Generation of Monoclonal Antibodies to Nucleic Acid Modifications

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ABSTRACT

Nucleic acid therapies and vaccines have increased in public interest since the first mRNA COVID-19 vaccine was granted EUA approval in 2020. RNA-based therapies, such as RNAi and mRNA, are designed to modulate protein synthesis in patients and have applications in oncology, gene therapy and the prevention of infectious diseases. These oligonucleotides often contain one or more structural modifications that increase their stability, uptake and effectiveness but may increase the likelihood of toxicity. Different modifications may be incorporated, in varying degrees, to the nitrogenous bases or to the sugar-phosphate backbone, with phosphorothioate (PS) replacing the natural phosphodiester bond being among the most common. Balancing the level of modification with the effectiveness of the therapeutic candidate often results in large oligonucleotide libraries that lead to increased time required for screening and associated reagent expense. In situ hybridization (ISH) is the traditional method for the detection and localization of potential oligonucleotides in cells and tissues. Typically, ISH methods require probes unique to each oligonucleotide candidate increasing development budgets and time. Alternatively, antibody-based immunoassays can provide robust detection methods with similar results as ISH assays.

Antibodies generated to common oligonucleotide modifications can be used to detect multiple oligo nucleotide candidates independent of sequence or structure. A panel of monoclonal antibody detection reagents to the phosphorothioate backbone modification can be a valuable tool to support the efforts of RNAi therapeutic and mRNA vaccine industries. Isolating multiple B-cells from immunized hosts, these antibodies have different binding characteristics, such as sensitivity and specificity, and may be optimized for various immunoassays. Our oligonucleotide-antigen preparations, hybridoma development and antibody characterization methods provide the analytical tools to advance nucleic acid therapies.

ANTIBODY DEVELOPMENT

Electrophoretic Mobility Shift Assay of ON-Conjugates

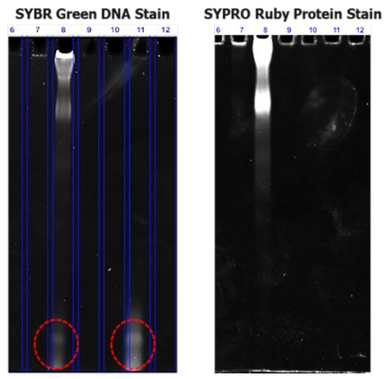

Figure 4. Mobility shifting assays confirming oligonucleotide conjugation to carrier protein. Oligo-carrier conjugations are confirmed by fluorescence staining. Both images are from the same agarose gel loaded with either ON-protein conjugate (lane 8) and ON only (lane 11) at equivalent amounts. On the left is SYBR green nucleic acid staining and on the right is SYPRO ruby protein staining. ON-Protein conjugates co-localize with both stains in the upper gel section, while unbound oligo can be seen migrating to the bottom of the gel (red circles added for clarity).

Fusion Events Resulting in PS-Specific Clones

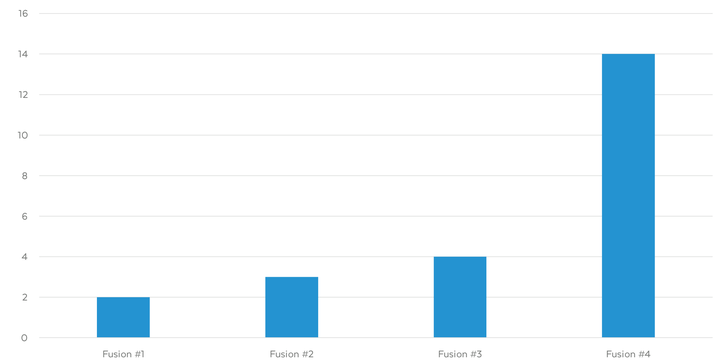

Figure 5. Successful hybridoma created for subsequent fusion events. The type of positive and negative controls during sera collection and post-fusion screening can play a significant role in identifying robust clones. Here we show how advancing the number of positive and negative controls, along with including known ON structures, can greatly increase the likelihood of creating hybridomas suitable for the detection of non-antigen ONs in immunoassays. Rockland has expertise in augmenting immunogens, adjuvants and administration routes to increase the likelihood of generation of highly specific antibodies.

INTRODUCTION

Oligonucleotides (ON) are short fragments of nucleic acid complementary to a specific mRNA or other types of nucleic acids, that can impede or amplify the expression of a gene, for instance by inhibiting or promoting transcription and translation of the corresponding protein. ON therapeutics and vaccines have been actively researched for 3 decades and broad approval of ON therapeutics is in process. The core problems with getting nucleic acid drugs to market have been and remain (1) poor stability and sensitivity to endo and exonuclease in cells and serum, (2) poor uptake into target cells or tissues, (3) off target effects due to partial complementation to an unintended target, and (4) immunogenicity or immuno-stimulation.

Over the last several decades development of ON technologies have addressed many of the central barriers to use of ON and mRNA. Chemical modifications make significant improvements into the stability of ON therapies without modifying the gene sequence and make potent targeting of gene-targets possible. In addition, targeted ligand-oligonucleotide conjugates, lipid nanoparticle (LNP) encapsulation, and antibody and small molecule conjugates show promise to improve oligonucleotide delivery and uptake. Reduction of off-target effects is challenging but improved sequence selection and chemical modifications have shown varying levels of success of decreasing toxicity while improving resistance to degradation. The innovation around ON has led to regulatory approval of anti-sense oligonucleotide and gapmer therapeutics and mRNA-based vaccines. Consequently, several clinical trials are underway for ON drugs to treat diseases including cancer, HIV/AIDS, Duchenne muscular dystrophy, and Cytomegalovirus retinitis.

Chemically modified nucleotides are the foundation to ON therapies and there are numerous nucleic acid modifications that have been developed. Figure 1 shows the modified inter-nucleotide linkages, as well as other alterations including morpholino, peptide nucleic acid (PNA), phosphoroacetate (PACE). The phosphorothioate (PS) backbone modification is the focus of this poster. It can affect binding affinity, but it inhibits degradation by nucleases and promotes interactions with blood proteins. The downside to PS is the toxicities associated with the binding to off-target proteins. Additional modifications can be made to 2' ribose positions and nucleic acid bases but are out of the scope of this work.

Rockland has experience successfully working with diverse nucleic acids chemical structures for antibody development. Rockland works closely with clients to properly understand the biochemical properties of the target nucleic acids and subsequently design appropriate antibody generation strategies. Presently there is a need to develop robust immunoassays to replace ISH-type assays, which monitor immunogenicity, absorption, distribution, metabolism and excretion, this requires development of antibody reagents. Since ON are not highly immunogenic this can be a difficult and time-consuming process.

Here we discuss the steps in the process for the development of an effective anti-oligonucleotide modification antibody, including: antigen design, immunization strategy, purification and manufacture. Nucleic acids by themselves are notoriously poor immunogens and are difficult to use unconjugated as an immunogen and as the analyte in immunoassays. However, by utilizing optimized methods for conjugation developed at Rockland, these limitations can be overcome with great success.

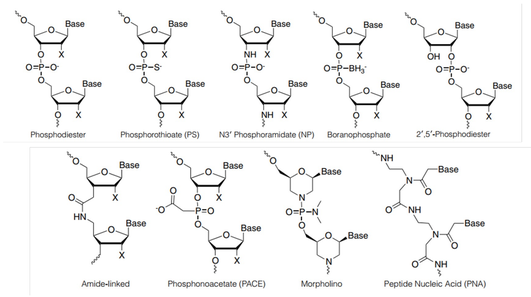

Figure 1. Examples of backbone chemical modifications

REAGENT PREPARATION STRATEGY

Standard Anti-Oligonucleotide Monoclonal Antibody Development

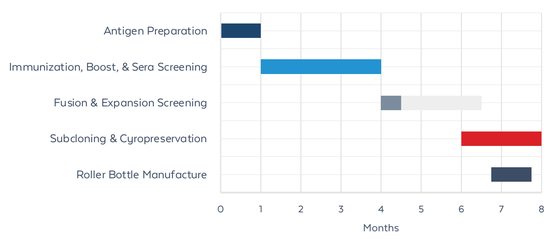

Figure 2. General timeline for anti-ON antibody reagent generation. The first phase of antibody generation relies on the quality of immunogen material. ON-carrier protein conjugates are effective when applied to the generation and purification of antibodies that specifically recognize nucleic acid-derived molecules. These ON-carrier protein conjugates also are useful in immunoassay screening for monoclonal selection. The use of carrier proteins is often necessary to induce immunogenicity to the ON especially since generating ON specific antibodies is a long process. The typical ON-antibody program is 2-4 times longer than comparable anti-peptide programs to generate titers suitable for use in downstream assay development.

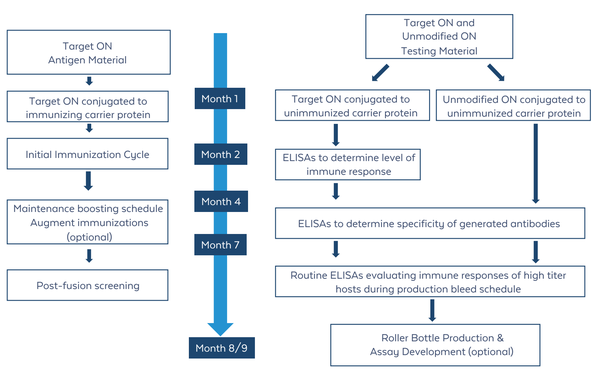

Figure 3. Flow diagram of ON antigen material and testing material. ON material is conjugated to carrier proteins according to project needs. A typical project requires 20-40 mg of target ON conjugated material be available for extended immunization cycles and ELISA screening, and an additional 10 mg of non-target ON conjugated material for screening.

ANTIBODY EVALUATION AND VALIDATION

Specificity of Anti-PS Antibodies Recognizing Target Modifications

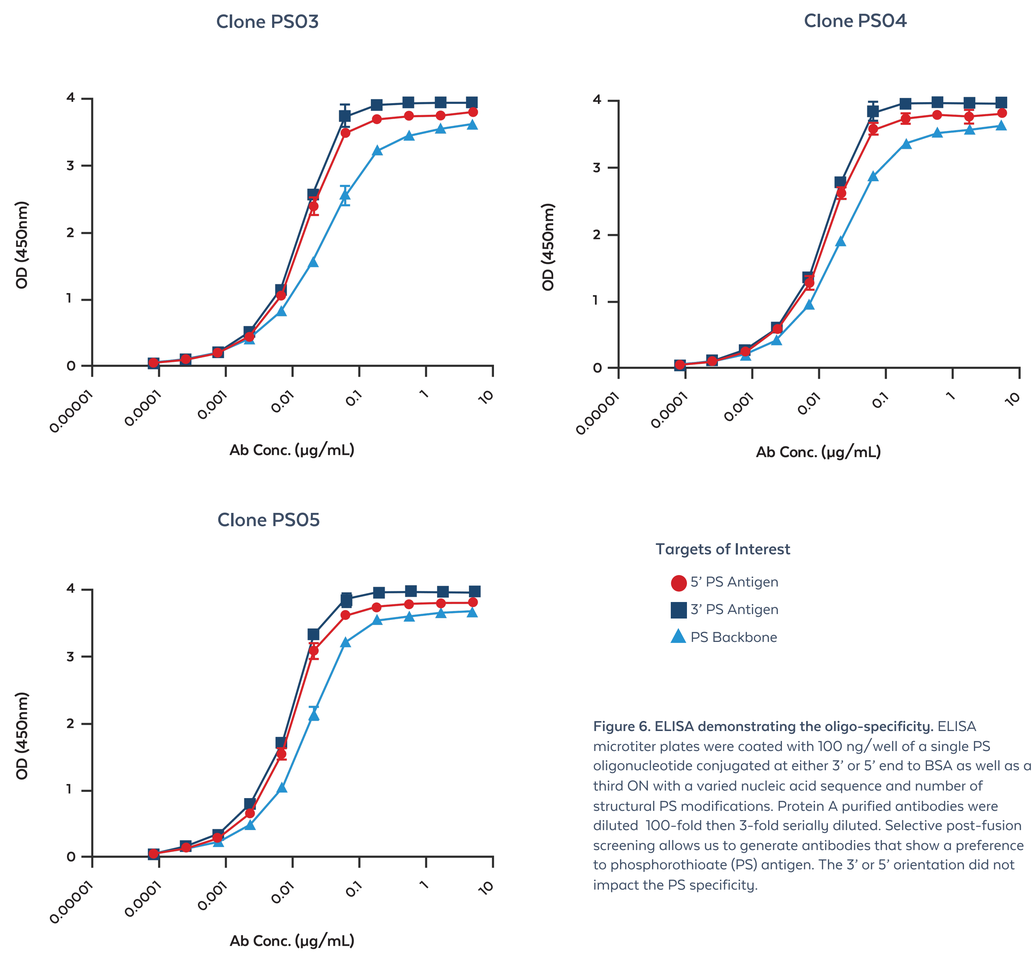

Figure 6. ELISA demonstrating the oligo-specificity. ELISA microtiter plates were coated with 100 ng/well of a single PS oligonucleotide conjugated at either 3' or 5' end to BSA as well as a third ON with a varied nucleic acid sequence and number of structural PS modifications. Protein A purified antibodies were diluted 100-fold then 3-fold serially diluted. Selective post-fusion screening allows us to generate antibodies that show a preference to phosphorothioate (PS) antigen. The 3' or 5' orientation did not impact the PS specificity.

CONCLUSION AND FUTURE DEVELOPMENT

In our work we set out to investigate the methods used to make anti-oligonucleotide antibodies. Here we tested methods for conjugation to carrier protein to make robust immunogen and screening reagents, tracked the trends of immune response to detect the PS modification, independent of the orientation of the location of the modification.

We were able to determine conditions that provided consistent ON-proteins conjugates thereby providing a high level of confidence in the input reagents for immunization. Also investigated was the diversity of immune response in mouse host animals using several different oligo-modifications. A common observation among the modified oligo antibody generation host cohorts was that advanced, additional screening techniques were needed to isolate a strong responder for hybridoma creation (diverse and high titer).

We also learned that it is common for the immune response to take 2 to 4 months longer than typical, and that the ability to create hybridomas from multiple hosts creates a survey of monoclonal antibodies with different sensitivity, specificity, and other binding characteristics. These differences create opportunities to generate reagents, highly optimized for specific immunoassays, as opposed to spending significant efforts to optimize assays to underperforming reagents.

Protein A purified monoclonal antibodies are a working solution to produce quality reagent for early method development. In future work, we will address additional anti-ON modification antibody targets with the goal of having a library of critical anti-ON modification antibody reagent panels.

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