APPLICATION GUIDE

ROCKLAND

Methods for Analysis of siRNA

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The distribution, localization, quantification, and trafficking of antisense oligonucleotides (ASOs) using ModDetect® antibodies has been recently reported.[1-5] These monoclonal antibodies (mAbs) are specific for chemical modifications, bind independently of nucleic acid sequence, and consist of panels that recognize commonly used modifications intended to stabilize ASO drugs (PS, 2′-MOE, 2′-OMe, and 2′-F). While prior work has focused on ASOs, here we extend the ModDetect® platform by presenting an optimized workflow for the sensitive and specific detection and quantification of small interfering RNA (siRNA) (see Fig 1).

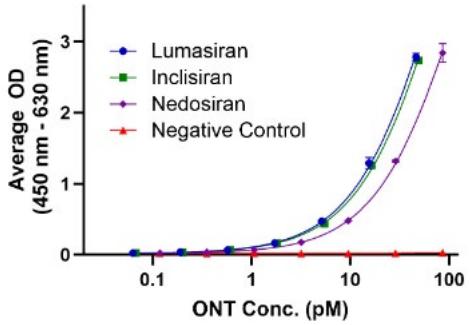

Fig 1. Quantification of three FDA-approved siRNAs using anti-OMe antibodies configured in a sandwich ELISA. PS-only modified negative control demonstrates specificity.

siRNAs represent an established therapeutic modality, recognized by the FDA as a platform technology[6] that leverages the endogenous RNA interference pathway to silence disease-associated genes. With several FDA-approved drugs and a growing clinical pipeline, siRNAs show strong potential across genetic, metabolic, and infectious diseases. Realizing this potential depends not only on effective delivery, but also on robust, sensitive, and sequence-independent methods for detection and quantification in biological systems. ModDetect mAbs enable such analysis through modification-specific recognition of siRNA.

Although anti-PS antibodies can be used for siRNA quantification, most siRNA designs incorporate a higher frequency of 2′-OMe modifications relative to PS linkages. This increased epitope density translates to improved assay sensitivity when using anti-OMe antibodies compared to anti-PS (see Fig 2). In addition, PS modifications are often localized to terminal regions of the sense and antisense strands, which may further limit antibody accessibility due to steric constraints. Accordingly, anti-OMe antibodies are recommended to maximize sensitivity and performance in siRNA detection assays.

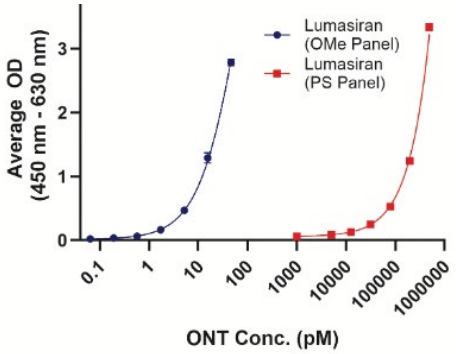

Fig 2. Comparison of siRNA lumasiran quantification in a sandwich ELISA using either anti-OMe or anti-PS mAbs. Greater sensitivity is observed using anti-OMe for capture and detection.

To enable quantitative analysis, a sandwich ELISA[7] was configured using unconjugated anti-OMe mAb for capture and a biotinylated anti-OMe mAb for detection. This format relies on binding to non-overlapping 2′-OMe epitopes within the siRNA duplex. Assay performance was verified using the FDA-approved siRNA therapeutics lumasiran, inclisiran, and nedosiran (Table 1).

Table 1. siRNA Materials

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

The protocol described here outlines the conditions used to generate these results; however, further optimization may be required based on the chemical composition of the siRNA to be quantified. The platform is broadly compatible provided non-overlapping OME epitopes are present within the siRNA and is highly reproducible (see Fig 3).

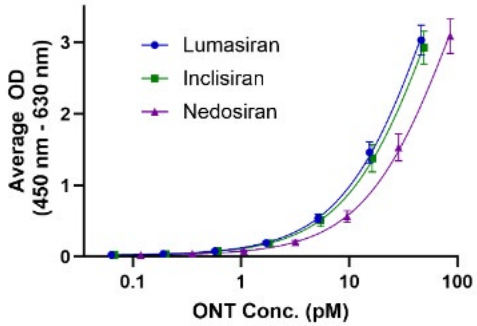

Fig 3. Reproducibility of three replicate experiments run over separate days each performed in triplicate. Error bars are shown.

In addition to quantitative measurement by sandwich ELISA, ModDetect anti-OMe antibodies enable spatial visualization of siRNA within cells by immunofluorescence microscopy, enabling visualization of intracellular distribution and localization. Because siRNAs typically contain multiple 2′-OMe modifications distributed along the duplex, anti-OMe mAbs serve as sequence-independent probes for intracellular detection across chemically diverse siRNA designs.

To demonstrate this application, HeLa cells were transfected with the siRNA lumasiran and subsequently stained using ModDetect anti-OMe mAb under optimized immunofluorescence conditions. This format allows direct visualization of OMe-modified siRNA following cellular delivery and fixation, supporting the use of ModDetect reagents beyond plate-based assays (see Fig 4).

Collectively, these results demonstrate that ModDetect anti-OMe mAbs provide a unified platform for both quantitative (ELISA) and spatial (immunofluorescence) analysis of siRNA in biological systems.

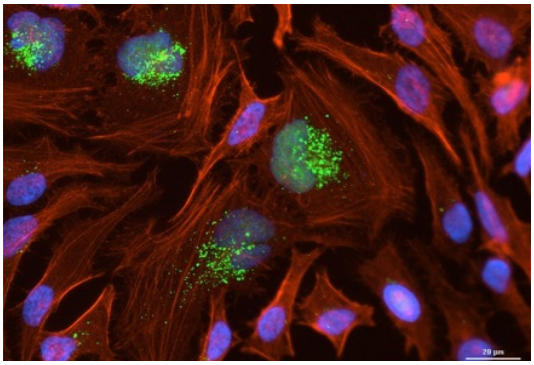

Fig 4. Detection of OMe-modified siRNA using anti-OMe mAbs by immunofluorescence. HeLa cells were cultured and transfected with 200 nM OMe modified lumasiran siRNA followed by detection of 2′-O-methyl modification using anti-OMe clone OME3. The antibody binds the siRNA in the cytoplasm in a pattern consistent 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).

Protocols

Anti-OMe Sandwich ELISA

Synthesize siRNA with more than one 2′-OMe modification. The 2′-OMe modifications must not be presented as overlapping epitopes, e.g., to minimize steric hindrance, to allow capture and detection. Signal may be proportional to the total number of 2′-OMe bonds present within the siRNA. Coat 96-well plates with 100 µL of ModDetect mAb clone OME3 *(Rockland, #200-301-NF7)* at 1.0 µg/mL in 0.1M sodium bicarbonate pH 9.5 and incubate overnight at 4°C. Wash three times with PBS-T *(Rockland, #MB-075-1000)* and then add 300 µL of Blocking Buffer for Fluorescent Western Blotting *(Rockland, #MB-070)* for 2 hours at room temperature. Dispense blocking buffer from wells and pat dry. Prepare a series of siRNA in sample buffer *(Rockland, #MB-070)* over the range of 0.06 pM (0.001 ng/mL) to 46 pM (0.8 ng/mL) concentrations by 3X serial dilution. Add 100 µL to each well in triplicates, followed by incubation for 2 hours at room temperature with agitation at 450 RPM. After three PBS-T washes, add 100 µL of biotinylated ModDetect mAb clone OME1 *(Rockland, #200-306-NF5)* prepared at 0.5 µg/mL followed by incubation for 1 hour at room temperature with agitation at 450 RPM. After three further PBS-T washes, add 100 µL of streptavidin-HRP *(Rockland, #S000-03)* at 0.125 µg/mL in sample buffer to each well for 30 minutes at room temperature protected from light with agitation at 450 RPM. After three additional PBS-T washes, add 100 µL of 3,3′,5,5′-tetramethylbenzidine *(Rockland, #TMBE-100)* and incubate for 30 minutes at room temperature in the dark. Stop the reaction by adding 100 µL of 1N HCl to each well and read the absorbance at 450 nm minus reference at 630 nm within 5 minutes.

Assay Parameters	Results	
	Lumasiran/ Inclisiran	Nedosiran
Range	0.063 – 46 pM (0.001 – 0.8 ng/mL)	0.12 – 86 pM (0.002 – 1.8 ng/mL)
Lower Limit of Quantification (LLOQ)	0.063 pM (0.001 ng/mL)	0.12 pM (0.002 ng/mL)
Upper Limit of Quantification (ULOQ)	46 pM (0.8 ng/mL)	86 pM (1.8 ng/mL)
Limit of Detection (LOD)	≤ 0.05 pM	
Coefficient of Determination (r^2)	≥ 0.98	
Assay CV%	≤ 20%	

Anti-PS Sandwich ELISA

Synthesize siRNA with more than one PS bond. The PS modifications must not be presented as overlapping epitopes, e.g., to minimize steric hindrance, to allow capture and detection by anti-PS antibodies. Ideally, PS modifications should be present at both the 5' and 3' ends of the siRNA on the sense and/or antisense strands. Signal may be proportional to the total number of PS bonds present within the siRNA. Coat 96-well plates with a mixture of ModDetect antibody clones PS04 and PS09 *(Rockland, PS04: #200-301-MV0; PS09: #200-301-MW3)* at 2 µg/mL each in 0.1M sodium bicarbonate pH 9.5 and incubate overnight at 4°C. Wash three times with PBS-T *(Rockland, #MB-075-1000)* and then add 300 µL of Blocking Buffer for Fluorescent Western Blotting *(Rockland, #MB-070)* for 2 hours at room temperature. Dispense blocking buffer from wells and pat dry. Prepare a series of siRNA in sample buffer *(Rockland, #MB-070)* over the range of 5 nM (0.087 µg/mL) to 500 nM (8.5 µg/mL) concentrations by 2.5X serial dilution. Further dilute a portion of the 5nM sample to 1 nM by performing a 5X dilution to establish the LOD sample. Add 100 µL to each well in triplicates, followed by incubation for 2 hours at room temperature with agitation at 450 RPM. After three PBS-T washes, add 100 µL of a biotinylated version of the same combined ModDetect antibodies *(Rockland, PS04: #200-306-MV0; PS09: #200-306-MW3)* prepared at 0.5 µg/mL each and added to the appropriate wells for 1 hour at room temperature with agitation at 450 RPM. After three further PBS-T washes, add 100 µL of streptavidin-HRP *(Rockland, #S000-03)* at 0.125 µg/mL in sample buffer to each well for 30 minutes at room temperature protected from light with agitation at 450 RPM. After three additional PBS-T washes, add 100 µL of High Definition 3,3',5,5'-tetramethylbenzidine *(Rockland, #TMBE-100)* and incubate for 30 minutes at room temperature in the dark. Stop the reaction by adding 100 µL of 1N HCl to each well and read the absorbance at 450 nm minus reference at 630 nm within 5 minutes.

Assay Parameters	Result
Range	5 – 500 nM (0.087 – 8.5 µg/mL)
Lower Limit of Quantification (LLOQ)	5.0 nM (0.087 µg/mL)
Upper Limit of Quantification (ULOQ)	491 nM (8.5 µg/mL)
Limit of Detection (LOD)	≤ 1.0 nM (≤ 0.017 µg/mL)
Coefficient of Determination (r^2)	≥ 0.98
Assay CV%	≤ 20%

Immunofluorescence

Wash cells once with room-temperature 1× phosphate-buffered saline (PBS) and fix in 4% paraformaldehyde (PFA) in PBS for 15 minutes at room temperature. Following fixation, wash samples twice with PBS. Permeabilize cells using 0.3% Triton™ X-100 in PBS for 10 minutes with gentle agitation, followed by three 5-minute washes in PBS. Reduce non-specific binding by incubating samples for 60 minutes at room temperature with gentle agitation in 5% goat serum prepared in 1× PBS. Sterile-filter (0.22 µm) Blocking Buffer prior to use. Dilute primary antibodies (*Rockland, OME3, #200-301-NF7*) at concentrations (1:50, 1:200, 1:500, 1:1000) in blocking buffer (PBS + 5% goat serum). Clarify antibody solutions by centrifugation prior to application. Incubate coverslips with primary antibody overnight at 4°C. Following primary antibody incubation, wash samples three times for 5 minutes in PBS containing 0.05% Tween-20, with gentle agitation. Dilute secondary antibody (*Rockland, Goat-Anti-Mouse Dylight™ 488, #610-141-121*) in blocking buffer (1:500 or 1:1000) and centrifuge prior to use. Incubate samples with secondary antibody for 1 hour at room temperature, protected from light. Following incubation, wash samples twice for 5 minutes with PBS-Tween (0.05%). Phalloidin counter-staining may be used as needed. Perform nuclear staining using NucBlue™ (*Hoechst, #33342*) for 5 minutes in PBS (without Tween) according to the manufacturer's instructions. Remove excess stain with one 5-minute PBS wash, followed by a brief rinse in deionized water to remove residual salts. Mount coverslips using Fluoromount-G (without nuclear stain) and seal with fast-drying nail polish prior to imaging.

ModDetect® Ordering Information

ModDetect 2′-O-Methyl (2′-OMe) Clones

Product	Unconjugated	Biotin
Clone OME1	200-301-NF5	200-306-NF5
Clone OME2	200-301-NF6	200-306-NF6
Clone OME3	200-301-NF7	200-306-NF7
Clone OME4	200-301-NF8	200-306-NF8
Clone OME5	200-301-NF9	200-306-NF9

ModDetect Phosphorothioate (PS) Clones

Product	Unconjugated	Biotin
Clone PS04	200-301-MV0	200-306-MV0
Clone PS09	200-301-MW3	200-306-MW3
	DL488	**DL549**
DyLight™ Clone PS04	200-341-MV0	200-342-MV0
DyLight™ Clone PS09	200-341-MW3	200-342-MW3

ModDetect Panels

Product	Format	Item No.
PS Panel	Unconjugated	KNA-100
PS Biotinylated Panel	Biotin	KNA-101
2′-MOE Panel	Unconjugated	KNA-200
2′-MOE Biotinylated Panel	Biotin	KNA-201
2′-OMe Panel	Unconjugated	KNA-300
2′-OMe Biotinylated Panel	Biotin	KNA-301

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