Immunoassays: an Orthogonal Approach for Localization, Quantification, and Trafficking of Oligonucleotide Therapeutic Drugs in Serum, Cells and Tissues

David P. Chimento[1], Amy L. Anderson[1], Ondrej Kostov[2], Lucia Yang[3], Adrian Krainer[3], Seth A. Farrier[1], Inês Fial[4], Peter Oliver[4] and Carl A. Ascoli[1]

[1]Rockland Immunochemicals, Inc., Limerick, PA, USA. [2]University of Colorado at Boulder, Boulder, CO, USA. [3]Cold Spring Harbor Laboratory (CSHL), Cold Spring Harbor, NY, USA.
[4]Nucleic Acid Therapy Accelerator (NATA), Harwell Campus, Oxford, UK. Correspondence to Carl Ascoli carl.ascoli@rockland.com.

1. ABSTRACT

Oligonucleotide therapeutics (ONTs) have increased in development since their inception in 1978. ONTs, such as antisense oligonucleotides (ASO) and small interfering RNA (siRNA), are short fragments of nucleic acid complementary to a specific mRNA and upon administration can modulate protein synthesis in patients. ONTs have been applied across diverse disease areas including oncology, neurology and infectious disease prevention. However, a significant number of hurdles remain to bring this class of drugs to more widespread use. One such limitation, improving the "drug-likeness" of ONTs, is facilitated by chemical modifications to the sugar-phosphate backbone and/or nitrogenous bases. Modifications are designed to increase ONT drug stability, uptake, and efficacy, but may increase the likelihood of toxicity. Chemical modifications include phosphorothioate (PS) modification of the phosphate portion of the backbone, adding substituent groups to the 2′ position of the ribose portion of the backbone, specifically 2′-O-methyl (OMe), 2′-O-methoxy-ethyl (MOE), and 2′-fluoro modifications of RNA, and peptide nucleic acids (PNA), locked nucleic acids (LNA), morpholino phosphoroamidate (PMO) modifications, and others.

A fundamental requirement for the ONT field is to generate reliable tools for their quantification in cells and tissue. We have developed, optimized, and validated a library of monoclonal antibody reagents that detect chemical modifications independent of nucleic acid composition, structure, strandedness, configuration, or platform[1]. These universal detection reagents can be used to localize and quantify ONTs in lysates and biofluids by various analytical assays[2]. While LC-MS/MS and ligand binding assays (LBA) are the mainstay of analytical assays for ONTs, immunoassays based on the antibodies described here represent an orthogonal approach to solving problems associated with demonstrating the safety and efficacy of this class of drug.

Here we report the ability to analyze ONTs in *in vitro* assays using panels of monoclonal antibodies specific for PS, 2′-MOE or 2′-OMe modifications. The performance of these antibodies demonstrates their utility as orthogonal analytical assays to support ADMET studies for ONT approval by regulatory agencies.

2. SUGGESTED 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 o PS containing ONTs
Other immunoassays	Various

Assays can be either primary assays or constitute an orthogonal approach to the collection of data intended to ensure the safety and efficacy of ONTs.

3. ANTIBODY PANELS

Clone ID	Chemical Modification	Isotype
PS01	Phosphorothioate (PS)	IgG_1 kappa
PS02		IgG_{2a} kappa
PS03		IgG_{2a} kappa
PS04		IgG_{2a} kappa
PS05		IgG_{2a} kappa
PS06		IgG_1 kappa
PS07		IgG_1 kappa
PS08		IgG_{2b} kappa
PS09		IgG_{2a} kappa
MOE1	2′-O-Methoxyethyl (2′-MOE)	IgG_1 kappa
MOE3		IgG_1 kappa
MOE4		IgG_1 kappa
MOE9		IgG_1 kappa
MOEC		Y_1K-Y_1K-$Y_{2a}K$ cocktail†
OMe1	2′-O-Methyl (2′-OMe)	IgG_1 kappa
OMe2		IgG_{2a} kappa
OMe3		IgG_3 kappa
OMe4		IgG_1 kappa
OMe5		IgG_{2a} kappa

†MOEC is a cocktail of three unique clones mixed in equimolar amounts.

4. ANTI-PS INTRACELLULAR LOCALIZATION (IF)

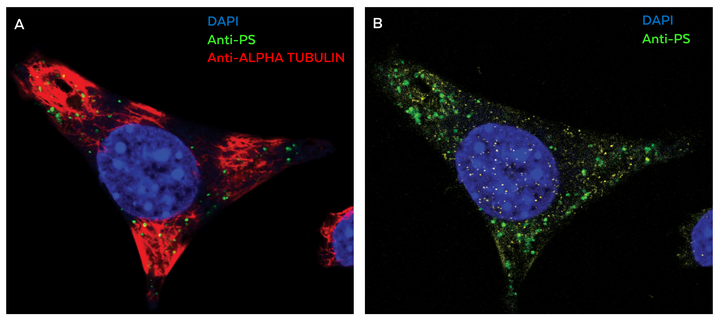


Fig 1. Intracellular localization of ASO using Anti-PS monoclonal antibody clone PS05. Mouse glioma cells (GL261) derived from C57 black mice were cultured and treated with ASO drug. After fixation with paraformaldehyde, cells were stained with DAPI (blue) and anti-PS antibody clone PS05 (green) either with (A) or without (B) staining using anti-alpha tubulin clone DMA1 (red). The anti-PS antibody was used at a 1:2000 dilution. Punctate cytoplasmic staining is consistent with endosomal storage of ASO within the cell, as expected for this ONT drug. Vehicle only treated cells showed no staining (not shown).

5. ANTI-PS CO-LOCALIZATION (IF) WITH CY5-CONJUGATED ASO

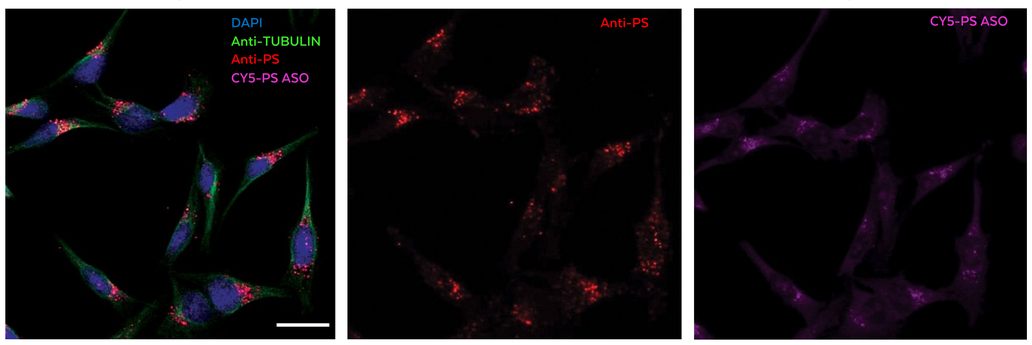


Fig 2. Colocalization of Cy5-conjugated ASO with Anti-PS monoclonal antibody clone PS04. HeLa cells were cultured and treated with 50 nM fully PS-modified ASO drug conjugated to the fluorochrome Cy5 at the 5′ end by transfection for 24 hours using 2 µL of Lipofectamine (Lipofectamine 2000, Thermo Fisher Scientific). After fixation with paraformaldehyde, cells were stained with DAPI (blue), anti-alpha tubulin (green), and anti-PS antibody clone PS04 (red). The fluorescence from Cy5-conjugated ASO is shown to colocalize with anti-PS antibody staining in the merged image. Images were captured using a Zeiss LSM 780 confocal laser scanning microscope. Maximum intensity projections. Scale bar, 25 µm. Conjugated ASOs were obtained from the laboratory of M. Caruthers, University of Colorado at Boulder.

6. PS QUANTIFICATION IN ONT DRUG

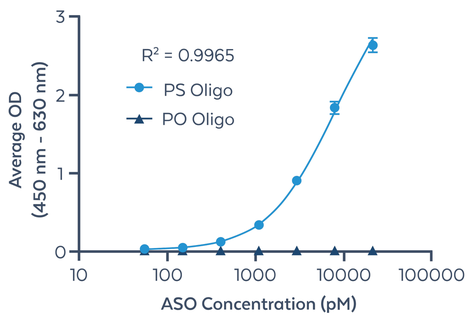


Fig 3. Quantification of fully PS modified ASO by ELISA using anti-PS monoclonal antibody (clone PS04). Non-modified PO oligonucleotide of the same sequence was used as a negative control (PO Oligo). The curve was generated using PS-modified ASO diluted in buffer from 55 pM to 21 nM in concentrations and subsequently detected using anti-PS antibody. A standard curve was plotted as the average OD result versus the log of ASO concentration in pM using a 4PL best fit formula. The LLOQ as defined as the lowest standard detected is 55 pM. The LOD was determined to be <11pM based on standard definitions of the term. The ULOQ as shown is 21 nM.

7. PS QUANTIFICATION IN HeLa WHOLE CELL LYSATE

Dual antibody immunometric assay (sandwich ELISA) used to capture and detect PS-modified ASO analyte present in HeLa whole cell lysates (WCL) after transfection and gymnotic delivery (both low and high ASO concentrations). Clone PS04 was used for capture and clone PS09 was used for detection. Untreated and PO ASO treated cells were used as negative controls. The PS-modified ASO is sandwiched between these two antibodies allowing for sensitive and specific detection.

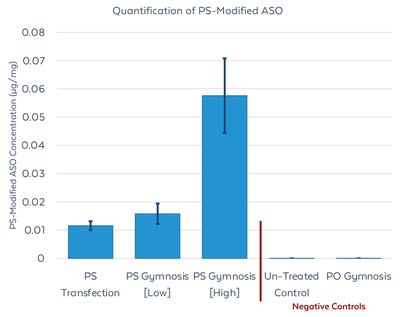


Fig 4. Quantification using standard curve. PS-modified ASO transfected HeLa WCL were quantified using a calibration curve designed for high sensitivity. Samples assayed as series of dilutions were plotted against calibrators to calculate concentration in source HeLa WCL material.

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

8. ANTI-PS DRUG RANKING

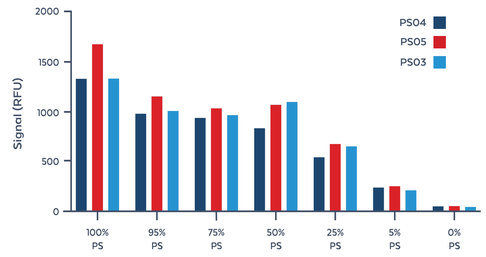
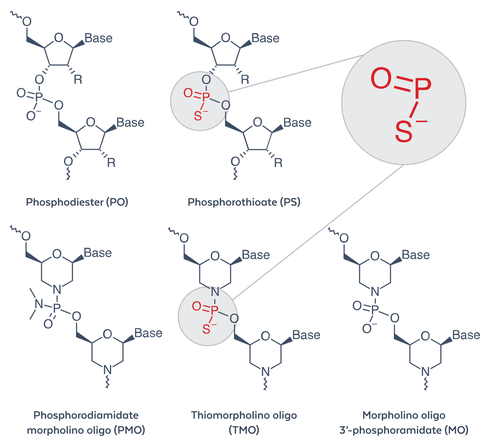


Fig 5. Downward trend of signal is observed by ELISA when anti-PS clones are incubated with ASOs possessing fewer PS bonds. The graph shows reactivity by ELISA when the degree of PS modification is lowered from 100% to 95%, 75%, 50%, 25%, 5% and 0%, respectively. As few as 1 modified base in 20 is detected above background. Anti-PS antibody (PS03, PS04, PS05) were diluted 1:10,000 for use.

9. ANTI-PS SPECIFICITY

Fig 6. Anti-PS Specificity. Antibodies to PS bind the oxygen-phosphorus-sulfur configuration of atoms common to both phosphorothioate and thiomorpholino[3] chemical modifications used to stabilize oligonucleotides. Both PS and TMO linkages are reactive with anti-PS based on their common structure (highlighted in red), but no reactivity is seen for PO, PMO, or MO based internucleotide linkages. Personal communication. M. Caruthers, University of Colorado at Boulder.



10. ANTI-PS TRAFFICKING (IF)

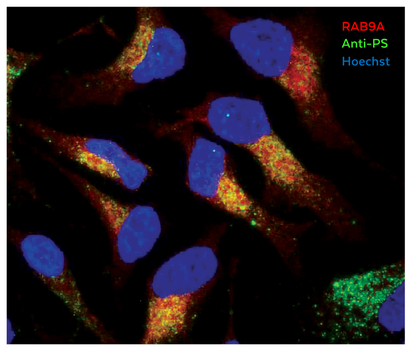


Fig 7. Intracellular trafficking. PS-modified ASO, detected by anti-PS antibody (clone PS03), was shown to colocalize with the RAB9A endosomal marker. HeLa cells were cultured, fixed with paraformaldehyde, reacted with anti-PS antibody (green) and anti-RAB9A (red), and counterstained with Hoechst (blue). Co-localization is seen for ONT and endosomes (yellow).

11. ANTI-MOE COLOCALIZATION (IF)

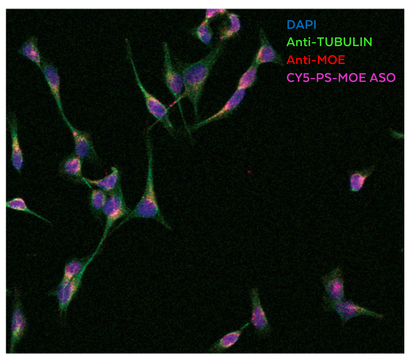


Fig 8. Colocalization of Cy5-conjugated PS-MOE ASO with anti-MOE antibody. HeLa cells were cultured and treated with 1 µM PS-MOE-modified ASO conjugated to the fluorochrome Cy5 by gymnotic uptake. After fixation with paraformaldehyde, cells were stained with DAPI (blue), anti-alpha tubulin (green), and anti-MOE antibody clone MOE-C (red). The fluorescence from Cy5-conjugated ASO (magenta) is shown to colocalize with anti-MOE antibody staining in the merged image.

12. ANTI-MOE BIODISTRIBUTION (IF)

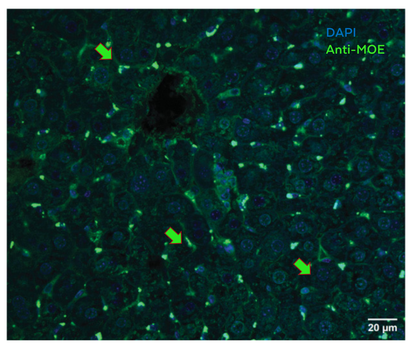


Fig 9. Biodistribution (IHC). Anti-MOE antibody (clone MOE4) detection of a 2′-MOE modified ASO 20-mer (5-10-5 gapmer containing ten MOE/PS bonds) delivered subcutaneously to mice (50 mg/kg, 72h). Liver tissue was immunostained with anti-MOE antibody (green) diluted 1:1,000 (overnight) and counterstained with DAPI (blue). Representative positive immunostaining indicates accumulation of the ASO in non-parenchymal cells surrounding hepatocytes (arrows). PBS-treated mice (negative control) showed no reaction (data not shown). Scale bar is indicated.

13. CONCLUSIONS

A limiting factor in the development of oligonucleotide drugs is the availability of analytical tools needed to perform assays required by regulatory agencies to assess efficacy and safety. Immunoassays empowered by the highly specific and sensitive antibodies described here represent an orthogonal approach to the classical assays used for analytical data collection. These tools offer considerable time and cost savings and may advance the rate at which these drugs are approved for use. Immunoassays like ELISA, IHC, and IF allow for the ranking of drug performance, quantification of ONTs, deposition of drug within tissue, the localization of drug within cells, and the assessment of dynamic trafficking within the cell. These antibodies can also serve as positive controls in both preclinical and clinical assays designed to assess immune response. We opine that highly specific antibody-based immunoassays capable of detecting as little as a single modified internucleotide linkage in 20 represents a significant advance and should be explored by the ONT field.

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14. REFERENCES

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3. Le BT, Paul S, Jastrzebska K, Langer H, Caruthers MH and Veedu RN. 2022. Thiomorpholino oligonucleotides as a robust class of next generation platforms for alternate mRNA splicing, Proc. Natl. Acad. Sci. U.S.A. 119 (36) e2207956119. https://doi.org/10.1073/pnas.2207956119.

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