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Strategies to Improve Assay Sensitivity to Quantify Therapeutic Oligonucleotides

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INTRODUCTION

Hybridization-based ELISA (enzyme-linked immunosorbent assay) and ECL (electrochemiluminescence) methods for quantifying therapeutic oligonucleotides have several advantages owing to their superior sensitivities, robustness, and lower costs. However, there is a high demand for assays with greater sensitivity with minimal to no sample clean-up in small sampling volumes. Herein, we present three cases where higher sensitivities were achieved by assessing various combinations of platforms, probe design, and signal amplification strategies. Additionally, such improvements have also resulted in a reduction of assay costs and workflow.

METHODS

Method 1

Transfer of a validated nuclease-dependent hybridization ELISA for quantifying an anti-sense oligo (ASO) from monkey to rabbit serum.

Original method characteristics:
- Sensitivity: 1 ng/mL
- Dynamic range: 100-fold
- LLOQ signal-to-noise ratio (SNR): 2.6
- Method observed a lower SNR at 1 ng/mL during transfer

Optimizations performed:
- Nuclease optimization (S1 nuclease to micrococcal nuclease)
- Use of Anti-digoxigenin antibody (platform switched to ECL)

Method 2

Development of a nuclease-dependent ELISA to quantify an antibody-phosphorodiamidate morpholio (PMO) conjugate in mouse tissue homogenates.

Optimizations performed:
- Multiple platforms/probes: single-probe ELISA vs dual-probe ECL
- Multiple protease treatment conditions

Method 3

Re-optimizing a qualified nuclease-dependent ECL method for PMO quantitation in mouse tissue homogenates.

Original method characteristics:
- Sensitivity: 1 ng/mL
- Dynamic range: 200-fold
- LLOQ SNR: 5

Optimizations performed:
- Probe optimization (assay switched to dual-probe format)
- Signal amplification (probe labeled with U-digoxigenin by terminal transferase for a 3' dAU tail + ruthenylated antibody)

RESULTS

Method 1

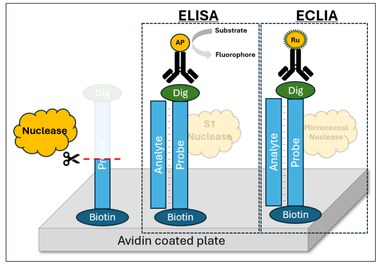


Figure 1. Schematic representation of the assay used in Method 1

Assay Performance:

Table 1. S1 Nuclease-Dependent ELISA in Monkey Serum

Sample Name	Nominal Conc. (ng/mL)	Instrument response	Measured Conc. (ng/mL)	%Bias	SNR
BL	NA	372.5	0.042	NA	NA
STD 1	1.00	957.0	0.985	-1.6	**2.6**
STD 8	100	41273.5	103	2.9	**110.8**

Table 2. Micrococcal Nuclease-Dependent ECLIA in Rabbit Serum

Sample Name	Nominal Conc. (ng/mL)	Instrument Response	Measured Conc. (ng/mL)	%Bias	SNR
BL	NA	107.5	NA	NA	NA
STD 1	0.500	634.5	0.504	0.8	**5.9**
STD 8	200	196015.0	198	-1.0	**1823.4**

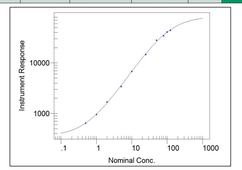


Figure 2. Representative calibration curve ELISA

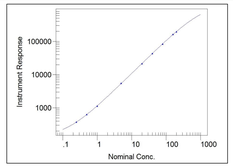
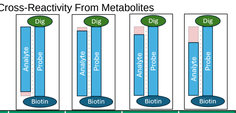


Figure 3. Representative calibration curve ECLIA

Table 3. Precision and accuracy (7 runs)

	LLOQ QC (0.500 ng/mL)	LQC (1.50 ng/mL)	MQC (10.0 ng/mL)	HQC (150 ng/mL)	ULOQ QC (200 ng/mL)
Mean	0.497	1.48	9.49	144	186
S.D.	0.0479	0.0823	0.375	6.31	7.74
%CV	9.6	5.6	4.0	4.4	4.2
%Bias	-0.6	-1.3	-5.1	-3.7	-7.2
%TE	10.2	6.8	9.0	8.1	11.4

Table 4. Cross-Reactivity From Metabolites

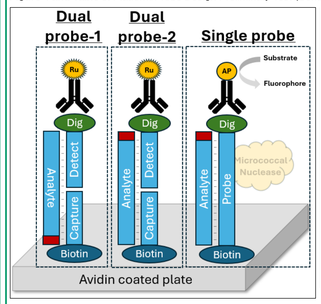


%Cross-Reactivity From	N-1 3' end	N-1 5' end	N-2 5' end	N-4 5' end
ELISA	40.5	38.1	19.4	7.0
ECLIA	53.0	42.2	1.6	0.3

Conclusion:
- Sensitivity: 0.5 ng/mL
- Dynamic range: 400-fold
- Superior SNR throughout the curve range
- 60% reduction in costs/plate
- 37.5% reduction in the workflow (from 8h to 5h)
- Overall reduction in cross-reactivity from N-2 and N-4 5' end metabolites.

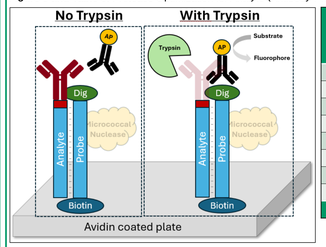
Method 2

Figure 4 and Table 5. Platform/Probe Design Preliminary Test (Control Free PMO)



Sample Name	Nominal Conc (ng/mL)	Instrument response		
		Dual probe set 1 (ECL)	Dual probe set 2 (ECL)	Single-probe (ELISA)
BL	NA	54.0	47.5	58.5
STD 1	0.100	69.0	171.0	890.0
STD 2	0.200	91.0	295.0	1658.5
STD 3	0.500	139.0	667.0	3829.0
STD 4	1.00	187.0	1271.0	7411.0
STD 5	4.00	646.0	4905.0	24693.0
STD 6	10.0	1833.0	12503.0	37538.5
STD 7	40.0	11268.0	48988.0	42283.5
STD 8	80.0	35196.0	101763.0	40127.5
STD 9	200.0	123786.0	230954.0	38796.5
STD 10	350.0	197478.0	284668.0	37541.0
STD 11	400.0	217999.0	292451.0	38431.0
LLOQ SNR		**1.3**	**3.6**	**15.2**

Figure 5 and Table 6. Protease Optimization for Analyte (Antibody-PMO)



Sample Name	Nominal Conc. (ng/mL)	Without Trypsin		With Trypsin	
		Signal	SNR	Signal	SNR
BL	NA	148	NA	50	NA
STD1	0.0300	91	0.6	162	**3.2**
STD2	0.0600	124	0.8	270	5.4
STD3	0.100	155	1.0	479	9.6
STD4	0.400	492	**3.3**	1574	31.5
STD5	1.00	1123	7.6	4026	80.5
STD6	4.00	4799	32.4	14752	295.0
STD8	10.0	12120	81.9	31001	620.0
Achievable LLOQ		**0.400**		**0.0300**	

Conclusion:
- Trypsin treatment reduces background and improved the signal output by at least 2- to 3-fold
- Sensitivity: 0.03 ng/mL
- Dynamic range: 333-fold
- MRD 1/2
- Multiple muscle tissue types (transgenic) qualified and analyzed against a curve prepared in mouse quadriceps

Table 7. Qualification of 6 Tissue Types

Tissue type	Neat	LLOQ QC (ng/mL)		HQC (ng/mL)		Pass
		0.0300		7.50		
	Conc ng/mL	Conc ng/mL	%Bias	Conc ng/mL	%Bias	(Y/N)
Heart	<LLOQ	0.0345	15.0	8.96	19.5	Y
Gastrocnemius	<LLOQ	0.0277	-7.7	7.87	4.9	Y
Quadriceps	<LLOQ	0.0309	3.0	7.62	1.6	Y
Tibialis anterior	<LLOQ	0.0294	-2.0	7.34	-2.1	Y
Diaphragm	<LLOQ	0.0329	9.7	7.26	-3.2	Y
Deltoid	<LLOQ	0.0348	16.0	6.93	-7.6	Y

Data shown from 1 lot/tissue for representative purposes only

Method 3

Figure 6 and Table 8. Assay performance: Micrococcal Nuclease-Dependent ECL in Mouse Tissues

Sample Name	Nominal Conc. (ng/mL)	Instrument Response	Measured Conc. (ng/mL)	%Bias	SNR
BL	NA	103	NA	NA	NA
STD 1	1.000	596	1.140	14	**5.8**
STD 10	200.0	96951.5	210.8	5.4	**941.3**

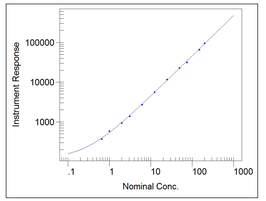


Table 9. Dig labeling

Sample ID	Conc. (ng/mL)	Single Dig	Terminal transferase tailing		
			15 min	30 min	45 min
BL	0	64.5	60.5	57	132
STD1	0.0100	157	216.5	249	354
STD2	0.0200	246	364.5	435.5	596.5
STD3	0.0400	417	679	816.5	1098.5
STD4	0.100	931	1626	2011.5	2535
STD5	0.400	3569	6529	7326	9815.5
STD6	1.000	8668.5	14709	17605.5	23798
STD7	4.000	33929	61548	73995	100430
STD8	8.000	66891.5	119811	139701	187443
STD9	10.00	86408	145670	173435	213239
LLOQ SNR		2.4	3.6	4.4	2.7
ULOQ SNR		1339.7	2407.8	3042.7	1615.4

Table 10. Assay performance: Dual Probe ECLIA in Mouse Tissues

Sample Name	Nominal Conc. (ng/mL)	Instrument response	Measured Conc. (ng/mL)	%Bias	SNR
BL	NA	49.5	NA	NA	NA
STD 1	0.0100	137	0.0102	2.0	**2.8**
STD 9	10.0	78976.5	10.3	2.9	**1595.5**

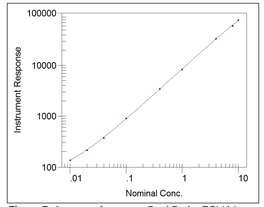


Figure 7. Assay performance: Dual Probe ECLIA in Mouse Tissues

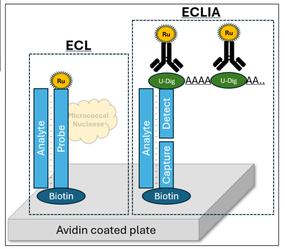


Figure 8. Assay performance: Dual Probe ECLIA in Mouse Tissues

Conclusion:
- 100-fold higher sensitivity
- Sensitivity: 0.01 ng/mL
- Dynamic range: 1000-fold
- MRD 1/4
- Amenable for transfer to human tissues with low sampling volumes

CONCLUSIONS

Bioanalytical methods for oligo therapeutics can be improved by assessing a combination of reading platforms, probe design, and signal amplification strategies. These improvements provide robust, cost- and time-efficient methods and serve as templates for developing methods for clinical studies requiring high sensitivities with low sampling volumes.