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3.2.S.3.1. ELUCIDATION OF STRUCTURE AND OTHER CHARACTERISTICS

This section contains information specific for formulations containing Original drug substance, which are discontinued. For information purposes, data/information supportive of the platform development approach for other presentations is maintained.

3.2.S.3.1.1. Introduction

This section describes the structure and characteristics of BNT162b2 drug substance (DS) which have been assessed using the analytical approaches outlined in Table 3.2.S.3.1-1. The analytical methodologies employed for BNT162b2 RNA drug substance characterization are capable of evaluating primary structure, including 5'-capping and 3'-poly(A) tail, and higher order structure. The results demonstrate that BNT162b2 RNA drug substance has the expected structure.

Analytical characterization was performed with BNT162b2 drug substance batch (20Y513C101), which is representative of the commercial process.

Table 3.2.S.3.1-1. Characterization Strategy for BNT162B2 Drug Substance

Characteristic	Analytical Approach	Methodology	Section References
Primary structure	Confirm expected RNA sequence at the oligonucleotide level	Reversed phase HPLC-UV and tandem mass spectrometry (LC/MS/MS) – of oligonucleotide fragments generated by RNAse T1 digestion	Section 3.2.S.3.1.2.1
	Confirm expected RNA sequence at the oligonucleotide level	4.2 1st ind Next Generation Sequencing Technology	Section 3.2.S.3.1.2.2
5'-Cap structure	Confirm the 5' capping structure and 5'-end profile	Reversed phase HPLC-UV and mass spectrometry (LC-UV/MS) analysis of purified 5′ terminal after RNaseH digestion	Section 3.2.S.3.1.3
Poly(A)-tail	Confirm the presence and determine the length of poly(A)-tail	Reversed phase HPLC-UV and mass spectrometry (LC-UV/MS) analysis of purified poly(A)-tail after Ribonuclease T1 digestion	Section 3.2.S.3.1.4
Higher order structure (HOS)	Spectroscopic analysis to confirm the presence and fingerprint of HOS	Circular dichroism (CD) spectroscopy	Section 3.2.S.3.1.5
Biological Activity	Confirm size of expressed protein Confirm spike protein expression	Western analysis Cell-based flow cytometry	Section 3.2.S.3.1.6

3.2.S.3.1.2. Primary Structure

3.2.S.3.1.2.1. LC/MS/MS - Oligonucleotide Mapping

The primary sequence of BNT162b2 DS was analyzed by LC/MS/MS - oligonucleotide mapping. BNT162b2 DS was digested with RNAse T1, and the resulting enzymatic fragments were separated by ion-paired reversed-phase high performance liquid chromatography (IP-RP-HPLC) with UV detection 4.2 1st ind (Figure 3.2.S.3.1-1 through Figure 3.2.S.3.1-5). 4.2 1st ind [redacted]

4.2 1st ind [redacted]

The 3′ terminal sequence region of BNT162b2 DS was detected by LC/MS/MS - oligonucleotide mapping, and the observed species are consistent with those observed by the Poly(A)-tail LC/MS methods described in Section 3.2.S.3.1.4. 4.2 1st ind [redacted] The 5′ terminal region including the 5′-Cap was characterized separately by LC/MS as described in Section 3.2.S.3.1.3.

The LC/MS/MS – oligonucleotide mapping results are summarized in Table 3.2.S.3.1-3 and demonstrate that BNT162b2 DS contains the correct sequence as predicted from the linear DNA template (Section 3.2.S.2.3 Source, History and Generation of Plasmids).

Figure 3.2.S.3.1-1. LC/MS/MS – Oligonucleotide Mapping of BNT162b2 DS (4.2 1st ind)

4.2 1st ind

Figure 3.2.S.3.1-2. LC/MS/MS – Oligonucleotide Mapping of BNT162b2 DS (4.2 1st ind)

4.2 1st ind

4.2 1st ind

Figure 3.2.S.3.1-3. LC/MS/MS – Oligonucleotide Mapping of BNT162b2 DS (4.2 1st ind)

4.2 1st ind

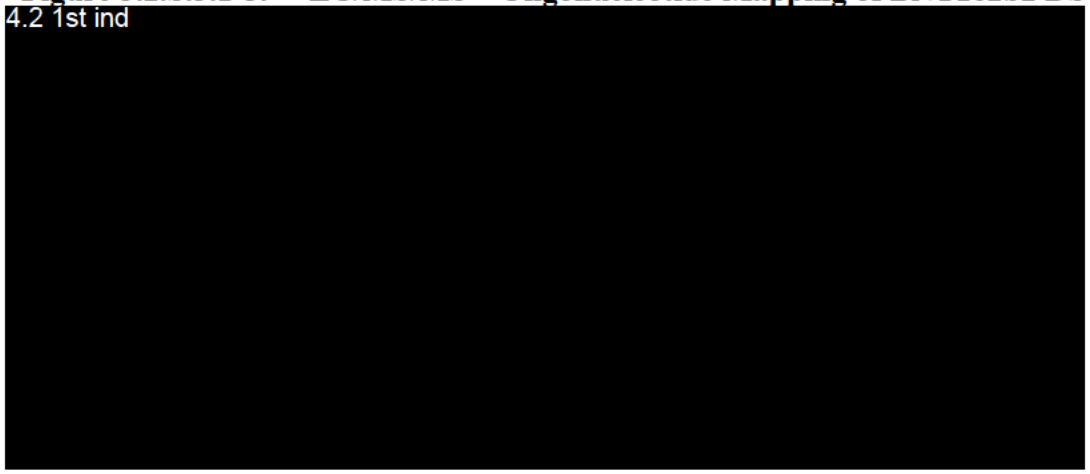
4.2 1st ind

Figure 3.2.S.3.1-4. LC/MS/MS – Oligonucleotide Mapping of BNT162b2 DS (4.2 1st ind)

4.2 1st ind

Figure 3.2.S.3.1-5. LC/MS/MS – Oligonucleotide Mapping of BNT162b2 DS (4.2 1st ind)

4.2 1st ind



4.2 1st ind

Table 3.2.S.3.1-2. LC-MS/MS – Oligonucleotide Mapping Sequence Coverage for BNT162b2

4.2 1st ind

[redacted]

Table 3.2.S.3.1-3. LC/MS/MS – Oligonucleotide Mapping Summary of BNT162b2 DS

Characteristics	Results
RNA sequence confirmation	BNT162b2 DS sequence coverage: Detected nucleotides represent 4.2 1st ind (Table 3.2.S.3.1-2)
RNA termini	4.2 1st ind - oligonucleotide mapping – see 3.2.S.3.1.3 for detailed analysis of 5' terminus 4.2 1st ind (see 3.2.S.3.1.4 for detailed analysis of PolyA tail)

3.2.S.3.1.2.2. Sequencing of RNA

In order to further confirm sequence identity, RNA sequencing for BNT162b2 DS was performed using the 4.2 1st ind Next Generation Sequencing (NGS) technology. BNT162b2 DS was subjected to NGS library preparation using an 4.2 1st ind [redacted] per manufacturer's recommendations.

4.2 1st ind [redacted]

4.2 1st ind [redacted]

4.2 1st ind [redacted]

Taken together, the RNA sequencing results further demonstrate that the BNT162b2 transcript generated during the *in vitro* transcription (IVT) process bears the correct RNA sequence as predicted from the linear DNA template (Section 3.2.S.2.3 Source, History and Generation of Plasmids).

Figure 3.2.S.3.1-6. BNT162b2 mRNA: RNAseq Coverage Plot

3.2.S.3.1.3. 5'-Cap Characterization by LC-UV/MS

The characterization of the 5' end capped (5'-Cap) and un-capped species of BNT162b2 DS was accomplished by ion-pair reversed-phase high performance liquid chromatography-ultraviolet light detection at 4.2 1st ind and online electrospray ionization mass spectrometry (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). 4.2 1st ind

analyzed by RP-HPLC/UV-ESI MS. In general, the ion-pair RP-HPLC-UV method separates species by the number of nucleotide residues, with shorter oligonucleotides eluting before longer oligonucleotides.

4.2 1st ind

Figure 3.2.S.3.1-7. 5'-Cap Assay UV Chromatogram of BNT162b2 DS

4.2 1st ind [redacted]

4.2 1st ind [redacted]

Figure 3.2.S.3.1-8. Mass Spectra of 5'-Cap, 5'-ppp and 5'-pp RNase Cleaved Fragments from BNT162b2 DS

Table 3.2.S.3.1-4. Accurate Mass Assignments for BNT162b2 DS 5′-Cap and non-Cap RNase Cleaved Fragments

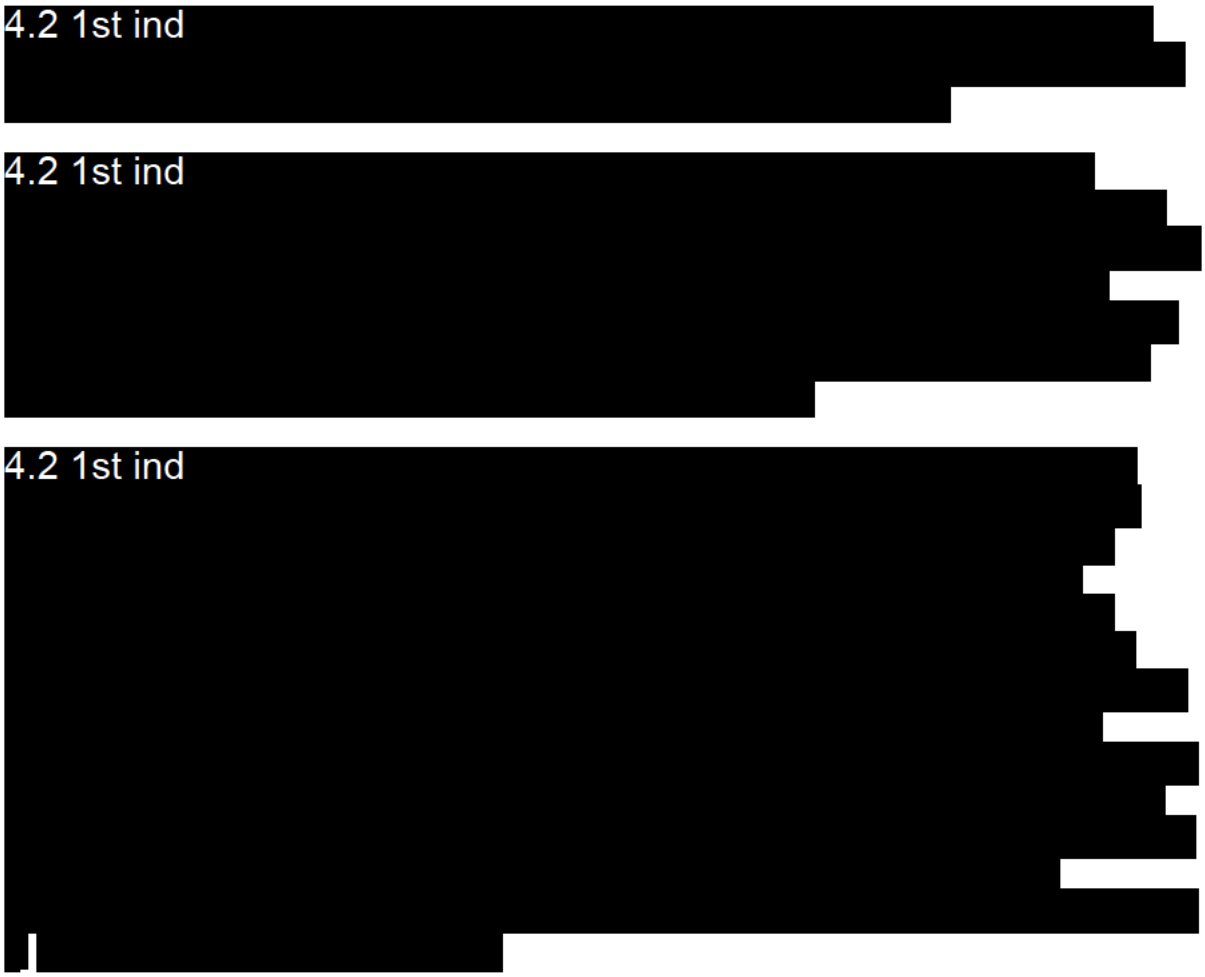
Species	Theoretical Monoisotopic Mass (Da)	Observed Mass (Da)[a]	Error (ppm)	Relative Abundance (%)[b]
4.2 1st ind				
4.2 1st ind				

3.2.S.3.1.4. 3′ Poly(A)-tail Characterization by LC-UV/MS

Analysis of the 3′ polyadenosine tail (poly(A)-tail) of BNT162b2 DS was accomplished by ion-pair reversed-phase high performance liquid chromatography with UV detection at 4.2 1st ind and on-line electrospray ionization mass spectrometry (RP-HPLC-UV/ESI MS or LC-UV/MS). The poly(A)-tail of BNT162b2 DS was cleaved off by ribonuclease T1 (RNase T1) followed by isolation via 4.2 1st ind affinity purification.

The plasmid-encoded poly(A)-tail of BNT162b2 DS, A30L70, measures 110 nucleotides in length, consisting of a stretch of 30 adenosine residues (A30 segment), followed by a 10 nucleotide linker sequence and another 70 adenosine residues (L70 segment). The RP-HPLC-UV profile of the extracted poly(A)-tail (Figure 3.2.S.3.1-9) 4.2 1st ind

4.2 1st ind



The LC-UV/MS results demonstrate that BNT162b2 DS contains the expected poly(A)-tail A30 and L70 segments 4.2 1st ind

Figure 3.2.S.3.1-9. RP-HPLC-UV Profile ([redacted]) of Extracted BNT162b2 DS Poly(A)-tail

Figure 3.2.S.3.1-10. Mass Spectrum of A30 Poly(A) Segment

Figure 3.2.S.3.1-11. Mass Spectrum of L70 Poly(A) Segment

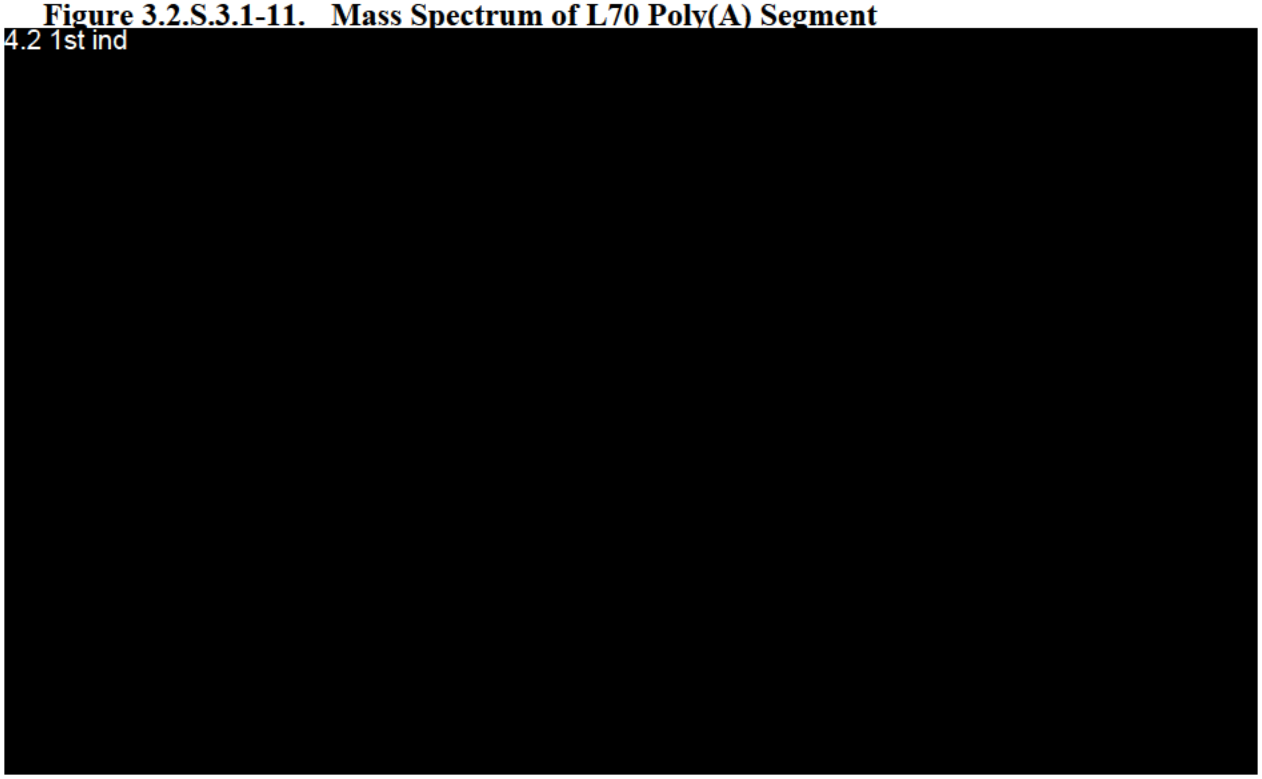


Table 3.2.S.3.1-5. Accurate Mass Assignments for BNT162b2 DS Poly(A)-tail

Poly(A) Segment	Sequence[a]	Number of Adenosines Observed (n)	Theoretical Masses (Da)[b]	Observed Masses (Da)[c]	Relative Abundance[d]
A30	4.2 1st ind				
L70					
	4.2 1st ind				

3.2.S.3.1.5. Higher Order Structure

The higher order structure of BNT162b2 mRNA 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.

Figure 3.2.S.3.1-12 CD spectrum of BNT162b2 mRNA

4.2 1st ind [redacted]

3.2.S.3.1.6. Biological Activity of BNT162b2 DS

The BNT162b2 RNA DS is formulated in lipid nanoparticles to create the drug product (DP). The lipid nanoparticle DP enables delivery of the RNA into host cells to allow expression of the SARS-CoV-2 S antigen and develop immune response. To characterize the biological activity of BNT162b2 RNA DS, a Western blot was used to evaluate the size of the expressed protein through transfection into HEK-293 cells in the presence of lipofectamine. In addition, a cell-based flow cytometry method was used to enumerate the population of cells which express the SARS-CoV-2 antigen encoded by the RNA in a DP sample.

3.2.S.3.1.6.1. Expressed protein size by Western blot

The protein size after in-vitro expression of BNT162b2 DS batch 20Y513C101 was determined using Western blot. 4.2 1st ind [redacted]

Figure 3.2.S.3.1-13. BNT162b2 Expressed Protein Size by Western Blot

4.2 1st ind [redacted]

4.2 1st ind [redacted] The results for DS batches R427-P020.2-DS, R438.P020-DS and R443.P020.2-DS are for information only.

4.2 1st ind [redacted]

Table 3.2.S.3.1-6. Theoretical protein size after BNT162b2 expression

	S Protein with Signal Peptide	Mature S Protein	S1 Subdomain	S2 Subdomain
4.2 1st ind	4.2 1st ind			
4.2 1st ind				

4.2 1st ind

Figure 3.2.S.3.1-14. Deglycosylated proteins after BNT162b2 transfection are consistent with the S1S2 protein

3.2.S.3.1.6.2. In Vitro Expression by Cell-Based Flow Cytometry

The in vitro expression of SARS-CoV-2 spike protein encoded by the RNA in BNT162b2 DS batch 20Y513C101, which has been formulated into lipid nanoparticle drug product (DP) was determined. DS batch 20Y513C101 was used in the production of DP lots EE8492 and EE8493. This analysis was performed using the method described in Section 3.2.P.5.2 Analytical Procedures – Cell-based Flow Cytometry.

Human embryonic kidney (HEK293T) cells were transfected with BNT162b2 DP. After incubation, the cells were harvested and transferred to assay plates. Transfected cells were stained, fixed, and permeabilized. Fixative was washed from the cells and a SARS-CoV-2 spike S1 primary antibody was added, which binds to any surface-expressed and intracellular SARS-CoV-2 S1 antigen. 4.2 1st ind Cells were analyzed by flow cytometry where the in vitro expression of the SARS-CoV-2 spike was determined as the percent positive cells (S1+) from the viable, single cell population. The results of this analysis are provided in Table 3.2.S.3.1-7.

Table 3.2.S.3.1-7. In Vitro Expression of BNT162b2 Drug Product

Drug Product Lot	Drug Substance Batch	In Vitro Expression (% positive cells)
EE8492	20Y513C101	4.2 1s
EE8493	20Y513C101	4.2 1s

3.2.S.3.1.7. Heightened Characterization of BNT162b2 Fragment Species

The BNT162b2 fragment profile is measured using 4.2 1st ind method, which enables quantitation of RNA integrity (intact RNA content). It is expected that a majority of the fragmented species are generated by premature transcriptional stops or mRNA hydrolysis. In the study described below, the fragmented species were evaluated for the presence of 5'-cap and poly(A) tail elements needed for protein expression, and the potential for fragment species to express truncated or off-target protein antigens was evalutated.

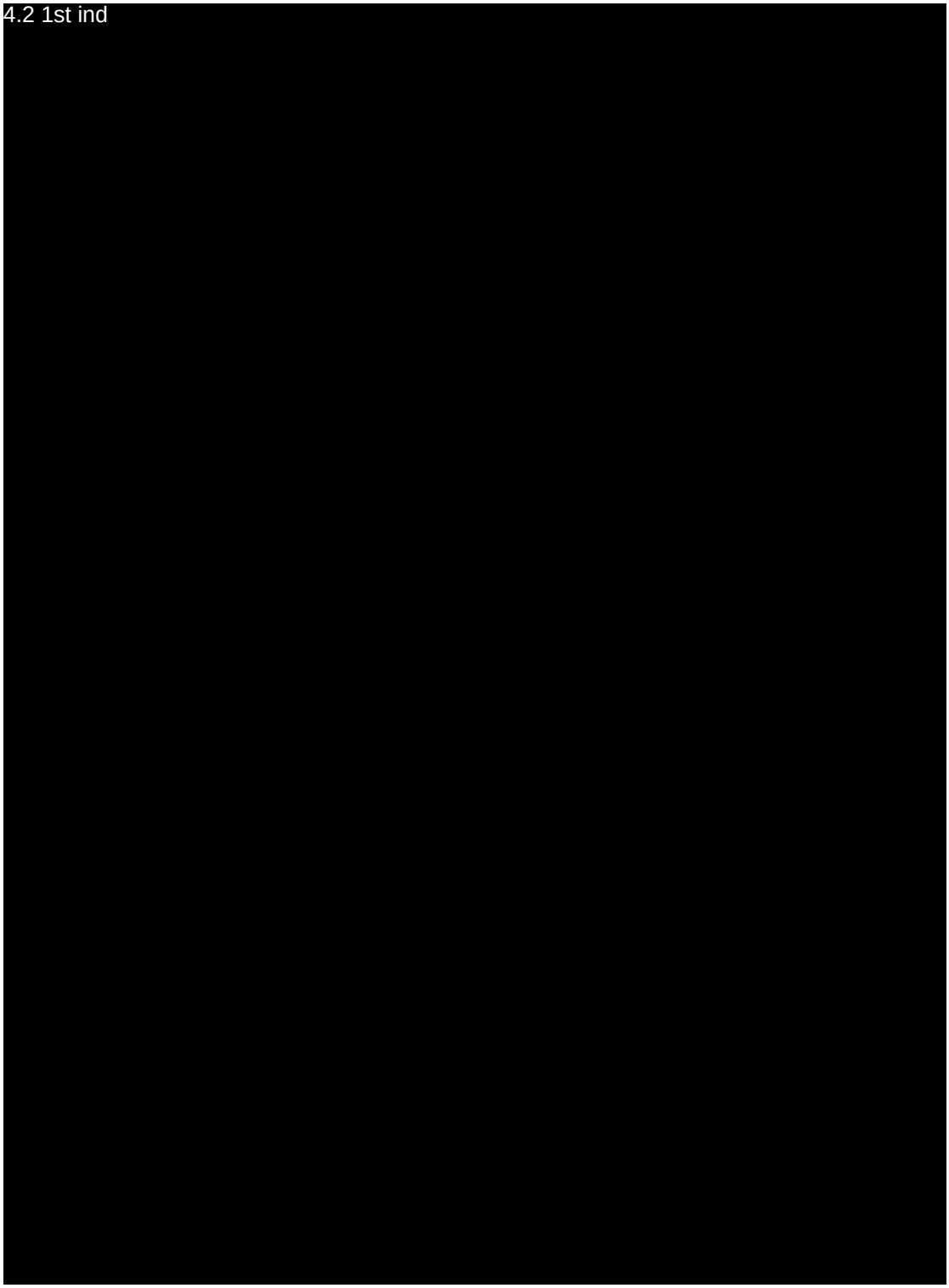
Additional characterization of intact and fragment species isolated using ion pairing (IP) RP-HPLC

DS batch R427-P020.2-DS (Process 1) and batch 20Y513C501 (Process 2) samples were fractionated using ion pairing RP-HPLC to further characterize the drug substance mRNA species. The samples of the active substance are representative of the finished product, as the fragment species profile is consistent between DS and DP, with only minor increases to the fragment content during DP manufacturing.

Figure 3.2.S.3.1-15 shows the ion pairing RP-HPLC chromatograms for batches R427-P020.2-DS and 20Y513C501. 4.2 1st ind

Figure 3.2.S.3.1-15. Ion Pairing RP-HPLC Chromatograms for DS batches R427-P020.2-DS and 20Y513C501

Figure 3.2.S.3.1-16. Fragment Analyzer Electropherograms of Peak 1, Peak 2, and Unfractionated DS



Peaks 1 and 2 from each DS batch (R427-P020.2-DS and 20Y513C501) 4.2 1st ind

Table 3.2.S.3.1-8. 5'-Cap Species Content

Sample	Early Non-Cap (%)	5'-Cap (%)	Late Non-Cap (%)
R427-P020.2-DS	4.2 1st ind		
Peak 1 (R427-P020.2-DS)			
Peak 2 (R427-P020.2-DS)			
20Y513C501			
Peak 1 (20Y513C501)			
Peak 2 (20Y513C501)			

Figure 3.2.S.3.1-17. 5'-Cap Chromatograms of BNT162b2 DS, Peak 1, and Peak 2 Samples

4.2 1st ind

4.2 1st ind

Quantitation of the poly(A) tail content by ddPCR 4.2 1st ind

Table 3.2.S.3.1-9. Poly(A) Tail Content

Sample	Poly(A) Tail Content (%)
R427-P020.2-DS	4.2 1st ind
Peak 1 (R427-P020.2-DS)	
Peak 2 (R427-P020.2-DS)	
20Y513C501	
Peak 1 (20Y513C501)	
Peak 2 (20Y513C501)	

4.2 1st ind

Figure 3.2.S.3.1-18. Poly(A) Length and Distribution of BNT162b2 DS, Peak 1, and Peak 2 Samples

4.2 1st ind

Assessment of the potential for translation into truncated S1S2 or other off-target proteins/peptides

To assess the potential for translation of mRNA fragments into truncated S1S2 proteins/peptides or other proteins/peptides, [redacted] orthogonal protein expression systems were utilized and translated proteins were evaluated by Western blot.

Figure 3.2.S.3.1-19. Full-Length Transcripts Require 5'-Cap for Translation

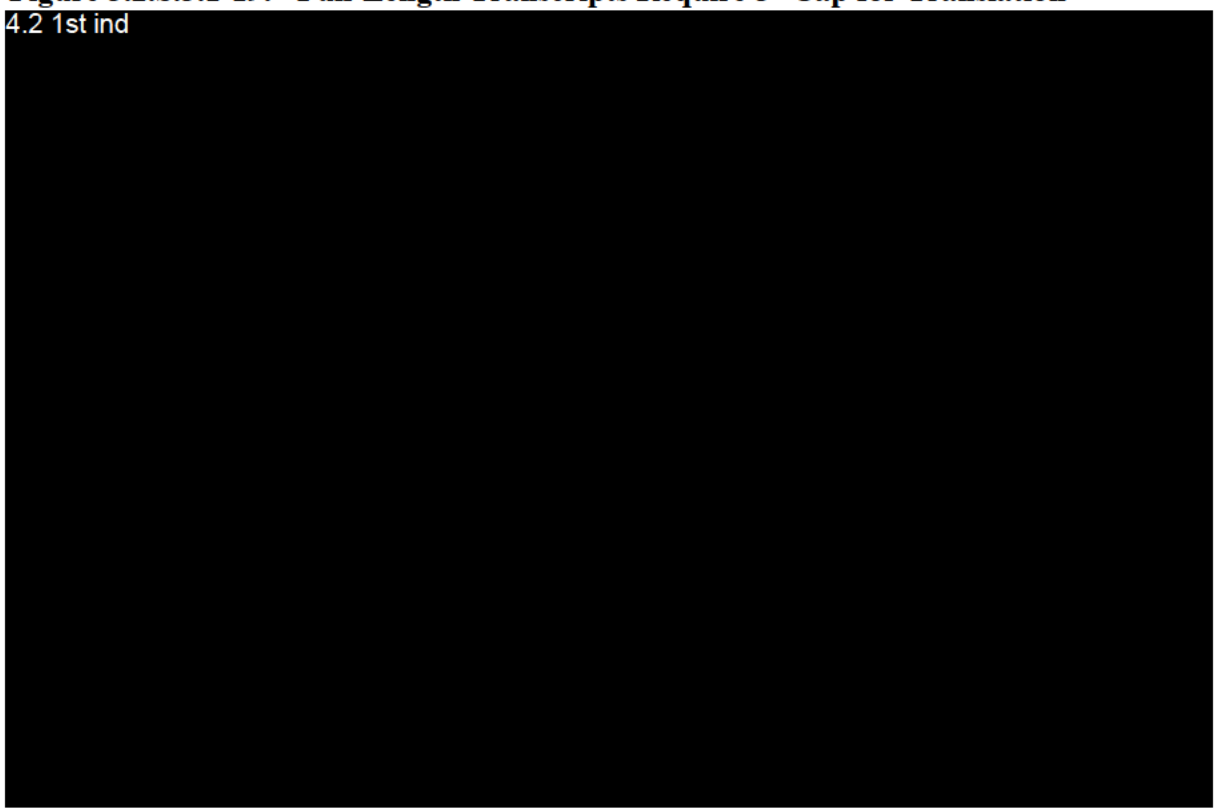


Figure 3.2.S.3.1-20. Full-Length Transcripts Require Poly(A) Tail for Expression

Consistent with the results using full-length mRNA transcripts, cells transfected with the starting DS batches (R427-P020.2-DS and 20Y513C501) or with 4.2 1st ind

Figure 3.2.S.3.1-21. Western Blot of Peak 1, Peak 2, and Starting DS Material

In a separate experiment, the potential for truncated transcripts to produce protein/peptides was evaluating using a cell-free in vitro expression system. The cell-free in vitro translation is performed using the commercially available Rabbit Reticulocyte Lysate (RRL) system, which is provided by 4.2 1st ind

4.2 1st ind

The RNA that was used in the in vitro translation study was produced as engineering run in the BioNTech Mainz / Rentschler manufacturing node generating representative material according to the commercial process (batch no: 1071509; 4.2 1st ind IVT reaction; 4.2 1st ind drug substance; expected protein size: approx. 4.2 1st ind). The batch data are presented in

Table 3.2.S.3.1-10. Batch data of the engineering run 1071509 (BioNTech Mainz/ Rentschler)

Parameter	Result
RNA integrity	4.2 1st ind
dsRNA	
Residual DNA template	
Identity of encoded RNA sequence	Identity confirmed
Appearance (Coloration)	Not more intensely colored than level [redacted] of brown (B) color standard
Appearance (Clarity)	4.2 1st ind
Content (RNA concentration)	
Bioburden	
Endotoxins	
pH	
5′-Cap	
Poly(A) tail	

BNT162b2 Process 2 batch 1071509 was intentionally degraded by exposure to elevated temperature, generating samples with various levels of fragmented species (Table 3.2.S.3.1-11). Fragment species in thermally degraded samples are generated by hydrolysis, which is the expected degradation pathway subsequent to in-vitro transcription

(i.e. in the DP manufacturing process). 4.2 1st ind [redacted]

Table 3.2.S.3.1-11. Thermally Degraded BNT162b2 Drug Substance

Sample	Degradation Time (min)	RNA Integrity (%)
1071509	4.2 1st ind [redacted]	
1071509-2		
1071509-4		
1071509-6		
1071509-9		
1071509-10		
1071509-20		

Figure 3.2.S.3.1-22. In vitro translation of degraded BNT162b2 by Western blot

4.2 1st ind [redacted]

The additional characterization data support the following conclusions:

- Fragment species observed by [redacted] are consistent across DS Process 1 and Process 2
  - Fragment species isolated using IP-RP-HPLC [redacted] contain 5'-cap, but lack poly(A) tail
  - IP-RP-HPLC [redacted]
- 5'-cap and poly(A) tail are required for translation of the full-length BNT162b2 transcript
- Evaluation of full-length and degraded/fragmented transcript expression in [redacted] orthogonal expression systems shows no evidence of truncated S1S2 or other proteins or peptides

Taken together, the characterization studies support the assessment that the BNT162b2 fragment species do not pose a risk of mRNA translation resulting in off-target proteins or peptides.

Literature References:

Benjamin D, Sato T, Cibulskis K, et al. Calling Somatic SNVs and Indels with Mutect2. bioRxiv 2019; 10.1101/861054.

Beverly M, Hagen C, Slack O. Poly A tail length analysis of in vitro transcribed mRNA by LC-MS. Anal. Bioanal. Chem. 2018; 410:1667-1677

Clopper C J, and Pearson E S. The Use of Confidence or Fiducial Limits Illustrated in the Case of the Binomial. Biometrika 1934; 26:404–413.

Poplin R, Ruano-Rubio V, DePristo M A, et al. Scaling accurate genetic variant discovery to tens of thousands of samples. bioRxiv 2018; 10.1101/201178.