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Strategies to Improve Assay Sensitivity for Bioanalysis of Therapeutic Oligonucleotides Through Ligand Binding Assays

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INTRODUCTION

Hybridization-based ligand binding assays (LBA) for quantifying therapeutics oligonucleotides from discovery to clinical studies are the preferred bioanalytical platforms owing to their high sensitivities and lower costs. Typically, these assays implement a single-probe nuclease-dependent or dual-probe approach (see **Figure 1** and **Figure 2** below). In the single-probe approach, unhybridized probes are cleaved by nucleases specific to single-stranded DNA (ssDNA), such as S1 nuclease or micrococcal nuclease. Both methods can be adapted onto conventional enzyme-linked immunosorbent assays (ELISA) or electrochemiluminescence (ECL) assays. While detection in ELISA is mediated through fluorescence generated through an enzymatic redox reaction, detection in ECL is measured through electrochemiluminescence generated by luminophores. The luminophores may be labeled on the detection probe for direct measurement (ECL assay) or onto an antibody against the detection probe (ECLIA).

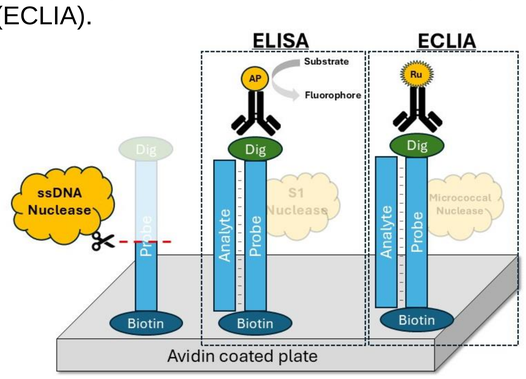


Figure 1. Schematic representation of the single-probe nuclease-dependent assay format. Capture facilitated through biotin; detection facilitated through digoxigenin + conjugated anti-digoxigenin antibody.

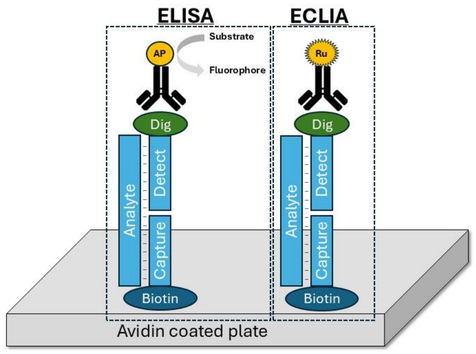


Figure 2. Schematic representation of the dual-probe assay format. Capture facilitated through biotin; detection facilitated through digoxigenin + conjugated anti-digoxigenin antibody.

There is an increasing demand for higher sensitivity in bioanalytical assays, as this allows more profound insight into the pharmacokinetic profiles for preclinical applications and allows quantitating potent compounds dosed at lower concentrations for clinical applications. Here, we present the strategies implemented for improving assay sensitivity for three bioanalytical methods 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.

CASE 1

Method transfer of a validated nuclease-dependent ELISA for anti-sense oligonucleotide (ASO) quantification in monkey serum to rabbit serum.

ELISA Performance

Table 1. S1 Nuclease-Dependent ELISA in Monkey Serum

Sample Name	Nominal Conc. (ng/mL)	Instrument response	Measured Conc. (ng/mL)	%Bias	Signal-noise ratio
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**

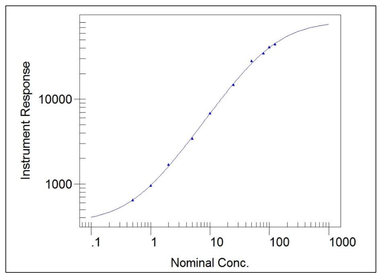


Figure 3. Representative calibration curve ELISA

ECLIA Performance

Table 2. Micrococcal Nuclease-Dependent ECLIA in Rabbit Serum

Sample Name	Nominal Conc. (ng/mL)	Instrument Response	Measured Conc. (ng/mL)	%Bias	Signal-noise ratio
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**

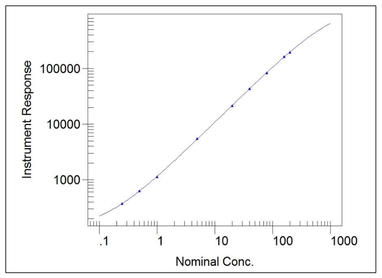
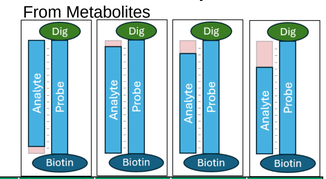


Figure 4. Representative calibration curve ECLIA

Table 3. Precision and Accuracy In ECLIA (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

ELISA in Monkey Serum—Parameters:

- S1 nuclease-based
- Sensitivity: 1 ng/mL
- Dynamic range: 100-fold
- LLOQ signal-noise ratio (SNR): 2.6
- ULOQ SNR: 110.8
- Workflow duration: ~8h
- Lower SNR at 1 ng/mL in rabbit serum observed

ECLIA in Rabbit Serum—Parameters and Conclusions:

- Micrococcal nuclease-based
- Sensitivity: 0.5 ng/mL
- Dynamic range: 400-fold
- LLOQ SNR: 5.9
- ULOQ SNR: 1823.4
- Workflow duration: ~5h
- 60% lower running cost
- Reduction in cross-reactivity from N-2 and N-4 5' metabolites

CASE 2

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

Assay Development (Free PMO):

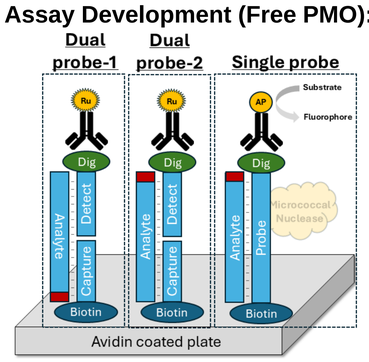


Figure 5. Platform/Probe Design Preliminary Test

Trypsin treatment (Antibody-PMO):

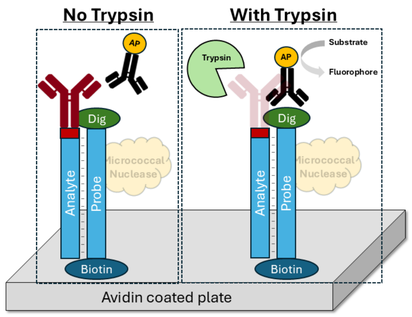


Figure 6. Protease optimization with conjugated payload (Antibody-PMO)

Table 7. Qualification of 6 Tissue Types

Tissue type	Neat	LLOQ QC (ng/mL)		HQC (ng/mL)		Pass
		0.0300		7.50		
	Conc	Conc	%Bias	Conc	%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 only

Table 5. Platform/Probe Design Preliminary Test

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

Table 6. Protease optimization with conjugated payload (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 improves 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

CASE 3

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

ECL Performance:

Table 8. Micrococcal Nuclease-Dependent ECL

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**

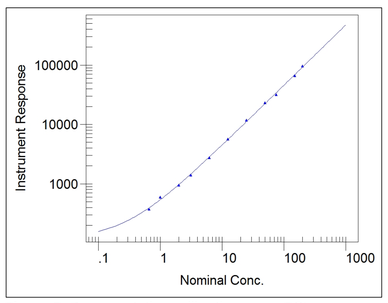


Figure 7. Representative calibration curve ECL

ECLIA Performance:

Table 9. Dual-Probe ECLIA

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**

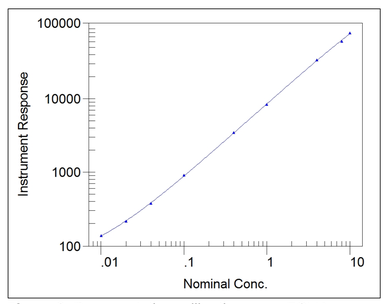


Figure 8. Representative calibration curve ECLIA

Table 10. Digoxigenin tailing optimization

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	23798
STD7	4.000	33929	61548	73995	100430
STD8	8.000	66891	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**

ECL Parameters:

- Micrococcal nuclease-based
- Sensitivity: 1 ng/mL
- Dynamic range: 200-fold
- LLOQ SNR: 5.8
- ULOQ SNR: 941.3

ECLIA Parameters and Conclusions:

- Dual-probe format
- Sensitivity: 0.01 ng/mL
- Dynamic range: 1000-fold
- LLOQ SNR: 2.8
- ULOQ SNR: 1595.5

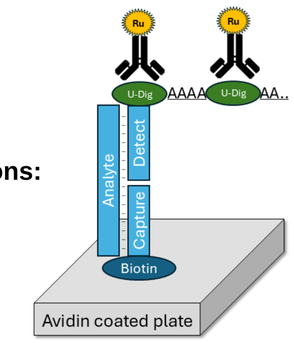


Figure 9. Optimized dual-probe ECLIA