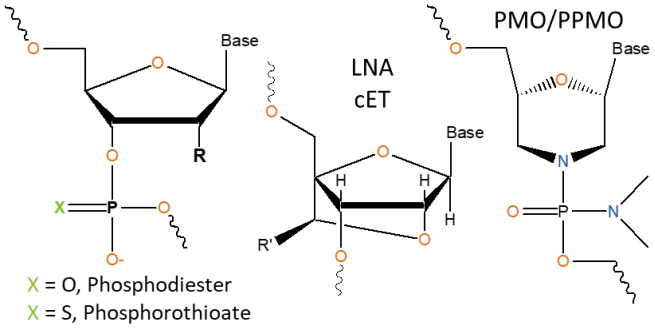
Analytical Method Development for Therapeutic Oligonucleotides

Experience, Flexible Service, and Quality

Pace Analytical Life Sciences (PLS) offers development services for the entire life cycle of analytical methods for therapeutic oligonucleotides. PLS adapts its services to meet the needs of drug developers at all stages from preclinical research through post-approval regulatory changes. Beginning with an Analytical Target Profile (ATP) defining the purpose and desired method performance, PLS scientists rationally design development experiments incorporating knowledge of each oligonucleotide, current technology, and Quality by Design (QbD) principles; our primary goal is to reduce the risk of costly program delays due to inadequate methods. Unlike methods from some Contract Manufacturing Organizations (CMO), all analytical methods at PLS are client owned. PLS also provides development reports that support transfer of a method to a PLS cGMP testing site or another receiving laboratory and subsequent validation. PLS cGMP services support a central laboratory testing model that provides better CMO flexibility and accountability.

Oligonucleotide Knowledge and Experience

The chemical structure, conjugations, and formulation of therapeutic oligonucleotides are constantly evolving. PLS experience goes beyond the chemically synthesized oligonucleotides illustrated in **Figure 1** to also include longer RNAs (gRNA and mRNA) and peptides, lipids, etc. used for formulation and delivery.



X = O, Phosphodiester
X = S, Phosphorothioate

R = -H, -F, -OH, $-OCH_3$, $-OCH_2OCH_2CH_2CH_3$, etc.

Figure 1. Diverse oligonucleotide chemistry requires rational experiments for efficient method development. Linkers at 3' or 5' ends including PEG, cholesterol, GalNAc, peptides, etc. increase the performance requirements of the needed analytical methods.

Knowledge of the biophysical and chemical properties of oligonucleotides, coupled with a conceptual understanding of analytical methods and the latest technology, is essential for efficient development. PLS understanding of oligonucleotide process and product related impurities is also important to aid interpretation of the detailed results thereby increasing confidence that promising results are not overlooked.

Analytical Methods for Oligonucleotides

The primary methods for determining the identity, assay, and oligonucleotide-related purity/impurity profile are listed in **Table 1**. The purity methods in Table 1 are critical to support CMC development programs; early development of these methods usually pay dividends by improving process and formulation development efforts. Methods for residual solvents, elemental impurities, water, and counterion testing, require minimal development while test methods for appearance, bacterial endotoxins, and microbial limits are usually fit for purpose without further development for a specific oligonucleotide.

Table 1. Primary methods for testing the identity, assay, and purity/impurity of therapeutic oligonucleotides.

Method	Single Strand	Duplex
Anion Exchange (AEX)	✓	✓
Ion-Pair Reverse Phase (IPRP)	✓	✓
Size Exclusion (SEC)	—	✓
Melting Temperature (T_M)	*	✓
LCMS	✓	✓
Sequencing (e.g. LC-MS/MS)	✓	✓
Capillary gel electrophoresis (CGE)	*	*

(*) The AEX and IRPP chromatography methods have replaced capillary gel electrophoresis for shorter oligonucleotides (< 50 nucleotides); CGE is still used for mRNA-based therapeutics. Observation of a melting temperature is essential for verifying the identity of duplex oligonucleotides but has been replaced by mass spectrometry methods for most single strand oligonucleotides. Non-denaturing chromatography methods (AEX or IPRP) may be needed as orthogonal methods for size exclusion chromatography.

Regulators Expect Orthogonal Purity Methods

While regulators have yet to publish guidance's specific to oligonucleotides, the originators of approved single and double stranded therapeutic oligonucleotides recently published their recommendations based on regulator feedback. The formation of impurities during each cycle of synthesis that are difficult or impossible to separate from the therapeutic oligonucleotide require orthogonal separation methods to resolve critical impurities for process control (**Figure 2**). PLS recommends performing development of orthogonal methods in parallel to coordinate the optimization of the collective specificity of each method thereby decreasing the risk that additional methods are later needed.

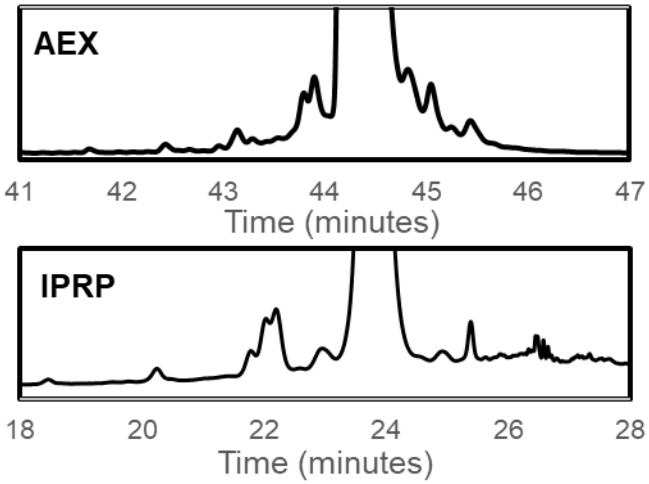


Figure 2. Orthogonal methods resolve more impurities collectively providing better manufacturing control.

Stability Indicating Methods and Impurity Profiles

The suitability of oligonucleotide methods for stability studies is demonstrated by conducting forced degradation studies. Regulators require the identification of the product related impurities formed during stability studies and the process related impurities formed during synthesis and purification. Impurity identification is best performed using mass spectrometry (MS) compatible methods such as IRPP methods (**Figure 3**). Identification of impurity peaks in AEX methods is best accomplished by co-injection of authentic impurities identified from an MS compatible method. In some cases, the identity of impurities in AEX methods is then deduced by comparing impurity levels in a comparability study. Alternatively, peaks from the AEX method are collected, desalted, and analyzed by LCMS to identify an impurity.

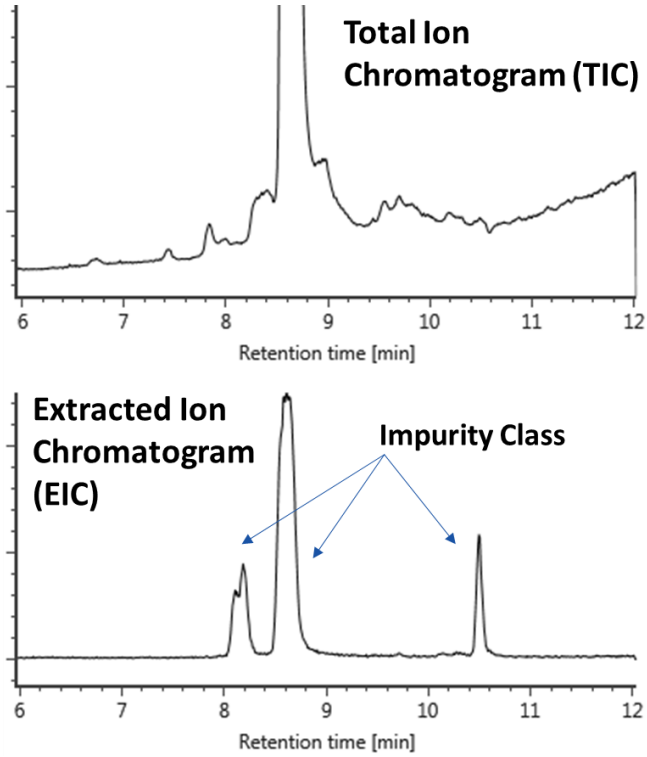


Figure 3. LCMS methods identify impurity peaks and can quantitate impurities coeluting with the main peak.

Method Transfer and Validation

The specificity (resolution) and sensitivity of chromatographic methods are important for method performance and key for their transfer and validation. **Figure 4** shows the IPRP chromatograms when 4 authentic impurities were spiked in at 0.05 to 0.5%. The best measure of LOQ is obtained when the spike level is near LOQ and is not subject to integration variability due to nearby impurities that are only partially resolved.

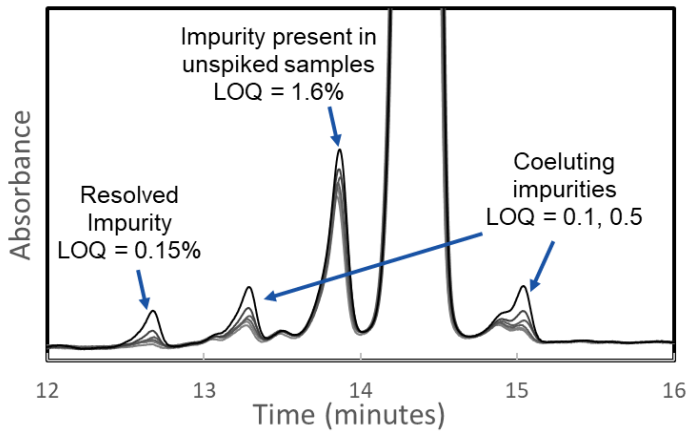


Figure 4. Validation experiments require careful design and selection of impurities when determining LOQ.