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### 2.3.S.3. CHARACTERISATION

#### 2.3.S.3.1. Elucidation of Structure and Other Characteristics

Section 3.2.S.3.1 Elucidation of Structure and Other Characteristics [Omicron'XBB.1.5) Variant] describes the structure, function and other characteristics of BNT162b2 Omicron XBB.1.5 variant drug substance (DS) which have been assessed using the analytical approaches outlined in Table 2.3.S.3.1-1. The analytical methodologies established for the mRNA platform were employed for Omicron XBB.1.5 variant DS characterization and are capable of evaluating primary structure, including 5'-capping and 3'-poly(A) tail, higher order structure and biological activity. The results demonstrate that Omicron XBB.1.5 drug substance has the expected structure and function.

Analytical characterization was performed with Omicron XBB.1.5 variant drug substance batch HD1999.

**Table 2.3.S.3.1-1. Characterization Strategy for BNT162b2 Omicron XBB.1.5 Drug Substance**

Characteristic	Analytical Approach	Methodology	Section References
Primary structure	Confirm expected RNA sequence at the oligonucleotide level	CCI	Section 2.3.S.3.1.2
Primary structure	Confirm expected RNA primary structure at the oligonucleotide level	Ion-pair reversed phase HPLC-UV and tandem mass spectrometry (LC/MS/MS) of oligonucleotide fragments generated by RNase T1 digestion	Section 2.3.S.3.1.1.1
	Confirm the presence and determine the length of poly(A)-tail		Section 2.3.S.3.1.2.2
Poly(A) Tail	Confirm the presence and determine the length of poly(A)-tail	Reversed phase HPLC-UV analysis of purified poly(A)-tail after Ribonuclease T1 digestion	Section 2.3.S.3.1.2.3
5'-Cap structure	Confirm the 5' capping structure and 5'-end profile	Ion-pair reversed phase HPLC-UV and mass spectrometry (LC-UV/MS) analysis of purified 5' terminus after RNaseH digestion	Section 2.3.S.3.1.2.4
Higher order structure (HOS)	Spectroscopic analysis to confirm the presence and fingerprint of HOS	Circular dichroism (CD) spectroscopy	Section 2.3.S.3.1.3
Biological Activity	Confirm size of expressed protein	Western blot analysis	Section 2.3.S.3.1.4

#### 2.3.S.3.1.1. Primary Structure

##### 2.3.S.3.1.1.1. Sequencing of RNA CCI

RNA sequencing (RNAseq) for BNT162b2 Omicron XBB.1.5 RNA DS was performed

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RNAseq results demonstrate that the BNT162b2 Omicron XBB.1.5 RNA transcript generated during the *in-vitro* transcription (IVT) process bears the correct RNA sequence as predicted from the linear DNA template.

#### 2.3.S.3.1.1.2. LC/MS/MS - Oligonucleotide Mapping

The primary structure of BNT162b2 Omicron XBB.1.5 RNA was analyzed by liquid chromatography tandem mass spectrometry (LC/MS/MS) - oligonucleotide mapping.

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The LC/MS/MS – oligonucleotide mapping results demonstrate that Omicron XBB.1.5 DS contains the correct sequence as predicted from the linear DNA template ([Section 3.2.S.2.3 Source, History and Generation of Plasmids \[Omicron \(XBB.1.5\) Variant\]](#)).

Further details are provided in [Section 3.2.S.3.1 Elucidation of Structure and Other Characteristics – \[Omicron \(XBB.1.5\) Variant\]](#).

#### 2.3.S.3.1.1.3. 3' Poly(A)-tail Characterization by LC/MS/MS – Oligonucleotide Mapping

Heightened characterization of the 3' polyadenosine tail (poly(A)-tail) of Omicron XBB.1.5 DS was accomplished CCI [REDACTED]

Further details are provided in [Section 3.2.S.3.1 Elucidation of Structure and Other Characteristics – \[Omicron\(XBB.1.5\) Variant\]](#)

The LC-UV/MS results demonstrate that Omicron XBB.1.5 DS contains the expected poly(A)-tail A30 and L70 segments CCI [REDACTED]

#### 2.3.S.3.1.1.4. 5'-Cap Characterization CCI [REDACTED]

The characterization of the 5' end capped (5'-Cap) and un-capped species of Omicron XBB.1.5 DS was accomplished by ion-pair reversed-phase high performance liquid chromatography-ultraviolet light detection at CCI [REDACTED] and online electrospray ionization mass spectrometry (IP-RP-HPLC/UV-ESI MS) or LC-UV/MS. Sample handling and chromatography follow the method described in [Section 3.2.S.4.2 Reversed Phase – High Performance Liquid Chromatography \(RP-HPLC\)](#).



Further details are provided in [Section 3.2.S.3.1 Elucidation of Structure and Other Characteristics – \[Omicron \(XBB.1.5\) Variant\]](#).

CCI [REDACTED]

#### 2.3.S.3.1.2. Higher Order Structure

The higher order structure of Omicron XBB.1.5 DS was characterized in solution using circular dichroism (CD) spectroscopy. CCI [REDACTED]

[REDACTED]

CCI



#### **2.3.S.3.1.3. Biological Activity of BNT162b2 Omicron XBB.1.5 DS**

The biological activity of Omicron XBB.1.5 DS requires the fully translated spike protein antigen. A Western blot analysis was used to evaluate the size of the expressed protein using *in vitro* translation.

#### **2.3.S.3.2. Impurities**

This section summarizes the impurities that are controlled and monitored during the drug substance manufacturing process.

Commercial scale batch testing results demonstrate that the process is capable of effectively and consistently delivering drug substance with acceptable levels of process and product related impurities and potential contaminants listed are listed in this section. Process validation data collected to date is provided for each impurity and will continue to be updated as testing for additional process validation batches is complete.

##### **2.3.S.3.2.1. Process-Related Impurities**

Process-related impurities are defined as impurities that originate from the manufacturing process and may be derived from reagents used in the in-vitro transcription and purification processes.

The process related impurities discussed in this section include residual DNA template.

##### **2.3.S.3.2.2. Residual DNA Template**

Residual DNA template is a process-related impurity derived from the linearized DNA template added to the in-vitro transcription reaction. Residual DNA template is further controlled through routine testing using the analytical procedure described in [3.2.S.4.2 Quantitative Polymerase Chain Reaction \(qPCR\)](#) and the Omicron (XBB.1.5) drug substance specification as described in [3.2.S.4.1 Specification – \[Omicron \(XBB.1.5\) Variant\]](#).

##### **2.3.S.3.2.3. Additional Process Related Impurities**

The additional process related impurities that were evaluated include nucleoside triphosphates (NTPs) and capping structure, small molecules, and enzymes. For further information about the evaluation of potential process-related impurities and result refer to [Section 3.2.S.2.6 Risk Assessment of Potential Process Related Impurities](#).

##### **2.3.S.3.2.2. Product-Related Impurities**

The product related impurities discussed in this section include double stranded RNA. Safety assessment of double stranded RNA was performed as part of specification setting. Refer to [3.2.S.4.5 Justification of Specifications \[Omicron \(XBB.1.5\) Variant\]](#). for information pertaining to levels of these impurities relative to patient safety.

### **Double Stranded RNA**

Double stranded RNA is a product-related impurity derived from the in-vitro transcription reaction. Double stranded RNA is further controlled through routine testing using the analytical procedure described in [3.2.S.4.2 Immunoblot](#) and the Omicron (XBB.1.5) drug substance specification as described in [3.2.S.4.1 Specification \[Omicron \(XBB.1.5\) Variant\]](#).

### **Potential Contaminants**

Potential contaminants are defined as any adventitiously introduced materials (e.g., chemical, biochemical, or microbial species) not intended to be part of the manufacturing process of the drug substance or drug product (ICH Q6B).

The potential contaminants that may be present in Omicron (XBB.1.5) drug substance are endotoxin and bioburden.

During manufacture of the drug substance, the manufacturing process has successfully been shown to effectively and consistently deliver drug substance with acceptable levels of the process and product related impurities and potential contaminants.