



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 documents¹ 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 (mAbs) deployed as immunoassays as an orthogonal approach to addressing these gaps.² 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. Individual mAb clones demonstrate differential binding affinities to their intended targets. The 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.³ 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.⁴⁻⁷ 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.

2. SUGGESTED/VALIDATED* USES

Analytical Assay	Purpose
Immunohistochemistry (IHC)*	Biodistribution
Immunofluorescence microscopy (IF)*	Intracellular localization
ELISA	Anti-drug antibody binding Immunogenicity studies positive control Drug ranking
Immunoprecipitation (IP)*	Protein binding in serum and/or tissue lysates
In vitro cell culture studies	Potency
Quantification studies*	To determine concentrations in biofluids
Compartmentalization studies*	To understand cytosolic and nuclear penetration of ONTs
3D cell culture studies*	To determine drug penetration into spheroids
Fluorescence-activated cell sorting (FACS)	To identify and separate cells based on presence of ONTs
Stereochemistry studies*	To assess the chiral make-up of PS containing ONTs
Other immunoassays	Various

Table 1. Suggested Immunoassays can be either primary assays or constitute an orthogonal approach to the collection of data intended to ensure the safety and efficacy of ONTs².

3. TARGETED MODIFICATIONS

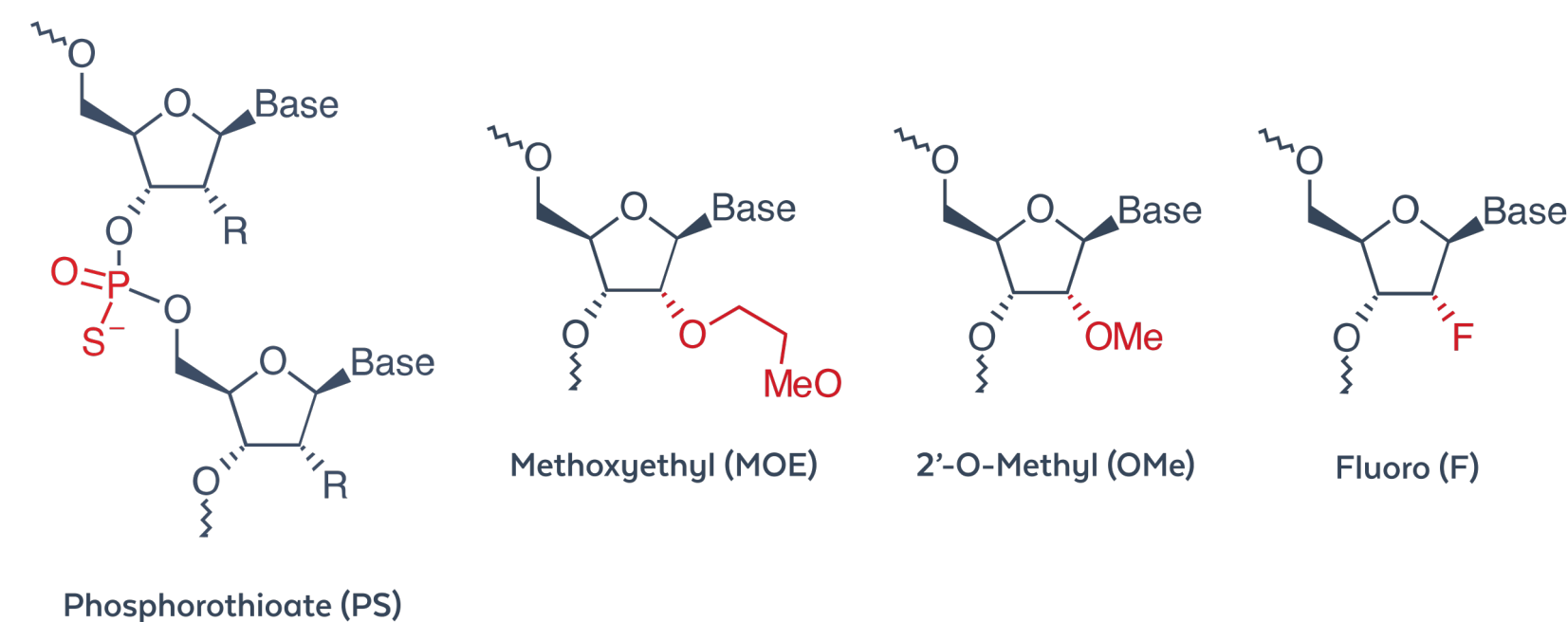


Fig 1. Common chemical modifications used to enhance the "drug likeness" of ONTs. PS, MOE, OMe and F modifications stabilize and enhance the efficacy of ONT drugs.

4. TARGETED DRUG MODALITIES

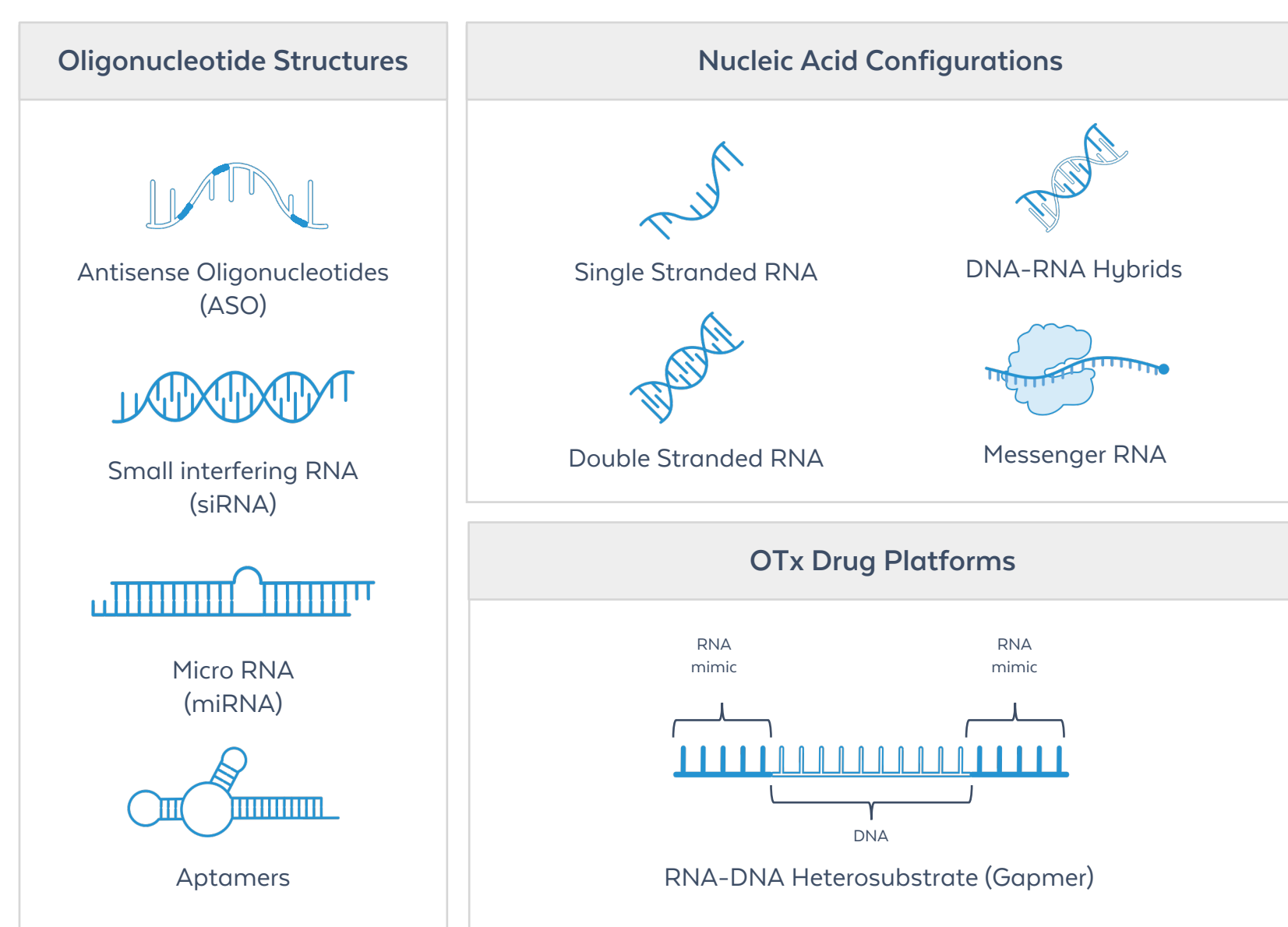


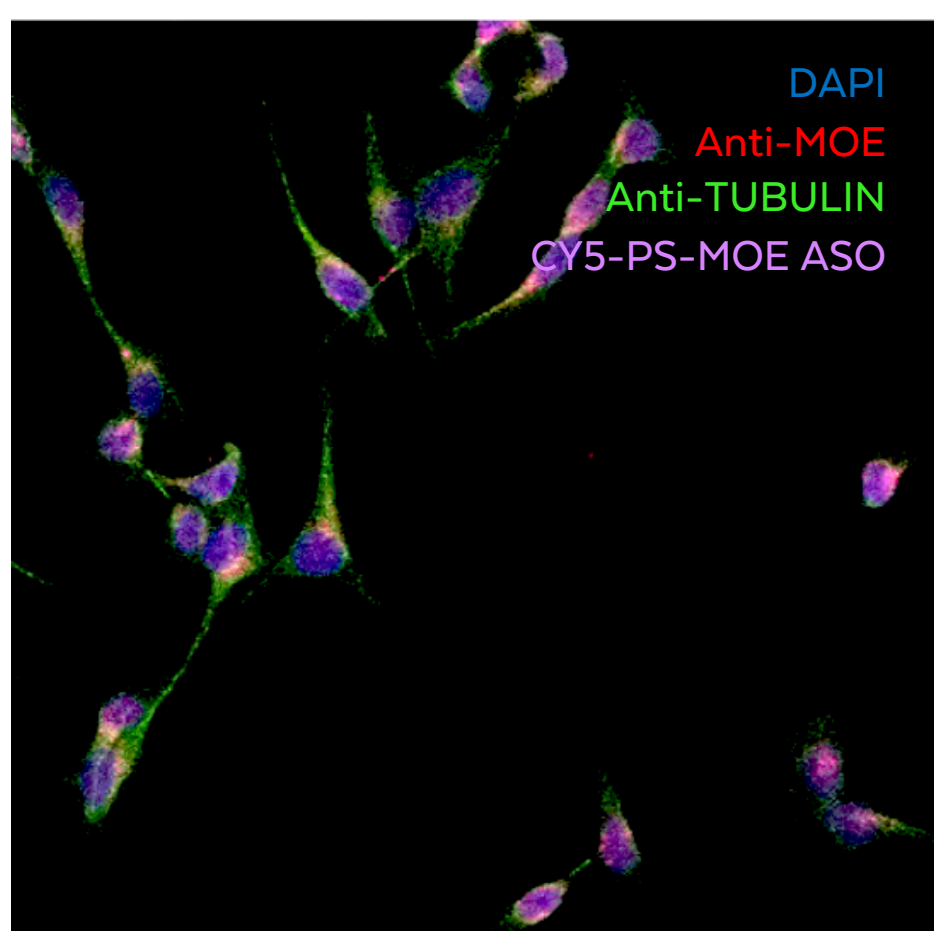
Fig 2. Developed monoclonal antibodies (ModDetect®) are analytical tools used to detect chemical modifications independent of nucleic acid composition, structure, strandedness, configuration, or platform.

12. IF ANTI-PS/ASO



Fig 8. 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 Hoechst. Cytoplasmic signal accumulation was consistent with endosomal compartmentalization (yellow). Image courtesy of Nucleic Acid Therapy Accelerator (NATA).³

13. IF ANTI-MOE/ASO



14. IF ANTI-OMe/siRNA

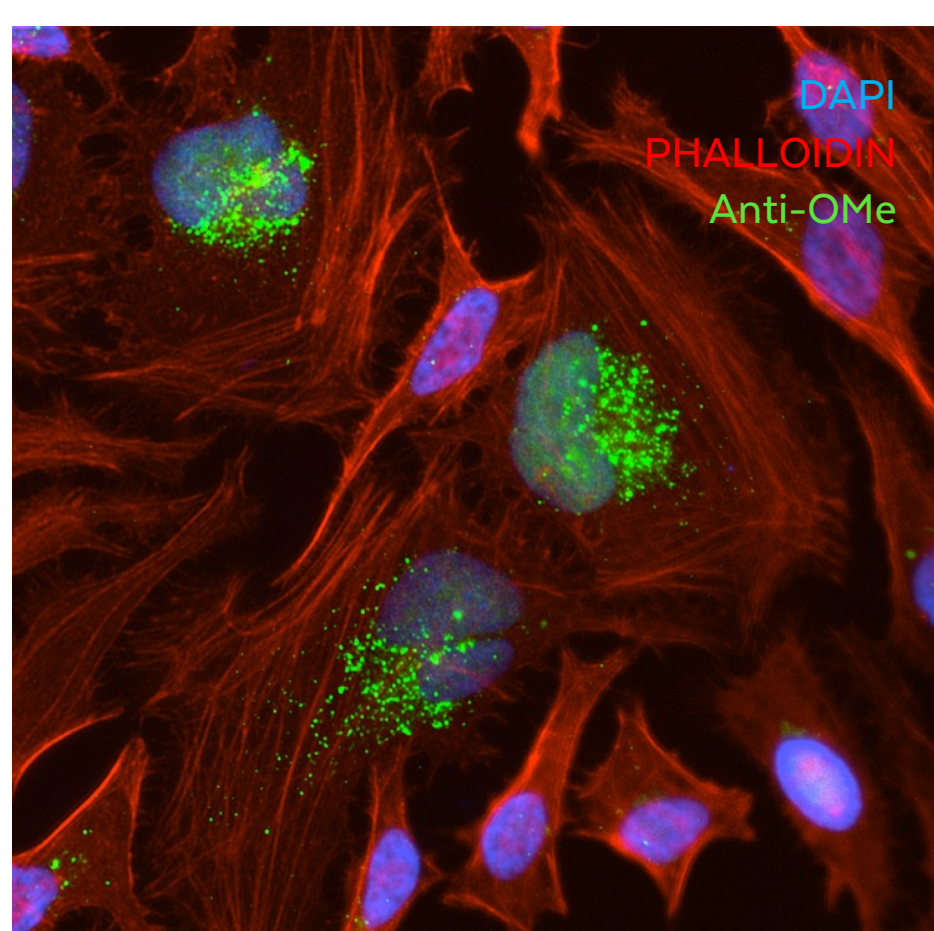


Fig 10. Detection of Ome siRNA with anti-Ome antibody. HeLa cells were cultured and transfected with 200 nM Ome modified lumasiran siRNA followed by detection of 2'-O-methyl modification using anti-Ome clone OME3. The antibody binds the siRNA in the cytoplasm in a pattern consistent with endosomal sequestration. Co-staining with phalloidin and DAPI provide structural context, confirming cellular location relative to cytoskeletal features and nuclei. Image courtesy of Charles Fisher (Rockland).

15. IF ANTI-F/siRNA

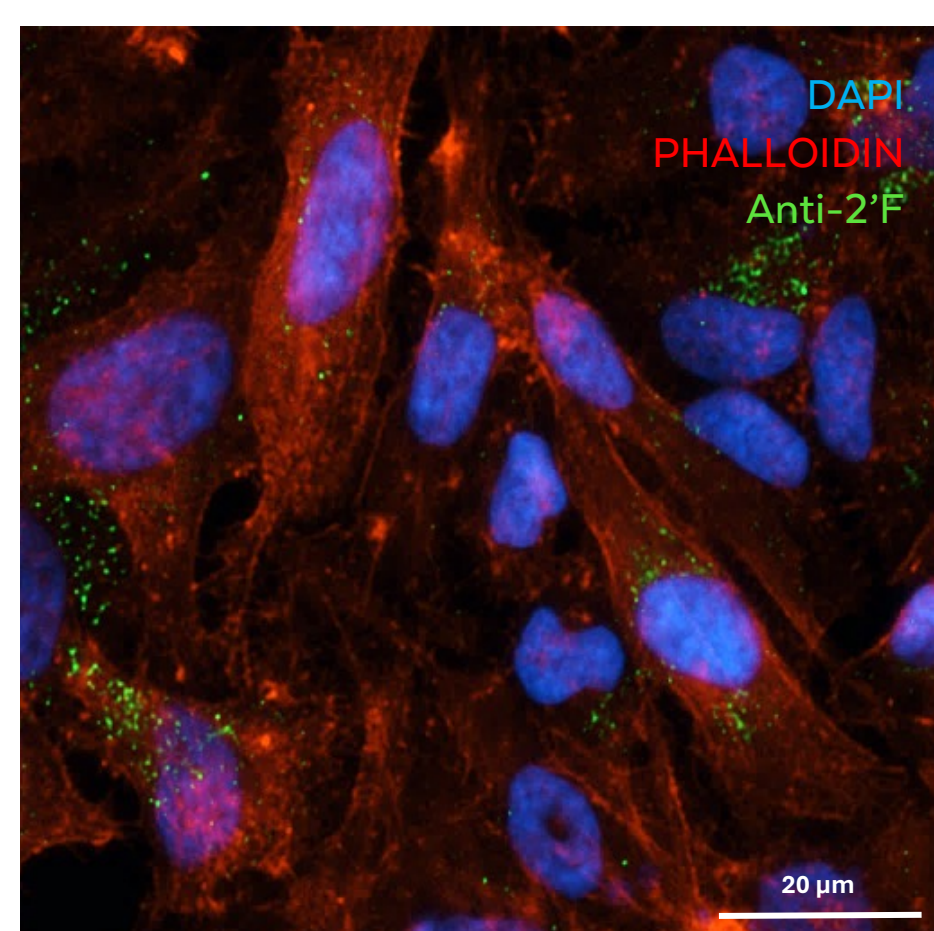
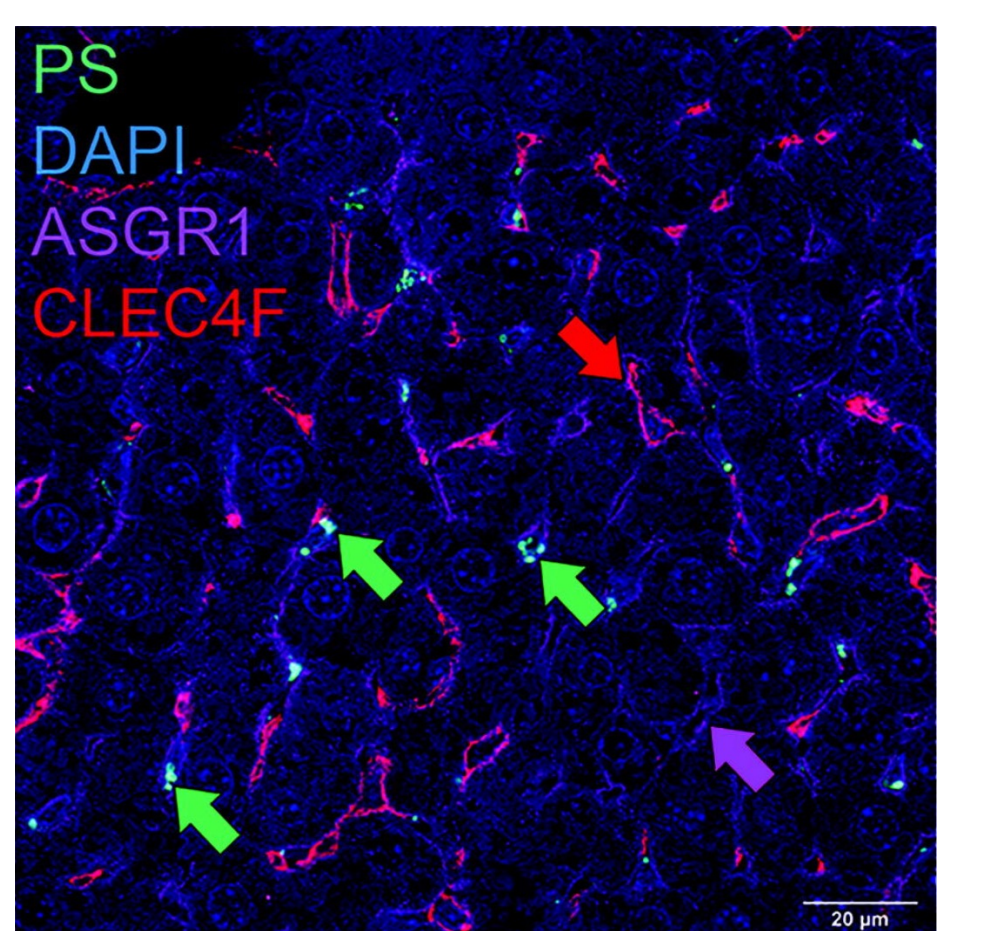
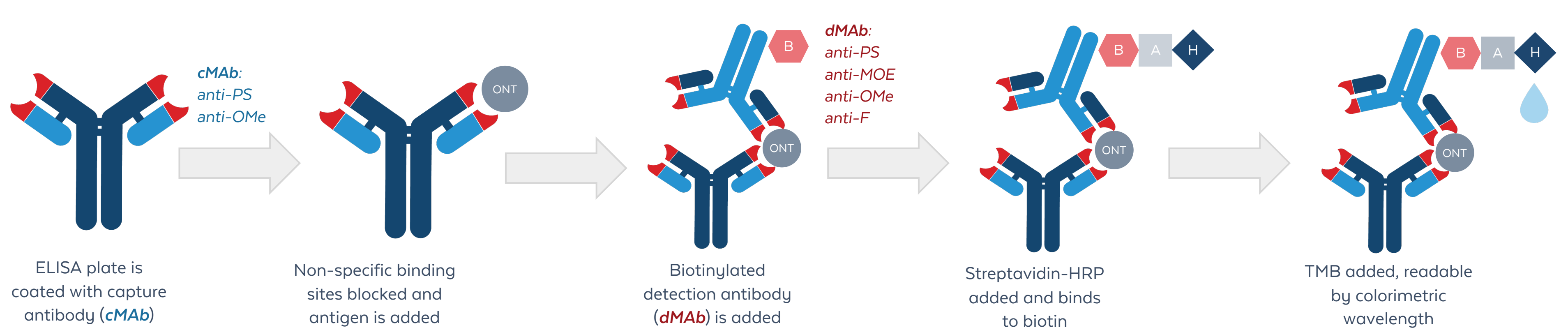


Fig 11. Detection of 2'F siRNA with anti-2'F antibody. HeLa cells were cultured and transfected with 200 nM 2'F modified nedosiran siRNA followed by detection of 2-deoxy-2-fluoro-D-ribofuranose modification using anti-2'F. The mAb binds the siRNA in the cytoplasm in a pattern consistent with endosomal sequestration. Co-staining with phalloidin and DAPI provide structural context, confirming cellular location relative to cytoskeletal features and nuclei. Image courtesy of Charles Fisher (Rockland).

16. TISSUE IHC ANTI-PS/ASO/LIVER



5. ELISA SCHEMATIC FOR QUANTIFICATION



6. MATRIX COMPATIBILITY

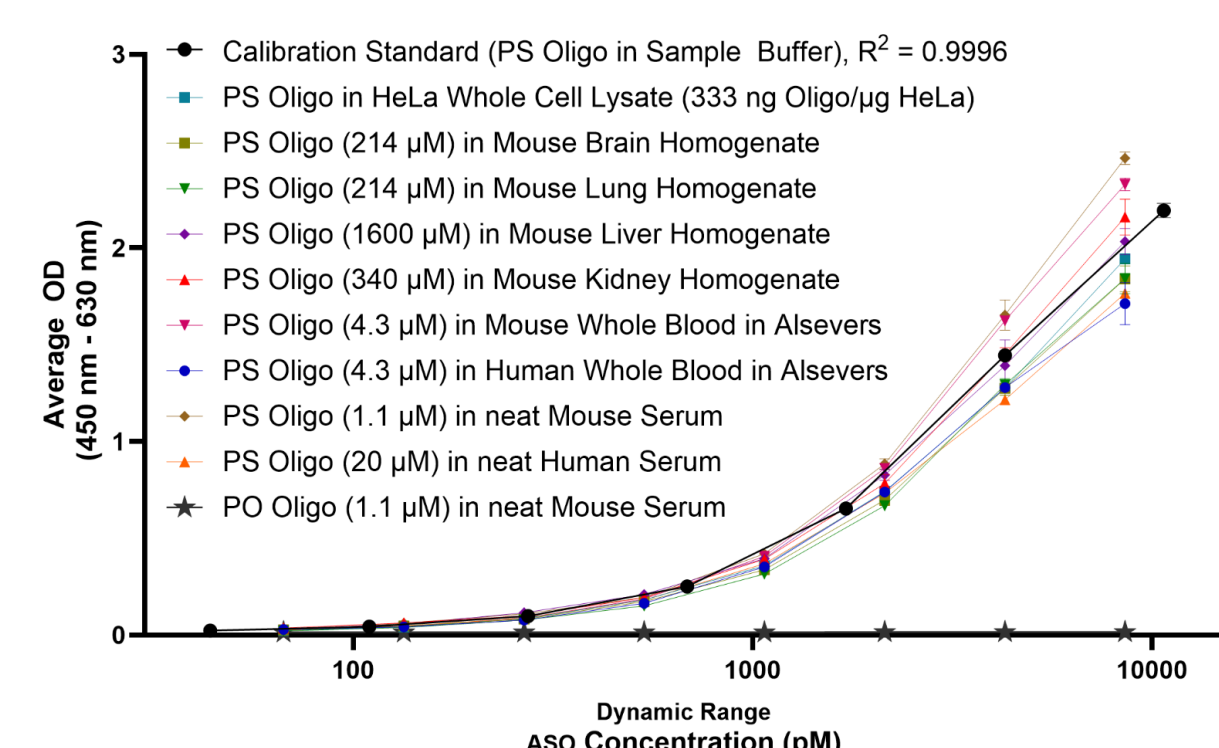


Fig 3. The quantification of fully PS-modified ASO by ELISA using anti-PS monoclonal antibody (clone PS04) in buffer and matrix. Matrix solutions spiked with PS-modified oligonucleotide and diluted 2-fold sequentially with Tris-based buffer to within range. Calibration Standard control curve generated 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.

Key Findings:
Functional Ranges in Human and Mouse Tissues

Well-suited to a wide range of biological matrices

9. siRNA QUANTIFICATION

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

siRNA Drug	Duplex Architecture	Modifications
Lumasiran ⁸	Canonical asymmetric GalNAc-ds-siRNA (21/23-mer) with 2-nt 3' antisense overhang	2'-OMe: 34 2'-F: 10 PS: 6
Inclisiran ⁹	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 ¹⁰	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

Quantification of siRNA by ELISA using ModDetect OMe Panel

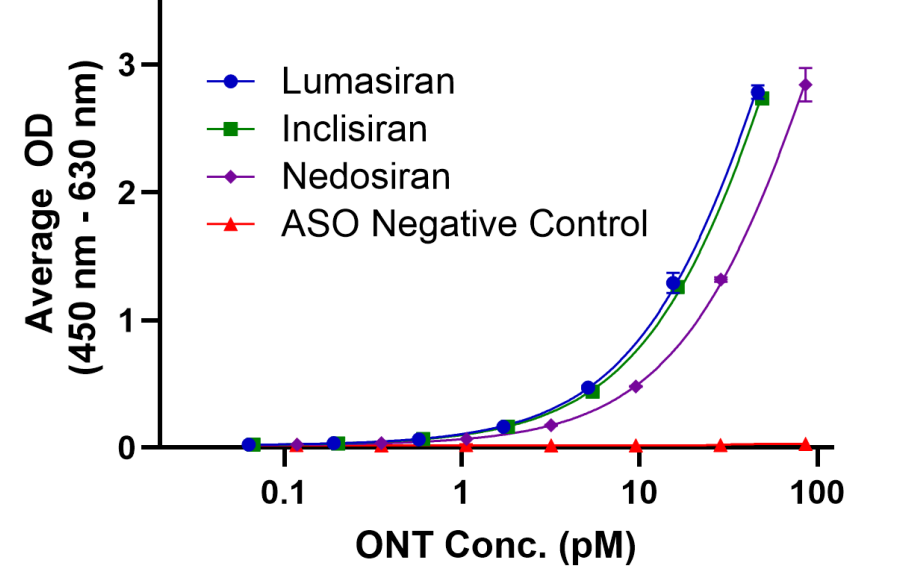


Fig 6. Sensitive detection and quantification of siRNA materials. ELISA utilizing a sandwich of anti-OME antibodies allows for universal detection of siRNA materials, demonstrated by the analysis of three unique ONTs.

10. ELISA DRUG SENSITIVITY

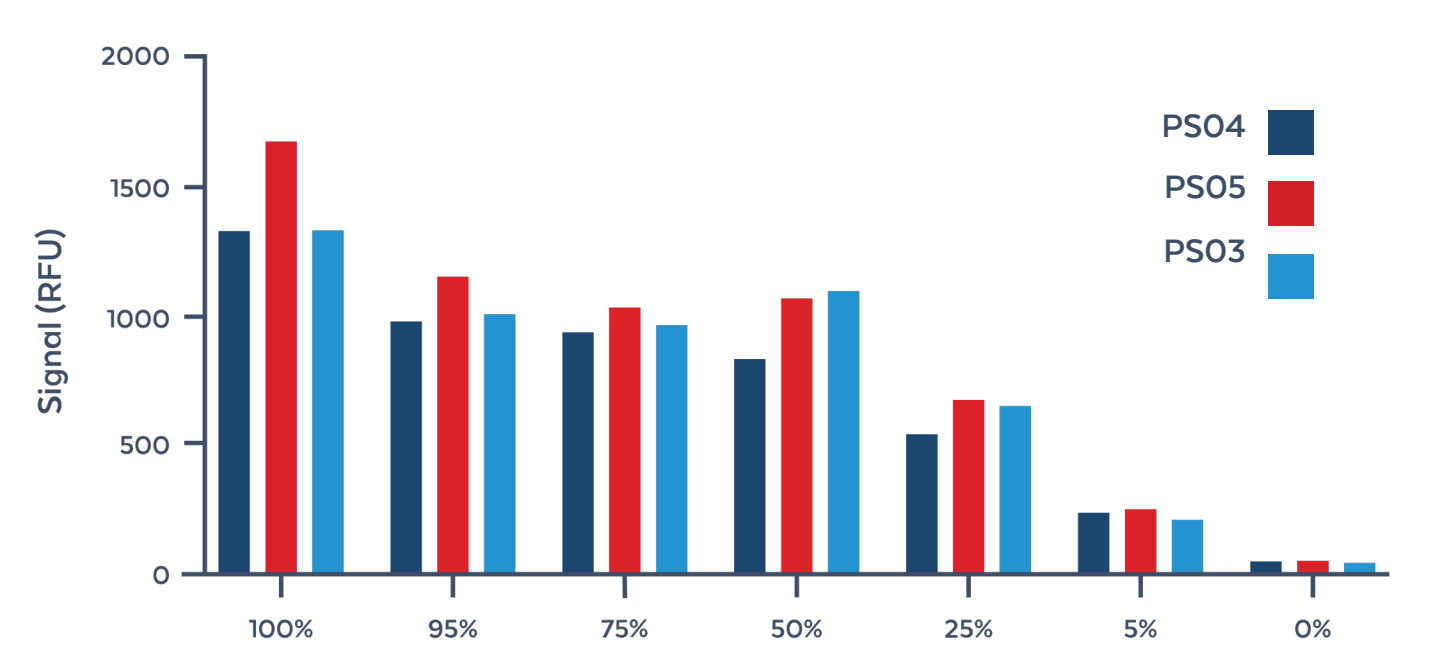


Fig 7. ELISA showing mAb signal correlates to the number of PS residues within ASO. Histogram is used to show reactivity of selected anti-PS mAbs to a synthesized ASO when the degree of PS modification is lowered from 100% to 95%, 75%, 50%, 25%, 5% and 0%, respectively. The ASO sequence is unchanged, only the number of PS linkages varies. While the signal is proportional to the number of linkages present, all mAbs tested in this series reacted with the ASO when as few as only 5% modified bases, e.g., 1 modified base out of a total of 20 bases, relative to baseline (0% data). Anti-PS mAbs were used at a 1:10,000 dilution followed by fluorescein conjugated anti-Mouse IgG secondary antibody.

11. ASSAY COMPARISONS

	ModDetect™ ELISA	LC-MS	hELISA	Branched DNA	qPCR
Sensitivity	~1 pM	~100 pM ⁵	~10 pM ⁶	~1 pM ⁷	~1 pM ⁸
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

Table 2. The ModDetect® platform delivers high sensitivity (~1 pM), fast throughput, and sequence independent detection for ASO quantification. The table above compares anti-PS antibodies for ASO detection to published values for LC-MS, hELISA, branched DNA, and qPCR analytical methods, highlighting the ability of ModDetect® to match leading methods in sensitivity while reducing complexity and eliminating the need for sequence-specific probes.

18. REFERENCES

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17. CONCLUSION

The utility of highly specific mAbs reactive to common chemical modifications (PS, MOE, OMe, F) used to stabilize ONTs is shown. Collectively referred to as ModDetect®, these mAbs bind ASOs, siRNA, mRNA and other modalities independent of sequence, and constitute an orthogonal approach to classical assays used 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.

USE FOR BEST DETECTION: ASO/anti-PS, siRNA/anti-OMe, mRNA/anti-F.

PATENT PENDING: The information presented is covered under U.S. Provisional Patent Application No.: 63/547,067.

ModDetect® is a registered trademark of Rockland Immunochemicals, Inc., Limerick, Pennsylvania, USA.