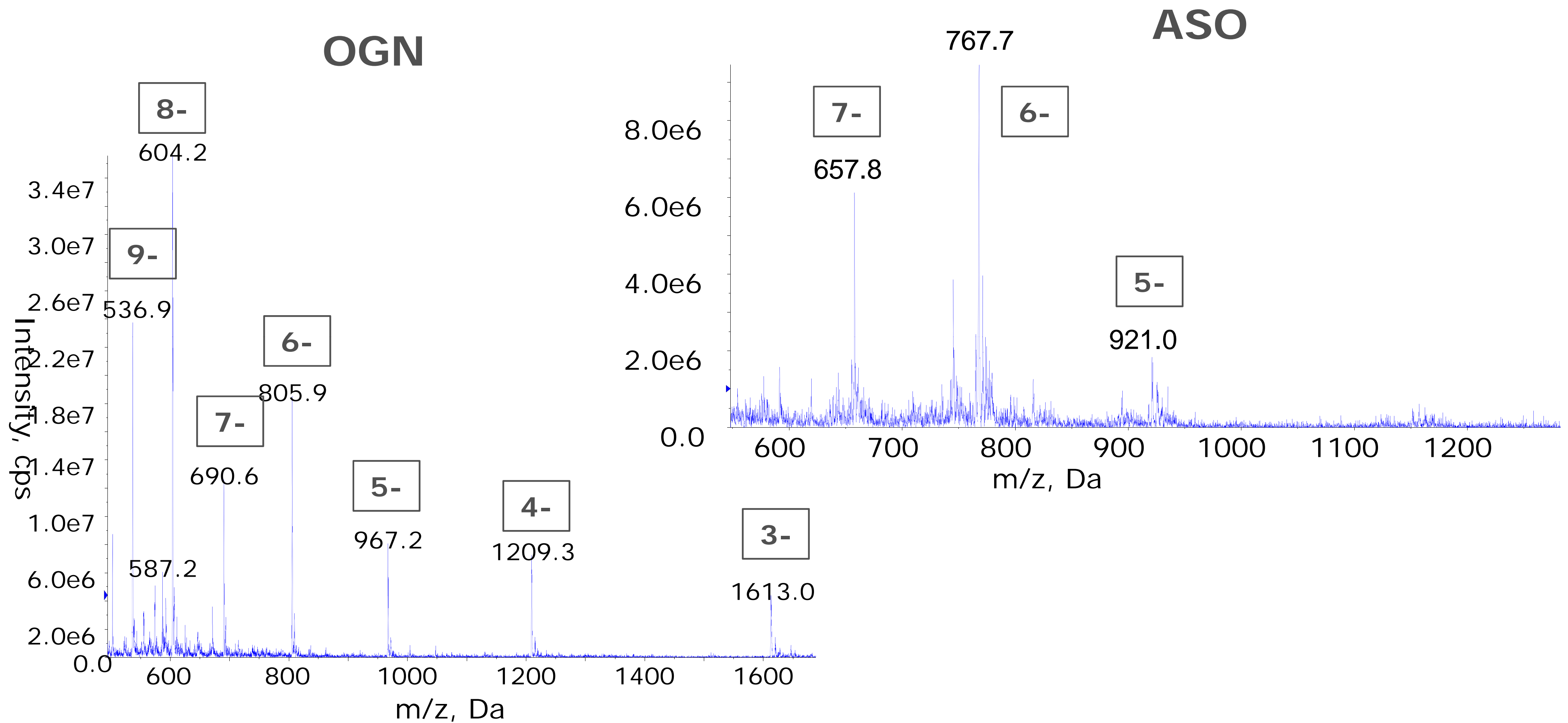
- Program supported @ CRO with hybridization Elisa assay
- Complementary LCMS(MS) assay(s)
  - Quantification of UD in plasma (range 0.5 μg/mL – 200 μg/mL)
  - Investigation of metabolites
- Challenges
  - Large acidic molecules
  - Specificity/selectivity (Na adducts, fragmentation)
  - Adsorption – protein binding
  - Chromatography – ion pairing
  - Sample preparation

Tuning: theoretical charge state distribution

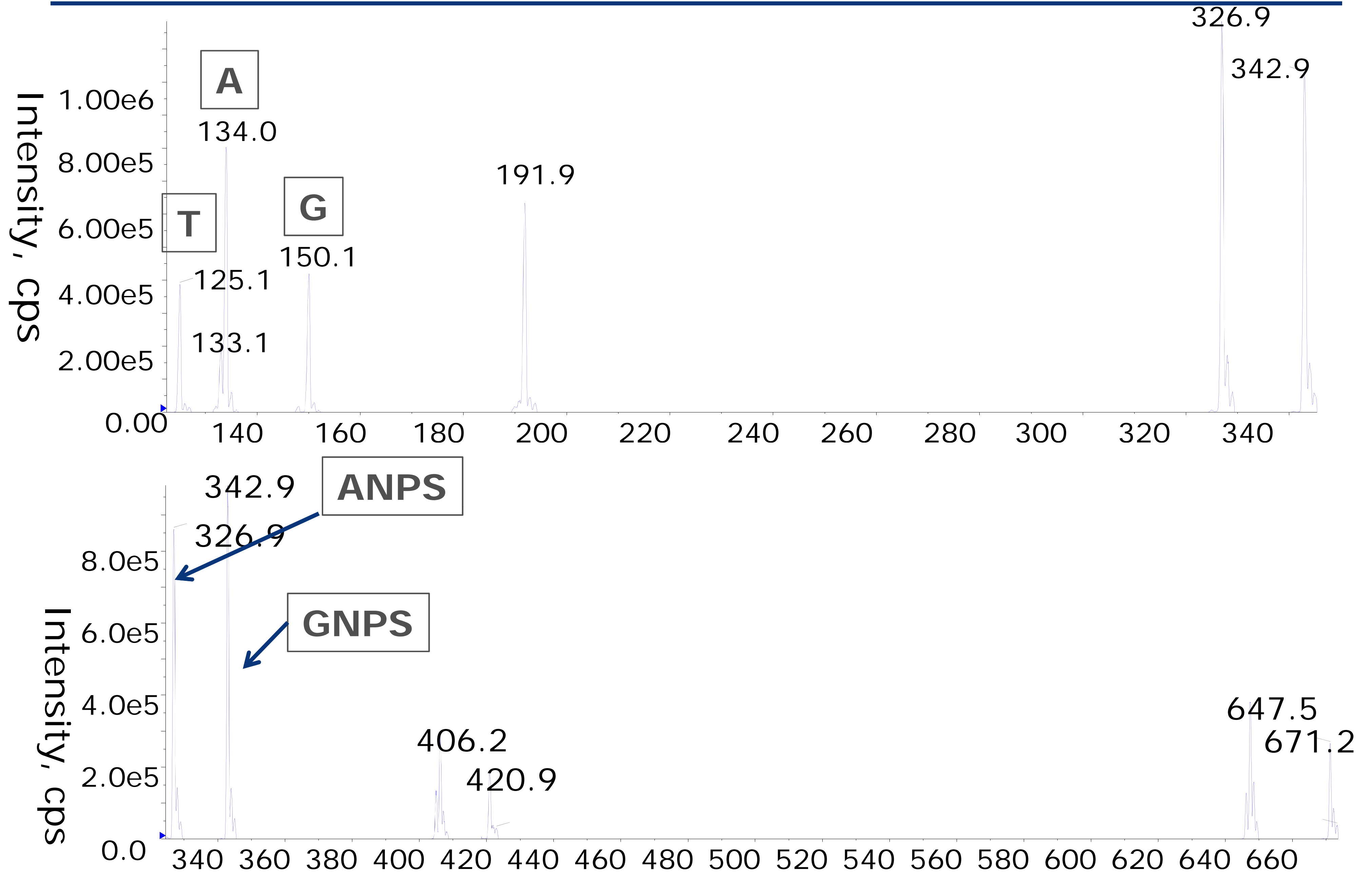
Name	OGN sequence	MW average	OGN Average m/z of charge states
FLP-NPS	TAGGGTTAGACAA	4610.25	575.27 (8-)
	13 mer		**657.59 (7-)**
			767.36 (6-)
			921.03 (5-)
Reference	ATCTATACAAGCTGTC	4840.22	536.79 (9-)
	16 mer		**604.01 (8-)**
			690.45 (7-)
			805.69 (6-)
			967.03 (5-)
			1209.04 (4-)

Tuning: negative mode

50 µg/mL in H_2O/MeOH/HFIP/TEA (70/30/1/0.1)



PIS of m/z 767.3



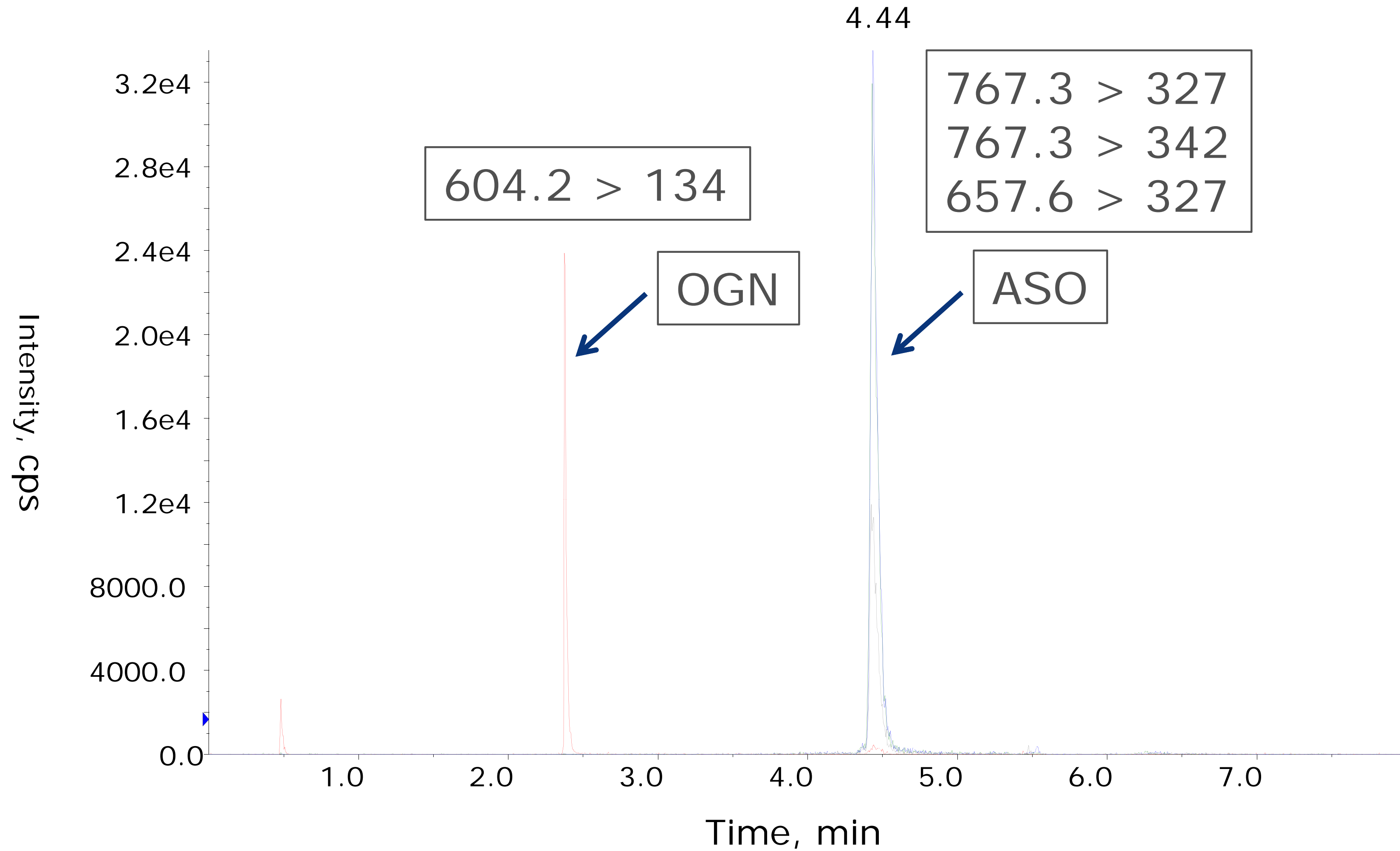
Chromatography

	Shimadzu LC30AD and SIL30ACMP
Column	Acquity BEH C_{18} **2.1 x 50 mm 1.7 µ**
Flow rate	300 µl/min
Solvent A	Water/**HFIP/TEA** (100/1/0.1; v/v/v/v)
Solvent B	**MeOH**/THF (70/30; v/v)

Time (min)	Solvent A	Solvent B
0.00	85	15
1.50	80	20
4.00	45	55
4.01	2	98
5.50	2	98
5.51	85	15

API4000	Q1 Mass (m/z)	Q3 Mass (m/z)	Dwell time (msec)
6-	767.3	327	75
		343	75
8-	604.2	134	75

Chromatogram



Chromatography

- **Observations:**
  - Shift in retention times
  - 50% decrease in response vs start @ end analytical run

- Variations:
  - ACN vs MeOH as solvent B
  - Removal of THF in solvent B
  - Addition of 1% HFIP and 0.1% TEA to B
  - Varying concentration of HFIP and TEA
  - Different modifiers/ion pairing reagents evaluated (DIEA, HA)

- Results: No substantial improvements

Chromatography

- No HFIP in mobile phase (post column addition) -> pH on column increases
  - Improved sensitivity
  - Response drift over analytical run
  - Peak shape deterioration and pressure increase
- Current method: with addition of HFIP and TEA in solvents volatility of HFIP requires frequent refreshment of solvents

Time (min)	Solvent A	Solvent B
0.00	100	0
1.00	80	20
4.50	65	35
4.51	2	98
5.50	2	98
5.51	100	0

	Shimadzu LC30AD and SIL30ACMP
Column	Acquity BEH C_{18} **2.1 x 50 mm 1.7 µ, 300A**
Flow rate	300 µl/min
Solvent A	Water/**HFIP/TEA** (100/0.5/0.1)
Solvent B	**MeOH**/THF/**HFIP/TEA** (70/30/0.5/0.1)

Considerations for sample treatment

- Adsorption - Lobind material
- Stability/solubility
  - Water soluble
  - Nuclease enzymes
- Protein binding (OGNs ionically bound)
  - 5% H_3PO_4
  - Chaotropic reagents (ureum, guanidine, lysis buffer)
  - Proteinase K
- Extraction from the matrix (plasma)
  - LL(chloroform/phenol)
  - SPE (ion pairing, ion exchange)
  - EtOH precipitation
  - Affinity purification

Sample preparation
LLE + SPE

- Extraction from plasma
  - **Phenol – chloroform**
  - **SPE-HLB**
- Protocol adapted from Ewles et al. 2014 Bioanalysis, 6, 447

LLE	**200 µL plasma + ASO**
	+ 500 µL Water/NH_3 (95/5)
	+ 200 µL ice-cold phenol/chloroform/IAA
	Mix 20 min – centrifuge – transfer 600 µL aqueous phase
SPE (HLB)	+ 600 µL Water/HFIP/TEA 100/2/0.2 - mix
	Condition SPE – transfer sample
	Wash
	Elute in 500 µL Water/ACN/TEA 40/60/1
	evaporate
	Reconstitute in 200 µL water/MeOH/HFIP/TEA 70/30/1/0.1

Low recovery

% recovery in chloroform/phenol extracts

sample	H_2O layer		Organic layer	
	ASO	OGN	ASO	OGN
Water + NH_4OH*	17	74	0	0
plasma + NH_4OH	11	65	-	-

* Ratio aqueous/organic 8/1

Sample preparation
SPE-AEX

200 µL plasma + 300 µL lysis buffer + ASO
Condition SPE column
Apply sample
Wash 2 x 2 mL wash buffer
elute 2 x 1 mL elution buffer
Evaporate - not complete dryness
Reconstitute in 300 µL water/HFIP/TEA

- Clarity OTX SPE columns (buffers delivered with starter kit)
- recovery only **7 %** for ASO (**50 %** for reference OGN)
- Evaporation and reconstitution -> 30 % loss
- pH 5.5 is critical for binding to Clarity OTX

Sample preparation SPE-OTX

Sample description	Lysis condition	% recovery ASO	% recovery reference OGN
20µl plasma + OGN mix + 200µl Lysisbuffer Clarity	30 min RT	**62**	**67**
20µl plasma + OGN mix + 20µl Guanidine 6M + 180µl PBS		53	**95**
20µl plasma + OGN mix + 2µl Protease K + 200µl PBS	15 min 50°C	**65**	**95**
20µl plasma + OGN mix + 200µl Lysisbuffer Clarity	<5 min, RT	28	55
	30 min, 50°C	**73**	**68**

Other strategies considered

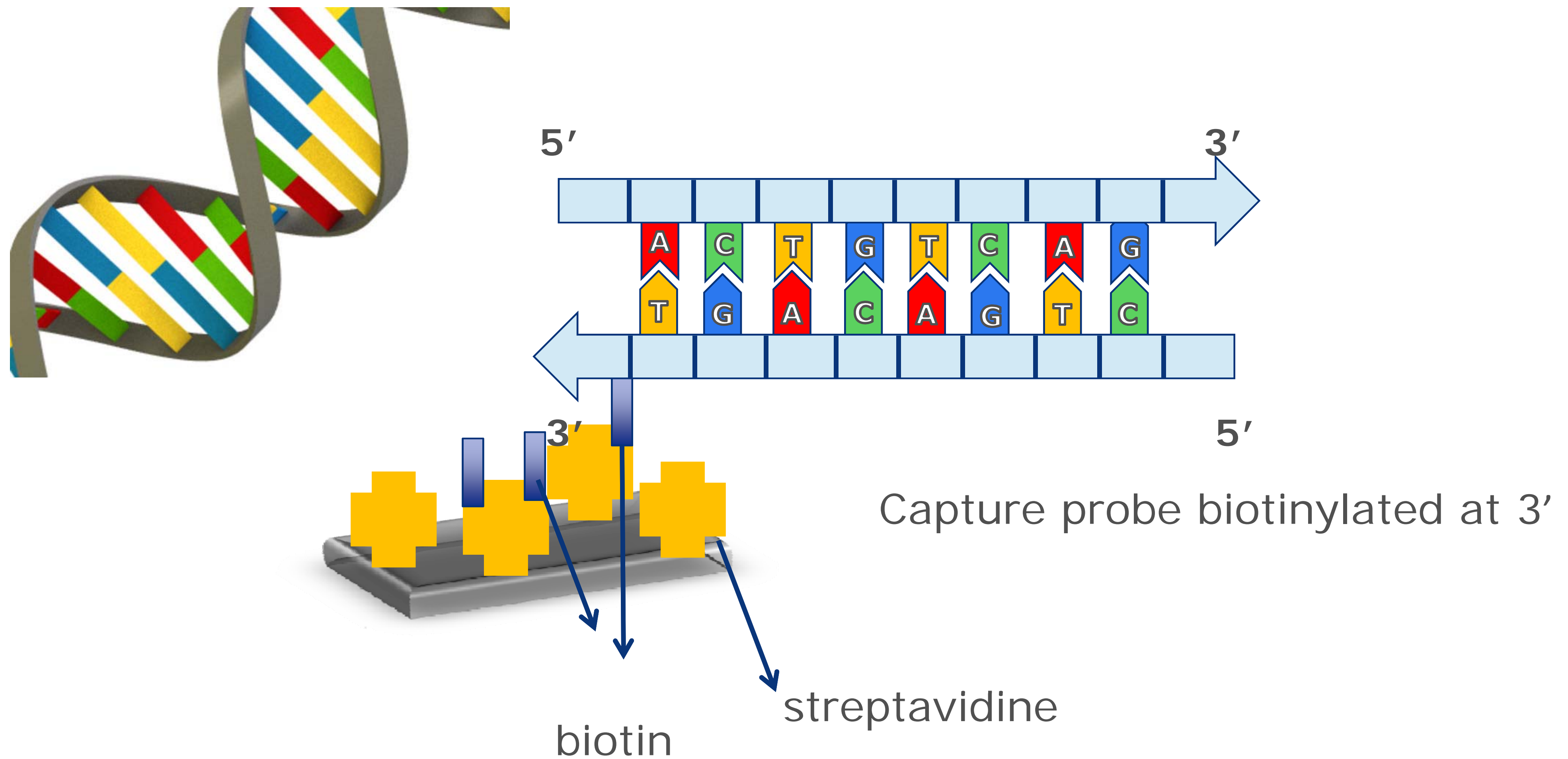
- Protein removal by precipitation
  - Add NH_4OH and CH_3CN
  - Evaporate
- Ethanol precipitation of DNA

- Protein removal
  - chloroform/phenol extraction
- Ethanol precipitation of DNA*

- Hybridization purification with complementary biotinylated probe
  - Streptavidin MSIA tips or streptavidin magnetic beads
  - Elution of the beads

* Chen and Bartlett, J Chrom A, 2013, 1288

Hybridization with biotinylated capture probe



Hybridization Protocol

- Plasma + Binding buffer + ASO
- Add capture probe and heat up to > Tm
- Cool to allow annealing
- Transfer to streptavidine coated MSIA or magnetic beads
- Wash with ice-cold buffers

- Elute
  - Under basic conditions (NaOH 0.2 M or NH_4OH)
  - At elevated temperature

Initial Results:

- Streptavidin – biotin reaction successful
  - No capture probe left following incubation with SA beads

- Hybridization as evaluated by mass spec not (yet) successful

Conclusions

- LC-MSMS development of this ASO is extremely challenging
- LC conditions controlled but robustness still dependent on the quality of the sample prep
- Sample preparation is critical step
  - +/- 60% extraction recovery (variable) with OTX protocol but no robust LCMS response
  - protein binding
  - removal of proteins before DNA isolation
  - Will hybridization offer the solution?
- Full scan HRMS or MALDI MS beneficial to evaluate shifts in charge state distribution or Na adducts or formation of degradation products?

Akcnowledgements

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Thank you

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