Bioanalysis of Oligonucleotides: Challenges and Solutions

14th JBF Symposium

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“Analysis of oligonucleotides from biological samples is a challenging analytical task …

[They] are either single or double stranded DNA or RNA molecules ... Chemically modified to reduce nuclease mediated degradation … Differ in length from approximately 15 up to >1000 nucleotides … And are sometimes conjugated with different ligands or other delivery systems ...

No single bioanalytical approach can be applied to cover all the different therapeutically relevant oligonucleotide classes.”

Welink, Jan, et al. 2018 White Paper. Bioanalysis 10.22 (2018): 1781-1801.

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Bioanalysis of oligonucleotides

qPCR

- Highly sensitive technology
- Susceptible to inhibition of polymerases
- Erroneous amplification of non-target sequences
- Cross-contamination by amplified target molecules
- Sample treatment is required

Chromatography

- Highly selective and specific
- Test full-length oligo and metabolites
- Wide dynamic range
- High reproducibility
- Excellent accuracy and precision
- Sensitivity: ~10-20 ng/mL
- Complex sample preparation

Hybridization Immunoassay

- Enhanced throughput
- Compatible with multiple types of analytes, matrix, delivery mechanisms
- Improved specificity (LNA)
- Sensitivity: ~pM with acceptable accuracy and precision

LC-MS for oligonucleotide quantification in biological matrices

- Hybridization ELISA (hELISA) is the most common platform for oligonucleotide quantification, but alternative technologies exist: hECL, LC-MS, LC-FL, LC-UV, qPCR/RT-qPCR, b-DNA, etc.
- Choice of platform depends on multiple variables: sensitivity, biotransformation/metabolism, reagent availability, modality, matrix, etc.

LC-MS

Advantages

- Good dynamic range
- LLE/SPE-based extractions, **no hybridization probes**
- High selectivity for simultaneous quantification of oligonucleotide and **metabolites**; both sense/anti-sense for siRNA
- High sensitivity for small oligonucleotides (<20 mer) and good sensitivity up to 40-50 mer

Drawbacks

- Typically less sensitive (low ng/mL LLOQ) compared to hELISA
- Extensive sample preparation time
- Ion-pairing reagents for LC, column degradation
- Lower throughput

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Common analytical challenges for oligonucleotides by LC-MS

Extraction

- Recovery – metabolites, negative backbone, solubility
- Protein binding – polar, disrupt protein binding
- Adsorption – low binding tubes/plates, no glass
- ISTD – typically analog oligonucleotide

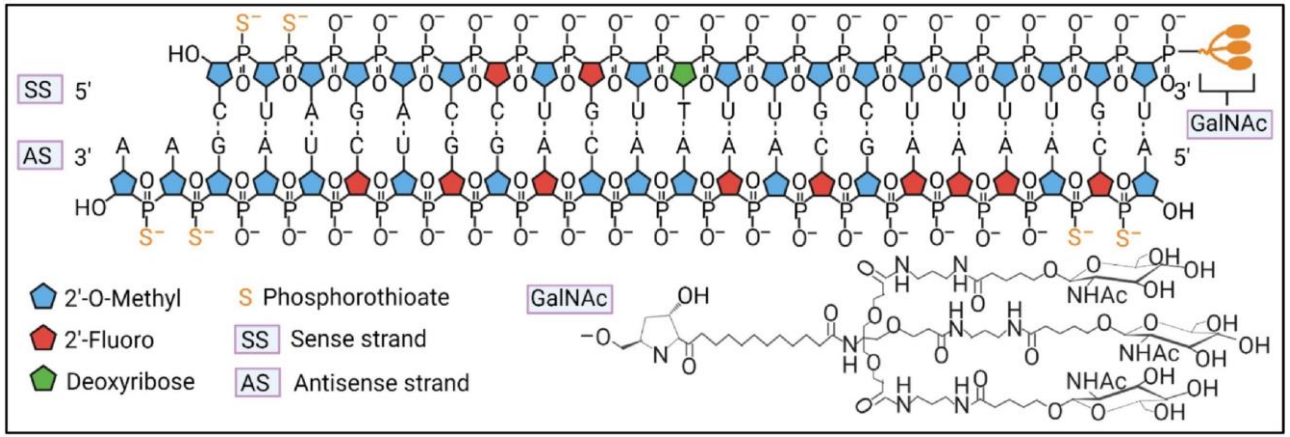
LC

- Retention – polyanionic, ion pairing reagents
- Separation – metabolites
- Peak shape – phosphorothioate stereocenter

MS

- Fragmentation – bases/nucleotides (m/z 100-350) and backbone (phosphodiester/phosphorothioate m/z 79/95), low selectivity, crosstalk
- Ionization – ESI(-), charge state distribution, adducts

Inclisiran



Migliorati et al. *Trends in Pharmacological Sciences* (2022).

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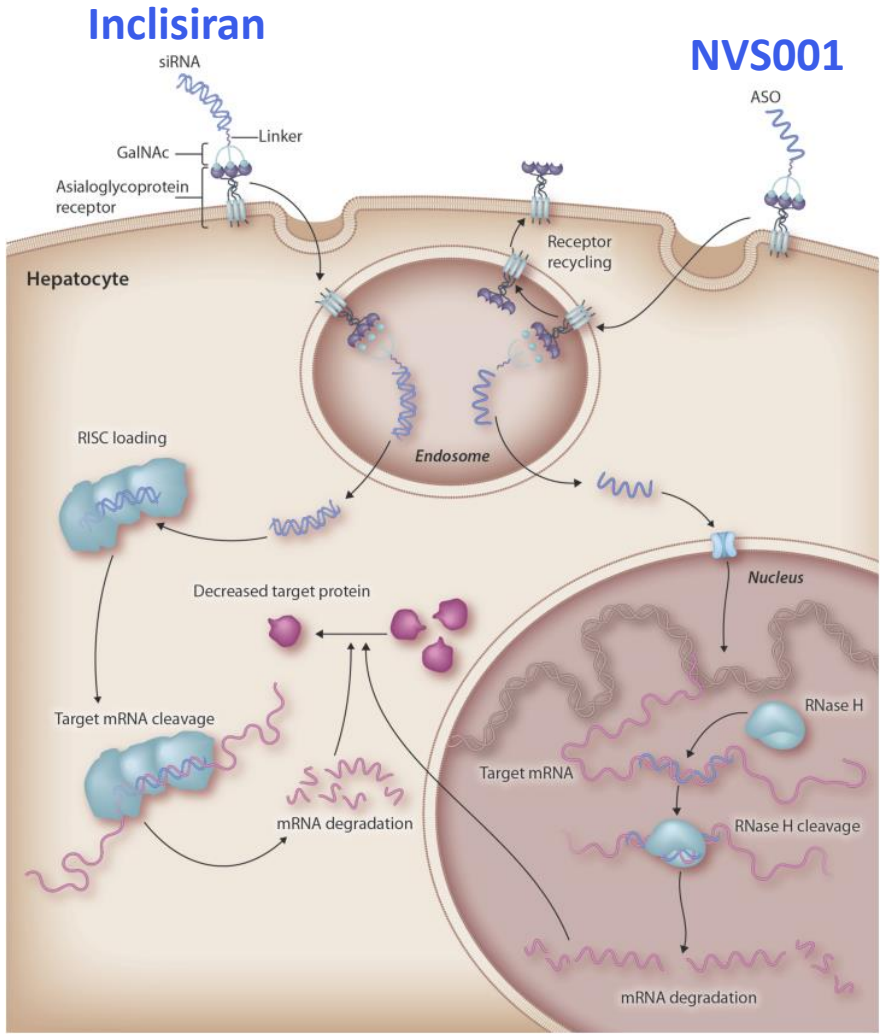
Oligonucleotide therapeutics for cardiovascular disease

NVS001

- NVS001 is an **antisense oligonucleotide-GalNAc** (ASO-GalNAc) conjugate (ASO-GalNAc) that inhibits production of apolipoprotein(a) [apo(a)]
- Apo(a) is a protein component of low-density lipoprotein cholesterol-like particle [LDL, Lp(a)] that is produced in the liver
- Lower Lp(a) levels leads to **lower circulating LDL-C** levels, which reduces the risk of cardiovascular disease; levels are genetically determined and cannot be controlled with diet/exercise

Inclisiran (aka Leqvio®, KJX839, A-120190/122088)

- Inclisiran is a **small interfering RNA-GalNAc conjugate** (siRNA-GalNAc) that inhibits production of proprotein convertase subtilisin/kexin type 9 (PCSK9)
- PCSK9 is a serine protease produced in the liver regulates low-density lipoprotein cholesterol (LDL-C) levels in plasma
- Lower PCSK9 levels leads to **lower circulating LDL-C** levels, which reduces the risk of cardiovascular disease



Debacker, Alexandre J., et al. *Molecular Therapy* 28.8 (2020): 1759-1771.

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Overview of NVS001 method development (human plasma)

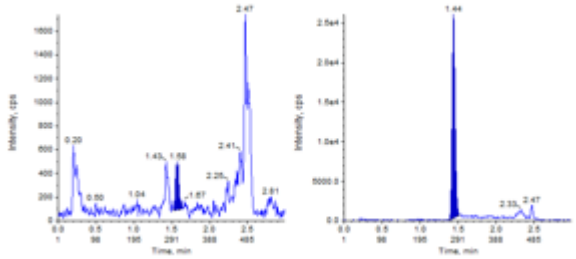
Initial methodology

- Human method **2.00 to 1,000** ng/mL, analog ISTD, MRM on **Sciex 6500+**
- Clarity® OTX SPE extraction, increased **aliquot volume 4X**
- DIPA/HFIP mobile phases, Waters Acquity BEH C18 column

Significant column clogging issue observed

- Batch couldn't be injected
- Tested 3X additional washes
- Tested higher Loading Buffer dilution
- Column clogging improved, but not resolved

Challenging sensitivity at LLOQ



Extraction

Aliquot **100** µL of plasma

Add 50 µL of Analog ISTD

Dilute with 250 **(600)** µL of Loading Buffer
(Guanidine HCl, Triton X-100, pH 2)

Load Phenomenex Clarity OTX™
(Mixed Mode Anion Exchange, No Ion Pairing, < 40mer)

Wash 3X **(6X)** with Ammonium Acetate pH 5.5, 50% MeCN

Elute with Ammonium Bicarbonate pH 9.5, 40% MeCN, 10% THF (BHT stabilized) into RNASecure

Evaporate and reconstitute in MeOH:Water:DIPA:HFIP

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Optimizing Clarity OTX recovery

- **Table 1** – Non-specific binding in post-spiking solutions; 135% recovery observed at LQC, likely due to lower curve range
  - Clarity OTX claims typical recovery is 80-90%
  - Poor sorbent loading and irreversible non-specific binding after dry down
  - Recovery in mouse plasma: ~75%
- **Table 2** – Modified post-spiking diluent to be 50 µg/mL BSA (aq); consistent but low recovery observed ~45%
- **Table 3** – Evaluated impact of dry-down; no loss on dry down was observed
- **Table 4** – Screened alternative elution solvents; no improvements; NVS001 likely lost during loading/wash
- **Significant column clogging continues**

Table 1 – Recovery with NSB

Sample	Analyte Peak Area	ISTD Peak Area	Sample	Analyte Peak Area	ISTD Peak Area
Mean Pre-LQC	9,069	34,325	**Mean Pre-HQC**	641,018	34,016
Mean Post-LQC	6,731	60,112	**Mean Post-HQC**	1,016,326	60,284
%Recovery	134.7	57.1	**%Recovery**	63.1	56.4

Table 2 – Recovery no NSB

Sample	Analyte Peak Area	ISTD Peak Area	Sample	Analyte Peak Area	ISTD Peak Area
Mean Pre-LQC	11,138	61,800	**Mean Pre-HQC**	735,268	53,540
Mean Post-LQC	23,099	111,772	**Mean Post-HQC**	1,776,907	109,239
%Recovery	48.2	55.3	**%Recovery**	41.4	49.0

Table 3 – Dry down

Sample	Analyte Peak Area	ISTD Peak Area	Sample	Analyte Peak Area	ISTD Peak Area
Post Pre-Dry LQC	23,599.2	113,737	**Post Pre-Dry HQC**	1,859,176	121,971
Post Post-Dry LQC	23,099.6	111,772	**Post Post-Dry HQC**	1,776,907	109,239
%Recovery	102.2	101.8	**%Recovery**	104.6	111.7

Table 4 – Elution solvents

Composition	C1	Original	C2	C3	C4	C5	C6
Ammonium Bicarb pH 9.5	100 mM	100 mM	100 mM	100 mM			
Acetonitrile	30	40	60		40	75	
THF	10	10	10	50	10	10	
Water	60	50	30	50	40	5	
Ammonium Hydroxide					10	10	10
Chloroform							45
Methanol							45
Recovery	47.5	50.8	49.8	43.9*	41.7	10.5	30.4
* No ISTD recovery observed							

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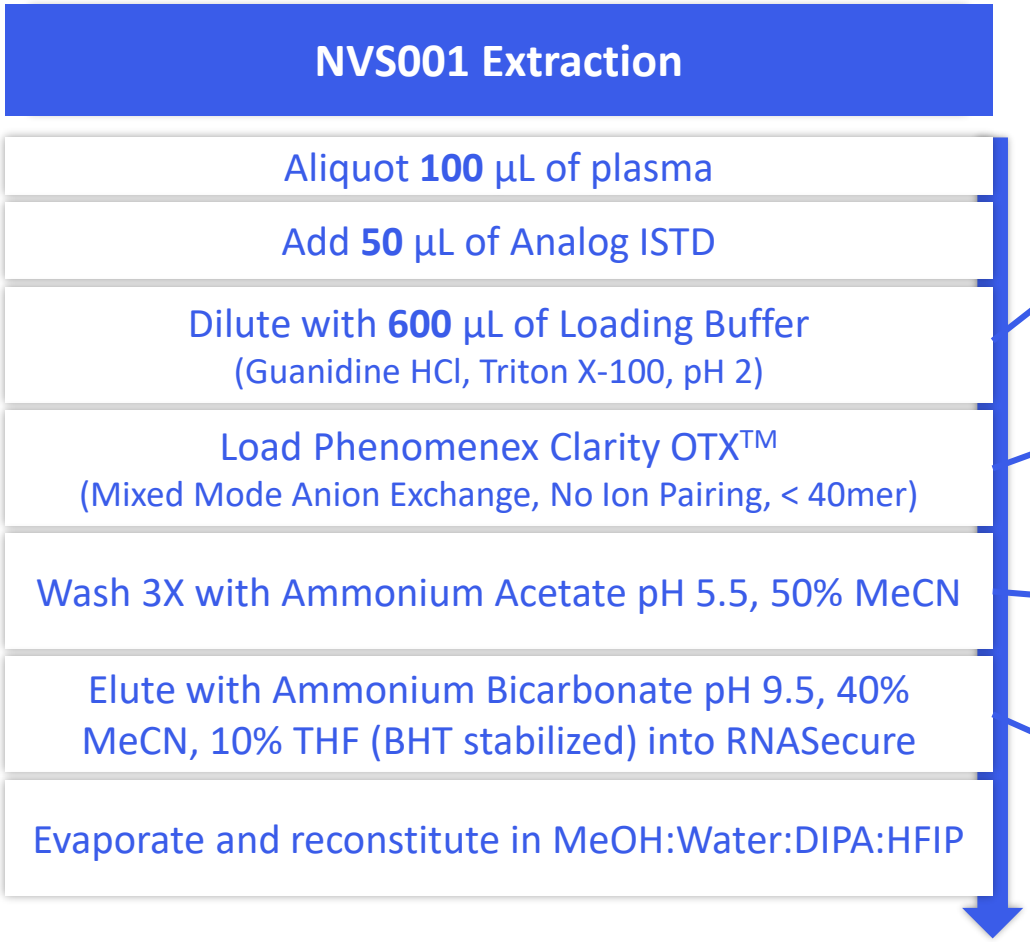
Optimizing Clarity OTX recovery



Buffer ratio is critical

Recovery increased to ~94% at 1:1 dilution (150 µL Buffer). Higher dilution ratios and wash buffer dilution were tested, column clogging not resolved.

Confirmed sorbent loading issue

Diluted sample flow through was re-extracted, significant amount of analyte present accounting for low recovery.

Alternative washing conditions unable to resolve column clogging

Alternative wash buffers screened and increased washing tested, column clogging not resolved.

Elution recovery can not be improved

Tested 3X elutions, no improvement. ~97% of analyte elutes in first fraction.

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Optimizing Clarity OTX recovery in the literature

Phenomenex® Clarity® OTX User's Guide (GU54341119_W)

- Clogging issue "Increase the volume of Lysis-Loading buffer; it is not uncommon to treat 1:4 (sample:LL buffer) prior to SPE."
- This recommendation was removed from v2.0 of the guide

Sun, Yuchen, et al. *Bioanalysis* 12.24 (2020): 1739-1756

- "Our data clearly demonstrated that **the lysis-loading buffer volume**, along with the salts used for the ASO wash step, are the most influential factors for the recovery rate."
- "Our unpublished finding indicates that the most appropriate lysis-loading buffer volume varies … **needs to be carefully tested during the respective method developments**."

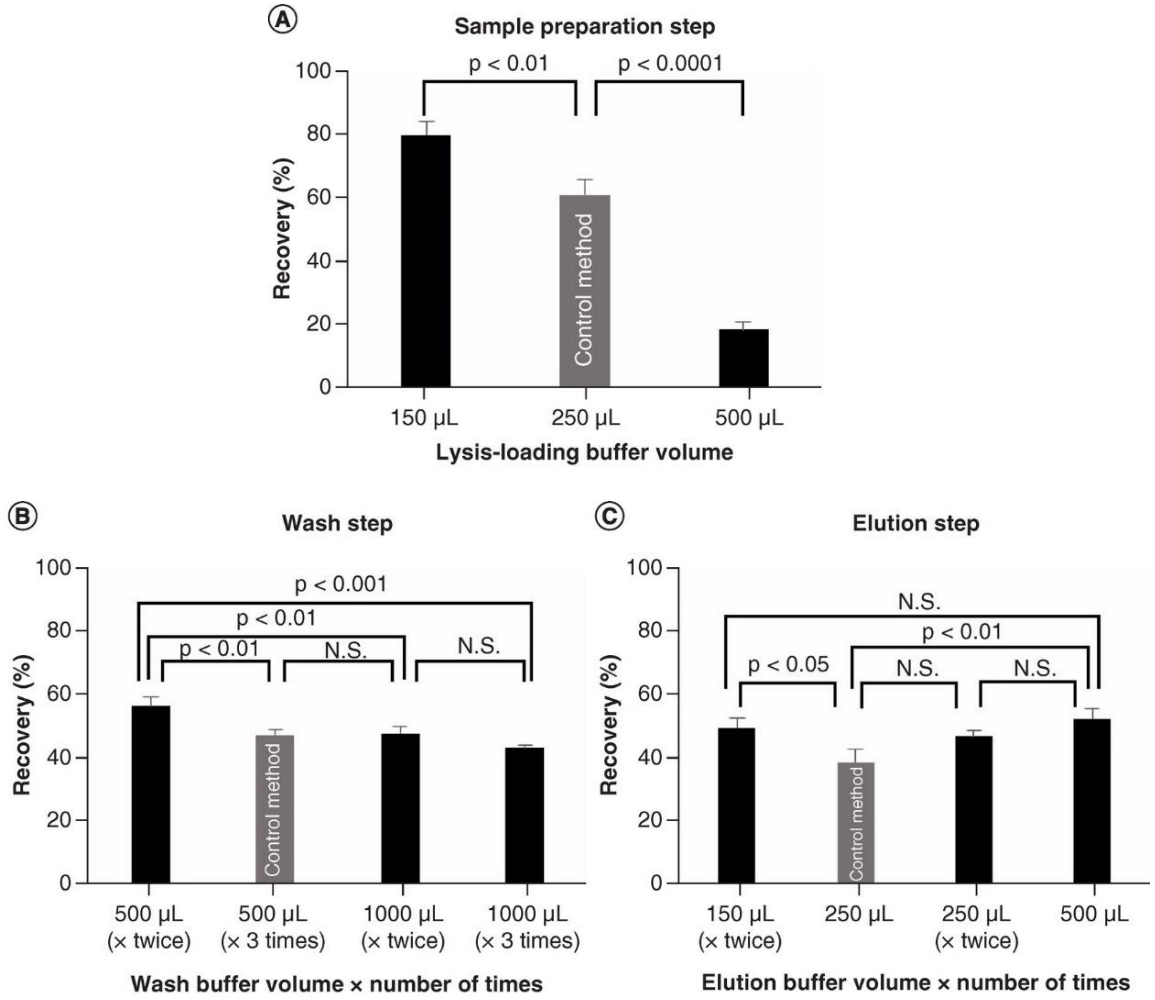

Fig. 4. from Sun, Yuchen, et al. (2020)

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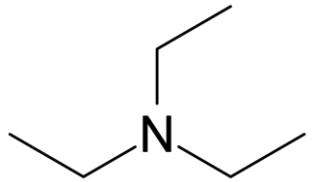
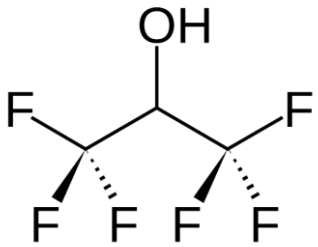
Challenges with chromatography and ion-pairing reagents

TEA



HFIP



Unresolved column clogging issue

- No clogging observed with 25 µL aliquot; clogging matrix related

Attempted to optimize ion-pairing reagents

- Increase signal, which would allow for reduced aliquot
- Alkylamine: ion-pair, neutralizes backbone; typically triethylamine (TEA)
- Perfluorinated alcohol: counter ion, acidic modifier to adjust pH, enhances ionization; typically HFIP
- Micellar-based chromatography at high alkylamine concentration and high pH (Li, Ning, et al. Journal of Chromatography A 1580 (2018): 110-119)

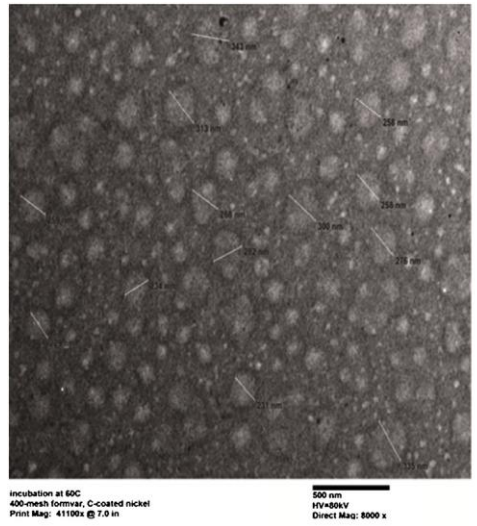


Fig. 4. from Li, Ning, et al. 2018

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Optimizing ion-pairing reagents

Alkylamine	Counter Ion	pH	Retention Factor (k')	Average Peak Area	Relative Signal	Notes
25 mM TEA	50 mM HFIP	9.3	3.5	174,718	65	High pH, low signal, micellar?
25 mM DIPA	50 mM HFIP	9.4	5.6	212,865	80	High pH, low signal
5 mM DIPA	25 mM HFIP	8.8	5.8	254,013	95	Acceptable
10 mM DIPA	25 mM HFIP	9.1	5.9	267,481	100	Acceptable, but higher pH
25 mM DIEA	50 mM HFIP	9.1	6.3	238,278	89	High pH
10 mM DIPA	**50 mM HFIP**	**8.7**	**6.9**	**250,960**	**94**	**From mouse method**
5 mM DIPA	100 mM HFIP	8.1	6.9	183,740	69	Low signal
5 mM DIEA	50 mM HFIP	8.4	7.8	259,149	97	ISTD co-eluting and MS crosstalk

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Alternative extractions to address column clogging

Alternative standard extractions for oligonucleotides

- Ledvina, Aaron R., et al. Bioanalysis 13.17 (2021): 1343-1353

Phenol: Chloroform: Isoamyl LLE

- Phenol denatures proteins, chloroform facilitates LLE and isoamyl reduces forming
- pH > 8 DNA/RNA in partitions into aqueous layer

LLE

- Additional clean-up and removes residual phenol

Oasis HLB SPE

- Ion-pairing based loading, washing and elution
- DIEA used to improve retention of polar GalNAc

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Alternative extractions to address column clogging

LLE + LLE - Phenol:Chloroform followed by DCM

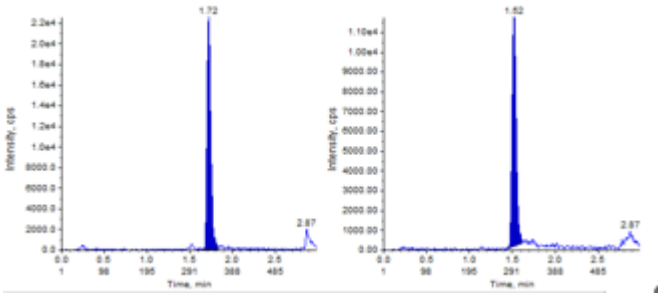
- Similar recovery observed to Clarity OTX with maximum transfer volumes
- Column clogging issue surprisingly **not** resolved, despite visually cleaner extracts
- Screened additional conditions to no success (alternative transfer volumes, reconstitution reagents)

LLE + SPE - Phenol:Chloroform followed by HLB SPE

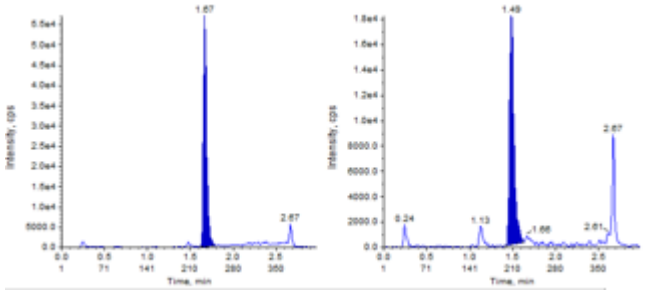
- Lower recovery observed relative to Clarity OTX (~60%)
- **Column clogging issue resolved**
- Surprisingly, overall signal was higher relative to Clarity OTX, despite lower recovery
- Method validated with no column issues

Sample	Analyte Peak Area	ISTD Peak Area	Sample	Analyte Peak Area	ISTD Peak Area
Mean Pre-LQC	13,876	60,972.2	Mean Pre-HQC	2,495,342	65,773.1
Mean Post-LQC	24,288	73,863.7	Mean Post-HQC	4,285,696	83,628.0
%Recovery	57.1	82.5	%Recovery	58.2	78.6

Clarity OTX
2.2e4 height
~66k area



LLE > SPE
5.5e4 height
~140k area



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Precision and accuracy for NVS001

Run	(2.00 ng/mL) Dilution=1	%Bias	(6.00 ng/mL) Dilution=1	%Bias	(50.0 ng/mL) Dilution=1	%Bias	(500 ng/mL) Dilution=1	%Bias	(800 ng/mL) Dilution=1	%Bias	(8000 ng/mL) Dilution=10	%Bias
P&A 1	2.07	3.5	5.17	-13.8	45.6	-8.8	528	5.6	824	3.0	7690	-3.9
	1.99	-0.5	5.45	-9.2	49.2	-1.6	486	-2.8	792	-1.0	8040	0.5
	2.03	1.5	5.66	-5.7	49.8	-0.4	480	-4.0	782	-2.3	7960	-0.5
	2.15	7.5	5.50	-8.3	47.0	-6.0	528	5.6	826	3.3	8040	0.5
	1.88	-6.0	&&4.61	-23.2	47.4	-5.2	493	-1.4	816	2.0	8070	0.9
	2.10	5.0	5.46	-9.0	46.7	-6.6	463	-7.4	842	5.3	7960	-0.5
P&A 2	1.91	-4.5	5.13	-14.5	50.2	0.4	546	9.2	801	0.1		
	1.82	-9.0	5.45	-9.2	50.1	0.2	533	6.6	819	2.4		
	2.17	8.5	5.58	-7.0	52.3	4.6	515	3.0	893	11.6		
	1.78	-11.0	6.06	1.0	50.1	0.2	528	5.6	&&924	15.5		
	1.91	-4.5	5.39	-10.2	46.8	-6.4	524	4.8	710	-11.3		
	1.85	-7.5	5.64	-6.0	50.6	1.2	479	-4.2	857	7.1		
P&A 3	2.19	9.5	5.86	-2.3	46.8	-6.4	518	3.6	845	5.6		
	2.30	15.0	5.73	-4.5	48.5	-3.0	495	-1.0	813	1.6		
	2.27	13.5	5.91	-1.5	48.9	-2.2	507	1.4	793	-0.9		
	2.14	7.0	5.74	-4.3	46.8	-6.4	490	-2.0	803	0.4		
	&2.52	26.0	5.77	-3.8	48.6	-2.8	526	5.2	800	0.0		
	2.26	13.0	5.43	-9.5	47.8	-4.4	513	2.6	820	2.5		
Mean Concentration (ng/mL)	2.07		5.53		48.5		508		820		7960	
Inter-run SD	0.197		0.334		1.77		22.8		45.3		140	
Inter-run RSD (%)	9.5		6.0		3.6		4.5		5.5		1.8	
Inter-run %Bias	3.5		-7.8		-3.0		1.6		2.5		-0.5	
n	18		18		18		18		18		6	

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Similar issues encountered with Inclisiran

History

- 10 to 10,000 ng/mL for both SS and AS, analog ISTD, HRMS
- Clarity OTX SPE extraction
- DIPA/HFIP mobile phases, Waters Acquity BEH C18 column

Recovery

- Known recovery issues from TQJ2HPP development
- Tested alternative Sample:Loading Buffer ratios
- Lower recovery with increasing Loading Buffer

Column clogging

- Phenomenex recommended extended and high positive pressure after slow loading
- Foaming, but no cross-well contamination
- **Column clogging issue resolved**
- Clarity OTX guide v2.0 (GU54341119_W) now recommends “Increase vacuum to 10-15” Hg immediately after loading sample on SPE media”

Loading Buffer (uL)	SS Peak Area	ISTD1 Peak Area	SS REC	ISTD1 REC
100	35,047,503	3,712,117	97	105
200	23,854,692	2,468,026	66	70
300	20,136,769	2,093,195	56	59
400	14,191,323	1,572,876	39	45
500	12,486,234	1,401,458	35	40
Mean Post-HQC	36,031,837	3,521,942		

Loading Buffer (uL)	AS Peak Area	ISTD2 Peak Area	AS REC	ISTD2 REC
100	42,842,089	5,325,185	97	101
200	26,121,389	3,238,089	59	61
300	21,380,654	2,673,032	48	51
400	15,846,061	1,853,759	36	35
500	13,912,493	1,669,433	32	32
Mean Post-HQC	44,125,283	5,288,676		

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Conclusions and lessons learned

Clarity OTX

- High positive pressure post-loading required to address column clogging
- Loading Buffer needs to be optimized
- Lengthy extraction time (~6 hours), high cost (~$800 per plate) and proprietary Loading Buffer

LLE-LLE

- Column clogging unresolved, additional investigation required
- Slightly shorter extraction (~5 hours), no SPE and no ion-pairing reagents

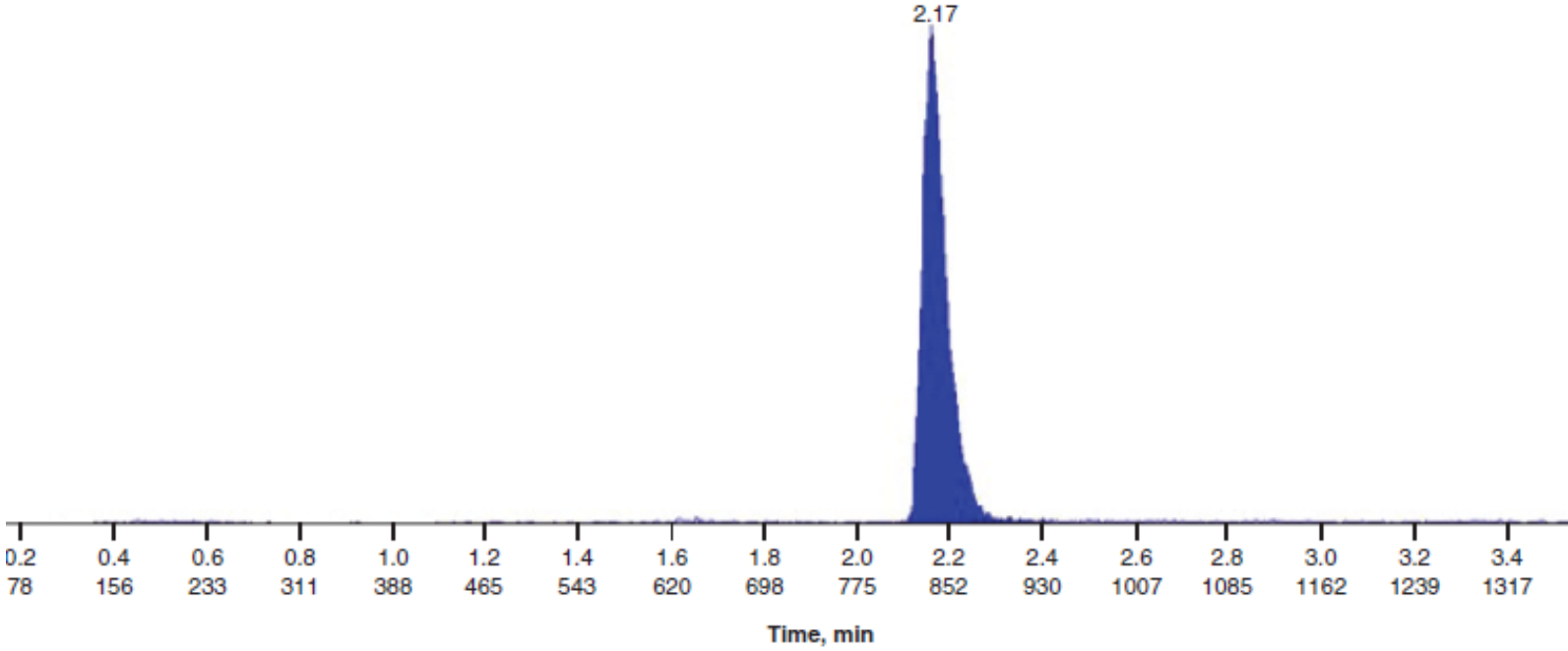
LLE-HLB SPE

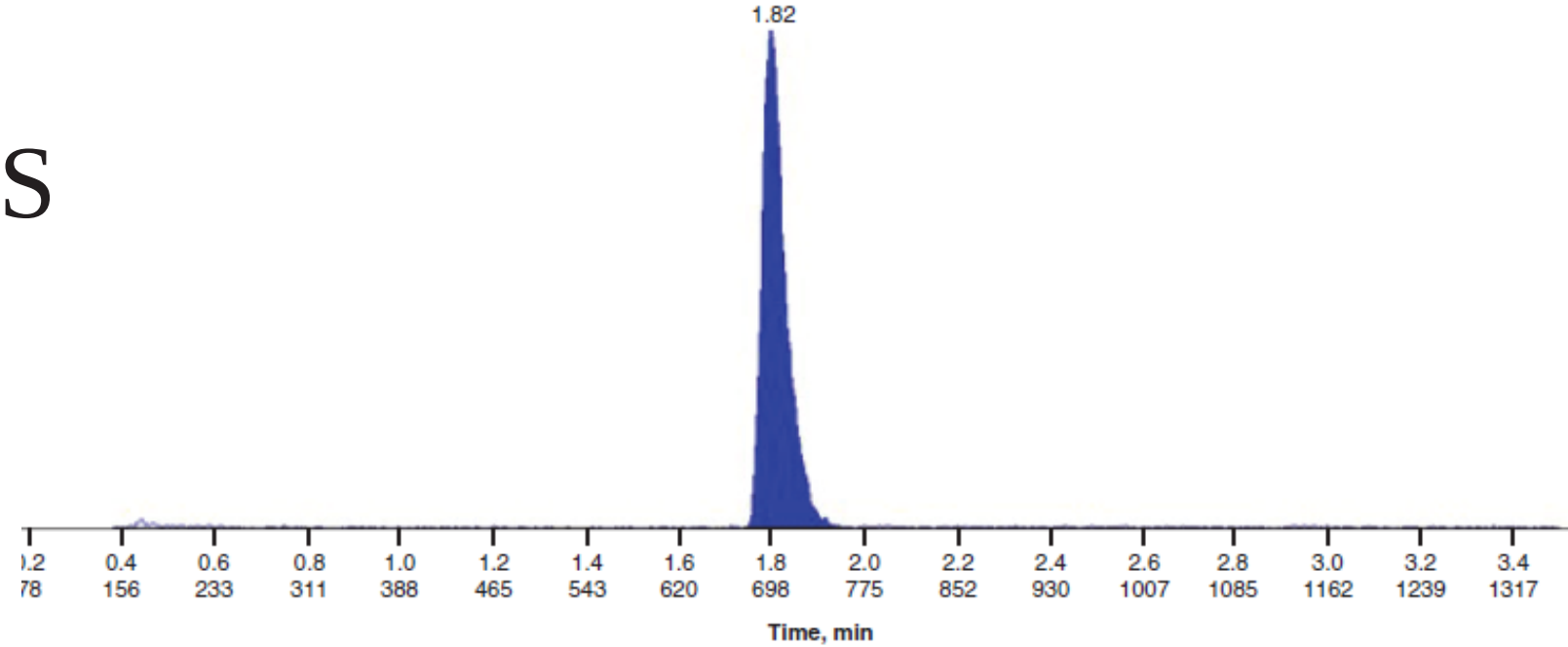
- Clean extracts, no column clogging
- Lower recovery, but higher sensitivity
- Lengthy extraction (~6hours), cost effective ($400 per plate) and requires ion-pairing reagents

Ion-pairing reagents

- Additional optimization recommended
- Avoid high alkylamine concentration, use HFIP to adjust pH

Complementary Analyses of Oligonucleotides via LBA and LC-MS





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Bioanalysis

High-sensitivity workflow for LC–MS-based analysis of GalNAc-conjugated oligonucleotides: a case study

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Aim: Mass-selective quantitation is a powerful attribute of LC–MS as a platform for bioanalysis. Here, a sensitive LC–MS approach has been validated for an oligonucleotide having chemical modifications (e.g., *N*-acetylgalactosamine [GalNAc] conjugated), to distinguish between the conjugated and unconjugated forms of the oligonucleotide, thereby enabling a nuanced view of the pharmacokinetic profile. **Results:** A high-sensitivity methodology for mass-specific measurement of AZD8233, a GalNAc-conjugated 16-mer oligonucleotide, using LLE-SPE with optimized LC conditions and detection of a low-mass fragment ion was successfully validated in the range of 0.20–100 ng/ml in human plasma. **Conclusion:** The AZD8233 LC–MS methodology adds valuable insight on the GalNAc linker's *in vivo* stability to the program and should be broadly applicable to oligonucleotides requiring high sensitivity and mass-selective measurement for quantitative discrimination from metabolites and endogenous interferences.

Bioanalysis (2021) 13(17), 1343–1353

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An oligonucleotide challenge

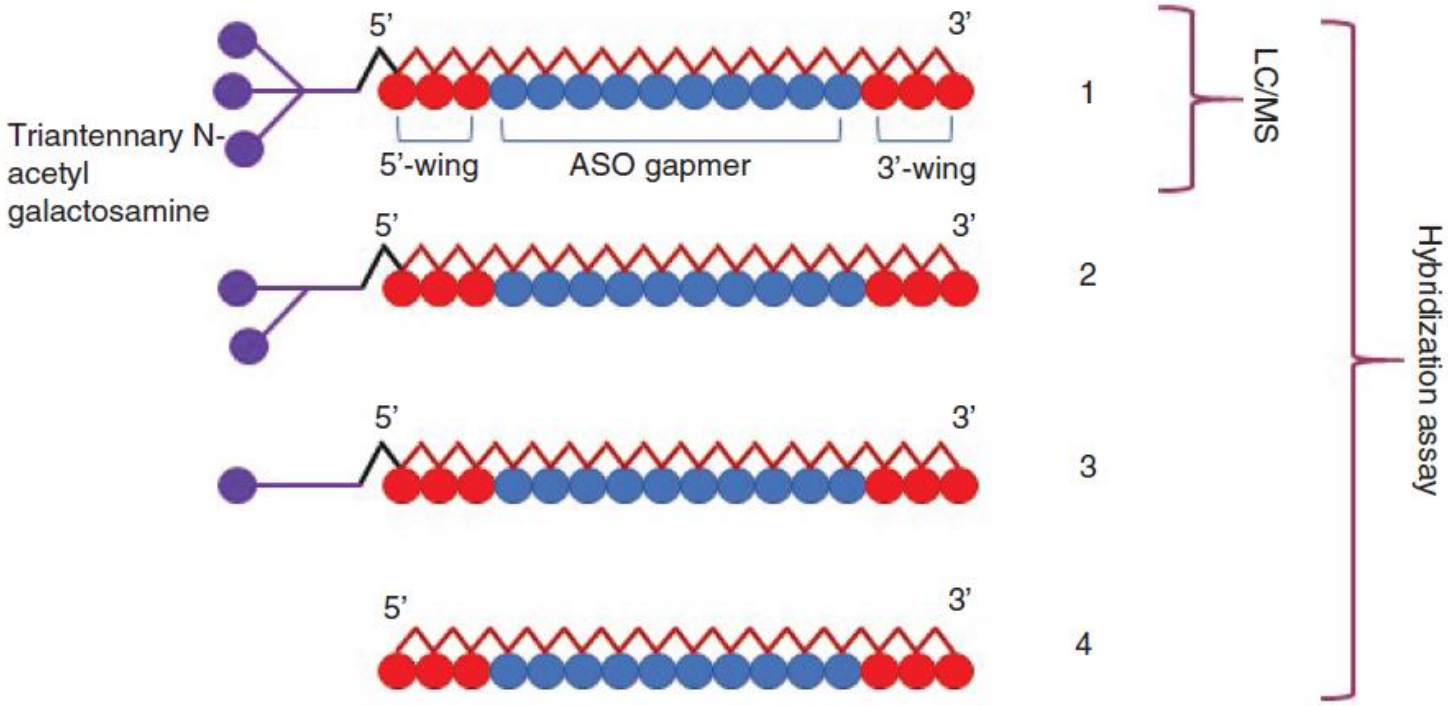
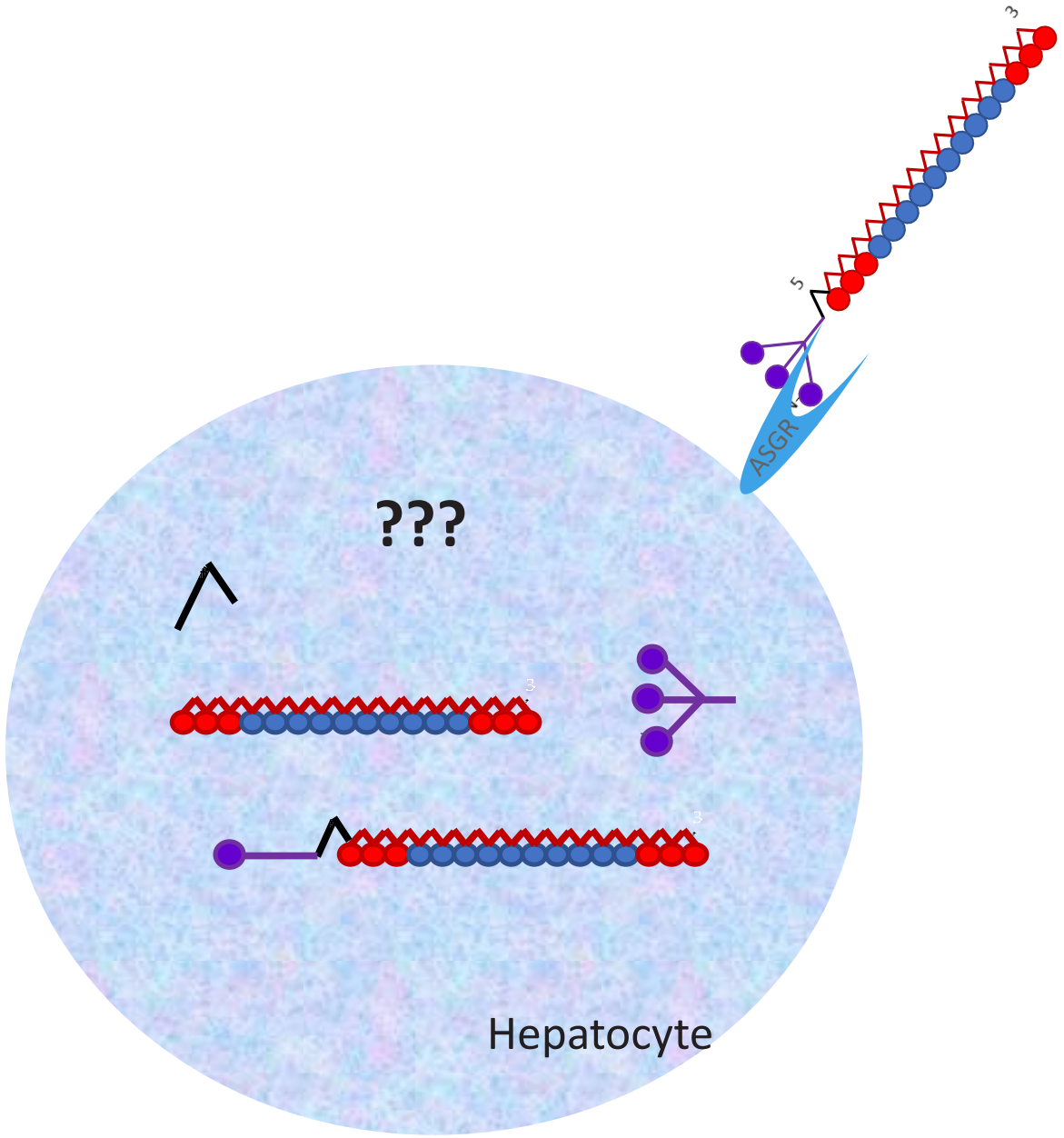


Figure 1. A highly selective LC/MS assay. Was developed and validated to assess low levels of AZD8233 **(1)** and to complement an hybridization assay assessing the levels of total full-length antisense oligonucleotide of AZD8233 without any differentiation between AZD8233, partially deglycosylated AZD8233 **(2 & 3)** and deglycosylated AZD8233 **(4)**.

What needs to be assessed?

- First GalNAc-conjugated ASO in the portfolio
- Modified form of the drug enhances liver uptake
- Little known about stability of the linker
- Total drug determined to be the critical value to monitor – but also want to understand concentration of the parent compound



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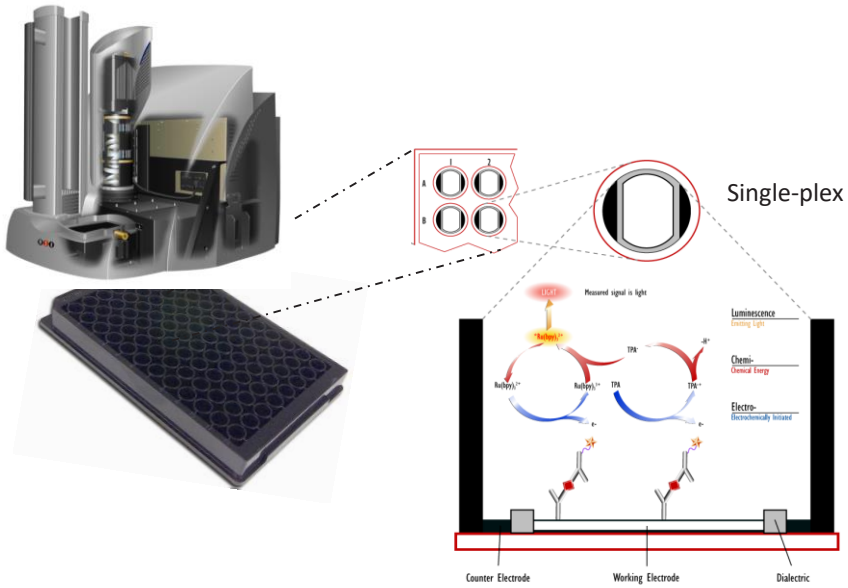
LBA method principles

- Format requires hybridized capture probe
- During the hybridization incubation, antibody interference is mitigated by sample heating and the addition of Proteinase K
- Following incubation, the hybridized mixture is added to a blocked and washed MSD® plate
- Following sample incubation, the plates are washed and a heated detector probe solution is added to the plate
- After the detector probe incubation, the plate is washed and MSD® Read Buffer is added
- MSD® QuickPlex® SQ120 platform is used



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LBA method performance

	ULOQ 70.0 ng/mL	HQC 52.0 ng/mL	MQC 3.50 ng/mL	LQC 0.156 ng/mL	LLOQ 0.0520 ng/mL
Mean	69.3	56.1	3.56	0.183	0.0574
S.D.	4.52	2.90	0.183	0.0255	0.00856
%CV	6.5	5.2	5.1	13.9	14.9
%Bias	-1.0	7.9	1.7	17.3	10.4
n	48	48	48	48	48
%TE	7.5	13.1	6.8	31.2	25.3

Sample Analysis Performance:
>85% run pass rate
>98% ISR pass rate

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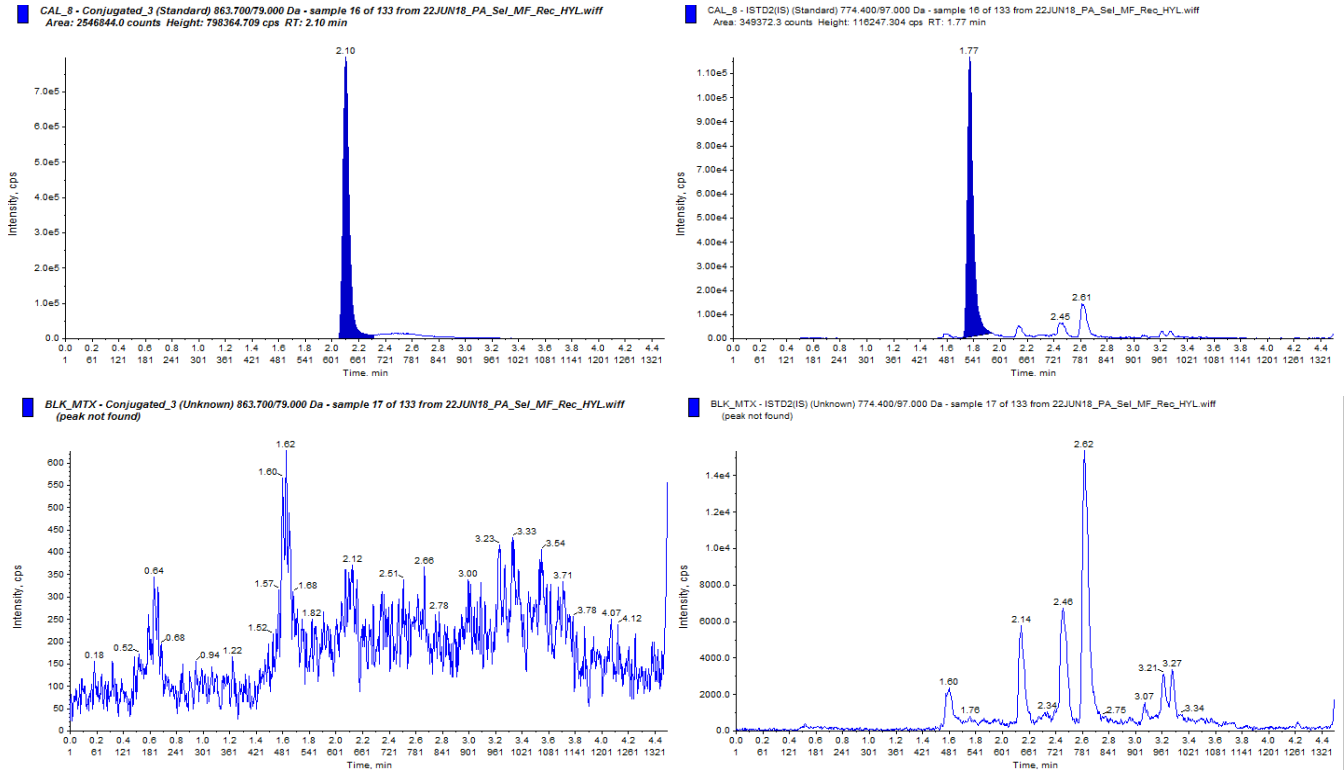
LC-MS method principles

- 250 µL aliquot
- BSA included to mitigate non-specific binding
- Liquid/liquid extraction followed by solid-phase extraction
- Ion-pairing reagent utilized for sensitivity (HFIP:DIPEA)
- Flow rate: 0.30 mL/min
- Sciex® 6500+ Triple quadrupole mass spectrometer



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LC-MS method performance

	Nominal concentration	LLOQ (0.2 ng/ml)	Low QC (0.6 ng/ml)	Low/mid QC (5 ng/ml)	Mid QC (50 ng/ml)	High QC (80 ng/ml)
Intra-assay (n = 6)						
1	Mean (ng/ml)	0.204	0.654	5.1	50.0	83.5
	Accuracy (%)	102	109	102	100	104
	Precision (%CV)	6.1	7.2	5.1	2.8	1.90
2	Mean (ng/ml)	0.192	0.657	5.4	54.0	85.6
	Accuracy (%)	96.0	109	108	108	107
	Precision (%CV)	2.7	2.6	2.4	3.0	2.8
3	Mean (ng/ml)	0.202	0.615	4.90	47.9	78.1
	Accuracy (%)	101	103	98.0	95.8	97.6
	Precision (%CV)	5.9	2.1	3.0	1.5	2.40
Inter-assay (n = 18)						
	Mean (ng/ml)	0.199	0.642	5.13	50.6	82.4
	Accuracy (%)	101	107	103	101	103
	Precision (%RSD)	3.2	3.6	4.9	6.1	4.7

Conclusions

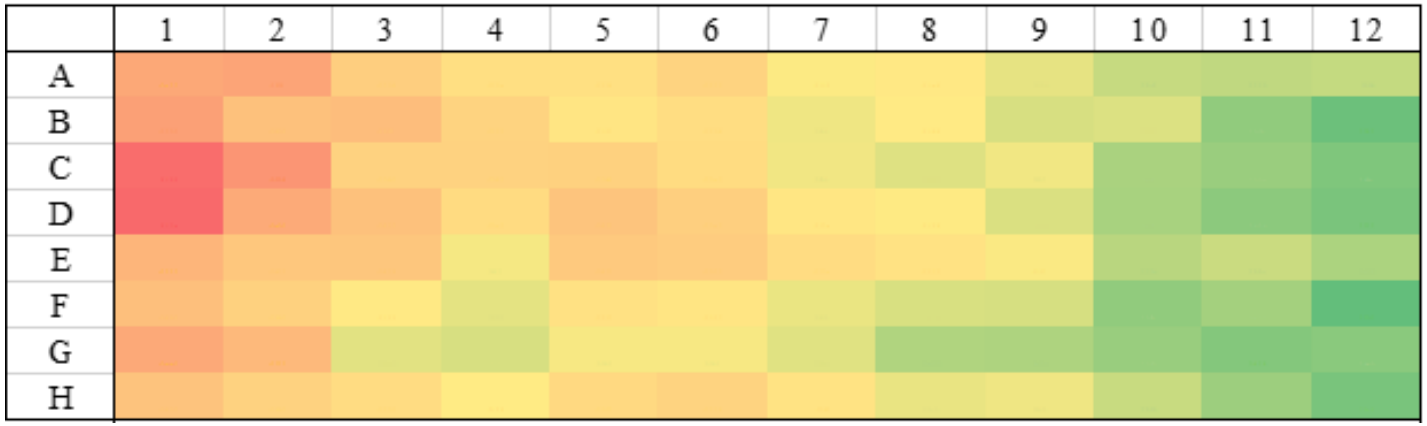
- 2 Methods for Quantitation of GalNAc-conjugated ASO were developed and validated
  - LBA methodology was used to assess total concentration of all glycosylated and unglycosylated forms
  - LC-MS methodology was used to assess the triantennary N-galactosylamine-modified oligonucleotide
  - No cross validation was performed – the assays were used to assess different questions
- A combination of LLE-SPE extraction, optimized LC conditions, and 6500+ triple quad mass spectrometer was utilized to fully validate an assay for a GalNAc-conjugated 16-mer oligonucleotide in human plasma having an LLOQ of 0.200 ng/mL
- The LC-MS assay was fully capable of selectively measuring the GalNAc-conjugated form in the presence of the non-conjugated form
- The MSD® immunoassay was able to monitor total drug down to 0.0520 ng/mL

* A different oligonucleotide program encountered issues with LBA assay performance and was successfully established using LC-MS

Optimizing and troubleshooting an aptamer hybridization ELISA

Case study: Dual probe assay for oligo drug

- Probes were obtained from a commercial vendor: Biotin- and Dig-labeled probes
- Assay framework was developed by client
- Development summary with commercial probes



Whole Plate Precision (% CV) approximately 5%

Intra-Run A&P – 6 QCs at Each Level				
	QC Bias (# out of 6)			
	0-10%	10-20%	>20%	Fail
ULOQ	0	1	5	5
HQC	1	4	1	1
MQC	6	0	0	0
LQC	6	0	0	0
LLOQ	6	0	0	0

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Optimizing and troubleshooting an aptamer hybridization ELISA

Case study: Dual probe assay for oligo drug, continued

- Development summary with commercial probes
  - Phase 1: 6 days, 7 runs for optimization
  - 1 day, 2 runs for qualification
  - Fails for QC bias
- Phase 2: 6 days, 14 runs for re-optimization
  - Probe concentrations increased
  - 2 days, 4 runs for qualification
  - Fails for QC bias, selectivity, stability
  - Other parameters barely passing
- Phase 3: 14 days, 9 runs for investigation
  - Fresh vs. frozen, location effects, incubation times
- 38 total runs without transitioning to validation

Run A – 6 QCs at Each Level				
QC Bias (# out of 6)				
	0-10%	10-20%	>20%	Fail
ULOQ	3	3	0	0
HQC	3	3	0	0
MQC	6	0	0	0
LQC	1	5	0	0
LLOQ - A	5	1	0	0
LLOQ - B	6	0	0	0

Dilution Linearity				
Bias (# out of 5)				
	0-10%	10-20%	>20%	Fail
In Range	0	4	1	1

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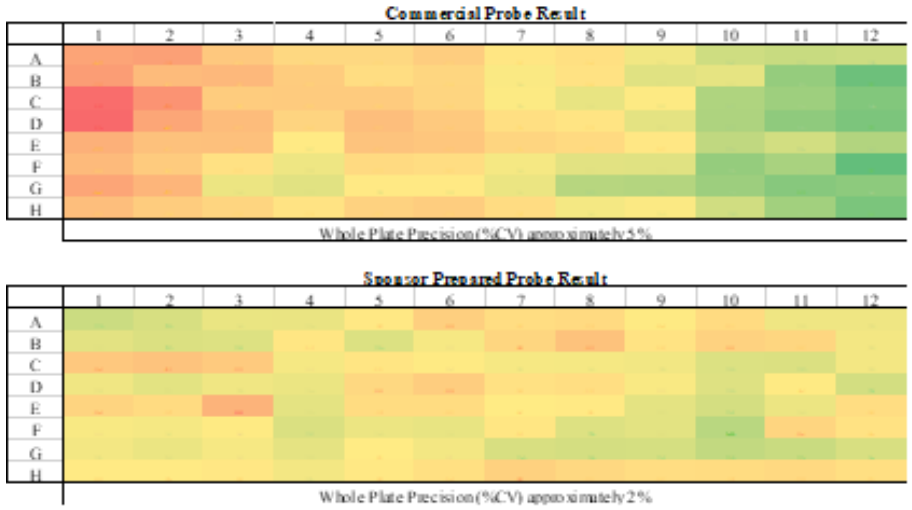
Optimizing and troubleshooting an aptamer hybridization ELISA

Case study: Dual probe assay for oligo drug, continued

- New probe is obtained from client due to:
  - High probe concentration
  - Vendor batch size
  - Vendor quality issues
- Development summary with client-made probes
  - Re-optimization 1: 2 runs over 2 days
    - New range (range remains 50-fold, shifted up by a factor of 2 from previous range)
  - Successful qualification
- 10 total runs to re-optimize and complete qualification



Run A – 6 QCs at Each Level				
QC Bias (# out of 6)				
	0-10%	10-20%	>20%	Fail
ULOQ	5	1	0	0
HQC	3	3	0	0
MQC	5	1	0	0
LQC	1	5	0	0
LLOQ	4	2	0	0

Run B – 3 QCs at Each Level				
QC Bias (# out of 3)				
	0-10%	10-20%	>20%	Fail
ULOQ	2	1	0	0
HQC	3	0	0	0
MQC	1	2	0	0
LQC	3	0	0	0
LLOQ	2	1	0	0

Run C – 2 QCs at H/M/L				
QC Bias (# out of 2)				
	0-10%	10-20%	>20%	Fail
HQC	2	0	0	0
MQC	1	1	0	0
LQC	1	1	0	0

Dilution Linearity				
Bias (# out of 6)				
	0-10%	10-20%	>20%	Fail
In Range	5	1	0	0

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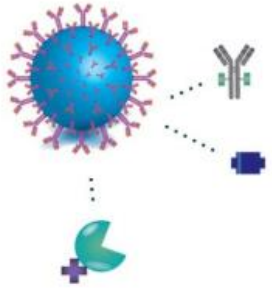
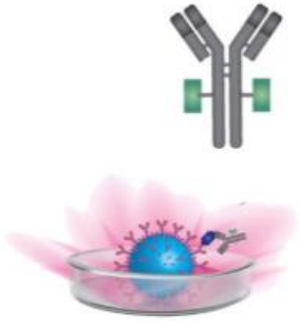
Simoa®: Single Molecule Array assay overview

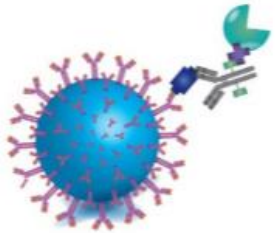
1 Paramagnetic particles coupled with antibodies designed to bind to specific targets are added to the sample.



2 Detection antibodies – capable of generating fluorescent product – are added.



3 The objective is to form an immunocomplex consisting of the bead, bound protein, and detection antibody.



4 At low concentrations, each bead will contain one bound protein, or none.

5 The sample is loaded into arrays, in the Simoa disc, consisting of more than 200,000 microwells – each large enough to hold one bead.

- Once the immunoassay portion is complete, the samples are added to the detector for the final stage
- Final stage = “LSI” = Load, Seal, Image

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https://www.quanterix.com/simoa-bead-technology

Case study: Development of hybridization assay in human samples by Simoa® vs. ECL

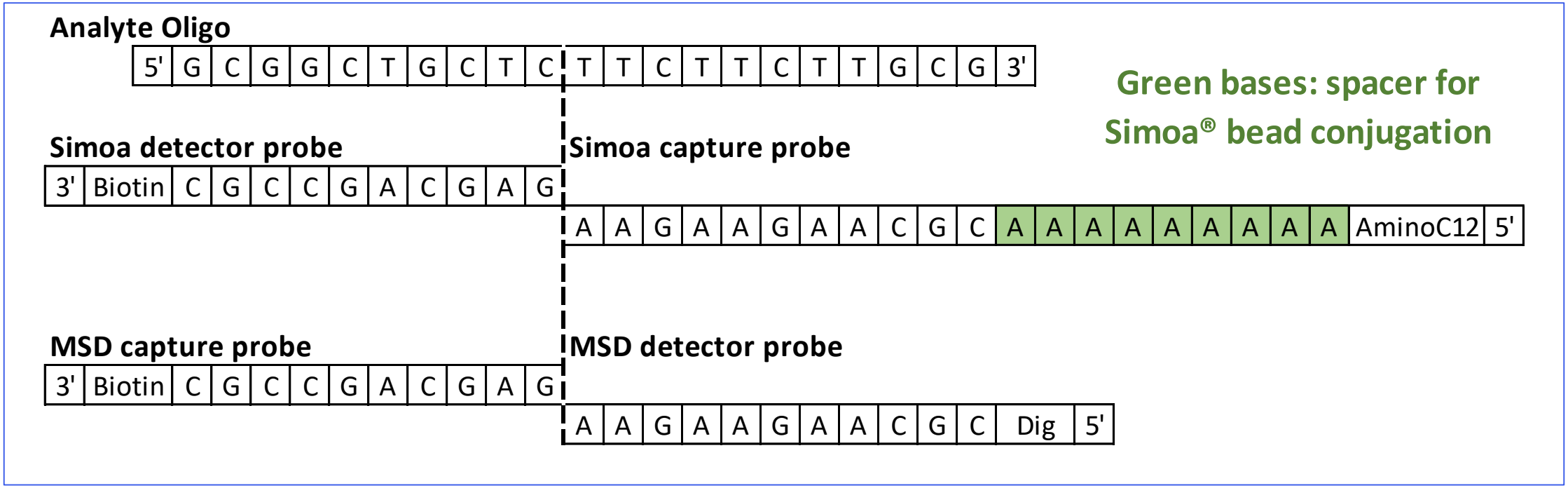
Motivation

- Lack of desired sensitivity for bioanalytical PK assays for oligonucleotide therapeutics
  - Both LC-MS and ligand-binding formats
  - Even 10-fold improvement would be beneficial

Proposal

- Develop sensitive method for oligonucleotide in human samples by Simoa®
  - 21-mer non-proprietary analyte
- ECL assay developed for the same analyte
- Compare the merit of the two assays regarding assay performance in accordance with regulatory guidance

Case study: Development of hybridization assay in human samples by Simoa® vs. ECL



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Case study: Development of hybridization assay in human samples by Simoa® vs. ECL

Simoa® Method Outline (in human serum)
1. Dilute all samples in buffer
2. Hybridization: Combine capture beads + diluted samples + detector probe and incubate (2 hours)
3. Wash beads and incubate with SBG enzyme (1hour)
4. Wash beads and load onto Simoa®
5. Plate reading (2-3 hours) **Total Assay Time = 7-8 hours** Readout signal: fluorescence Reported raw data: AEB (Average number of Enzyme per Bead)

ECL Method Outline (in human serum)
1. Hybridization: Incubate capture probe + samples (1.5 hours)
2. Add hybridized probe/samples to streptavidin coated MSD plate and incubate (1-2 hours)
3. Wash plate and add digoxigenin-labeled detector probe (1 hour)
4. Wash plate and add Sulfo Tag-labeled anti-digoxigenin detection antibody (1 hour)
5. Wash plate and read **Total Assay Time = 5-6 hours** Readout signal: electrochemiluminescence Reported raw data: RLU

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Simoa® challenges and troubleshooting

Critical reagents

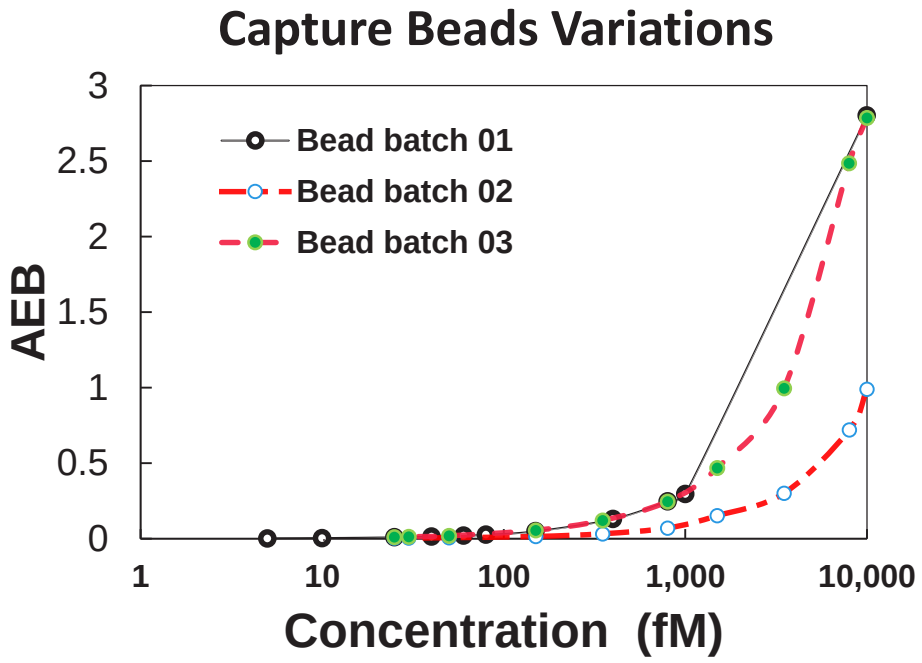
- Capture Probe Conjugated Beads
  - Conjugated beads bridging (EDC condensation reaction)
  - Conjugated beads stability to be evaluated in study
- Enzyme contamination (increased background)

Simoa® operation and detection

- Assay throughput:
  - Single well operation/reading
  - Sample tested as triplicates
- Error detection (~0.8%) caused by signal interference

Extensive dilution

- Super low concentrations of qualified range
  - Material stability
  - Need supports from automated liquid handling system
- Accuracy and precision challenge in validation



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Simoa® vs. ECL: Assay qualification

	Simoa®	ECL
Lower Limit of Quantitation:	100 fM	4,000 fM
Upper Limit of Quantitation:	8,000 fM	2,000,000 fM
Sample Requirement per Test:	25 µL	150 µL
MRD	1/14	1/3.3
Plate Type:	Simoa® HD 96-well plate	MSD 96-well Streptavidin SECTOR Plate
Capture Reagent:	Capture probe conjugated magnetic beads	Biotinylated capture probe
Detection Reagent:	Biotinylated detector probe	Primary: Digoxigenin-labeled probe Secondary: Sulfo Tag-labeled anti-Dig
Curve Fit; Weighting Factor:	4-PL; $1/y^2$	4-PL; $1/y^2$
Method Selectivity:	Pass	Pass
Dilution Linearity:	1/500,000	1/40,000

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Simoa® vs. ECL: Assay qualification

Selectivity (normal matrix)

Simoa®	LLOQ Spike Conc.:	30.0		HQC Spike Conc.:	7500		LLOQ Spike Conc.:	100		HQC Spike Conc.:	6000	
Sample	Results (fM)	%Bias	%CV	Results (fM)	%Bias	%CV	Results (fM)	%Bias	%CV	Results (fM)	%Bias	%CV
Individual 01	27.1	-9.7	14.0	6350	-15.3	0.6	97.7	-2.3	4.6	6290	4.8	2.8
Individual 02	28.4	-5.3	1.6	7260	-3.2	0.7	96.5	-3.5	5.7	6390	6.5	6.7
Individual 03	30.0	0.0	3.3	8140	8.5	6.1	106	6.0	5.6	6560	9.3	0.6
Individual 04	35.0	16.7	0.5	8540	13.9	3.1	113	13.0	1.5	6730	12.2	6.5
Individual 05	29.1	-3.0	9.6	7910	5.5	2.1	94.1	-5.9	5.5	6320	5.3	7.1
Individual 06	30.1	0.3	21.1	7820	4.3	1.1	95.7	-4.3	1.1	6700	11.7	1.8
Individual 07	26.2	-12.7	0.4	6960	-7.2	7.0	92.3	-7.7	4.2	5640	-6.0	3.0
Individual 08	33.8	12.7	1.0	6690	-10.8	3.1	114	14.0	4.4	5990	-0.2	3.0
Individual 09	37.6	**25.3**	2.2	8490	13.2	1.4	104	4.0	3.8	6400	6.7	0.5
Individual 10	29.8	-0.7	5.8	6800	-9.3	7.2	102	2.0	6.3	6250	4.2	10.0
Pool*	34.9	16.3	0.0	NA	NA	NA	100	0.0	9.0	6740	12.3	11.4

ECL	Unspiked	LLOQ Spike Conc.:	4000		HQC Spike Conc.:	1600000	
Sample	Results (pM)	Results (fM)	%Bias	%CV	Results (fM)	%Bias	%CV
Individual 01	<LLOQ	4060	1.5	3.9	1411512	-11.9	0.7
Individual 02	<LLOQ	4138	3.5	2.5	1457476	-8.8	2.9
Individual 03	<LLOQ	4316	8.0	1.4	1497897	-6.3	3.4
Individual 04	<LLOQ	801	**-80.0**	4.4	318818	**-80.1**	4.1
Individual 05	<LLOQ	4433	10.8	0.8	1515762	-5.0	1.2
Individual 06	<LLOQ	4176	4.5	0.2	1524896	-5.0	3.3
Individual 07	<LLOQ	3959	-1.0	1.1	1429832	-10.6	0.7
Individual 08	<LLOQ	4029	0.8	1.7	1537166	-3.8	1.2
Individual 09	<LLOQ	4161	4.0	3.2	1459914	-8.8	1.9
Individual 10	<LLOQ	4060	1.5	5.6	1498715	-6.3	0.4
Pool*	<LLOQ	4356	9.0	4.5	1435015	-10.0	0.5

Selectivity Results:

- **Simoa®**
  - Range I: 9 of 10 passing samples
  - Range II: 10 of 10 passing samples
- **ECL**
  - 9 out of 10 passing samples

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Conclusion

- Simoa® and ECL hybridization assays developed for human matrix
  - Sensitive assays, meeting regulatory standards
- Simoa® and ECL comparison
  - Both assays qualified for accuracy & precision, selectivity and dilution linearity
  - ~40x improvement in sensitivity for Simoa® vs. ECL
- Challenges for Simoa® in regulated clinical studies
  - Robustness and reproducibility
  - Stability
  - Assay throughput

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Acknowledgements

NVS001 and Inclisiran LC-MS

Labcorp Team

- Brendan Powers
- Nicholas Saichek
- Doug Carhuff (MD/VAL chemist)

Novartis Team

- Wenkui Li
- Xuejun Sun
- Yunlin Fu

Simoa Analysis

Labcorp Team

- Jill Uhlenkamp
- Bingbing Wang
- Erica Simmons
- Barry Peterson

GalNAc-Conjugated Oligonucleotides

Labcorp Team

- Aaron Ledvina
- Matthew Ewles
- Paul Severin
- David Good
- Jill Uhlenkamp
- Bingbing Wang

AstraZeneca Team

- Cecilia Arfvidsson

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Thank you

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