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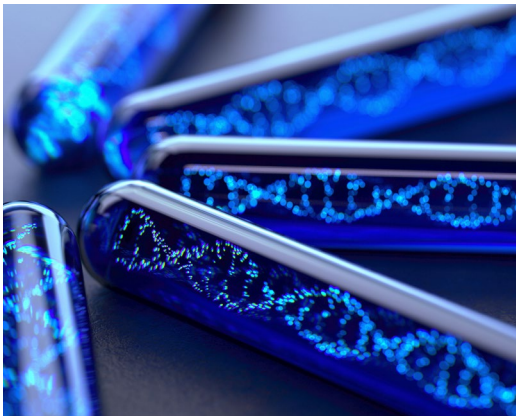
ANTISENSE OLIGONUCLEOTIDES

Antisense oligonucleotides (ASOs) require robust and sensitive bioanalytical assays for their quantitation in increasingly complex biological matrices, such as eye, liver, or brain tissues. The bioanalytical assay needs to ensure no interference at low concentrations.Hybridization-based assays are an effective and accurate approach to quantify parental ASOs in circulation, and in targeted tissue.



Recent Advances in ASO Research

Antisense oligonucleotides are short, synthetic, single-stranded oligodeoxynucleotides, widely used for altering RNA expression to reduce, restore, or modify protein expression via several distinct mechanisms. This protein modification technology holds great potential to change the therapeutic landscape for both neurological and non-neurological conditions (among others), as research progressively disentangles the relevant pathogenic mechanisms and provides an ideal platform to test oligonucleotide-based strategies. Signs of the success of such strategies include the FDA approval of oligonucleotide therapeutics for treatment of diseases, such as spinal muscular atrophy[1,2], and several others currently in clinical trials. Other oligonucleotide-based therapeutics in development include siRNAs and miRNAs which target the mRNA of different cancer-promoting genes.[3]

The results of the above studies have shown that improvement in ASO chemical modifications and delivery systems are required to increase their efficiency in the clinic, since they directly impact tissue-specific targeting, cell entry, stability, and potency.[3] ASOs that have an extracellular targeting will require special attention to enhance their half-life and solubility in plasma, and prevent their degradation. In recent years, these improvements have begun to materialize, with new generations of RNA-based therapeutics, such as the N-Acetylgalactosamine (GalNAc) conjugates, offering reduced toxicity *in vivo,* improved potency and pharmacokinetic profiles, and lower off-target activity.[4,5]

Regulatory Considerations

No ICH or FDA regulatory guidelines specifically address expectations or standards for oligonucleotide products. Nonclinical development and safety evaluations of oligonucleotide therapeutics have generally followed **small molecule regulatory guidelines**. Until such time as specific guidelines are issued, it is important for bioanalytical service providers to work from a position of thorough knowledge and understanding, and ensure that potential challenges are adequately addressed.

ASOs have different mechanisms of action and, as a result, diverse toxicology profiles may be possible, which highlights the importance of conducting a thorough safety toxicology study. The potential toxicities can be due to interactions between the ASO molecule and other molecules (including proteins) as a result of Watson-Crick base pairing to unintended nucleic acids, or through independent mechanisms.[6] ASOs are typically not immunogenic in nature. However, when assessing the risk of developing an immunological response to the drug, it is important to take into account whether an endogenous counterpart is present and whether it is similar to the drug. In this case, any immune response generated against the drug will spread to the endogenous counterpart, which may potentially cause a safety concern.[7] In case oligonucleotides are covalently linked to a given ligand, immunogenicity to the ligand linker or the oligonucleotide would need to be evaluated as a precaution. Therefore, it is important to characterize the anti-drug antibody (ADA) response in preclinical and clinical studies and report any ADA-positive samples as a risk-based approach.

Bioanalytical Considerations

ADAs and Metabolite Interference

ADAs are important to detect in preclinical and clinical studies. A potential exists for any protein that interacts with ASOs to interfere with quantification of the ASO. Consequently, it is important to also evaluate the potential interference of ADA on study sample quantitation. Should ADAs interfere with quantitation of the ASO, conditions need to be optimized to reduce interference and regain accurate quantitation, and ensure proper PK interpretation. Several methods can be employed during hybridization to help degrade any binding proteins, including ADAs.

It is imperative to determine if the hybridization ELISA method is selective for the parent compound, or if metabolites potentially quantitate as well. To make this determination, it is recommended to analyze a variety of shortened metabolites during validation to assess if any will quantitate, recognizing that longer metabolites, such as N-1 and N-2, are more likely to do so than shorter metabolites. If/when metabolite quantitation is observed, selectivity for the parent compound may be increased through appropriate probe design and the addition of S1 nuclease.

ASOs Quantitation in Tissues

ASOs may not be evenly distributed in a given tissue, and ASO concentrations may differ depending on the sampling site. To ensure successful incurred sample reproducibility, a whole sample tissue homogenate is highly recommended, to ensure the consistency between sample extractions, taking into consideration sample size/volume, and the type of tissue in question.

The steps to fully homogenize/extract tissue samples should be assessed during assay development, prior to assay qualification, to ensure complete homogenization of study samples occurs. While soft tissues like brain or spinal cord can easily be homogenized using simple bead-based methods, other tissues like liver, kidney, or gut tissue may need a second round to ensure a proper homogenization. Failure to fully homogenize samples could result in the ASO remaining trapped in tissues, resulting in under-recovery of sample ASO concentrations.

The stability of ASOs in one tissue matrix may be different from another due to specific tissue characteristics, and stability should always be assessed in each matrix during method qualification or validation.

Method Sensitivity

Hybridization-based methods provide the best reported assay sensitivity and throughput, compared with other bioanalytical methods for ASOs. They are also less time consuming, as they require little or no sample clean-up. They have been widely used for the quantitative analysis of ASOs to support both TK and PK evaluations. The table below illustrates the differences with other bioanalytical methods used in the industry (Table 1).

Table 1. Quantitation of ASOs by different bioanalytical methods.

Parameters	Hybridization Assays	PCR Assays	LC-MS/MS Assays
Sample Preparation	ASO isolation not required	ASO isolation is required	ASO extraction is required
Throughput	Can be automated	Medium to high	Medium
Sensitivity	High sensitivity	Highest sensitivity	Potentially acceptable
Interference/Specificity	High specificity for parent ON May have cross-reactivity with N-1 and N-2 metabolites	May be prone to contaminations	Can quantitate all metabolites and parent ON
Method Validation	Follow 2018 FDA guidance on Bioanalytical Method validation for LBA[8]	No current guidance	Follow 2018 FDA guidance on Bioanalytical Method validation for LC-MS/MS[8]

The importance of using a highly sensitive bioanalytical method stems from different critical parameters for the preclinical or clinical study:

1. Advancements in drug delivery and chemical modifications of ASOs have improved drug stability and potency, resulting in a reduction in the number of required doses, and amount of drug required per dose. This, by extension, will require more sensitive methods to quantitate the drug.

2. The ability to quantitate ASOs to very low concentrations impacts calculation of half-life; more sensitive assays will produce more accurate readouts.

3. Timepoints close to the end of the dosing cycle are expected to have lower concentrations in various tissues. Highly sensitive methods will produce the most accurate readings, allowing for an accurate PK profile even in late timepoints.

4. ASO concentrations should be determined in all specific tissues, depending on the targeted therapeutic disease, as this will impact the dosing strategy when the development program advances from preclinical to clinical phases. As some tissues will not have high concentrations of the ASO, the sensitivity of the assay is important to accurately quantitate ASOs in these tissues.

Meso Scale Discovery (MSD™) multiplexing technology is a promising electroluminescence-based detection system (ECLIA), making use of a microtiter plate, with favorable sensitivity and range of detection. It has been our experience that shifting from a colorimetric to ECLIA platform significantly increases the dynamic range of the assay and, in turn, sensitivity.

ALTASCIENCES' CASE STUDIES

Case Study 1: Resolving Issues with Matrix Effect

As we were developing a hybridization ELISA assay to quantitate ASOs in human feces to support a first-in-human clinical trial, issues were encountered with assay selectivity. High blank and LLOQ signals were observed in two of five individual lots, impacting selectivity, as difference in signal between lots was so high (up to fivefold) that the selectivity evaluation did not meet the acceptance criteria of 80% (see Table 2). In these initial experiments four individual lots of human feces were pooled to be used for the calibration curve and QCs.

Table 2. Nominal concentrations of ASO in individual lots unspiked or spiked at LLOQ and HQC.

Nominal ASO Concentration (ng/mL)	Blank	LLOQ		HQC	
	0.0000	0.3500		75.00	
Individual Lot #	**ASO Concentration (ng/mL)**	**ASO Concentration (ng/mL)**	**%RE**	**ASO Concentration (ng/mL)**	**%RE**
1	0.3782*	0.7640*	118.3	84.32	12.4
2	0.3665*	0.6617*	89.1	89.47	19.3
3	BLQ	0.3894	11.3	79.18	5.6
4	BLQ	0.3963	13.2	80.28	7.0
5	BLQ	0.4075	16.4	89.21	18.9

BLQ: Below Lower Limit of Quantitation (<0.3500 ng/mL)

*: % Deviation Unacceptable for QCs

Two different hypotheses were evaluated:

1. The failed evaluation is due to the nature of the matrix (feces) which may have more interference in some individual lots.

2. The method hybridization conditions are not optimal and require more optimization.

To address the first hypothesis, 12 individual lots of human feces were pooled together (instead of four) to prepare the calibration curve and QCs, and they were tested against the same five individual lots, either unspiked or spiked, at both LLOQ and HQC. Results shown in Table 3 indicate that the unspiked blank matrix from those same two lots fell below the limit of quantitation; however, the LLOQ concentrations remained above recovery. %RE was at 26.9% and 31.6% respectively for the first and second lot. We concluded that increasing the number of lots used to prepare the calibration curve and QCs was important to represent the true background of tissue sample and to reduce the nominal ASO concentration in the individual lots.

Table 3. Nominal concentrations of ASO in individual lots unspiked or spiked at LLOQ and HQC, following modification to calibration curve and QCs.

Nominal ASO Concentration (ng/mL)	Blank	LLOQ		HQC	
	0.0000	0.3500		75.00	
Individual Lot #	**ASO Concentration (ng/mL)**	**ASO Concentration (ng/mL)**	**%RE**	**ASO Concentration (ng/mL)**	**%RE**
1	BLQ	0.4440*	26.9	86.32	15.1
2	BLQ	0.4607*	31.61	84.27	12.4
3	BLQ	0.3674	5	76.98	2.6
4	BLQ	0.3691	5.5	78.24	4.3
5	BLQ	0.4129	18	87.67	16.9

BLQ: Below Lower Limit of Quantitation (<0.3500 ng/mL)

*: % Deviation Unacceptable for QCs

To further optimize the assay and better eliminate the matrix effect, the method MRD was increased and the LLOQ was raised threefold, to 1ng/g. This resulted in complete elimination of the matrix effect when the same individual selectivity lots were tested again, as illustrated in Table 4.

Table 4. Nominal concentrations of ASO in individual lots unspiked or spiked at LLOQ and HQC, after increasing the MRD and LLOQ.

Nominal ASO Concentration (ng/mL)	Blank	LLOQ		HQC	
	0.0000	1.0000		75.00	
Individual Lot #	**ASO Concentration (ng/mL)**	**ASO Concentration (ng/mL)**	**%RE**	**ASO Concentration (ng/mL)**	**%RE**
1	BLQ	1.108	10.8	80.57	7.4
2	BLQ	1.083	8.3	72.57	-3.2
3	BLQ	1.068	6.8	86.85	15.8
4	BLQ	1.198	19.8	74.82	-0.2
5	BLQ	1.073	7.3	85.12	13.5

BLQ: Below Lower Limit of Quantitation (<0.3500 ng/mL)

These results indicate that matrix interference was impacting the lower range of the assay. Creating a larger pool for calibration curve and QC, and increasing both LLOQ and MRD levels, were important to resolve the method selectivity issue.

Case Study 2: Stability of ASO in Tissue Matrix

ASO concentrations in monkey and rat plasma were successfully developed and validated to support preclinical studies. Our objective was to transfer the previously developed bioanalytical method into human gut tissue. During the method transfer and pre-validation stage, all critical evaluations for selectivity, specificity, precision and accuracy, dilution linearity, and prozone were acceptable. The stability of the ASO in gut tissue at the homogenization stage was tested following three freeze/thaw cycles (Table 5A) and at room temperature (22 °C Nominal) (Table 5B) for 26.9 hours. In both evaluations, the ASO was under-recovering at both the QC1 and QC3 levels. As gut tissues were suspected to be rich in bacterial flora with high levels of nucleases, proteinase K was added during sample spiking. The addition of proteinase K did not improve the recovery in either case.

Table 5 A. Freeze/thaw stability (3 F/T Cycles) for analyte in human gut tissues (homogenate).

	Stability QC Without Proteinase K		Stability QC with Proteinase K	
	QC1	QC3	QC1	QC3
	6.938*	719.7*	1.343*	501.1*
	3.367*	380.3*	2.025*	519.5*
N	2	2	2	2
Nominal Concentration (ng/g)	37.5	1875	37.5	1875
Mean	5.152	550	1.684	510.3
SD	2.525	240	0.4827	13.02
%CV	49	43.6	28.7	2.6
%RE	-86.3	-70.7	-95.5	-72.8

Table 5 B. Analyte stability in thawed matrix stored at room temperature (22 °C nominal) for 26.9 hours in human gut tissues (homogenate).

	Stability QC Without Proteinase K		Stability QC with Proteinase K	
	QC1	QC3	QC1	QC3
	3.662*	435.2*	1.844*	445.4*
	2.494*	419.3*	1.298*	403.3*
N	2	2	2	2
Nominal Concentration (ng/g)	37.5	1875	37.5	1875
Mean	3.078	427.3	1.571	424.3
SD	0.8259	11.29	0.3861	29.71
%CV	26.8	2.6	24.6	7
%RE	-91.8	-77.2	-95.8	-77.4

*: % Accuracy Unacceptable for QCs

As the stability of the drug was impacted at the homogenization step, we concluded that sample extraction should be performed immediately following homogenization to prevent any possible ASO degradation. To confirm the time period of ASO stability in gut tissue homogenate, the drug was spiked in gut tissue homogenate at QC1 and QC3 level and tested at room temperature (22 °C nominal) for 1, 2, 3, and 4 hours. Table 6 illustrates that the drug is stable up to four hours. These results indicate that gut tissue homogenate should not be frozen/thawed; instead, the extraction should be done within four hours of the homogenization procedure.

Table 6. ASO stability at room temperature (22 °C nominal) at different time points (1 hr, 2 hr, 3 hr, and 4 hr) in human gut tissues (homogenate).

	Stability QC 1 hr		Stability QC 2 hr		Stability QC 3 hr		Stability QC 4 hr	
	QC1	QC3	QC1	QC3	QC1	QC3	QC1	QC3
	36.47	1742	34.09	1679	36.53	1571	32.11	1530
	37.09	1760	33.65	1633	37.49	1650	35.41	1500
	33.48	1617	39.48	1444	37.02	1637	32.46	1476
N	3	3	3	3	3	3	3	3
Nominal Concentration (ng/g)	37.5	1875	37.5	1875	37.5	1875	37.5	1875
Mean	35.68	1706	35.74	1585	37.01	1619	33.33	1502
SD	1.929	77.84	3.244	124.3	0.4836	42.63	1.812	26.86
%CV	5.4	4.6	9.1	7.8	1.3	2.6	5.4	1.8
%RE	-4.8	-9	-4.7	-15.4	-1.3	-13.6	-11.1	-19.9

Conclusions

New generation ASOs have better potency and bio-distribution. Thus, for concentration determination, high method sensitivity is required to achieve an accurate PK profile at the preclinical stage, which will drive decisions on clinical dosing. By increasing the sensitivity of the assay, other issues may arise, such as selectivity or metabolite interferences, and so it is even more critical to evaluate all types of interferences in the bioanalytical method during method development. Finally, since there are no ICH or FDA regulatory guidelines for the validation of ASO products, our strategy is to follow the general principles outlined in FDA 2018[8] as a starting point for method validation, where applicable.

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ABOUT ALTASCIENCES

Altasciences is an integrated drug development solution company offering pharmaceutical and biotechnology companies a proven, flexible approach to preclinical and clinical pharmacology studies, including formulation, manufacturing, and analytical services. For over 25 years, Altasciences has been partnering with sponsors to help support educated, faster, and more complete early drug development decisions. Altasciences' integrated, full-service solutions include preclinical safety testing, clinical pharmacology and proof of concept, bioanalysis, program management, medical writing, biostatistics, clinical monitoring, and data management, all customizable to specific sponsor requirements.

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