Case Study: A Fast, Novel Strategy To Select an Internal Standard When Developing an Oligonucleotide LC-MS Quantification Method

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Introduction

Antisense oligonucleotide therapies are currently being investigated for the treatment of genetic and neurodegenerative diseases. They have a shorter drug development time as compared to traditional small molecule drugs and antibody-based large molecule drugs. An oligonucleotide can vary in length but typically consists of about 20 nucleotides that bind to target RNA through Watson-Crick base pairing. By binding to the target RNAs, an oligonucleotide can alter gene expression and prevent the translation of proteins to achieve a therapeutic effect. Similar to protein-based therapies, hybridization immunoassays, such as enzyme-linked immunosorbent assays (ELISAs), and liquid chromatography coupled with mass spectrometry (LC-MS) methods are required in preclinical development to determine the toxicological, pharmacokinetic, and metabolic effects of the oligonucleotide in animal models prior to use in human trials. However, due to the unique polar properties of oligonucleotides, developing a sensitive and robust bioanalytical quantification assay is challenging due to extraction of molecules from various biological matrices, as well as LC-MS method development. In addition, lack of a stable isotopically labeled internal standard (IS) creates another layer of challenges for quantification method development. In this presentation, we propose a fast, novel strategy to select an analog IS during development of an LC-MS quantification assay for two antisense oligonucleotides (ASO).

Experiments

In order to promptly select an analog IS for two ASOs, Alliance Pharma worked on building an IS library that includes generic duplex DNAs of various lengths (from 16 to 40 mers) since it generally covers the length of majority of oligonucleotides that are being developed as pharmaceutical therapies. After comparing different vendors, seven ISs of generic duplex DNAs (16, 20, 24, 28, 32, 36, and 40 mers; **Table 1**) were selected and synthesized by Thermo Fisher Scientific using randomized sequences generated by an in-house developed algorithm. The guanine-cytosine content of these ISs is ~50%.

In the case study, two ASOs (ASO1 with MW 7196.34 amu and ASO2 with MW 6816.92 amu) were used as oligonucleotides to evaluate the IS library. The linearity study with all seven ISs was used for IS screening. The data of the IS screening (**Figure 1**) indicates that both ASO1 and ASO2 had the best linear responses using the 24-mer generic IS with the MW of 7325.8 amu. Other ISs gave quadratic responses for both oligonucleotides across the tested curve range. This 24-mer IS was thus selected for both oligonucleotides for further evaluation like inter- and intra- precision and accuracy (**Tables 2** and **3**). All results indicate that the selected 24-mer IS with the molecular weight closest to both oligonucleotide works very well.

Besides the challenge of IS selection, other challenges like peak tailing, carryover, and low sensitivity were encountered during method development. The multiple phosphate groups in the oligonucleotides resulted in weak retention and peak tailing using most of the common LC columns. However, the Waters™ XBridge™ Premier Oligonucleotide BEH C_{18} column along with a mobile phase consisting of hexafluroisopropanol (HFIP) and diisopropylamine (DIPA) showed symmetric and reproducible peaks with sufficient resolution and sensitivity, and low baseline (**Figures 2** and **3**). In order to eliminate carryover effect, a high column temperature of 70˚C was used. A low-high-low zig-zag gradient was applied at the end of the LC gradient to successfully remove the n-dodecyl-β-D-maltoside (DDM) detergent from the lysis buffer (**Figure 4**). In order to maximize sensitivity, different extraction methods including protein precipitation extraction (PPE), solid-phase extraction (SPE), and liquid-liquid extraction (LLE) were compared, and the LLE using 25:24:1 (*v/v/v*) phenol/chloroform/isoamyl alcohol was adopted. Intra-day accuracy (%bias) and intra-day precision (%CV) for the 3 runs were within the acceptance criteria range of 80.0% to 120% (**Tables 2** and **3**). Therefore, the assay was successfully developed and qualified within a serum concentration range of 200 to 80,000 ng/mL.

Results

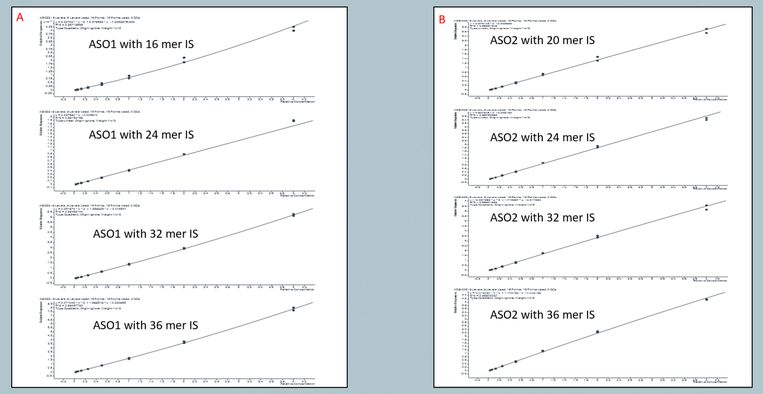

Figure 1: Analog internal standard screening for ASO1 (A, MW 7196.34 amu) and ASO2 (B, MW 6816.92 amu)

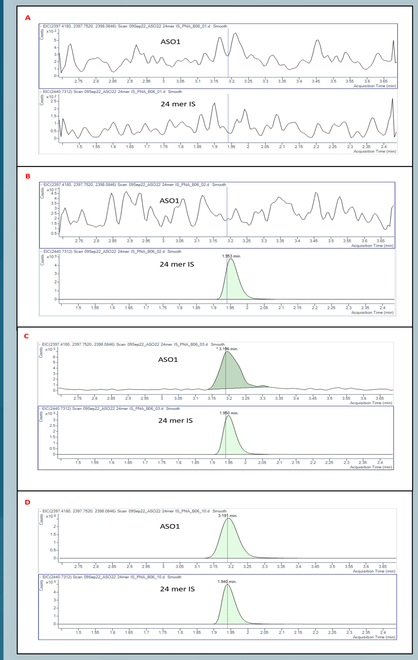

Figure 2: Representative chromatograms of matrix blank (A), control-zero (B), LLOQ-QC (C), and ULOQ-QC (D) samples for ASO1

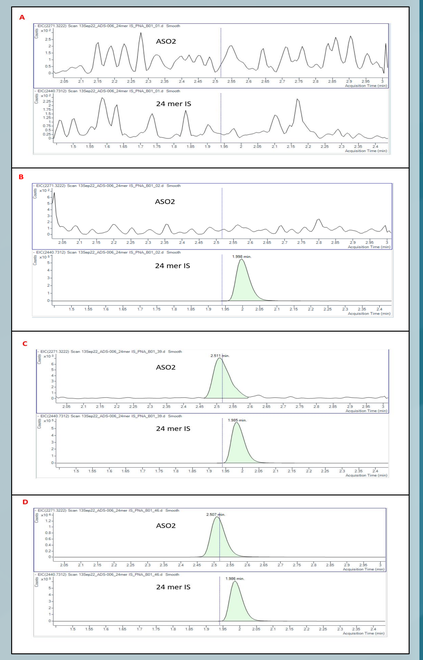

Figure 3: Representative chromatograms of matrix blank (A), control-zero (B), LLOQ-QC (C), and ULOQ-QC (D) samples for ASO2

Results (*Cont.*)

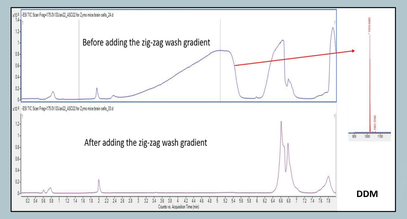

Figure 4: Total ion chromatogram comparisons before and after the implementation of a low-high-low zig-zag wash gradient for DDM cleanup

Table 1: List of the generic DNA internal standard library

Length	Sequence	Oligo type	MW
16 Mer	GAATACTACATATTAG	DNA	4888.28
20 Mer	GCGAACTGACGAACAGGAAT	DNA	6184.11
24 Mer	TGCCTTTCTGAGACTATCTTGGCG	DNA	7325.81
28 Mer	TGTCTTCAATAGCGGTGTTACAGACCA	DNA	8274.45
32 Mer	TCCAGGAACGACCCAATTGCATACACCTTGCA	DNA	9722.4
36 Mer	AGCTGCTGCGGGATGAATTTATTAGCCAAGCTACGG	DNA	11140.3
40 Mer	AAGCTCCAGGAGGTAGATTGCTTCCATACACGCATGTGTA	DNA	12320.1

Table 2: Inter- and intra- accuracy and precision for ASO1

Run ID		ASO1 Concentration (μM)							
		LLOQ-QC	DEV	LQC	DEV	MQC	DEV	HQC	DEV
		0.0320	(%)	0.0800	(%)	0.800	(%)	3.20	(%)
1		0.0368	15.0	0.0913	14.1	0.822	2.8	3.67	14.7
		0.0351	9.7	0.0894	11.7	0.860	7.5	3.57	11.6
		0.0363	13.4	0.0894	11.8	0.794	-0.8	3.69	15.4
		0.0380	18.9	0.0869	8.6	0.837	4.7	3.61	12.8
		0.0370	15.6	0.0901	12.6	0.837	4.6	3.75	17.0
		0.0374	17.0	0.0899	12.3	0.864	8.0	3.77	17.7
	n	6		6		6		6	
	Mean	0.0370		0.0890		0.836		3.68	
	SD	0.00102		0.00145		0.0257		0.0755	
	CV(%)	2.8		1.6		3.1		2.1	
	Accuracy(%)	115.6		111.3		104.5		114.9	
2		0.0247	-22.9	0.0642	-19.8	0.841	5.1	3.74	16.8
		0.0318	-0.5	0.0669	-16.4	0.804	0.5	3.82	19.3
		0.0300	-6.3	0.0688	-13.9	0.844	5.5	3.75	17.3
		0.0334	4.3	0.0742	-7.2	0.772	-3.5	3.75	17.2
		0.0312	-2.4	0.0678	-15.2	0.846	5.7	3.77	17.7
		0.0258	-19.5	0.0616	-22.9	0.752	-6.0	3.82	19.4
	n	6		6		6		6	
	Mean	0.0290		0.0670		0.810		3.77	
	SD	0.00350		0.00430		0.0407		0.0357	
	CV(%)	12.1		6.4		5.0		0.9	
	Accuracy(%)	90.6		83.8		101.3		117.9	
3		0.0341	6.5	0.0720	-10.0	0.807	0.8	3.699	15.6
		0.0323	1.0	0.0686	-14.2	0.856	6.9	3.726	16.4
		0.0314	-1.9	0.0672	-16.0	0.776	-3.0	3.816	19.2
		0.0319	-0.2	0.0707	-11.6	0.845	5.6	3.767	17.7
		0.0309	-3.5	0.0734	-8.3	0.845	5.6	3.711	16.0
		0.0326	1.9	0.0746	-6.7	0.861	7.6	3.835	19.8
	n	6		6		6		6	
	Mean	0.0322		0.0711		0.832		3.76	
	SD	0.00111		0.00282		0.0333		0.0567	
	CV(%)	3.4		4.0		4.0		1.5	
	Accuracy(%)	100.6		88.9		103.9		117.5	
Inter-assay:									
	n	18		21		21		21	
	Overall Mean	0.0328		0.0759		0.826		3.74	
	SD	0.00372		0.0104		0.0339		0.0704	
	CV(%)	11.3		13.7		4.1		1.9	
	Accuracy(%)	102.6		94.9		103.2		116.8	

Table 3: Inter- and intra- accuracy and precision for ASO2

Run ID		ASO2 Concentration (μM)							
		LLOQ-QC	DEV	LQC	DEV	MQC	DEV	HQC	DEV
		0.0320	(%)	0.100	(%)	0.800	(%)	3.20	(%)
1		0.0332	3.7	0.0958	-4.2	0.846	5.8	3.09	-3.3
		0.0347	8.5	0.0939	-6.1	0.831	3.9	3.15	-1.5
		0.0336	4.9	0.0946	-5.4	0.820	2.5	3.22	0.5
		0.0332	3.8	0.0985	-1.5	0.824	3.0	3.07	-4.2
		0.0325	1.5	0.0951	-4.9	0.846	5.7	3.18	-0.5
		0.0343	7.3	0.0942	-5.8	0.872	9.0	3.12	-2.4
	n	6		6		6		6	
	Mean	0.0340		0.0950		0.840		3.14	
	SD	0.00082		0.00168		0.0193		0.0562	
	CV(%)	2.4		1.8		2.3		1.8	
	Accuracy(%)	106.3		95.0		105.0		98.1	
2		0.0351	9.6	0.0960	-4.0	0.900	12.6	2.96	-7.4
		0.0355	11.0	0.1013	1.3	0.865	8.1	3.05	-4.7
		0.0352	10.1	0.1044	4.4	0.900	12.5	3.00	-6.4
		0.0379	18.5	0.0958	-4.2	0.904	13.0	3.10	-3.1
		0.0346	8.3	0.1074	7.4	0.898	12.2	3.04	-5.0
		0.0342	6.9	0.0986	-1.4	0.878	9.7	3.07	-3.9
	n	6		6		6		6	
	Mean	0.0350		0.1010		0.891		3.04	
	SD	0.00130		0.00470		0.0157		0.0509	
	CV(%)	3.7		4.7		1.8		1.7	
	Accuracy(%)	109.4		101.0		111.4		94.9	
3		0.0332	3.6	0.103	3.4	0.836	4.5	2.905	-9.2
		0.0345	7.9	0.092	-8.1	0.876	9.5	2.956	-7.6
		0.0338	5.7	0.098	-1.6	0.883	10.3	3.038	-5.1
		0.0363	13.3	0.096	-3.9	0.879	9.8	2.918	-8.8
		0.0266	-16.8	0.101	1.5	0.897	12.1	2.908	-9.1
		0.0383	19.6	0.101	1.4	0.912	14.0	2.973	-7.1
	n	6		6		6		6	
	Mean	0.0338		0.0988		0.880		2.95	
	SD	0.00396		0.00424		0.0255		0.0511	
	CV(%)	11.7		4.3		2.9		1.7	
	Accuracy(%)	105.6		98.8		110.1		92.2	
Inter-assay:									
	n	18		21		21		21	
	Overall Mean	0.0343		0.0982		0.870		3.04	
	SD	0.00246		0.0042		0.0298		0.0939	
	CV(%)	7.2		4.3		3.4		3.1	
	Accuracy(%)	107.1		98.2		108.8		95.1	

Conclusions

- A fast, novel strategy was proposed to select a commercially available IS from Alliance Pharma's oligonucleotide IS library for development of an oligonucleotide LC-MS quantification method.
- The optimized oligonucleotide extraction and LC-MS method showed success of analog IS screening for two different oligonucleotides using Alliance Pharma's oligonucleotide IS library.
- In addition, the best IS candidates appeared to have molecular weights and retention times close to those of the target oligonucleotide.
- Building a generic DNA IS library is efficient, effective and economic.

This method/strategy can serve as a powerful tool for oligonucleotide quantification in various biological matrices.