Detection and Quantification of PS-Modified ASO in Biological Matrices using ModDetect™ Panel

Seth Farrier, Vidal de la Cruz, Kent Morgan, Todd Giardiello, and Carl Ascoli

Correspondence to Seth Farrier seth.farrier@rockland.com. Rockland Immunochemicals, Inc., Limerick, PA, USA.

1. ABSTRACT

The rapid advancement of oligonucleotide therapeutics (ONTs) has created a growing need for sensitive and reliable detection methods to support their development and clinical application including orthogonal approaches to the collection of analytical data. ONTs such as antisense oligonucleotides (ASOs) and small interfering RNAs (siRNAs) offer promising treatments for a wide range of genetic disorders, but their development is hindered by challenges in quantifying their distribution, stability, and transport in complex biological matrices. As outlined in FDA guidance documents, there is a significant need for more robust immunogenicity assays and analytical tools to evaluate drug metabolism and pharmacokinetics (DMPK) for ONTs. Effective assays are essential for understanding the absorption, distribution, metabolism, excretion, and toxicology (ADMET) properties of these drugs[1]. To address these gaps, we developed and optimized an ELISA-based platform to detect PS-modified ONTs in multiple biological matrices. This platform demonstrates both high-range and low-range capabilities, providing a robust tool for quantifying ONTs in complex biological environments such as serum, blood, and tissue homogenates. The assay shows high repeatability and sensitivity, essential for reliable monitoring of therapeutic delivery, stability, and potential off-target effects. The assays presented here form the foundation of a broader analytical platform that could be extended to identify protein-binding partners using techniques such as Biolayer Interferometry (BLI) and immunoprecipitation. By leveraging these tools, it may be possible to monitor changes in the binding profiles of free and matrix-bound oligonucleotides, offering insights into metabolic alterations and protein interactions that could impact therapeutic efficacy. These advancements have the potential to overcome limitations in assays intended to support the clinical translation of ONTs.

2. INTRODUCTION

Matrix effects in biological fluids present significant challenges in detection of PS-modified oligonucleotides by ELISA due to the solution's complex composition. High protein content can bind ONTs non-specifically, reducing their availability for detection. Additionally, endogenous nucleases or enzymes may degrade ONTs, compromising assay sensitivity. Non-specific interactions with lipids, salts, and other biomolecules can interfere with antibody binding and enzymatic reactions, leading to background noise or altered signal intensity. Furthermore, cross-reactivity with assay components may result in false positives or variability in quantification. To address these challenges, strategies such as optimized sample preparation, dilution techniques, optimization of assay parameters, and/or use of matched calibration matrices are essential for improving assay accuracy and reliability.

In this study, we demonstrated utilization of ModDetect™ antibodies[2-4] in a Sandwich ELISA to test a variety of biological matrices. Throughout the study, the following matrices have been examined:

Matrices	
Mouse Serum (#D108-00-0500)	**Human Serum** (#D119-00-0500)
Mouse Whole Blood (#R308-0050)	**Human Whole Blood** (#R314-0050)
Mouse Brain Homogenate (#W10-000-T004)	**Mouse Lung Homogenate** (#W10-000-MQ1)
Mouse Liver Homogenate (#W10-000-T020)	**Mouse Kidney Homogenate** (#W10-000-T017)
HeLa Whole Cell Lysate (WCL) (#W09-000-364)	

3. ELISA SCHEMATIC

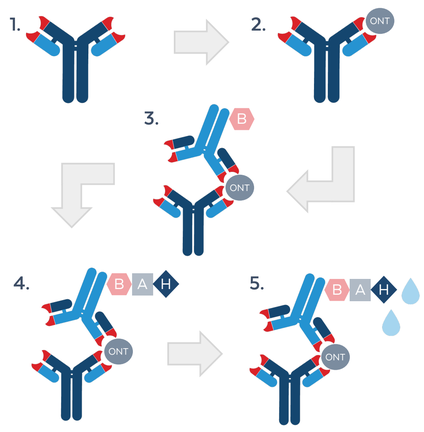


Fig 1. Schematic of a Sandwich ELISA Assay. (1) Multi-well ELISA plate is coated with capture antibody. (2) Non-specific binding sites blocked with blocking reagent and antigen is added. (3) Plate is washed to remove unbound antigen and biotinylated detection antibody is added. (4) Streptavidin-HRP is added and binds to biotinylated detection antibody. (5) TMB substrate is added and converted into measurable color to determine antigen quantity.

4. HIGH RANGE & LOW RANGE ASSAY

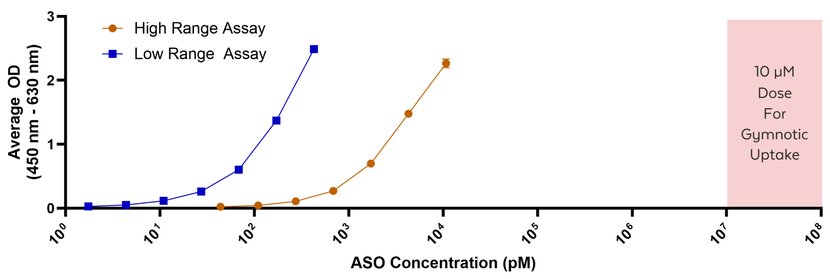


Fig 2. Variations in testing parameters using ModDetect™ Panel allows for customizable analysis methods. In this study, both "high-range" (suited for higher range) and "low-range" (suited for sensitivity) assays have been established and used throughout testing.

**Affinity constant as measured against 5Biotin_MOE/PS Gapmer.*

Low Range Assay

Parameter	Specification
Goodness of fit (4PL)	$R^2 \geq 0.98$
Range of Standard Curve	**1.7 - 428 pM (0.016 - 4.0 ng/mL)**
Precision, intra- and inter- assay CV%	≤ 20%
Sensitivity, Lower Limit of Quantitation (LLOQ)	≤ 1.7 pM (≤ 0.016 ng/mL)
Sensitivity, Lower Limit of Detection (LOD)	**< 0.9 pM (< 0.008 ng/mL)**
Detection Antibody	PS09 (K_D= < 1.0 pM*)

High Range Assay

Parameter	Specification
Goodness of fit (4PL)	$R^2 \geq 0.98$
Range of Standard Curve	**44 - 10,700 pM (0.4 - 100 ng/mL)**
Precision, intra- and inter- assay CV%	≤ 20%
Sensitivity, Lower Limit of Quantitation (LLOQ)	≤ 44 pM (≤ 0.4 ng/mL)
Sensitivity, Lower Limit of Detection (LOD)	**< 11 pM (< 0.1 ng/mL)**
Detection Antibody	PS04 (K_D = 1.5 pM*)

5. DETECTION IN MATRIX

Matrix buffer study examining minimum allowable dilution of matrix that would yield acceptable precision and accuracy of PS-modified ASO. Calibration Standard preparation was prepared in tris-based buffer and examined against Serum matrix solutions prepared in a similar manner (substituting buffer for respective serum solution).

Key Findings:
≤ 0.1% Optimum Serum Concentration

Dynamic Range in 100% Matrix using 0.1% Dilution:
44 - 10,700 nM (0.4 - 100 µg/mL) (High Range Assay)
1.7 - 428 nM (0.016 - 4.0 µg/mL) (Low Range Assay)

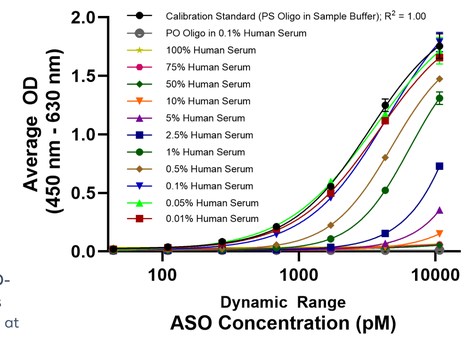


Fig 3. Overlay of multiple experiments examining concentration of Human Serum (#D119-00-0500) for detection and quantification of PS-modified ASO. At high concentrations, signal is depressed; however, optimal signal is obtained as matrix concentration is decreased, starting at approximately 0.1%.

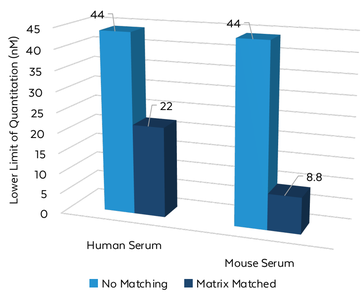
6. IMPROVED ASSAY SENSITIVITY WITH MATRIX MATCHING

Calibrating the assay with a solution of diluted matrix in buffer (**Matrix Matching**) decreased lower limit of quantitation as compared to an assay using a buffer calibration. Dilute sample in matrix-fortified buffer to replicate a like material.

Key Findings:
Improved Assay Sensitivity

Sensitivity customizable to individual matrix
Allows higher matrix concentration

We observe a Lower Limit of Quantitation when matrix matching is performed compared to 0.1% allowable matrix concentration. When utilizing matrix matching techniques, reported at least 2x decrease in LLOQ concentration.



Lower LLOQ = Greater Sensitivity

7. MATRIX COMPATIBILITY

Dilutions of each matrix solution were spiked with a known concentration of PS-modified ASO. The spiked matrix solution is then diluted with Tris-based buffer to within the calibration range of the assay.

Key Findings:
Functional Dynamic Ranges in Human and Mouse Tissues

Well-suited to a wide range of biological matrices

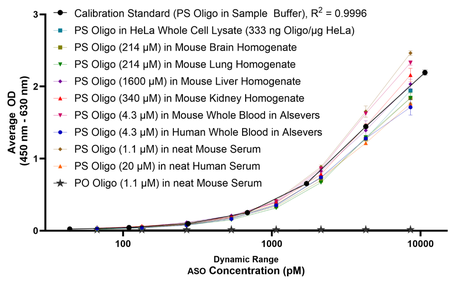
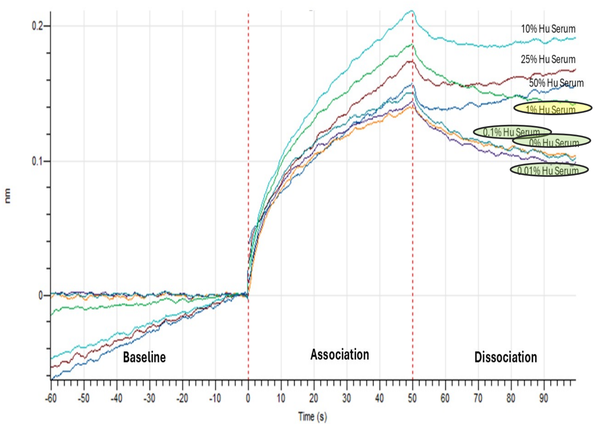


Fig 4. The quantification of fully PS-modified ASO by ELISA using anti-PS monoclonal antibody (clone PS04) in buffer and matrix. Matrix solutions spiked with PS-modified oligonucleotide and diluted 2-fold sequentially with Tris-based buffer to within range. Calibration Standard control curve generated with PS-modified ASO diluted in Tris-based buffer over a range of 44 pM to 10.7 nM by 2.5-fold serial dilution and subsequently detected using biotin-conjugated anti-PS monoclonal antibody and Streptavidin-HRP.

8. BIO-LAYER INTERFEROMETRY

Biolayer Interferometry (BLI) enables real-time, label-free measurement of ASO binding kinetics and affinity, preserving native molecular interactions. Its high-throughput format and low sample requirements make it ideal for screening multiple ASO candidates or targets. BLI allows for flexible assay design, including specificity assessments and compatibility with complex biological matrices.

Matrix Concentration	Binding Constant %Difference to 0% Matrix
50%	100.0%
25%	100.0%
10%	80.6%
1%	22.7%
0.1%	18.9%
0.01%	3.8%
0%	0.0%



Key Findings:
Optimal Binding at ≤ 0.1% Matrix

Orthogonal approach consistent with ELISA

Fig 5. Bio-Layer Interferometry (BLI) using PS-modified ASOs immobilized on AR2G sensors was performed against constant ModDetect™ antibody PS04 in various human serum dilutions for screening experiment. Affinity was inconsistent with control at higher serum concentrations, with notable re-binding during dissociation. Results aligned with ELISA data, indicating matrix interference above certain thresholds, and acceptable parallelism to control at 0.1% and 0.01% serum—while shape retention at 1% support defined matrix matching potential. Results are color-coded in the table by deviation from control: green indicates closest agreement, with increasing divergence represented by yellow, orange, and red (unacceptable)

9. REPRODUCIBILITY

Unaltered triplicate experiments are completed on multiple days and assessed for deviations to determine reproducibility. This is critical in assay development.

Key Findings:
Intra- and Inter- Assay Reproducibility

Repeatable with negligible variation in day-to-day assays with replicates run in triplicate, ensuring consistent, reliable results

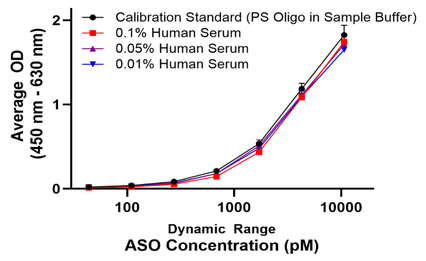


Fig 6. Triplicate experiments of PS-modified ASO in Human Serum dilutions. Quantification of fully PS-modified ASO by ELISA using anti-PS monoclonal antibody (clone PS04) in buffer and Human Serum solutions over range of 44 pM to 11 nM at 2.5-fold serial dilutions. Experiment tested in triplicate at 0.1%, 0.05%, and 0.01% of specified Matrix. No meaningful differences are observed for each experiment.

10. IF OF HELA CELLS

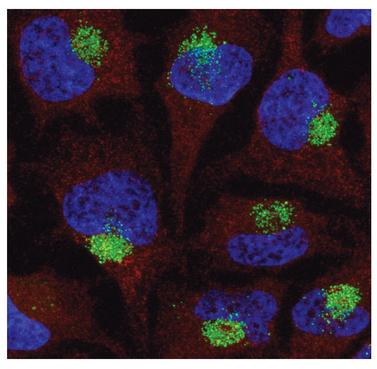
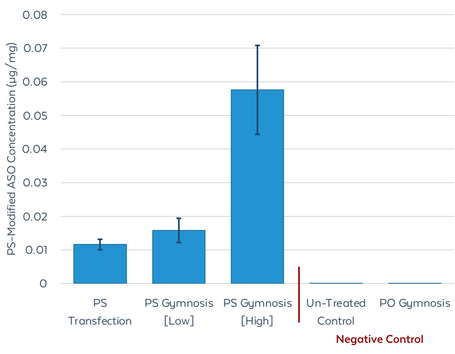


Fig 7. IF microscopy using anti-PS mAb clone. HeLa cells were cultured and treated with 100 nM of a fully phosphorothioate (PS)-modified 16-mer MALAT-1 antisense oligonucleotide (ASO) via gymnosis for 72 h. After PFA fixation, cells were incubated with anti-PS antibody in PBS (1:1,000 dilution, green) and an anti-α-tubulin antibody (red), followed by nuclear counterstaining with DAPI (blue). Cytoplasmic signal accumulation was consistent with endosomal compartmentalization. Image courtesy of Nucleic Acid Therapy Accelerator (NATA)[2]

11. QUANTIFICATION IN HELA WHOLE CELL LYSATE

Real-world HeLa Whole Cell Lysate (WCL) treated with PS-modified ASO by transfection and gymnotic delivery were tested by Low Range Assay. Samples were treated as unknowns and analyzed by ELISA.

Fig 8. Unknown HeLa WCL samples assayed against calibration curve utilizing low-range assay. Samples assayed as series of dilutions and plotted against calibrators to calculate concentration in source HeLa WCL material.



HeLa WCL Material	ASO (µg) in HeLa WCL (mg)
PS Transfection	0.0116 µg/mg
PS Gymnosis [Low]	0.0158 µg/mg
PS Gymnosis [High]	0.0576 µg/mg
Untreated Control	Not Detected
Non-Modified PO Gymnosis	Not Detected

12. SPECIFICITY

In each biological matrix, ModDetect™ panel of antibodies demonstrate a high degree of specificity by binding only to PS-modified ASO, yielding no signal for unmodified ASO or unspiked matrix.

Key Findings:
Specific to PS-modified ASO ONLY

No reaction to unmodified ASO or untreated matrix

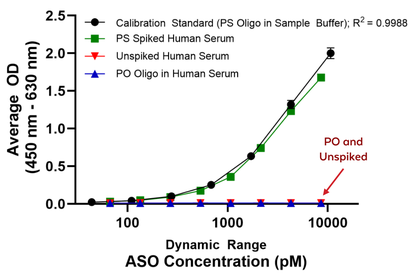


Fig 9. Analysis of PS-Modified Oligo and unmodified (PO) Oligo spiked into Human Serum, as well as unspiked Human Serum, against buffer calibration curve. High degree of correlation between PS-modified ASO spiked matrix to calibration curve, while Unspiked and Unmodified ASO spiked Human Serum yielded zero ASO signal.

13. METHOD COMPARISON

Comparison to other methods utilized for the quantification of ASOs.

	ModDetect™ ELISA	LC-MS	hELISA	Branched DNA	qPCR
Sensitivity	~ 1 pM	~100 pM[5]	~10 pM[6]	~1 pM[7]	~1 pM[8]
Cost	$	$$$$	$-$$	$$	$$
Throughput	●	●	●	●	●
Complexity	Sequence independent Universal capture based on modification	Requires intensive sample preparation, expensive instruments, and skilled operators	Additional hybridization and incubation steps Customized probes	Multiple hybridization and signal amplification steps Customized probes	Requires nucleic acid extraction, primer/probe design, and precise thermal cycling Customized probes

14. CONCLUSION

Our study demonstrates that (1) ELISA-based detection of PS-modified ONTs across diverse biological matrices is achievable; (2) detection is practical in terms of ease of use and cost-effectiveness; and (3) assay sensitivity rivals that of classical methodologies. By overcoming known challenges associated with matrix effects this optimized assay platform maintained high sensitivity and reproducibility, confirming its utility for preclinical and potentially clinical applications. These findings support the broader implementation of this approach as a reliable tool for quantifying ONTs in complex sample types.

15. REFERENCES

1. U.S. Food & Drug Administration. Clinical Pharmacology Considerations for the Development of Oligonucleotide Therapeutics - Guidance for Industry. June 2024. https://www.fda.gov/media/159414/download
2. Chimento DP, Anderson AL, Fial I and Ascoli CA. 2025. Bioanalytical Assays for Oligonucleotide Therapeutics: Adding Antibody-Based Immunoassays to the Toolbox as an Orthogonal Approach to LC-MS/MS and Ligand Binding Assays. Nucleic Acid Ther 2025;35:6-15. https://doi.org/10.1089/nat.2024.0065
3. Fial, I., Farrier, S. A., Chimento, D. P., Ascoli, C. A., Wan, X., & Oliver, P. L. (2025). Characterizing antibodies targeting antisense oligonucleotide phosphorothioate and 2′-O-methoxyethyl modifications for intracellular trafficking and biodistribution studies. Nucleic Acid Ther 2025;35(4):168-181. https://www.liebertpub.com/doi/10.1177/21593337251361396
4. Kashyap D, Cadeddu M, Oliver PL, Milne TA, and Booth MJ. (2025) Harnessing BET-Bromodomain Assisted Nuclear Import for Targeted Subcellular Localization and Enhanced Efficacy of Antisense Oligonucleotides. J. Am. Chem. Soc. 147 (32), 29478-29488. DOI: https://doi.org/10.1021/jacs.5c09544
5. Jiang, D., Li, P., & Yuan, L. (2024). Bioanalysis of free antisense oligonucleotide payload from antibody-oligonucleotide conjugate by hybridization LC-MS/MS. Bioanalysis, 16(15), 791-800. https://doi.org/10.1080/17576180.2024.2368339
6. Haegele JA, Boyanapalli R, Goyal J. Improvements to Hybridization-Ligation ELISA Methods to Overcome Bioanalytical Challenges Posed by Novel Oligonucleotide Therapeutics. Nucleic Acid Ther. 2022 Aug;32(4):350-359. https://doi.org/10.1089/nat.2021.0100
7. Mahajan, S., Zhao, H., Kovacina, K., Lachacz, E., Hoxha, S., Chan, J., & Liang, M. (2022). High-sensitivity Quantification of Antisense Oligonucleotides for Pharmacokinetic Characterization. Bioanalysis, 14(9), 603-613. https://doi.org/10.4155/bio-2022-0035
8. Shin M, Meda Krishnamurthy P, Devi G, Watts JK. Quantification of Antisense Oligonucleotides by Splint Ligation and Quantitative Polymerase Chain Reaction. Nucleic Acid Ther. 2022 Feb;32(1):66-73. https://doi.org/10.1089/nat.2021.0040