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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 (KP.2) Variant] describes the structure, function and other characteristics of BNT162b2 Omicron KP.2 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 KP.2 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 KP.2 drug substance has the expected structure and function.

Analytical characterization was performed with Omicron KP.2 variant drug substance batch LL6148.

Table 2.3.S.3.1-1. Characterization Strategy for BNT162b2 Omicron KP.2 Drug Substance

Characteristic	Analytical Approach	Methodology	Section References
Primary structure	Confirm expected RNA sequence at the oligonucleotide level	CCI	Section 2.3.S.3.1.1.1
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.3.1.1.2
	Confirm the presence and determine the length of poly(A)-tail		Section 2.3.S.3.1.1.3
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.3.1.1.3
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 RNase H digestion	Section 2.3.S.3.1.1.4
Higher order structure (HOS)	Spectroscopic analysis to confirm the presence and fingerprint of HOS	Circular dichroism (CD) spectroscopy	Section 2.3.S.3.1.2
Biological Activity	Confirm size of expressed protein	Western blot analysis	Section 2.3.S.3.1.3

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 KP.2 mRNA DS was performed CCI

CCI

CCI

CCI

RNAseq results demonstrate that the BNT162b2 Omicron KP.2 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 KP.2 RNA was analyzed by liquid chromatography tandem mass spectrometry (LC/MS/MS) - oligonucleotide mapping. Omicron KP.2 DS was digested with RNase T1, and the resulting enzymatic fragments were separated by ion-pair reversed-phase high performance liquid chromatography (IP-RP-HPLC) with UV detection at CCI

CCI

The LC/MS/MS - oligonucleotide mapping results demonstrate that Omicron KP.2 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 \(KP.2\) Variant\]](#)).

Further details are provided in [Section 3.2.S.3.1 Elucidation of Structure and Other Characteristics – \[Omicron \(KP.2\) 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 KP.2 DS was accomplished CCI

Further details are provided in [Section 3.2.S.3.1 Elucidation of Structure and Other Characteristics – \[Omicron \(KP.2\) Variant\]](#)

The LC-UV/MS results demonstrate that Omicron KP.2 DS contains the expected poly(A)-tail A30 and L70 segments CCI

2.3.S.3.1.1.4. 5'-Cap Characterization by LC-UV/MS

The characterization of the 5' end capped (5' -Cap) and un-capped species of Omicron KP.2 DS was accomplished by ion-pair reversed-phase high performance liquid chromatography-ultraviolet light detection at CCI 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).

CCI

Further details are provided in [Section 3.2.S.3.1 Elucidation of Structure and Other Characteristics – \[Omicron \(KP.2\) Variant\]](#).

CCI

CCI

CCI

2.3.S.3.1.2. Higher Order Structure

The higher order structure of Omicron KP.2 DS was characterized in solution using circular dichroism (CD) spectroscopy. A CD spectrum is a measure of differential absorption of the left- and the right-circularly polarized light by the test article, which arises due to structural asymmetry. The ordered structure of mRNA yields a CD spectrum that may contain positive and/or negative signals, while the absence of a CD signal generally indicates a lack of ordered structure.

CCI

2.3.S.3.1.3. Biological Activity of BNT162b2 Omicron KP.2 DS

The biological activity of Omicron KP.2 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 drug substance specification as described in 3.2.S.4.1 Specification – [Omicron].

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] 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 drug substance specification as described in 3.2.S.4.1 Specification [Omicron].

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 (KP.2) 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.