Altasciences, 575 Armand-Frappier, Laval, Québec, H7V 4B3, Canada
Altasciences.com | contact@altasciences.com

Development of a Hybridization LC-MS Methodology for Quantitation of siRNA in Cynomolgus Monkey

Jean-François Dupuis[1], Long Yuan[2], Jean-Nicholas Mess[1] and Kevork Mekhssian[1]

[1]Altasciences, Laval, QC, Canada; [2] Biogen, Cambridge, MA, USA

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INTRODUCTION

Small interfering RNA (siRNAs) are short, double-stranded oligonucleotides that can regulate gene expression by inducing mRNA degradation (**Figure 1**). Quantitative bioanalysis in various biological matrices is critical to understanding the PK/PD parameters of therapeutic oligonucleotide drug candidates. Hybridization LC-MS/MS has been recently established as a successful methodology for quantifying single-stranded oligonucleotides in plasma. This approach relies on capturing the oligonucleotide of interest with a full-length complementary DNA probe, followed by analysis with LC-MS/MS (**Figure 2**). Applying the same workflow to double-stranded siRNA presents additional challenges, as the probe competes with the sense-strand of the siRNA. This study presents the first adaptation of the hybridization LC-MS/MS workflow for quantifying a double-stranded oligonucleotide (siRNA1).

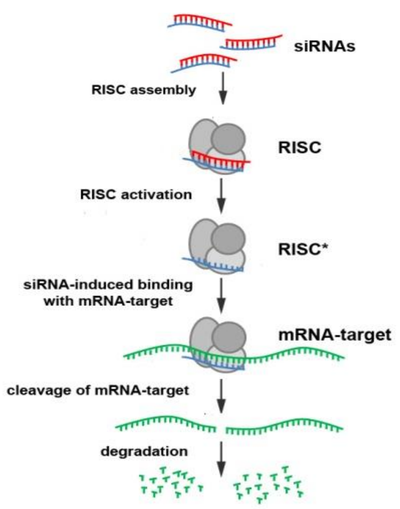

Figure 1. siRNA mechanism of action (Image adapted from Petrova N.S *et al.,* 2013)

METHODS

HYBRIDIZATION LC-MS/MS

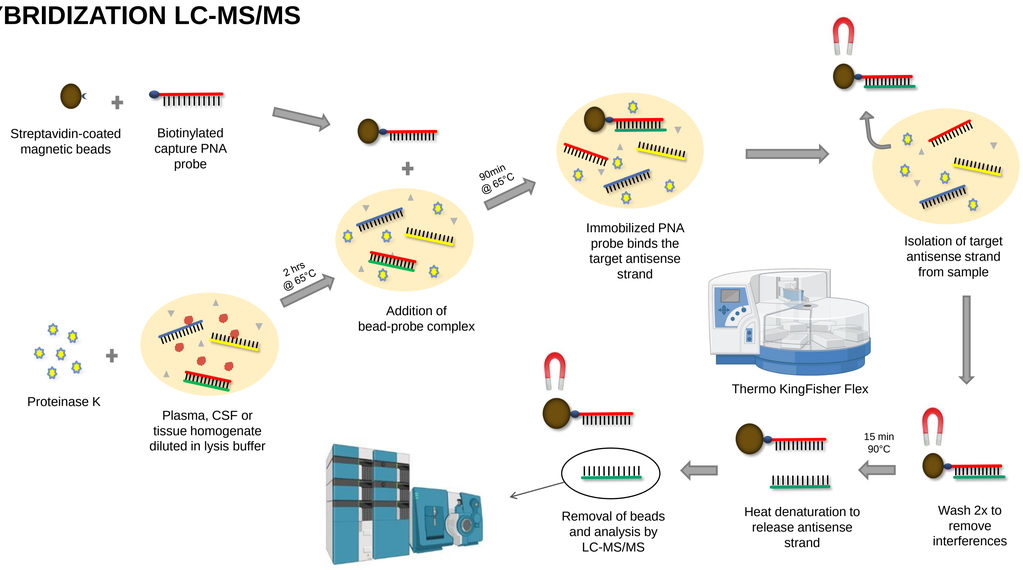

Figure 2. Hybridization LC-MS/MS workflow for the quantitation of siRNA in biological matrices

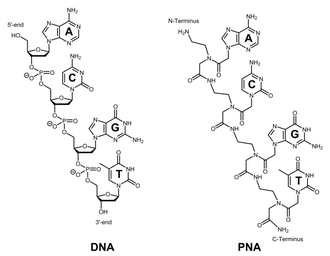

Figure 3. Deoxyribonucleic acid (DNA) and peptide nucleic acid (PNA) structures

Table 1. Analyte and Internal Standard Parameters

Oligo	MW (kDa)	Length (bp)	Chemistry	MRM
AS1 (siRNA1 antisense strand)	6.9	21	Mixed backbone with 2'-OMe and 2'-F modification	629 / 95
Internal Standard	7.9	20	Uniform MOE with PS backbone	879 / 95

RESULTS

In the hybridization step, a full-length complementary DNA-based probe is typically employed to capture single-stranded oligonucleotides because of its high affinity and specificity. This DNA-based probe achieved near-complete recovery of the antisense strand (AS1) of siRNA1, independent of temperature and salt concentration (**Figure 4, left**). However, under the same conditions, recovery of the double-stranded siRNA was low at lower temperatures and high salt concentrations due to the competition from the siRNA1 sense-strand (**Figure 4, right**).

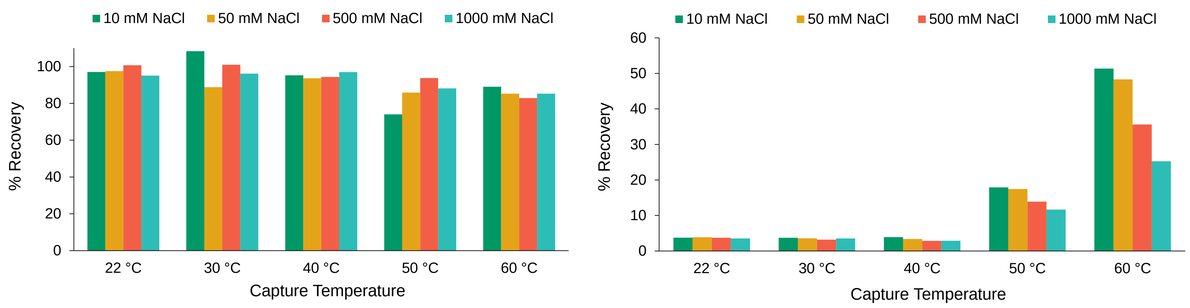

Figure 4. Capture efficiency (% Recovery) of AS1 (left) and siRNA1 (right) using a DNA-based probe at different temperature and salt concentrations

The recovery of siRNA1 utilizing PNA and DNA-based probes was compared. The PNA capture probe offers a key advantage due to its lack of a negatively charged deoxyribose phosphate backbone, which eliminates electrostatic repulsion with the siRNA1 antisense strand. Moreover, the higher melting point of the PNA:RNA complex allows for more efficient annealing at elevated temperatures. Under conditions of 65 ºC and 10 mM NaCl (**Figure 5**), the PNA probe achieved a 90% recovery compared to 55% with the DNA probe. The PNA-probe conjugated beads were then incorporated into the extraction workflow, and the assay was evaluated for precision and accuracy (**Table 2**).

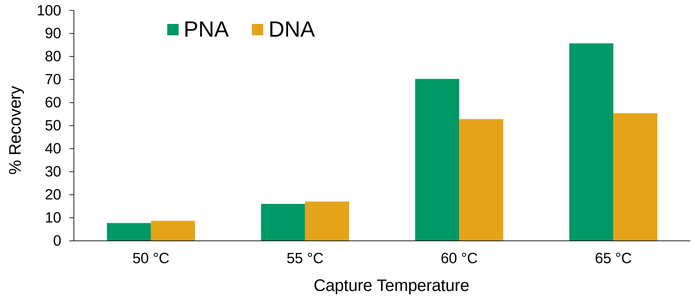

Figure 5. Capture efficiency (% Recovery) of siRNA1 using PNA- versus DNA-based probe at increasing temperature

Table 2. Precision and Accuracy of siRNA1 in Cyno Plasma Extracted Using the PNA Probe

QC Sample	LLOQ QC 2.00 ng/mL	LQC 6.00 ng/mL	MQC 500 ng/mL	HQC 750 ng/mL
1	1.98	5.87	527.11	681.34
2	1.94	6.50	441.75	674.75
3	2.05	5.91	464.04	756.63
4	2.29	5.56	469.18	753.45
Mean	2.07	5.96	475.52	716.54
S.D.	0.16	0.39	36.40	44.55
n	4	4	4	4
% C.V.	7.6	6.6	7.7	6.2
% Nominal	**103.3**	**99.3**	**95.1**	**95.5**

CONCLUSION

An analytical method for the quantitation of double-stranded siRNA1 using a PNA-based probe was successfully developed. The method is accurate and precise across a range of 2.00 to 1000.00 ng/mL in cyno plasma. With minor adjustments, the assay was subsequently qualified in CSF and tissues and used to support a nonclinical study in cynomolgus monkeys.

REFERENCE

Yuan, L. Dupuis, J-F, Mekhssian ,K. A Novel Hybridization LC-MS/MS Methodology for Quantification of siRNA in Plasma, CSF and Tissues Samples *Molecules* 2023