Specific Quantitation of Antisense Oligonucleotides in Plasma Using MSD® Electrochemiluminescence, Hybridization-Based ELISA, and HPLC-MS/MS Platforms

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INTRODUCTION

Antisense oligonucleotides (ASOs) are short, chemically modified, single-stranded DNA/RNA oligonucleotides that specifically target to genes of interest and regulate target protein biosynthesis. Steered by recent approval of several such drugs[1], ASOs have emerged as a promising therapeutic platform attracting a flock of biopharmaceutical companies[2]. To support the preclinical and clinical investigation of pharmacokinetic behaviors of ASO drugs, it is essential to develop a simple, specific, and sensitive method for accurate quantification of these agents in biological matrices. Frontage Laboratories methods for ASO quantification:

- MSD electrochemiluminescence (MSD ECL)
- hybridization-based ELISA (HELISA)
- HPLC-MS/MS

Here we compare the sensitivity, selectivity, precision, and accuracy of these three detection methods.

MATERIALS AND METHODS

Materials:

ASO analyte: 20-mer oligonucleotide with sequence of 5'-GGC TAA ATC GCT CCA CCA AG-3'[3]. Capture probe: 3'-biotinylated 29-mer DNA oligonucleotide designed with the first 20-mer sequence from the 3'-end complementary to ASO analyte. Detection probes: 9-mer DNA oligonucleotides complementary to the 5'-end overhang of the capture probe with 5'-end phosphorylated and 3'-end digoxigenin/ruthenium labeled.

MSD® ECL and HELISA Methods:

Hybridization buffer containing 2 nM capture probe incubated at 95 °C for 5 min and cooled down immediately on ice to disrupt secondary structures.

Human plasma standards and QCs were mixed with an equal volume of capture probe and incubated at 42 °C for 90 min for hybridization.

0.25% Triton-X added to mixture to reduce non-specific hybridization.

90 µL hybridized mixture was loaded onto an MSD-GOLD Streptavidin plate (for MSD ECL) or a NeutrAvidin-coated High Binding Capacity plate (HELISA) and incubated at 37 °C for 30 min.

After wash, 100 µL ligation solution containing 7.5 U/mL T4 DNA Ligase, 1 nM detection probe, and 1 mM ATP was added and incubated at 18 °C overnight for ligation.

Unligated DNA was removed by treatment with 60 U/well of S1 nuclease for 2 h at 37 °C.

MSD plates were read on an MSD Image Sector S 600 in 2X Read Buffer. Digoxigenin-labeled probe was detected and quantitated by an alkaline phosphatase (AP)-conjugated anti-Digoxigenin antibody (1:2,000) and AttoPhos® AP Fluorescent Substrate System.

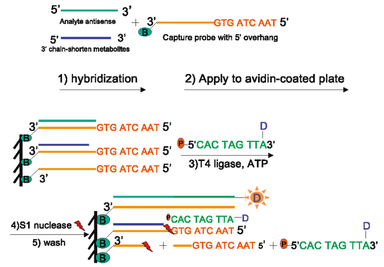
HPLC-MS/MS Method:

A mixture of 50 µL ASO standards with 50 µL human plasma, 10 µL IS, and 900 µL equilibration buffer (50 mM NH_4OAc, pH 5.5) were loaded to WAX SPE cartridges after conditioning.

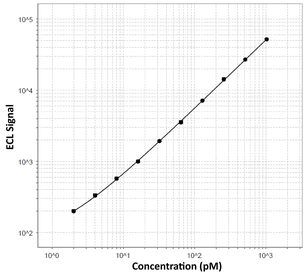
The eluent containing analyte was evaporated to near dryness and reconstituted with 150 µL of 50 µM EDTA.

20 µL of reconstituted solution was injected on HPLC-MS/MS system.

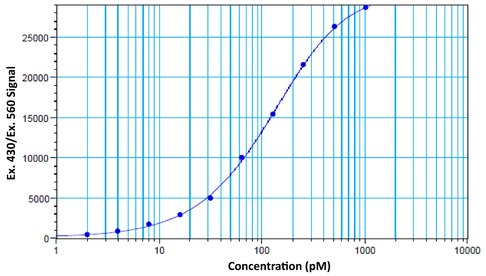
Illustration of MSD ECL and HELISA Methods (Adapted from Ref. 3)



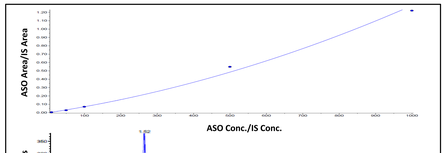
A Representative Calibration Curve of the MSD ECL Method

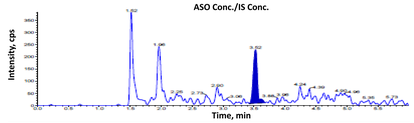


A Representative Calibration Curve of the HELISA Method



HPLC-MS/MS Method





Sample Name	Sample Type	Dilution Factor	Analyte Peak Name	Accuracy (%)	Analyte Concentration (nM)	Calculated Concentration (nM)
WAX DB 1	Unknown	1.00	II. Trans	N/A	N/A	#DIV/0!
WAX Blk 1	Unknown	1.00	II. Trans	N/A	N/A	No Peak
WAX STD 2	Standard	1.00	II. Trans	102.12	10.00	10.212
WAX STD 3	Standard	1.00	II. Trans	88.76	50.00	44.379
WAX STD 4	Standard	1.00	II. Trans	99.91	100.00	99.907
WAX STD 5	Standard	1.00	II. Trans	109.70	500.00	548.487
WAX STD 6	Standard	1.00	II. Trans	96.83	1000.00	968.287

Representative Standard Data of MSD ECL and HELISA

		Std 10	Std 9	Std 8	Std 7	Std 6	Std 5	Std 4	Std 3	Std 2	Std 1
	Conc. (pM)	2.00	4.00	8.00	16.00	32.00	64.00	128.00	256.00	512.00	1024.00
MSD ECL	#1	1.95	4.05	8.07	15.24	31.73	59.64	128.47	259.67	511.01	1021.27
	#2	2.01	4.25	8.33	15.81	32.43	62.77	128.81	267.11	520.26	1019.61
	Intra-run Mean	1.98	4.15	8.20	15.53	32.08	61.21	128.64	263.39	515.64	1020.44
	Intra-run SD	0.046	0.141	0.181	0.407	0.495	2.210	0.241	5.261	6.540	1.173
	Intra-run %CV	2.3	3.4	2.2	2.6	1.5	3.6	0.2	2.0	1.3	0.1
	Intra-run %RE	-1.0	3.7	2.5	-3.0	0.3	-4.4	0.5	2.9	0.7	-0.3
HELISA	#1	1.89	4.26	8.97	16.15	29.25	66.90	127.97	249.13	489.50	876.34
	#2	2.01	4.43	9.31	16.42	28.83	67.19	125.92	259.83	548.41	1222.61
	Intra-run Mean	1.95	4.34	9.14	16.28	29.04	67.04	126.95	254.48	518.96	1049.47
	Intra-run SD	0.080	0.121	0.241	0.192	0.300	0.203	1.455	7.568	41.660	244.851
	Intra-run %CV	4.1	2.8	2.6	1.2	1.0	0.3	1.1	3.0	8.0	23.3
	Intra-run %RE	-2.6	8.6	14.2	1.8	-9.2	4.8	-0.8	-0.6	1.4	2.5

Inter-Run Precision and Accuracy of MSD ECL and HELISA (n=5)

QC Level	Conc. (pM)	MSD ECL		HELISA	
		%CV	%RE	%CV	%RE
ULOQ	1024.00	3.2	-1.0	8.3	-6.8
HQC	768.00	2.2	1.4	8.9	-13.4
MQC	500.00	2.2	0.2	6.6	-6.6
LQC	12.00	4.4	-2.7	13.8	10.1
LLOQ	4.00	7.5	-11.0	3.6	1.3

Selectivity Test Results of MSD ECL and HELISA

	SeL Samples	Note	Spiked ASO Conc. (pM)	ASO Conc. Found (pM)	%CV	%RE
MSD ECL	Sel01	LLOQ Level	4.00	3.82	4.5	-4.4
	Sel02	LLOQ Level	4.00	3.61	1.3	-9.9
	Sel03	LLOQ Level	4.00	3.64	2.5	-9.0
	Sel04	LLOQ Level	4.00	3.54	0.4	-11.5
	Sel05	LLOQ Level	4.00	3.69	2.9	-7.7
	Sel06	LLOQ Level	4.00	3.55	1.7	-11.2
	Sel07	LLOQ Level	4.00	3.41	4.1	-14.8
	Sel08	LLOQ Level	4.00	3.67	2.9	-8.2
	Sel09	LLOQ Level	4.00	3.28	0.5	-18.0
	Sel10	LLOQ Level	4.00	3.34	1.4	-16.4
HELISA	Sel01	LLOQ Level	4.00	3.51	0.6	-12.4
	Sel02	LLOQ Level	4.00	4.11	2.0	2.8
	Sel03	LLOQ Level	4.00	3.67	3.7	-8.3
	Sel04	LLOQ Level	4.00	3.77	1.7	-5.7
	Sel05	LLOQ Level	4.00	3.79	2.3	-5.3
	Sel06	LLOQ Level	4.00	3.55	6.1	-11.2
	Sel07	LLOQ Level	4.00	3.32	2.6	-17.0
	Sel08	LLOQ Level	4.00	3.63	0.2	-9.3
	Sel09	LLOQ Level	4.00	3.20	2.7	-20.0
	Sel10	LLOQ Level	4.00	3.66	0.4	-8.5

DISCUSSION AND CONCLUSION

Frontage has three different platforms for the quantification of ASOs: For our MSD ECL and HELISA methods, the lower limit of detection (LLOD) and LLOQ were found to be 2 pM (12 pg/mL) and 4 pM (24 pg/mL) respectively, the highest sensitivity ever reported. Both assays showed a linear signal increase from 2 pM to 1024 pM and high selectivity in plasma, with 100% passing rate (%RE ≤ 20%). Furthermore, by fine-tuning the concentrations of the capture and detection probes we were able to adjust the range of quantification. MSD ECL outperformed over HELISA due to its enhanced precision and accuracy of calibration curves and QCs and dilution linearity (data not shown).

The HPLC-MS/MS method had a sensitivity of 10 nM (61 ng/mL), much lower than that of MSD ECL and HELISA methods. However, as HPLC-MS/MS has an unparalleled high specificity against shortened ASO metabolites, it can serve as a powerful tool for ASO quantification in tissues with drug accumulation.

REFERENCES

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