Application Note

Quality Standards for DNA phosphoramidite raw materials

Introduction

Therapeutic oligonucleotides have experienced rapid expansion since their first approval in 1998 [1]. Since then, there have been over a dozen additional approvals, with several more in late-stage clinical development for a wide range of therapeutic indications. These synthetic oligonucleotides are classified as either antisense oligonucleotides (ASOs), small interfering RNA (siRNAs), microRNA (miRNAs) aptamers, or DNAzymes. They all use modified nucleic acid molecules known as phosphoramidites as raw materials. Phosphoramidites are short chains of nucleotides used as building blocks for the synthesis of DNA oligonucleotides. Each phosphoramidite is composed of the following **(Figure 1)**:

- A variable group that protects amino groups in the nitrogenous base (pink)
- A phosphoramidite moiety on the 3′ position of the sugar moiety (purple)
- A Dimethoxytrityl (DMT) group protecting the 5′ hydroxy group (green)
- A diisopropylamino group serves as a leaving group (yellow)
- A 2-cyanoethyl group (orange)

The phosphoramidite moiety consists of two groups: a diisopropylamino group which is removed during synthesis and links the phosphoramidites to the growing oligonucleotide chain, and a 2-cyanoethyl group that protects the second hydroxyl on the phosphorus (III) center. The P (III) atom in a nucleoside phosphoramidite is chiral, and therefore exists as a mixture of two diastereomers.

Figure 1. Example structure of a standard 2′ deoxyadenosine phosphoramidite [2]

During oligonucleotide synthesis, side reactions can occur on residual reactive sites such as hydroxyl and amino groups as the oligonucleotide chain length increases. To prevent these side reactions, nucleotides are modified with protecting groups. Benzoyl (Bz) and isobutyryl (iBu) groups protect the amino group of the nucleobases adenine, cytosine, and guanine, dimethoxytrityl (DMT) protects the 5′-hydroxyl group of the sugar moiety and diisopropylamino and 2-cyanoethyl (CE) groups prevent side reactions at the 3′-hydroxyl group of the sugar moiety. Since thymine has a less reactive secondary amine group, an amino protecting group is not necessary.

Oligonucleotide synthesis consists of four major steps: deprotection, coupling, oxidation, and capping, adding each nucleotide from 3′ to 5′ to the growing chain until the desired sequence is achieved. The final oligonucleotide product is then cleaved from the solid support and purified to remove any remaining impurities or incomplete sequences.

It is important to note there are limitations to the length of synthetic oligonucleotides that can be reliably synthesized. These limitations are due to the occurrence of side reactions, such as incomplete coupling or unintended modifications, which become more prevalent as the length of the oligonucleotide increases. As a result, the synthesis of longer oligonucleotides may require additional purification steps and can be more challenging to achieve with high yield and purity.

To support quality assessment of these raw materials, USP has developed five DNA phosphoramidite Reference Standards (RSs) that can help manufacturers control variability and to look at impurity profiles using various analytical techniques, including ^{31}P NMR, RP-HPLC and LC-MS **(Table 1)**.

As shown in this application note, raw materials were sourced from various vendors and evaluated with the aim to identify any potential variations in quality using various analytical techniques against the five USP RSs. The reproducibility, sensitivity, and robustness of the methods were also tested.

Table 1: General information for the USP DNA phosphoramidite Reference Standards

Name	**iBu dG β-Cyanoethyl Phosphoramidite**	**T β-Cyanoethyl Phosphoramidite**	**Bz dA β-Cyanoethyl Phosphoramidite**	**5-Me Bz dC Cyanoethyl Phosphoramidite**	**Bz dC Beta Cyanoethyl Phosphoramidite**
USP Catalog #	1152030	1152031	1152032	1152033	1152034
Base Protection	Isobutyryl	N/A	Benzoyl	Benzoyl	Benzoyl
Molecular Formula	$C_{44}H_{54}N_7O_8P$	$C_{40}H_{49}N_4O_8P$	$C_{47}H_{52}N_7O_7P$	$C_{47}H_{54}N_5O_8P$	$C_{46}H_{52}N_5O_8P$
Molecular Weight	839.93 g/mol	744.83 g/mol	857.95 g/mol	847.95 g/mol	833.92 g/mol
CAS #	93183-15-4	98796-51-1	98796-53-3	105931-57-5	102212-98-6

For more information, please visit www.usp.org/biologics/oligonucleotides

Methods

Purity by reverse-phase high-performance liquid chromatography (RP-HPLC)

The chromatographic purity analysis was performed on a C18 column (250 × 4.6 mm, 5 µM particle size) at ambient temperature using gradient elution. 0.1M triethylammonium acetate (TEAA) in water (pH 7.0 ± 0.1) and acetonitrile were used as mobile phase A and B at flow rate of 1 mL/min. Samples were prepared in acetonitrile and injected at a concentration of ~1.0 mg/mL. Purity is calculated using %TDA.

Purity by phosphorus-31 nuclear magnetic resonance (^{31}P NMR)

The ^{31}P NMR spectra were recorded at 202 MHz using zgig (proton decoupled) pulse program with 1024 scans. The acquisition parameters were set to 1.5 sec. acquisition time (AQ), 2.0 sec. Relaxation delay (D1), a spectral width (SW) of 300 ppm, a spectral offset (O1) of 100 ppm, a pulse width (P1) of 12-14 µsec and 4 dummy scans (DS). 5% H_3PO_4 in D_2O was used as system suitability solution and samples were prepared at ~0.3 g/mL in 1% TEA (v/v) in $CDCl_3$. The signal obtained with the system suitability solution was used as a reference to verify the chemical shift.

Identification by mass spectrometry (MS)

This is a flow injection MS method, and no column is required. Sample was isostatically eluted with mobile phase 100% acetonitrile at a flow rate of 0.5 ml/min. Mass spectra were acquired in the ESI positive polarity mode (ESI+) on Orbitrap mass spectrometer. The scan range was set to 150–2000 m/z at a default resolution of 120,000. Samples were prepared at ~ 0.1 mg/mL in mobile phase acetonitrile (100%).

Results and discussion

The five DNA phosphoramidite Reference Standards were submitted for multi-laboratory collaborative studies employing multiple techniques to characterize each, including checking their purity and identity. The raw materials from different vendors were also evaluated and tested across multiple laboratories for their quality and use in manufacturing oligonucleotide drug substances. The collaborative study involved joint efforts between multiple parties, which required pooling of expertise across diverse group of researchers, institutions, and organizations. These studies helped us to validate the feasibility and viability of the idea by demonstrating the success of the concept and then the suitability of these materials as USP standards.

During the proof of concept (PoC) study, the analytical methods donated by external sponsors were initially evaluated and optimized by a USP internal lab to support the subsequent multi-center collaborative study. In addition to the four standard DNA phosphoramidites with unmodified bases (dA(bz), dG(iBu), T, and dC(bz) phosphoramidites, one non-standard DNA phosphoramidite (5-Me-dC(bz)) was also evaluated for suitability as a USP Reference Standard. Multiple labs were recruited to fully characterize the bulk candidate materials using optimized methods from the PoC study (RP-HPLC, ^{31}P NMR and MS). USP provided aliquots of bulk materials to the labs for testing. Lab reports and raw data from each participating lab were submitted to USP for review and approval. The results from these studies are described below.

A reverse-phase liquid chromatography (RP-HPLC) method was used to evaluate material purity. As shown in **Figure 2**, the DNA phosphoramidites were separated as double peaks which represent the two diastereomers. The lower chromatogram depicts a zoomed-in view of the impurities with good resolution between the peaks of interest. Percentage of isomers I and II were quantitated separately but reported as a sum for purity. The purity results for all five DNA phosphoramidite RSs were consistent among the three testing labs. **Table 3** shows the results for each of the RSs from each individual testing lab. Purity level for all five DNA phosphoramidite candidate materials dG, T, dA, dC and 5-Me dC were ≥ 99.0%, which shows quality superior to typical requirement of ≥98.0%. However, the purity of 5-Me dC is 99.10%, slightly lower than the four unmodified amidites (≥ 99.5%), which manifests that more modification steps may lead to challenges to purge the impurities from the material. Another difference observed with 5-Me dC is the late elution (≥ 20 min) compared with the four standard DNA phosphoramidites dG, T, dA and dC eluted within 6-12 minutes in a 30-minute run.

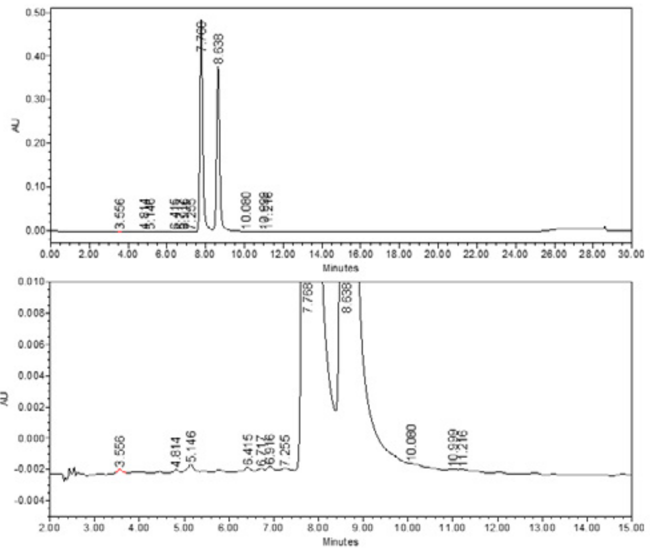


Figure 2. Representative Chromatograms for Bz dA Beta-Cyanoethyl Phosphoramidite.
Top: full scale and Bottom: zoomed scale.

Table 3: HPLC purity results from collaborative study

Labs	HPLC Purity Results (%TDA, as is basis)				
	dG	**T**	**A**	**dC**	**5-Me dC**
1	99.75	99.42	99.77	99.64	99.13
2	99.66	99.47	99.73	99.63	99.08
3	99.75	99.55	99.47	99.56	99.09
Average	**99.72**	**99.48**	**99.66**	**99.61**	**99.10**
%RSD	0.29	0.30	0.29	0.04	0.03

Nuclear Magnetic Resonance (NMR) spectroscopy is a powerful analytical technique that can provide structural information for compound characterization. ^{31}P NMR can also be used as an orthogonal method to further assess material purity by detecting the P(III) contents in DNA phosphoramidites. As each phosphoramidite contains one chiral phosphorus (III) center, P(III) main peaks exhibited as a mixture of two diastereomers in ^{31}P NMR analysis as shown in **Figure 3**. Non-amidite P(III) impurities were below detection limits in all five DNA phosphoramidite candidate materials, and P(V) impurities were found at a level of less than 1%. The purity results obtained by ^{31}P NMR match that of RP-HPLC assessment. **Table 4** shows the testing results for each of the RSs.

In addition, ^{1}H NMR was employed to confirm the structure of all five DNA phosphoramidite RSs for identification purposes (data not shown).

Table 4: Summary of ^{31}P purity results

^{31}P NMR Purity Results (% area, as is basis)					
% Area	**dG**	**T**	**A**	**dC**	**5-Me dC**
Main	99.57	99.57	99.78	99.19	99.61
P(III)	ND	ND	ND	ND	ND
P(V)	0.43	0.43	0.22	0.81	0.40

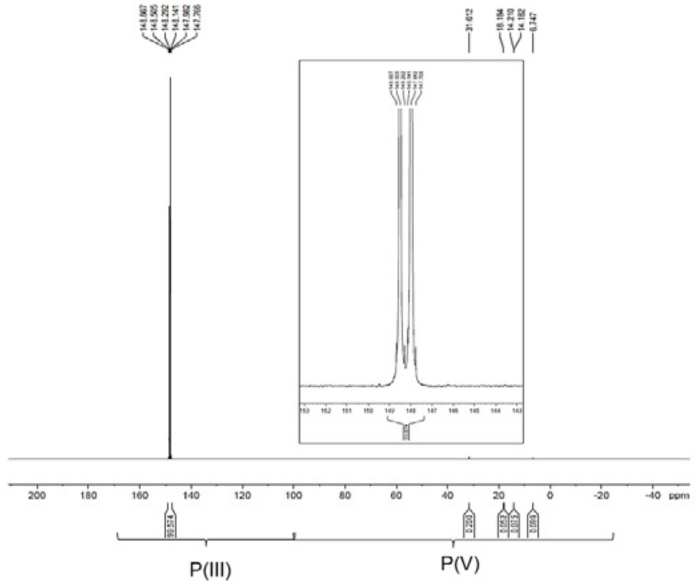


Figure 3. Representative ^{31}P NMR for iBu dG Beta-Cyanoethyl Phosphoramidite: P(III) impurities correspond to the sum of relative response for signals from ~100 to 169 ppm, excluding the main components at ~150 ppm; P(V) impurities correspond to the signals from -25 to 99 ppm.

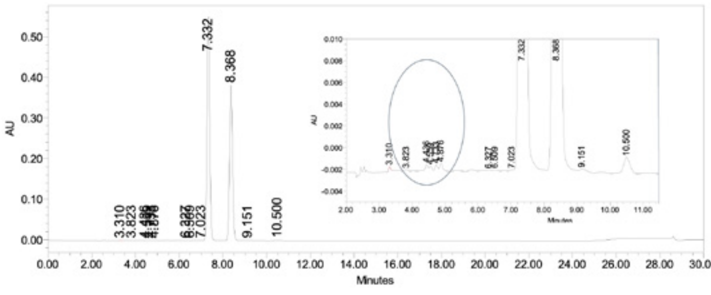
LC-MS was performed to measure mass for the DNA phosphoramidites by two collaborative labs. The LC conditions used by the two labs were slightly different, but their results were consistent. The experimental data show good agreement with the calculated theoretical masses **(Table 5)**.

Table 5: Comparison of theoretical mass and calculated mass by LC-MS

$[M+H]^+$	Theoretical *m/z*	Observed *m/z*	
		Lab 1	Lab 2
dG	840.3844	840.3848	840.3843
T	745.3361	745.3362	745.3359
dA	858.3739	858.3743	858.3740
dC	834.3626	834.3628	834.3628
5-Me dC	848.3783	848.3786	848.3785

Water content and residual solvents are also part of quality control, as it may interfere with the coupling step during oligonucleotide synthesis and lead to a reduction in yield. Water and residual solvents in USP DNA phosphoramidite RSs have been measured and taken into consideration in calculating the assigned value of the candidate materials using mass balance method. Mass balance calculation determined that each mg of USP DNA phosphoramidite RS contains no less than 0.98 mg of corresponding DNA phosphoramidites.

Raw materials sourced from various vendors were also evaluated to identify any potential variations in quality. The phosphoramidite structure and corresponding mass information using LC-MS has been calculated (data not shown). When testing the same set of samples from two different vendors on RP-HPLC and ^{31}P NMR, variability in quality of these critical raw materials was observed. Although the percent purity for each phosphoramidite was above specification limits, the differences in the impurity profile can be seen as shown in **Figure 4 and 5**. When assessing product purity, it is essential to minimize these impurities as they can result in inaccurately determined overall product purity and should be closely monitored (refer to **Table 6 and Table 7)**.



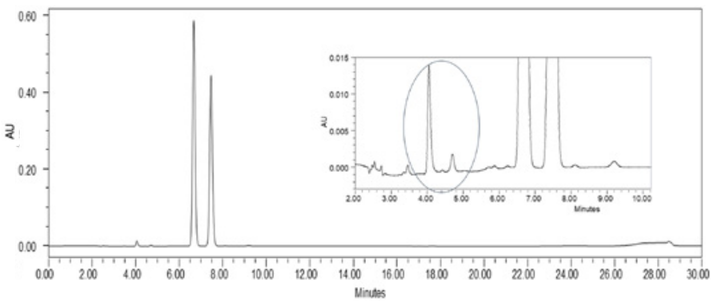
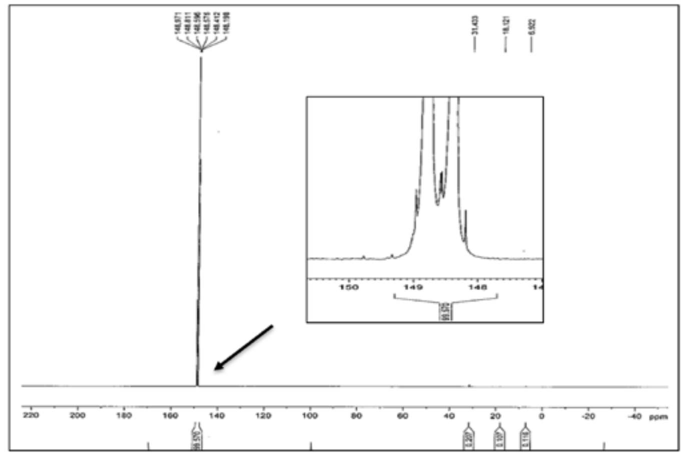


Figure 4. Representative Chromatograms for T Beta-Cyanoethyl Phosphoramidite from vendor A (left) and vendor B (right)



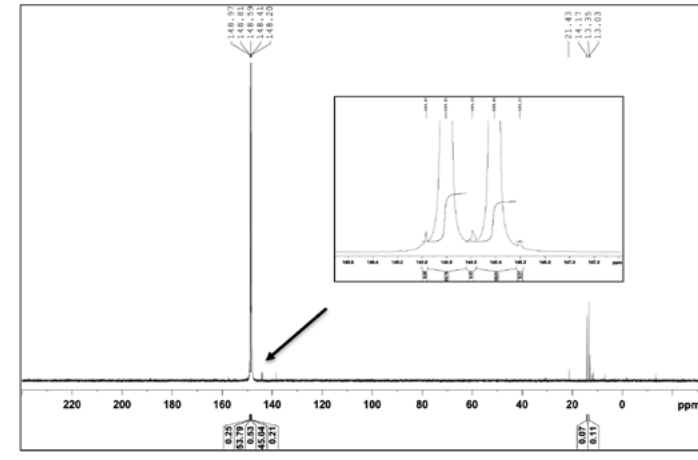


Figure 5. Representative ^{31}P NMR for T Beta-Cyanoethyl Phosphoramidite from vendor A (left) and vendor B (right)

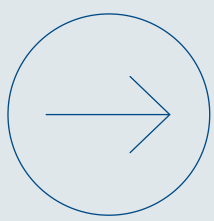
Conclusion

With the growing interest in oligonucleotide therapeutics, stringent quality control of phosphoramidite starting materials is important for achieving a high-quality final oligonucleotide product. DNA phosphoramidites, as the starting materials for synthesis of DNA or DNA-containing oligonucleotides, are incorporated into oligonucleotide chains through an iterative synthetic cycle. Trace amounts of impurities in the starting materials will impact product integrity and efficacy. Currently, lack of regulatory guidelines that specifically address the quality expectations for oligonucleotide products could potentially lead to inconsistent product qualities among different manufactures. A controlled raw material supply chain and established vendor partnerships ensure consistency and security of supply.

By following the methods mentioned in this note, in conjunction with our RSs, you can confidently assess the quality of raw materials for use in manufacturing your oligonucleotide therapies.

The USP DNA phosphoramidites Reference Standards can support the quality assessment of raw materials throughout the product life cycle, serving as:

- A primary RS to compare and control variability of raw materials
- System suitability for analytical methods
- Internal assay control
- Impurity analysis
- Development of platform technologies
- Proficiency controls for method transfer and staff training



The USP DNA phosphoramidite Reference Standard(s) provide users with options to select the best fit-for-purpose starting materials for their specific analytical needs. This commitment ensures that customers can consistently rely on the quality and consistency of our products, thereby enhancing their overall satisfaction and confidence in our organization's offerings.

Table 6: RP-HPLC purity results from two different vendors

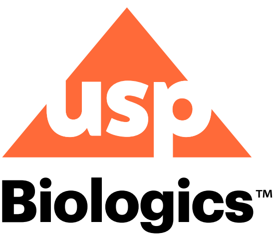
Sample	CoA Specification Limits	Vendor A Observed Purity %	Vendor B Observed Purity %
iBu dG β-Cyanoethyl Phosphoramidite	≥98.0%	99.6	98.6
T β-Cyanoethyl Phosphoramidite	≥98.0%	99.6	99.3
Bz dA β-Cyanoethyl Phosphoramidite	≥98.0%	99.8	99.5
Bz dC Beta Cyanoethyl Phosphoramiadite	≥98.0%	99.2	98.9

Table 7: Phosphoramidite structure and theoretical mass values compared with [M+H]+ and [M+Na]+ mass calculated for each vendor

Sample	Structure	Theoretical Mass Value (Dalton)		Vendor A Experimental Mass Value (Dalton)		Vendor B Experimental Mass Value (Dalton)	
		$[M+H]^+$	$[M+Na]^+$	$[M+H]^+$	$[M+Na]^+$	$[M+H]^+$	$[M+Na]^+$
iBu dG β-Cyanoethyl Phosphoramidite		840.4	862.3	840.4	862.4	840.2	862.2
T β-Cyanoethyl Phosphoramidite		745.3	767.3	745.3	767.3	-	767.1
Bz dA β-Cyanoethyl Phosphoramidite		858.4	880.4	858.4	880.4	858.3	880.2
Bz dC Beta Cyanoethyl Phosphoramiadite		834.4	856.3	834.4	856.3	834.2	856.2

References

1. Moumné L, Marie AC, Crouvezier N. *Oligonucleotide Therapeutics: From Discovery and Development to Patentability. Pharmaceutics.* 2022 Jan 22;14(2):260. doi: 10.3390/pharmaceutics14020260. PMID: 35213992; PMCID: PMC8876811.
2. Twist Bioscience. (n.d.). *Phosphoramidite Chemistry for DNA Synthesis | Twist BioScience.* https://www.twistbioscience.com/blog/science/simple-guide-phosphoramidite-chemistry-and-how-it-fits-twist-biosciences-commercial



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