Use of the N-PLEX™ Platform for the Detection of Antisense Oligonucleotides (ASOs) in Plasma

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1 Introduction

The number of FDA-approved drugs that are revolutionizing treatment strategies for cancer and other diseases is on the rise. One of the categories of drugs that is gaining prominence is antisense oligonucleotides (ASOs), which are modified, single-stranded DNA or RNA molecules (generally less than 35 nucleotides). These ASOs are often complementary to specific RNA or DNA sequences and produce alteration of the protein corresponding proteins. Due to their short length, however, it is difficult to measure ASOs in circulation for PK studies. N-PLEX™ MS/MS is frequently used to detect ASOs in biological samples, which provides good specificity but low sensitivity. Hybridization ELISA techniques have also been described and have lower sensitivity, but greater sensitivity is still needed in many cases to enable thorough PK studies.

We sought to address this limitation by developing methodology around Meso's newly developed nucleotide detection platform, N-PLEX. This primary goal was to establish reliable solutions that could measure antisense oligonucleotides with detection on the N-PLEX platform to allow for high-sensitivity detection of ASOs through chemiluminescence. One of the most common biological matrices that is used for PK studies, including ASOs, is plasma. Therefore, this methodology was developed and optimized for detection of ASO plasma samples, in which sub-pM detection was achieved in all three techniques discussed: OLA-mediated detection, T4 ligation-mediated detection, and detection on an iRrieve Protection assay.

2 Methods

2.1 N-PLEX™ Technology

N-PLEX™ technology is a chemiluminescent detection technology that utilizes SULFO-TAG™ labels that emit light upon electrochemical stimulation initiated at the electrode surfaces of MultiLUM™ and MultiSPOT™ receptors.

2.2 Electrochemiluminescence Technology

Minolta-specific background and strong responses to analytes yield high signal-to-background ratios. The stimulation mechanism (electrolysis) is decoupled from the response signal (light), rendering mass stability.

Surface coatings can be customized. Surface coatings can be customized.

2.3 Back of the N-PLEX Platform

N-PLEX plates contain 15 unique capture oligonucleotides, each of which is bound to its corresponding spot on the electrode surface. Detection of a nucleic acid sequence of interest is accomplished by hybridization of one or more probes with sequence complementarity to these capture oligos and the nucleic acid of interest, followed by detection via electrochemiluminescence (ECL). Electrochemiluminescent interactions with SULFO-TAG® minimize background. Streptavidin, hybridization, and detection are completed using Meso proprietary buffers and solutions.

Model ASO

For the development of methodology for ASO detection on the N-PLEX platforms, a model DNA oligo was selected for each of the techniques assessed. The model ASO that was chosen for this study is a 25mers DNA oligo with the following sequence:

5′-GAGACAGTGCAGGCAGGCGGC-3′

Plasma Samples

Naive male C57BL/6J mice plasma samples in sodium citrate were purchased from BioIVT (Westbury, NY). For experiments involving the use of plasma, exogenous ASO was added to high calcium (3.5 mM) and univalent electrolyte trials.

ASO Detection with Amplification Via the Oligonucleotide Ligation Assay (OLA)

The oligonucleotide ligation assay (OLA) used two probes that are complementary to the first 10 or last 10 nucleotides of the ASO. One probe contained a strict specific sequence at the 5′ end that allowed for hybridization to the OLA probe, while the other probe contained a phosphorothioate group on the 5′ end that served as an anchor for ligation and subsequent elongation. The hybridized DNA ligase was used for multiplex rounds of ligation of the two probes when appropriately hybridized to the ASO. The ligated probes were hybridized to specific capture oligos on the N-PLEX plates to allow for detection via electrochemical detection bound to SULFO-TAG.

ASO Detection Via T4 Ligation-mediated Ligation

T4 ligase-mediated detection of the ASO used the same probes as OLA-mediated detection. However, T4 DNA ligase was used and only allowed for one round of ligation of the two probes when appropriately hybridized to the ASO. The ligated probes were hybridized to specific capture oligos on the N-PLEX plates to allow for detection via electrochemical detection bound to SULFO-TAG.

ASO Detection Via RNase Protection Assay

The RNase protection assay utilizes a single-stranded probe for the detection of the modeled ASO on the N-PLEX platform. This complementary DNA probe contained a 5′ nucleotide sequence that was complementary to the plate-bound capture probe followed by an RNA portion that was complementary to the model ASO and a biotin on the 3′ end for detection via streptavidin bound to SULFO-TAG. Plasma samples were pretreated with RNaseA reagent (ThermoFisher Scientific) and treated to 70 °C for 10 minutes to inactivate endogenous RNaseA enzymes. The probe was hybridized to both the ASO and the plate. RNaseA was added to degrade any single stranded RNA sequence. Therefore, any RNA in the probe not fully protected by the ASO was degraded and the signal released from the DNA portion of the probe, resulting in a detectable signal via electrochemical bound to SULFO-TAG.

3 Results: OLA-mediated ASO detection

Optimized Protocol

A 10-point calibration curve was generated by spiking ASO into buffer or murine plasma, serially diluting with 4-fold dilutions (166 pM top of curve) and hybridized with probes and T4 DNA ligase. Four blots were used for each OLA (LLODest). OLA was performed with the following protocol: 2 min at 95 °C and 30 cycles of 30 s at 95 °C and 5 min at 37 °C. N-PLEX plates were blocked for 30 min at 37 °C before OLA incubation.

3.1 Plasma Samples

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3.2 Results: OLA-mediated ASO-detection

3.3 Results: T4 Ligase-mediated ASO-detection

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3.4 Results: RNase Protection Assay

Optimized Protocol

A 10-point calibration curve was generated by spiking ASO into buffer or murine plasma, serially diluting with 4-fold dilutions (166 pM top of curve) and hybridized with probes and T4 DNAligase. Four blots were used for each OLA (LLODest). OLA was performed with the following protocol: 2 min at 95 °C and 30 cycles of 30 s at 95 °C and 5 min at 37 °C. N-PLEX plates were blocked for 30 min at 37 °C before OLA incubation.

3.5 Results: Reproducibility Testing

Optimized Protocol

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3.6 ASO Detection Methodology Comparisons

<table>
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<th>Technique</th>
<th>Sample Type</th>
<th>Detection Limit (fM)</th>
<th>R2</th>
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4 Conclusions

These data indicate that, paired with various techniques, the N-PLEX platform is a viable option for highly sensitive detection of ASOs in plasma. Highly reproducible results were obtained for each method described.

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