Robust Methods for Anti-Oligonucleotide Antibody Generation

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ABSTRACT

Oligonucleotide therapies have gained momentum in the pharmaceutical industry since the approval of Fomivirsen, an anti-sense oligonucleotide therapeutic, in 1998. Oligonucleotides are nucleic acid fragments designed to influence gene products and therefore the biochemical processes within patients. Oligonucleotides often contain one or more structural modifications that increase their stability, uptake and effectiveness. The variety of therapeutic modes of action increases the value of oligonucleotides, exemplified by success in cell and gene therapies for liver and eye malignancies and in the treatment of cancers. Strategies include gene specific antisense oligonucleotides to disrupt the transcription of the defective gene; or using siRNA to induce cleavage of the mRNA transcript, disrupting expression of the bad gene. Additionally, different oligonucleotide modifications may be incorporated (e.g. nucleoside fluorination, or to the sugar-phosphate backbone components) with phosphorothioate backbone replacement being the most common.

Oligonucleotide therapeutics is a relatively immature field. Biotech and Pharma companies currently rely on conventional immunoassays as part of the bioanalytical strategy supporting clinical development and this requires the creation of de novo immunogenicity and anti-drug antibody assays to support required safety and quality standards. Critical to the development of these bioanalytical assays are antibody reagents specific to the modified-oligonucleotide. Making modified-oligonucleotide specific antibodies is not trivial, and known problems include poor antigen-immunogenicity and the antibody's ability to differentiate the modified-therapeutic from endogenous molecules. Here we summarize our expertise developing oligo-specific antibody reagents to a number of different target chemistries. Our methods, including oligonucleotide-antigen preparations and qualification, immunization strategies as well as characterization of highly specific antibodies, provide the bioanalytical tools to support development of oligo-based therapeutics.

ANTIBODY DEVELOPMENT

Electrophoretic Mobility Shift Assay of ON-Conjugates

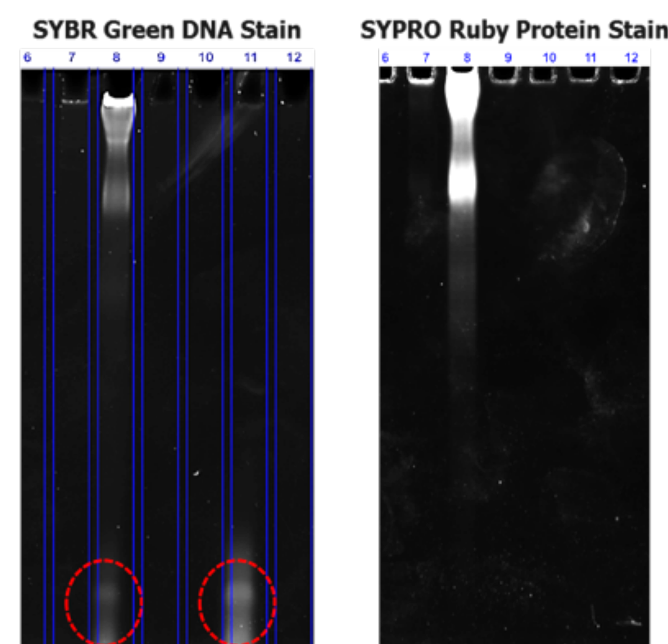


Figure 4 Mobility shifting assays confirming oligonucleotide conjugation to carrier protein. Oligo-carrier conjugations are confirmed by fluorescence staining. Both images are from the same agarose gel loaded with wither ON-protein conjugate (lane 8) and ON only (lane 11) at equivalent amounts. On the 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).

Time for development of titer Response for Different Antigen Types

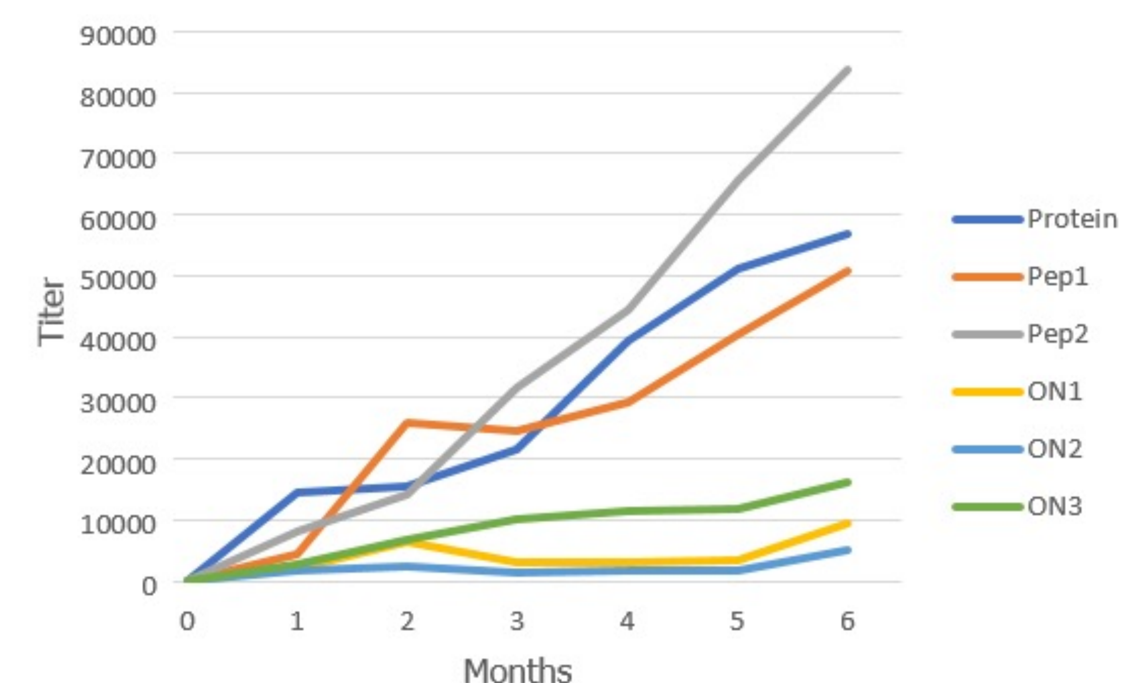


Figure 5 ELISA titers demonstrating rabbit host immune responses to different antigen types. The type of antigen can play a significant role in immunogenicity, even when conjugated to similar carrier proteins. Here we show variation in antibody generation programs for whole protein antigen (Protein), carrier protein conjugated peptide antigen (Pep1 and Pep2) and carrier protein conjugated oligonucleotide antigen (ON1, ON2 and ON3). Rockland has expertise in augmenting immunogens, adjuvants and administration routes to increase the likelihood of generation of high titer anti-sera.

INTRODUCTION

Anti-sense oligonucleotides (ASO) are short fragments of nucleic acid complementary to a specific mRNA or other types of nucleic acids, that can impede the expression of a gene, for instance by inhibiting transcription and translation of the corresponding protein. ASO therapeutics has been actively researched for 3 decades and broad approval of ASO therapeutics seems with reach. 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 (OTE) due to partial complementation to an unintended target, and (4) immunogenicity or immuno-stimulation. Over the last several decades development of ASO technologies have addressed many of the central barriers to use of ASO. 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, nanoparticle encapsulation, and antibody and small molecule conjugates show promise to improve oligonucleotide delivery and uptake. Reduction of OTE is challenging but improved sequence selection and chemical modifications has shown a level of success. Lastly, immunogenicity is poorly understood and can derive from multiple pathways most notably toll-like receptor engagement. The innovation around ON has led to regulatory approval of Vitravene/Fomivirsen, the first anti-sense oligonucleotide therapeutic (AON). Consequently, several clinical trials are underway for AON 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 phosphorothioate (PS) modified inter-nucleotide linkages, as well as other alterations including morpholino, peptide nucleic acid (PNA), phosphoroacetate (PACE). Our work will highlight They can affect binding affinity, but it positively affects sensitivity to nucleases and promotes interactions with blood proteins. backbone modification has been the focus recent work on ASOs.

Presently there is a need to develop robust assays to monitor for immunogenicity or immunostimulation and this requires development of antibody reagents. Since the oligos are not highly immunogenic this can be a difficult and time-consuming process. To date we have generated antibody reagents against multiple diverse nucleic acids chemical structures and modifications including conventional oligonucleotides (12-15 bases), DNA-RNA hybrids, PS and PdiS modified ASO backbones, single stranded oligonucleotide mixtures (cocktails), single stranded RNA (ssRNA) double stranded RNA (dsRNA), nucleotides and nucleosides.

Here we discuss the steps in the process for the development of an effective anti-oligonucleotide antibody, including: antigen design, immunization strategy, purification and manufacture. Nucleic acids by themselves are notoriously poor immunogens and are difficult to use unconjugated as and 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.

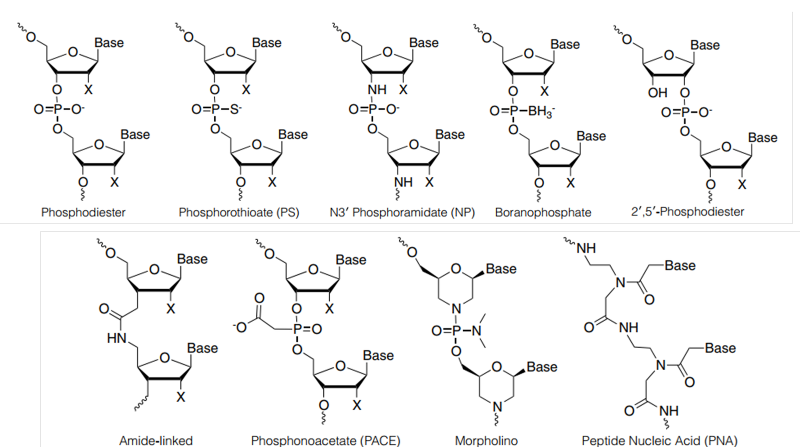


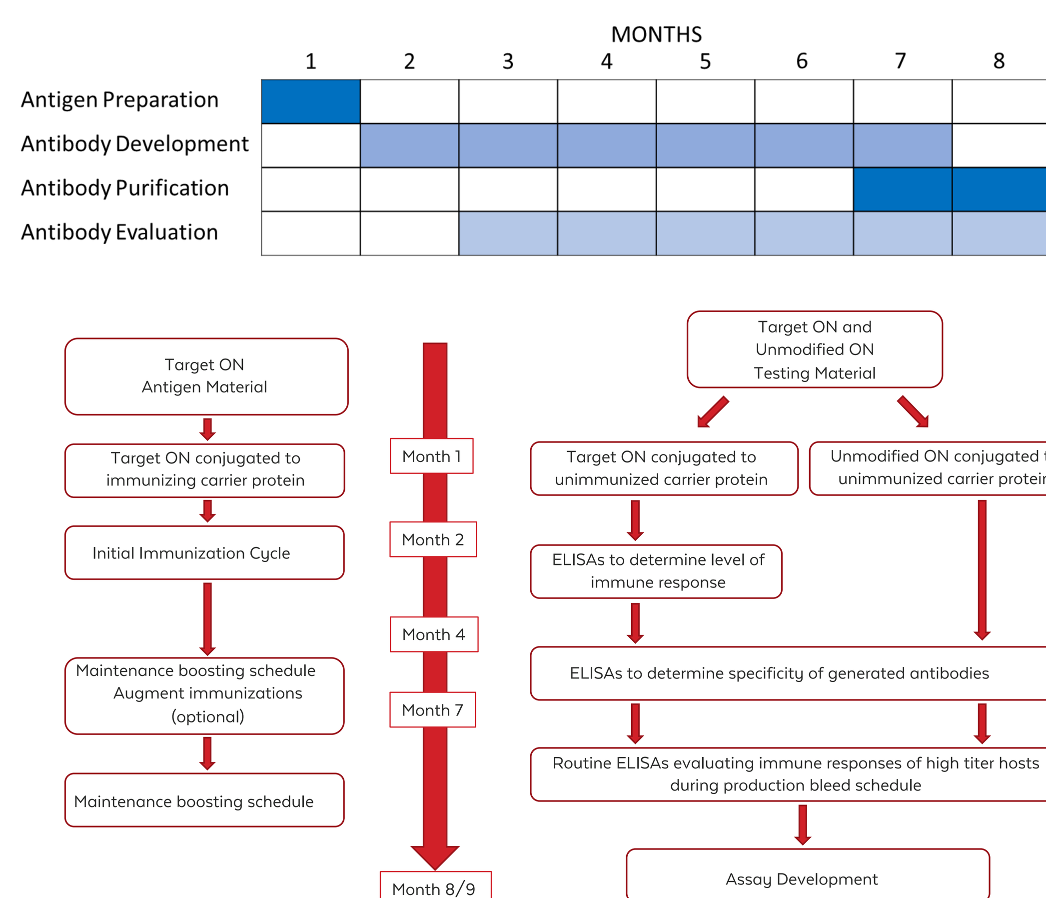
Figure 1 Examples of internucleotide chemical modifications

REAGENT PREPARATION STRATEGY

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 are also useful in immunoassay screening during antisera development and also in 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 3-4 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 30-50 mg of target ON conjugated material be available for extended immunization cycles and ELISA testing.

Standard Anti-Modified Oligonucleotide Timeline and Critical Reagent Preparation and Strategy



ANTIBODY EVALUATION AND VALIDATION

Diversity of Anti-ON Titer Response in a 9-host Cohort

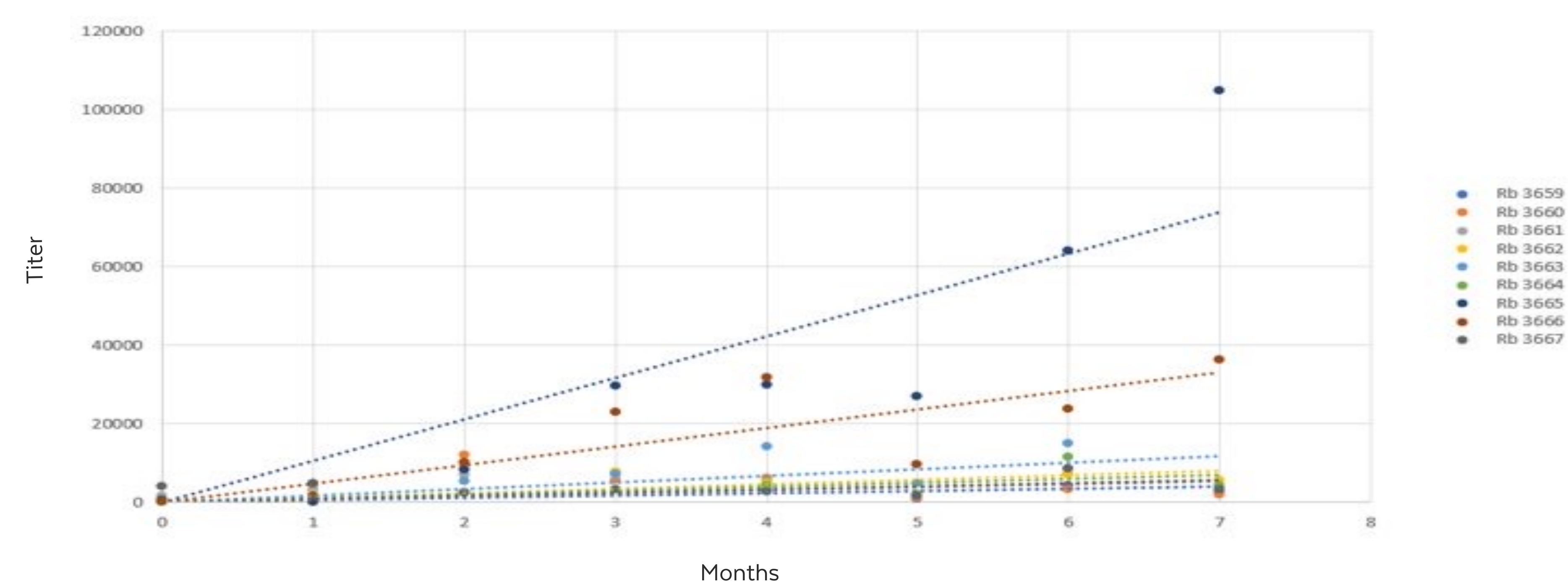


Figure 6 ELISA titer demonstrating the trend of immune response in a 9-rabbit cohort. ONs are poorly immunogenic, to overcome this Rockland applies strategies to increase immunogenicity and increase the diversity of the potential immune responses. Standard anti-ON programs begin with 9 animal cohorts, increasing the likelihood some of the hosts will produce high titer anti-sera. Figure 6 shows the trending of ELISA titers during an 8-month anti-ON antibody generation program. All hosts were immunized and test samples collected according an approved protocol. Host animals 3665 and 3666 showed titers sufficient to be moved to a production bleed schedule, and anti-sera was supplied for assay development.

ELISA Titration Curves of Rabbit Anti-Phosphorothioate Backbone

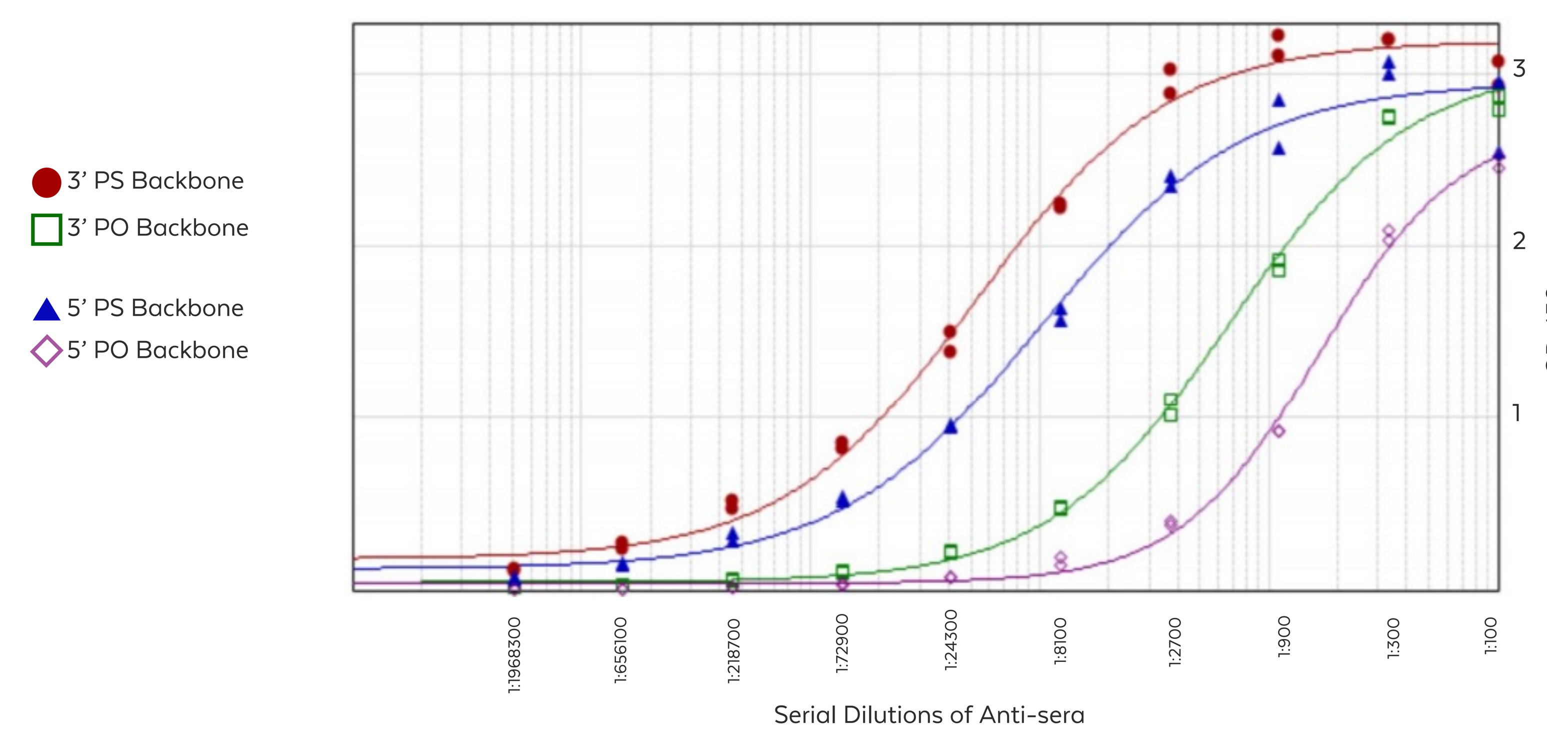


Figure 7 ELISA demonstrating the oligo-specificity. ELISA microtiter plates were coated with 100 ng/well of a single PO or PS oligonucleotide conjugated at either 3' or 5' end to BSA. The 4 ONs have identical nucleoside sequences. Antisera from a single rabbit was diluted 100-fold then 3-fold serially diluted. At 60 days into the immunization cycle, antibodies begin to show a preference to phosphorothioate (PS) antigen over biological phospho-diester (PO) oligonucleotides. 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 as well as the level of specificity to PS vs PO nucleotide linkages.

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 rabbit host animals using a number of different oligo-modifications (including DNA, RNA phosphorothioate and phosphoro-dithioate, and halogenated nucleotides). A common observation among the modified oligo antibody generation projects was that additional host animals beyond the typical 3 animal cohort are necessary to find a strong responder (rapid and high titer).

We also learned that it is common for the immune response to take 2 to 3 months longer than typical, and that 1-2 animals could be early responders while some animals show either a delayed or non-immune response.

We attempted several types of purification of the anti-ON antibodies. Our data suggested that new antibody purification methods are needed because traditional methods are not suitable when working with nucleic acid ligands. Presently antisera and protein A purified antibodies are a working solution to produce quality reagent for early method development, and current affinity purification methods require optimization. The core problem is co-elution of intact or fragmented target-ON. In future work we will address the anti-ON antibody affinity purification problem with the goal of having a bulk purification strategy for critical anti-ON antibody reagents.

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