Detection and Quantification of PS, MOE, OMe, or F Modified ASO or siRNA in Biological Matrices Using Modification-Specific Antibodies in Immunoassays

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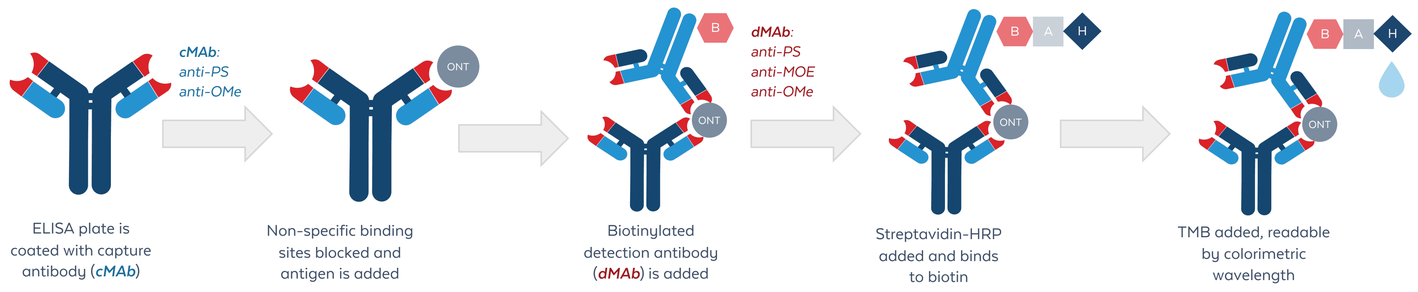
1. ABSTRACT

The rapid advancement of oligonucleotide therapeutics (ONTs) has created a growing need for sensitive and reliable detection methods to support ONT development and clinical application including orthogonal approaches to the collection of analytical data. Antisense oligonucleotides (ASOs) and small interfering RNAs (siRNAs) offer promising treatments for a wide range of genetic disorders, rare diseases, and cancer, but their development is hindered by challenges in assessing their distribution, stability and transport in complex biological matrices. Recent FDA guidance documents[1] call for more robust 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) of these drugs. We have developed and optimized panels of monoclonal antibodies (mAb) deployed as immunoassays as an orthogonal approach to addressing these gaps. Collectively referred to as ModDetect®, these mAbs are specific for most common chemical modifications present on ONTs including phosphorothioate (PS), 2'-O-methoxyethyl (MOE), 2'-O-methyl (OMe) or 2'-fluoro (F). The mAbs display specificity to their intended target, bind independently of nucleic acid sequence, and are functional in a variety of immunoassays intended to collect bioanalytical data relevant to DMPK[2-6]. Individual mAb clones demonstrate differential binding affinities to their intended targets. The two most used applications for these mAbs include: (1) visualization to determine biodistribution or intracellular localization, and (2) quantification to measure the amount of drug present in tissue or lysates. For example, imaging studies show the presence of ONTs in drug-targeted cell types in tissues, and/or the precise location of ONTs within cells by multiplexing using antibodies to subcellular markers. Likewise, in an optimized ELISA-based platform we detect PS, MOE, OMe, or F-modified ONTs in multiple biological matrices. The use of ModDetect® antibodies avoids altered pharmacokinetics observed when ONTs are directly conjugated to reporters. These immunoassays form the foundation of an analytical platform that could overcome limitations in classical assays and advance clinical translation of ONTs.

2. ELISA SCHEMATIC



3. ELISA RANGE/PARAMETERS

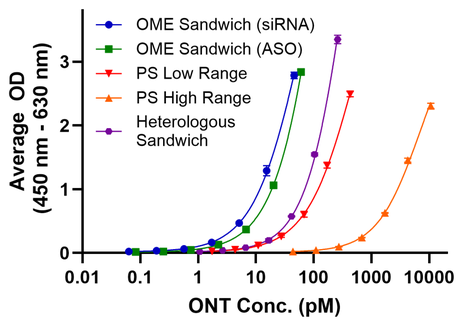


Fig 1. Variations in testing parameters using ModDetect®. Panel allows for customizable analysis methods. Optimization of differing modification targets and/or assay parameters (including panel capture / detection antibodies) allows for sensitive assays for various ONT materials.

Key Findings:
Assay Versatility

Method parameters adaptable for ONT materials to optimize sensitivity

Parameter	PS Low Range	PS High Range	OMe Sandwich (siRNA)	OMe Sandwich (ASO)	Heterologous Sandwich
Target Analyte	PS-Modified ASO	PS-Modified ASO	PS/OME/F Modified siRNA	OME Modified ASO	PS/MOE Modified Gapmer
Range of Standard Curve	1.7 – 428 pM (0.016 – 4.0 ng/mL)	44 – 10,700 pM (0.4 – 100 ng/mL)	0.06 – 46 pM (0.001 – 0.8 ng/mL)	0.08 – 61 pM (0.001 – 0.6 ng/mL)	1.1 – 260 pM (0.008 – 2.0 ng/mL)
Lower Limit of Quantitation (LLOQ)	≤ 1.7 pM (≤ 0.016 ng/mL)	≤ 44 pM (≤ 0.4 ng/mL)	≤ 0.06 pM (≤ 0.001 ng/mL)	≤ 0.08 pM (≤ 0.001 ng/mL)	≤ 1.1 pM (≤ 0.008 ng/mL)
Lower Limit of Detection (LOD)	< 0.9 pM (< 0.008 ng/mL)	< 11 pM (< 0.1 ng/mL)	< 0.01 pM (< 0.0002 ng/mL)	< 0.02 pM (< 0.0002 ng/mL)	< 0.2 pM (< 0.002 ng/mL)
PS/MOE Sandwich cMAb/dMAb	PS04 / PS09	PS04 / PS04	OME3 / OME1	OME1 / OME3	PS04 / MOEC

4. MATRIX COMPATIBILITY

Key Findings:
Functional Ranges in Human and Mouse Tissues

Well-suited to a wide range of biological matrices

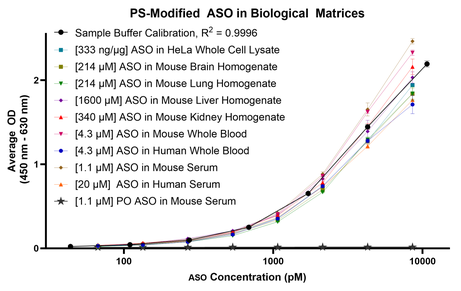


Fig 2. The quantification of fully PS-modified ASO by ELISA using anti-PS monoclonal antibody (clone PS04) in buffer and matrix. Matrix solutions spiked with PS-modified oligonucleotide and diluted 2-fold sequentially with Tris-based buffer to within range. Calibration Standard control curve generated 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.

5. QUANTIFICATION IN HELA WCL

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

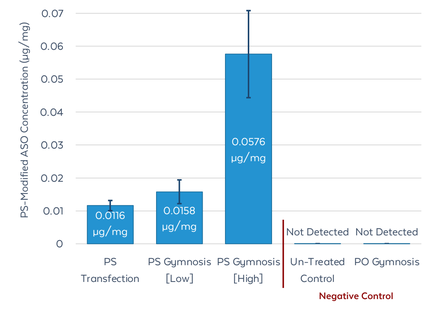


Fig 3. Unknown HeLa WCL samples assayed against calibration curve utilizing low-range assay. Samples assayed as series of dilutions and plotted against calibrators to calculate concentration in source HeLa WCL material.

6. MATRIX MATCHING SENSITIVITY

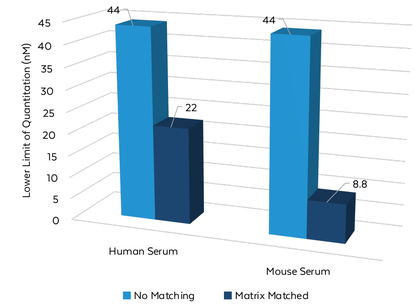
Key Findings:
Improved Assay Sensitivity

Sensitivity customizable to individual matrix
Allows higher matrix concentration

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.

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. siRNA QUANTIFICATION

siRNAs vary in length, architecture, and type and degree of modification. By utilizing an anti-OME sandwich ELISA, a universal method has been shown to be effective for the detection and quantification of siRNA regardless of differences with a high sensitivity, precision, and accuracy.

siRNA Drug	Duplex Architecture	Modifications
Lumasiran[7]	Canonical asymmetric GalNAc-ds-siRNA (21/23-mer) with 2-nt 3' antisense overhang	2'-OMe: 34 2'-F: 10 PS: 6
Inclisiran[8]	Canonical asymmetric GalNAc-ds-siRNA (21/23-mer) with 2-nt 3' antisense overhang	2'-OMe: 31 2'-F: 12 PS: 6 DNA: 1
Nedosiran[9]	Dicer-substrate GalNAc-ds-siRNA (~22-mer guide with 36-mer passenger) (~58 nt total)	2'-OMe: 35 2'-F: 19 PS: 6 RNA: 4

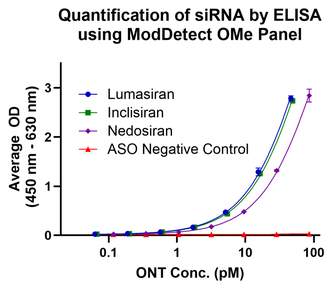


Fig 4. Sensitive detection and quantification of siRNA materials. ELISA utilizing a sandwich of anti-OME antibodies allows for universal detection of siRNA materials, demonstrated by the analysis of three unique ONTs.

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.

Key Findings:
Optimal Binding at ≤ 0.1% Matrix

Orthogonal approach consistent with ELISA

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%

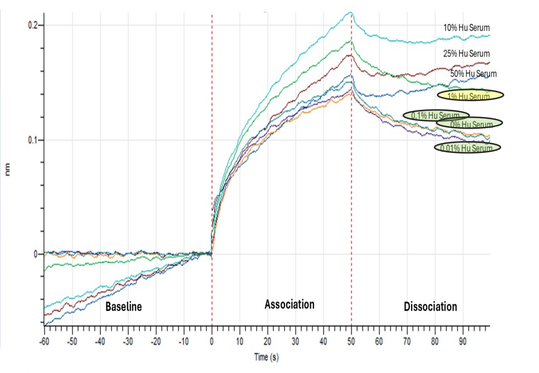
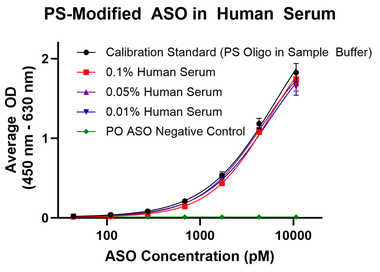


Fig 5. Bio-Layer Interferometry (BLI) using PS-modified ASOs immobilized on AR2G sensors was performed against constant ModDetect® antibody PS04 in various human serum dilutions for screening experiment. Affinity was inconsistent with control at higher serum concentrations, with notable re-binding during 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 & SPECIFICITY



Key Findings:
Intra- and Inter- Assay Reproducibility with specificity towards target modification

Repeatable with negligible variation in day-to-day assays with replicates run in triplicate, ensuring consistent, reliable results
No reaction to unmodified ASO

Fig 6. Aggregate of Unaltered Triplicate experiments of PS-modified ASO in Human Serum dilutions with unmodified ASO Negative Control. 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 over multiple days at 0.1%, 0.05%, and 0.01% of specified Matrix. No meaningful differences or deviations are observed for each experiment, while unmodified ASO spiked Human Serum yielded zero ASO signal.

10. IF ANTI-PS/ASO

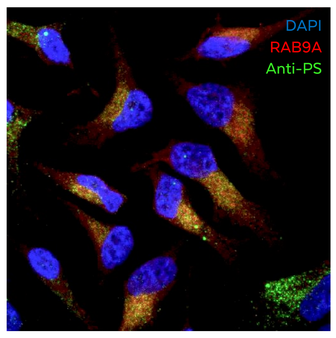


Fig 7. Intracellular trafficking. PS-modified ASO (100 nM) reacted by HeLa cells by gymnosis for 72 h followed by detection using anti-PS antibody (clone PS03) diluted 1:1000. Anti-PS is shown to colocalize with the RAB9A endosomal marker. HeLa cells were cultured, fixed with paraformaldehyde, reacted with anti-PS antibody and anti-RAB9A, and counterstained with DAPI. Cytoplasmic signal accumulation was consistent with endosomal compartmentalization (yellow). Image courtesy of Nucleic Acid Therapy Accelerator (NATA).[2]

11. IF ANTI-MOE/ASO

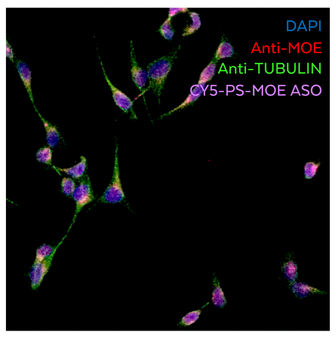


Fig 8. Colocalization of Cy5-conjugated PS-MOE ASO with anti-MOE antibody. HeLa cells were cultured and treated with 1 µM PS-MOE-modified ASO conjugated to the fluorochrome Cy5 by gymnotic uptake. After fixation with paraformaldehyde, cells were stained with DAPI, anti-alpha tubulin, and anti-MOE antibody clone MOE-C. The fluorescence from Cy5-conjugated ASO is shown to colocalize with anti-MOE antibody staining in the merged image. Image courtesy of Nucleic Acid Therapy Accelerator (NATA).[2]

12. IF ANTI-OMe/SIRNA

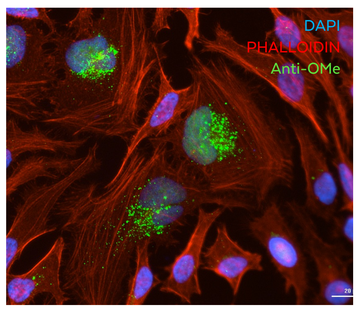


Fig 9. Detection of OMe siRNA with anti-OMe antibody. HeLa cells were cultured and transfected with 200 nM OMe modified lumasiran siRNA followed by detection of 2'-O-methyl modification using anti-OMe clone OME3. The antibody binds the siRNA in the cytoplasm in a pattern consistent with endosomal sequestration. Co-staining with phalloidin and DAPA provide structural context, confirming cellular location relative to cytoskeletal features and nuclei. Image courtesy of Charles Fisher (Rockland).

13. IHC ANTI-PS/ASO/LIVER

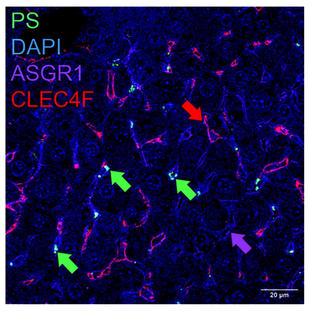


Fig 10. Detection of MOE/PS ASO with anti-MOE antibody. 2'-MOE modified gapmer with PS bonds was delivered subcutaneously to mice and fixed liver tissue was immunostained with anti-PS (clone PS03). Green arrows show accumulation of ASO in nonparenchymal cells surrounding hepatocytes. Red arrow show Kupffer cells (anti-CLEC4F) and magenta arrows show hepatocyte membranes (anti-ASGR1). Image courtesy of Nucleic Acid Therapy Accelerator (NATA).[2]

14. CONCLUSION

The utility highly specific mAbs reactive to common chemical modifications used to stabilize ONTs (PS, MOE, OMe, F) is shown. Collectively referred to as ModDetect®, these mAbs bind ASOs and siRNA independent of sequence, and constitute an orthogonal approach to classical assays to quantify and visualize this class of drug. Quantification by ELISA is achieved across diverse biological matrices. Localization in tissue shows the deposition in targeted cell types, and sequestration within cells. These findings support the broader implementation of this technology as an orthogonal approach to support ONT safety and efficacy.

15. REFERENCES

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