# Selection and Evaluation of Hybridization Capture Probes for LC/MS Analysis of Oligonucleotides

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## Introduction

LC/MS bioanalysis of oligonucleotides has had its historical challenges in all areas of the workflow including extraction, liquid chromatography, and mass spectrometry detection. Among the primary pain points for the extraction of oligonucleotides is poor recovery from nonspecific binding or poor extraction efficiency using the most common extraction approaches. Recent publications of a more specific biotinylated probe hybridization approach have addressed these challenges, however, there hasn't been much presented on the specific advantages for different probe types that can be used for this hybridization work. Although there has been a focus on DNA, LNA, and PNA probe design with research to demonstrate the specific attributes each offers, there has been limited discussion on the overall impact on recovery and interference from these different probes. Biotinylated probe design has been focused on limiting self-hybridization of the probe while maintaining a complimentary sequence with a sufficiently high score to out-compete any interferences from matrix or from the sense strand in siRNA modalities while keeping the melting temperature (Tm) of the hybridized duplex low enough to ensure recovery from the streptavidin beads. Recently, while developing an assay for a siRNA complex using the LNA approach, we observed interferences from the probe to the antisense strand when it was analyzed by mass spectrometry. We modified the melting temperature to release the streptavidin/biotinylated hybridized to avoid releasing most of the biotinylated probe/antisense complex, but the limited release still had enough of the probe in the final extracts to cause interferences in the LLOQ samples. Modifying LC conditions helped to resolve the interference, but those changes were not entirely successful due to peak shape issues with the internal standard. After reviewing the probe design, we implemented an alternate PNA probe with the intention that any residual PNA probe would be easier to resolve chromatographically.

## Overview and Methodology

Biotinylated PNA and LNA probes were selected based on the predicted physical properties of a complementary sequence generated by manufacturer-supplied computational tools. In general, probes were selected if their predicted RNA melting temperature was >80°C, their guanine and cytosine (GC) content was between 30-50%, and the likelihood of self-hybridization was minimal. This ensured that they were easily extracted via magnetic bead separation on the Thermo Kingfisher platform.

LNA probes were further scrutinized by ensuring that there were no stretches of greater than 4 LNA nucleotides and these stretches were spaced out to avoid clustering. Cytosine and thymine LNA nucleotides were most impactful for governing melting temperatures. PNA probes were selected if they had fewer than 6-purine (adenine and guanine) stretches and <50% purine content.

All probes were then screened for recovery and potential interferences and/or suppression relative to the target siRNA. In this instance, LNA probes were ruled out based on a co-eluting interference with the target at the mass transition which would impact linearity at LLOQ concentrations.

A 25.0uL aliquot of rat plasma fortified with the target siRNA was placed into a 96 well Kingfisher plate followed by 100 mL of loading buffer, Tris digestion buffer and TCEP. Each sample was then fortified with the PNA probe and Proteinase K. The plate was incubated at 65°C for approximately one hour and then cooled to hybridize the PNA probe to the antisense strand. Streptavidin-functionalized magnetic beads were then added to the samples to bind the probe to the bead. The plate was processed on the Kingfisher and the antisense strand was melted off the streptavidin beads at 95°C to a clean 96 well DNA LoBind plate containing 100 µL of an aqueous solution and 10.0 mL of working IS. Extracts were stored at 5°C until analysis via Sciex API 7500 with an ESI (-) source using a Thermo DNApac column (2.1 x 50mm, 4.0 um) heated to a temperature of 85°C. A simple linear gradient was run at 0.400 mL/min from 10% organic to 35% organic over 2.5 minutes to elute the target siRNA.

## Method Development

Initial method development mimicked the LNA probe selection and workflows described in the paper authored by Karan Agrawal (Bioanalysis-2023-0079), paying close attention to the self-hybridization score and maximizing the melting temperatures of the LNA-AS complex to be above 80°C but below 90°C. To determine the final methodology for extraction of the AS strand while keeping the aliquot size at 25.0 mL, we ran experiments to determine conditions for the LNA capture probe, the amount of Proteinase K required, the digestion time, and time required to anneal the capture probe to the antisense strand.

After determining the initial extraction conditions, it was noted that interferences were observed in the IS only samples that were determined to be from the presence of free LNA-probe in the final extracts. Conditions were optimized for the "melting" step on the Kingfisher, but the interference was not eliminated. LC conditions were modified to minimize the interference but optimal peak shape for both the ASO and the IS could not be achieved while still separating the interference (Figures 1-3). We then revisited the overall probe design and looked to the probe type with the theory that a PNA probe rather than a LNA probe might eliminate the interferences with LC conditions common to oligonucleotides since the backbone of the PNA is neutral instead of negative. After redesigning the capture probe from LNA to PNA we were able to obtain extraction conditions that had both high recovery and no interference from the capture probe.

**Figure 1** Reagent Blank

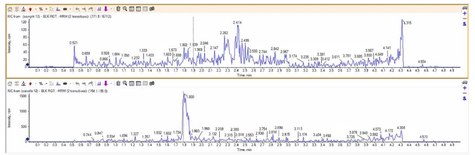


**Figure 2** Reagent Blank with LNA Probe

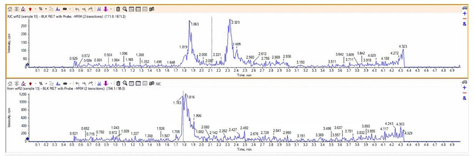
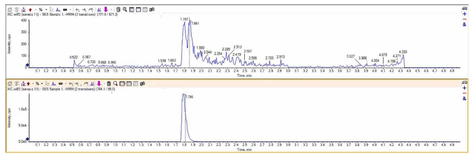


**Figure 3** Reagent plus Int. Std., with 5 ng/mL siRNA and LNA Probe



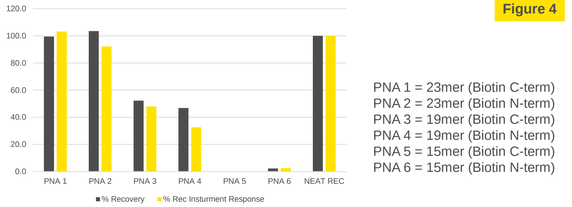
## PNA Probe Evaluation

Both PNAs and LNAs are able to bind to complementary RNA sequences with a relatively high affinity when compared to DNAs. Overall, PNAs have a neutral charge versus the negative charge that is observed with LNAs which leads to poor solubility of a PNA probe in aqueous conditions. PNAs will also aggregate when there is high purine content in the sequence, therefore long purine stretches should be avoided to increase water solubility. Finally, the longer the PNA sequence you have, the less water soluble the strand may be in aqueous solutions leading to lower recovery of the ASO. Therefore, the following strategies should be considered when designing a complementary PNA probe.

- Reduce purine content, especially G bases
- Avoid long purine stretches
- Avoid self-complementary sequences to reduce PNA/PNA self hybridization

To develop the siRNA assay with a PNA probe, six probes were designed to evaluate performance based on the number of complementary base pairs and solubility. The siRNA of interest was 23 base pairs in length and the probes ranged from 23 base pairs down to 15 base pairs in length (PNA1-PNA6). We also looked at differences between putting the biotin tag onto either the N-terminus or C-terminus side of the PNA probe. Both specificity and Tm increased for the hybridized duplex as the length increased with the goal to maximize specificity while keeping the Tm low enough to release the AS strand for detection. The amount of G bases was kept consistent to minimize the variables and keep solubility to length of the PNA. Overall, the length nor the site of biotin conjugation of the PNA strand was not a determinant in solubility or Tm, but the length was a factor in overall recovery which is most likely due to specificity of the PNA to the AS strand (Figure 4). As predicted, the neutral backbone of the PNA strand eliminated interference from the probe to the AS strand with the established LC ion pairing conditions (Figure 5).

**Figure 4**




PNA 1 = 23mer (Biotin C-term)
PNA 2 = 23mer (Biotin N-term)
PNA 3 = 19mer (Biotin C-term)
PNA 4 = 19mer (Biotin N-term)
PNA 5 = 15mer (Biotin C-term)
PNA 6 = 15mer (Biotin N-term)

**Figure 5**

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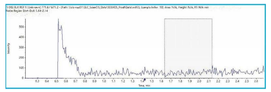
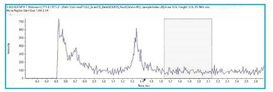
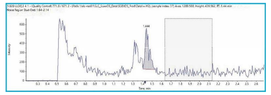


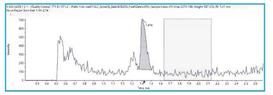
**Matrix Blank**



**0.25 ng/mL in Matrix**



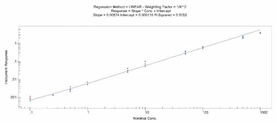
**0.50 ng/mL in Matrix**



## Final Method

Extraction

- Aliquot 50 µL of calibrators and QCs to KingFisher plate
- Add 10 µL of PNA probe (7.5 µM)
- Add 100 µL of Clarity OTX Lysis Buffer
- Add 200 µL Digest Buffer (100mM Tris-HCl, 250 mM NaCL, 10 mM TCEP and 10 µM EDTA in water)
- Add 5 uL of Proteinase K (20 mg/mL solution)
- Incubate at 60 °C for 1 hour
- To wash plates 1 and 2, add 500 µL of 100mM Tris-HCl, 500 mM NaCL, 1 mM EDTA in water
- To the elution plate, add 100 uL of 1%HFIP, 0.1% DIPEA, and 10 µM in water
- Place sample plate into kingfisher
- Remove sample plate at programed pause step and cool to room temp for 15 minutes
- Add 30 µL of MyOne Streptavidin C1 magnetic beads at 10 mg/mL
- Add 300 µL of Capture Buffer (100mM Tris-HCl, 500 mM NaCL, 1 mM EDTA, 0.05% Tween-20 in water)
- Place prepared wash plates, elution plate, and sample plate back into KingFisher and resume protocol
- Remove elution plate containing extracted ASO and add 50 µL of internal standard
- Analyze via LC/MS




| | LLOQ 2 | LLOQ 1 | LQC | MQC | HQC |
|---|---|---|---|---|---|
| Theor. Conc. | 0.250 | 0.500 | 1.50 | 15.0 | 80.0 |
| Found Conc. | | | | | |
| #1 | & 0.307 | 0.508 | && 1.16 | 14.8 | 71.6 |
| #2 | & 0.316 | 0.455 | 1.28 | 13.8 | 84.4 |
| #3 | & 0.304 | & 0.345 | 1.51 | 14.9 | 85.6 |
| #4 | 0.263 | 0.500 | 1.48 | 14.1 | 74.1 |
| #5 | 0.275 | 0.581 | 1.57 | 14.0 | 86.7 |
| #6 | & 0.304 | 0.518 | 1.62 | 16.0 | 79.7 |
| | | | | | |
| Mean | 0.295 | 0.485 | 1.44 | 14.6 | 80.4 |
| S.D. | 0.0208 | 0.0794 | 0.179 | 0.817 | 6.33 |
| %CV | 7.1 | 16.4 | 12.4 | 5.6 | 7.9 |
| %Theoretical | 118.0 | 97.0 | 96.0 | 97.3 | 100.5 |
| %Bias | 18.0 | -3.0 | -4.0 | -2.7 | 0.5 |
| n | 6 | 6 | 6 | 6 | 6 |

## Conclusions

Among the typical comments received from pharmaceutical companies considering a LC/MS hybridization approach for the analysis of siRNA are those surrounding the complications involved in the design of the capture probe. Although a workflow has been documented with regards to the physical attributes of the complimentary strands melting point and its own self hybridization score, there is little documented with regards to avoiding interference from the capture probe to the analyte of interest. Here we consider the removal of potential interference by using capture probes of the opposite polarity to the analyte of interest to fully resolve any potential contribution back to the analyte. The use of biotinylated capture probes for the extraction of oligonucleotides has produced clean extracts, high recovery, and low LLOQs, but there is still more research to be done to make the design of the capture probes more efficient, reducing the overall time required to develop a method. Here we outline another approach that can be used in cases where the LNA capture probe may produce interference to the oligonucleotide being analyzed in the method.