SPECIFIC OBLIGATION (SO#1)

In order to complete the characterisation of the active substance and finished product, the MAH should provide additional data. The following data are requested in order to complete the information on the active substance and finished product characterisation.

a) Additional data is to be provided to further characterise the truncated and modified mRNA species present in the finished product both from process 1 and 2. Data are expected to cover batches used in clinical trials (for which the characterisation data could be available earlier) and the PPQ batches. These data should address results from ion pairing RP-HPLC addressing 5'cap levels and presence of A30 and L70 in the poly(A) tail. These data should further address the potential for translation into truncated S1S2 proteins/peptides or other proteins/peptides. Relevant protein/peptide characterization data for predominant species should be provided. Any homology between translated proteins (other than the spike protein) and human proteins that may, due to molecular mimicry, potentially cause an autoimmune process should be evaluated. **Due date: July 2021. Interim reports: March 2021, and on a monthly basis.**

b) The analysis of the main peak of the RNA integrity test representing the full-length RNA, should be also undertaken using the ion pairing RP-HPLC addressing 5'cap levels and presence of A30 and L70 in the poly (A) tail. **Due date: July 2021. Interim report: March 2021**

c) Additional data for the active substance are to be provided to confirm the identities of the observed Western Blot (WB) bands obtained by the *in vitro* expression assay. Protein heterogeneity, resulting in broad bands on the WB and uncertainties in the theoretical intact molecular weight of the spike protein, is assumed to be due to glycosylation. Therefore, to further confirm protein identities, enzymatic deglycosylation of the expressed proteins followed by WB analysisshould be performed. Correlation with the calculated molecular weights of the intact S1S2 protein should be demonstrated. **Due date: July 2021. Interim report: March 2021**

RESPONSE to SPECIFIC OBLIGATION

Response to (a) and (b):

The BNT162b2 fragment profile, as measured using the Fragment Analyzer CGE method, was demonstrated to be consistent between Process 1 and Process 2 drug substance (DS) in the previously submitted Response Q002 – Quality – Major Objection 04-Dec-2020 (seq 0003). The associated safety assessment revealed that the likelihood of fragmented transcripts being expressed into proteins is low, with a rationale that a majority of the fragmented species are generated by premature transcriptional stops or mRNA hydrolysis.

As such, the fragmented species predominantly do not contain both 5'-cap and poly(A) tail elements needed for protein expression.

The requested additional characterization data are provided below, along with new data that conclusively demonstrate that the fragmented mRNA species do not pose a risk for expression of truncated or other proteins.

Additional characterization of 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 intact and fragment 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 1 shows the ion pairing RP-HPLC chromatograms for batches R427-P020.2-DS and 20Y513C501. For both batches, two peaks are observed by IP-RP-HPLC. Fragment Analyzer electropherograms (Figure 2) demonstrate that Peak 1 consists almost entirely of fragmented species, consistent with the data provided previously in Assessment of Responses to CHMP Q01 – Quality 11-Dec-2020 (seq 0006). The Peak 2 electropherogram demonstrates that the predominant species is consistent with the main peak observed by fragment analyzer, with lower levels of fragments compared with the starting material.

Figure 1. Ion Pairing RP-HPLC Chromatograms for DS batches R427-P020.2-DS and 20Y513C501

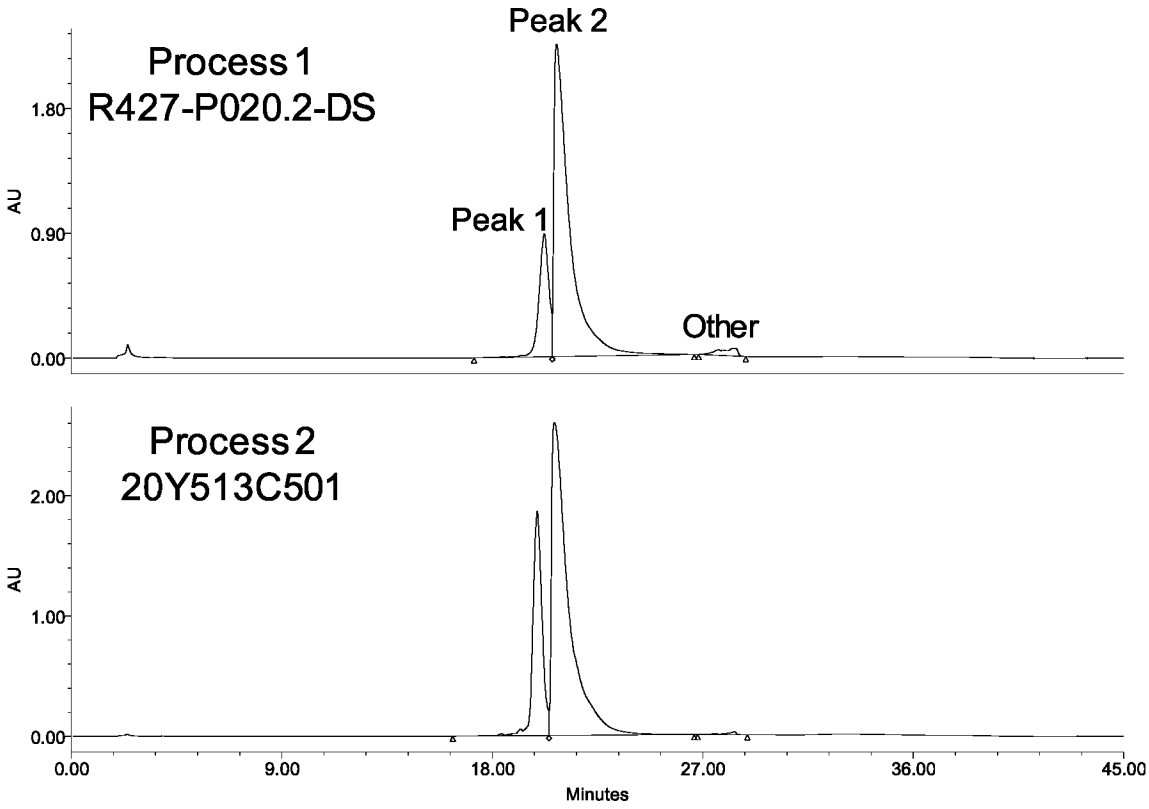
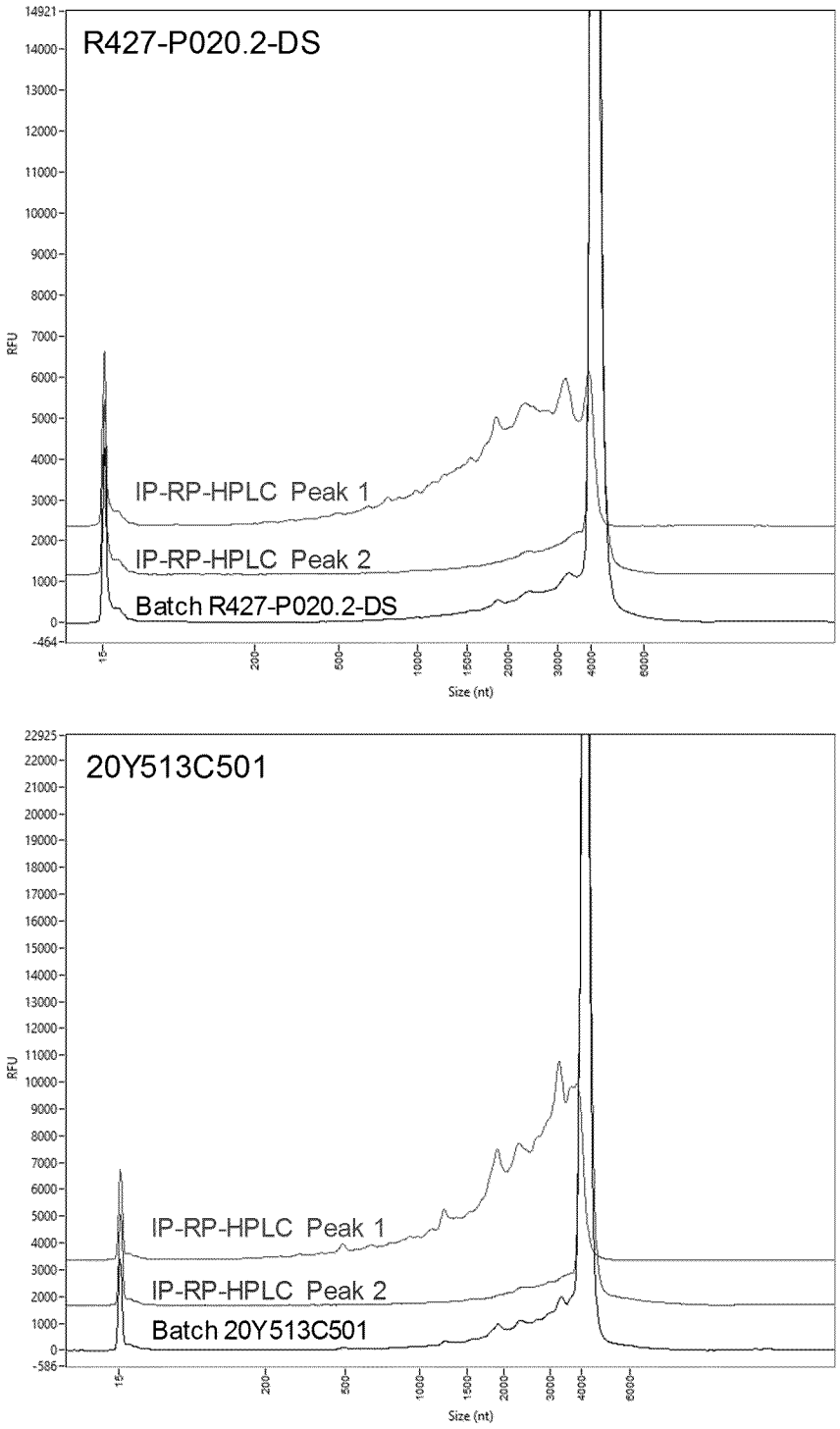


Figure 2. Fragment Analyzer Electropherograms of Peak 1, Peak 2, and Unfractionated DS

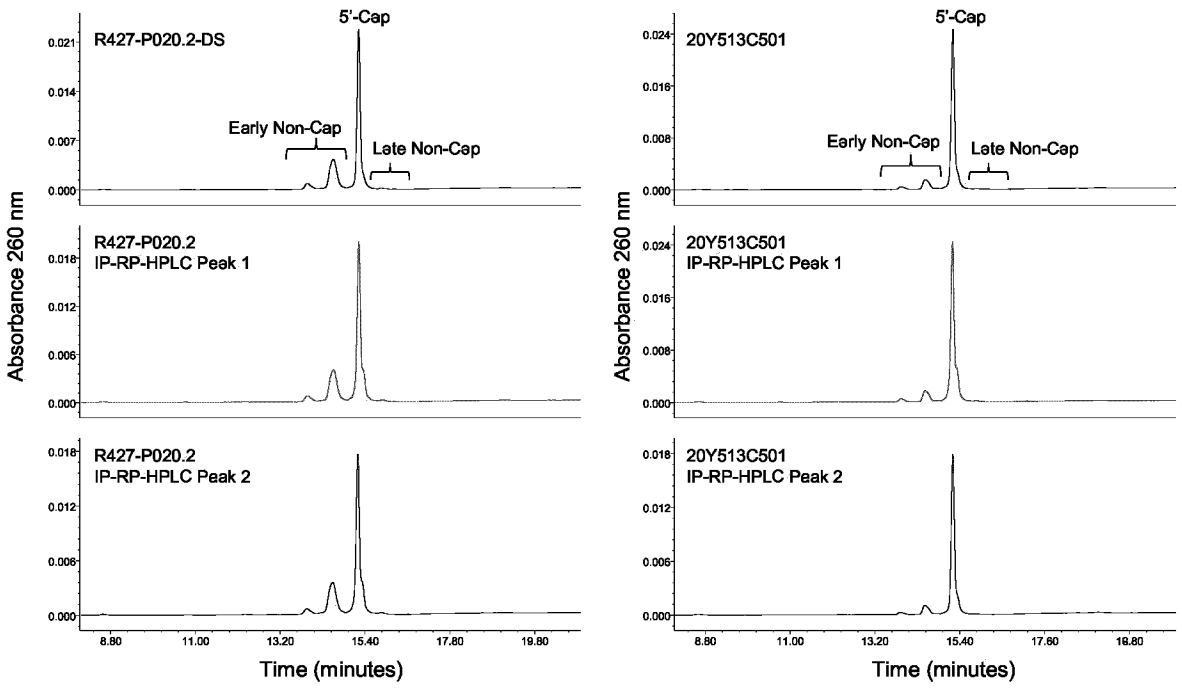


Peaks 1 and 2 from each DS batch (R427-P020.2-DS and 20Y513C501) were then isolated and characterized to compare 5'-cap and poly(A) tail length to that of unfractionated material. Table 1 shows that 5'-cap and non-cap species content is unchanged across Peak 1, Peak 2, and unfractionated starting materials, consistent with previous characterization data. Additionally, comparable 5'-cap chromatographic profiles were observed for all six samples (Figure 3).

Table 1. 5'-Cap Species Content

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

Figure 3. 5'-Cap Chromatograms of BNT162b2 DS, Peak 1, and Peak 2 Samples



In contrast to 5'-cap, characterization of the poly(A) tail length and distribution by IP-RP-HPLC demonstrates that the fragment species in Peak 1 lack poly(A) tail, while the Peak 2 and unfractionated starting DS samples show the expected length and distribution for the BNT162b2 poly(A) tail (Figure 4). These results support the conclusion that a significant proportion of fragment species in BNT162b2 are 5'-capped at levels consistent with the

intact transcript, but predominantly lack the poly(A) tail, likely arising from premature transcriptional stops during production.

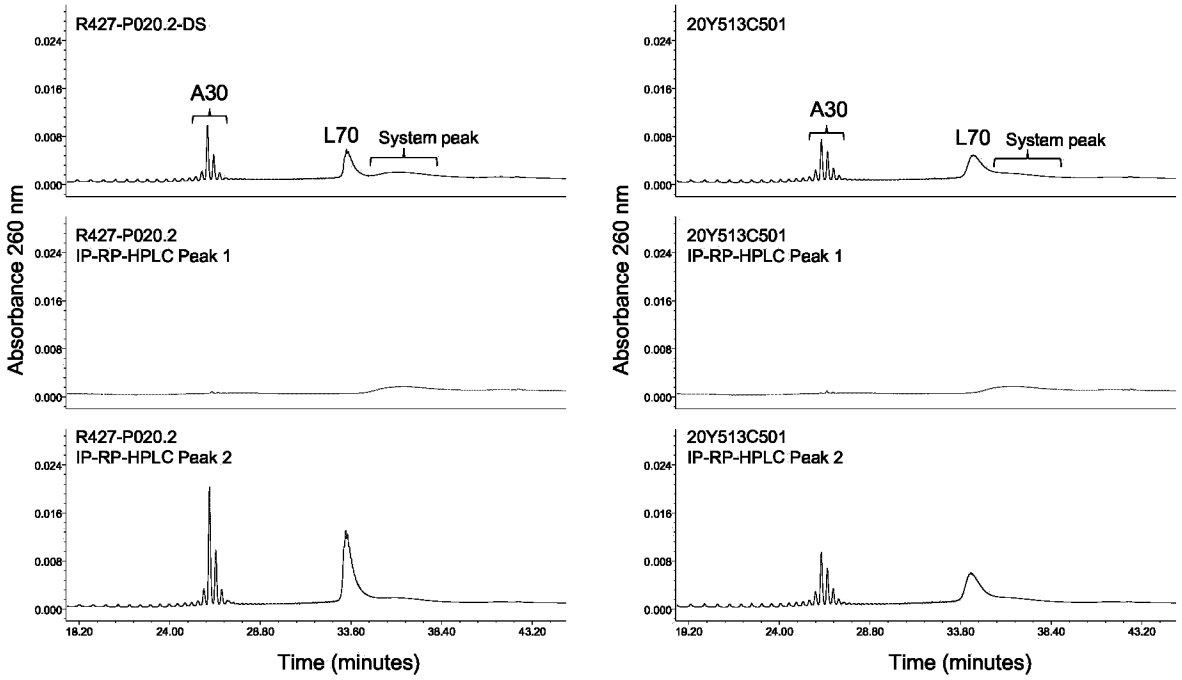
Quantitation of the poly(A) tail content by ddPCR further confirms that the fragment species in Peak 1 lack significant poly(A) tail content (< LOQ), while the Peak 2 and starting DS materials for both R427-P020.2-DS and 20Y513C501 contain [redacted] poly(A) tail content (Table 2). Additionally, the poly(A) tail content in Peak 2 fractions is consistently higher than the starting material, indicating enrichment of intact (i.e. poly-adenylated) transcripts in these samples.

Table 2. Poly(A) Tail Content

Sample	Poly(A) Tail Content (%)
R427-P020.2-DS	[redacted]
Peak 1 (R427-P020.2-DS)	
Peak 2 (R427-P020.2-DS)	
20Y513C501	
Peak 1 (20Y513C501)	
Peak 2 (20Y513C501)	

LOQ = Limit of Quantitation

Figure 4. Poly(A) Length and Distribution of BNT162b2 DS, Peak 1, and Peak 2 Samples



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, two orthogonal protein expression systems were utilized and translated proteins were evaluated by Western blot.

As shown previously in Section 3.2.S.2.6 Development History and Comparability Assessment, Western blot of BNT162b2-transfected cell lysates demonstrates expression of the S1S2 protein antigen. In this previous study, truncated protein species were not observed by Western blot in DS batches that contained up to 40% fragment species.

To test the hypothesis that transcripts require both 5'-cap and poly(A) to support protein translation, full-length transcripts lacking either 5'-cap or poly(A) tail were transfected into HEK-293 cells, and the resulting cell lysates were analyzed by Western blot using detection antibodies specific for the S1 or S2 domain. In contrast to BNT162b2 DS, which shows the expected banding pattern for the S1S2 protein antigen, S1S2 protein bands were not detected for the transcripts lacking either the 5'-cap (Figure 5) or the poly(A) tail (Figure 6). This control experiment using full-length transcripts demonstrates that both 5'-cap and poly(A) tail are required for protein expression.

Figure 5. Full-Length Transcripts Require 5'-Cap for Translation

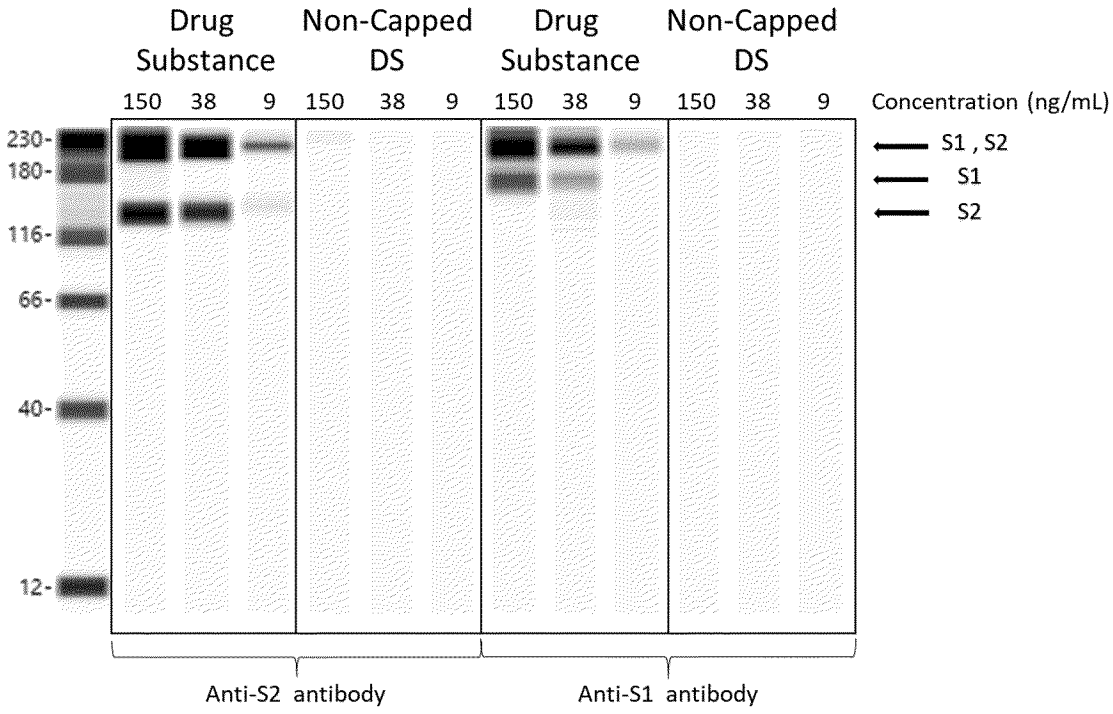
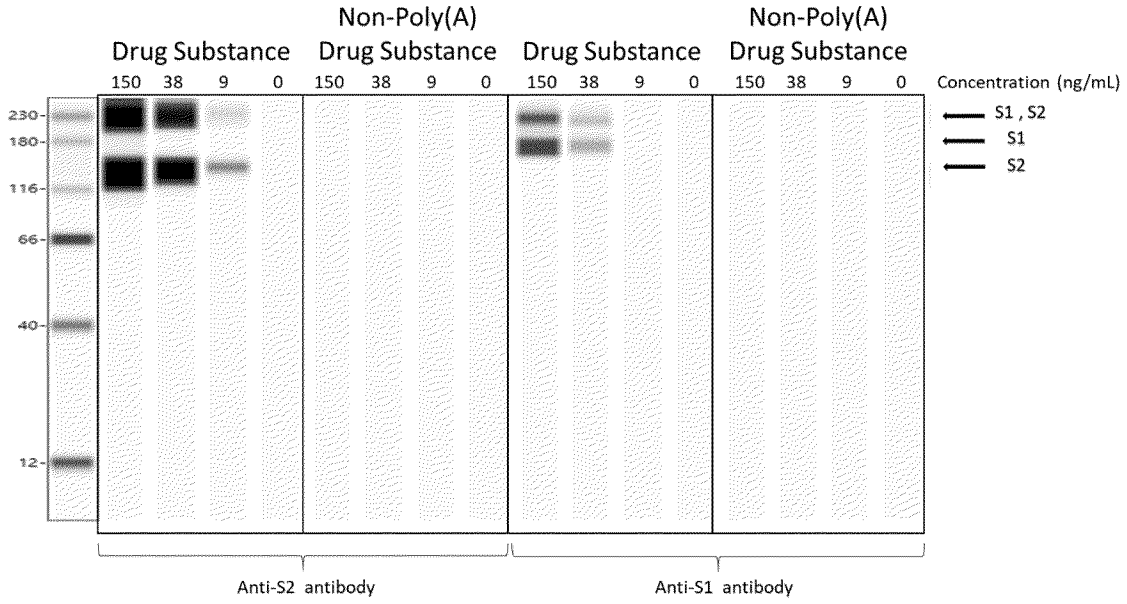
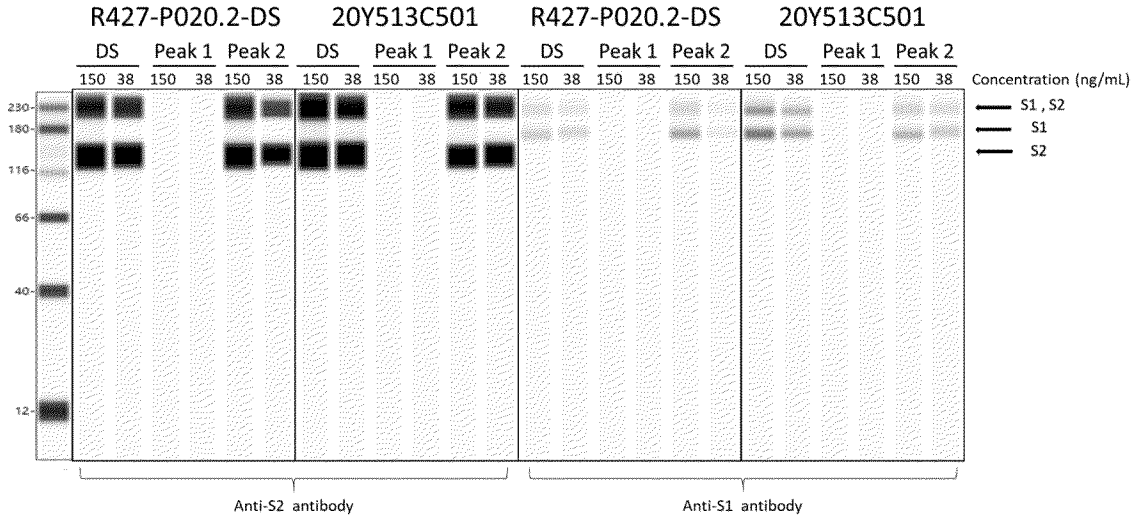


Figure 6. Full-Length Transcripts Require Poly(A) Tail for Expression



Consistent with the results using full-length mRNA transcripts, cells transfected with the starting DS batches (R427-P020.2-DS and 20Y513C501) or with Peak 2 material (isolated using IP-RP-HPLC above) show comparable spike protein expression by Western blot, whereas cells transfected with the Peak 1 material comprised of fragmented species do not show evidence of protein expression (Figure 7). The results are consistent for both Process 1 and Process 2 batches, and both show a dose-dependent expression response for the unfractionated DS and Peak 2 samples.

Figure 7. Western Blot of Peak 1, Peak 2, and Starting DS Material

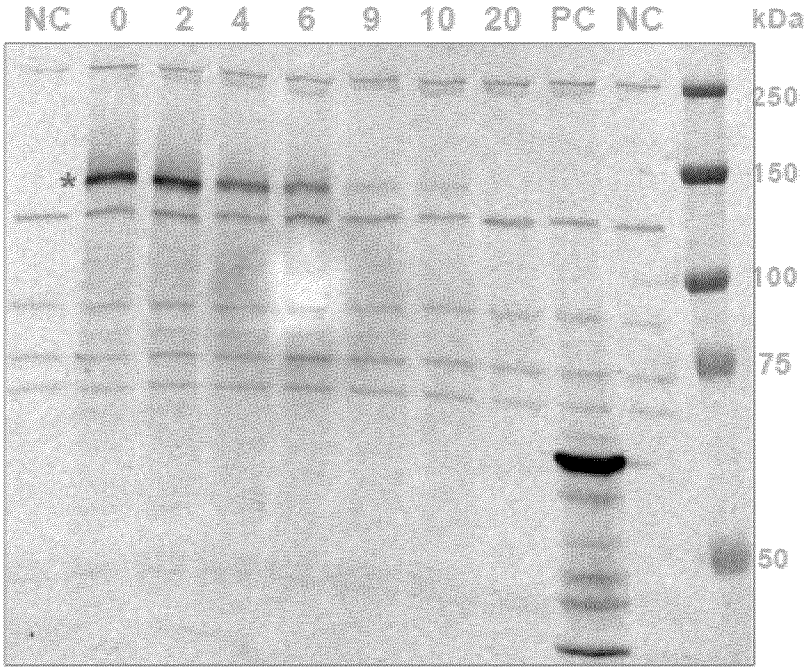


In a separate experiment, the potential for truncated transcripts to produce protein/peptides was evaluating using a cell-free in vitro expression system. BNT162b2 Process 2 batch 1071509 was intentionally degraded by exposure to elevated temperature, generating samples with various levels of fragmented species (Table 3). 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). The starting and degraded materials were subjected to in vitro expression using rabbit reticulocyte lysate, wherein biotinylated lysine is incorporated into newly translated proteins and peptides for detection by Western blot. This approach enables detection of both truncated S1S2 or other proteins/peptides, if present, without the need for protein/peptide-specific detection antibodies. After in vitro expression, the non-degraded BNT162b2 sample produced a protein approximately 140 kDa, which is consistent with the expected size of the aglycosylated S1S2 protein (denoted with a "*" in Figure 8 and additionally discussed in the response to part (c) below). For degraded samples, RNA integrity correlated with Western blot band intensity for the full-length protein; however, no truncated or other protein species were detected beyond the background bands observed in the negative control sample (NC).

Table 3. Thermally Degraded BNT162b2 Drug Substance

Sample	Degradation Time (min)	RNA Integrity (%)
1071509	0	
1071509-2	2	
1071509-4	4	
1071509-6	6	
1071509-9	9	
1071509-10	10	
1071509-20	20	

Figure 8. In vitro translation of degraded BNT162b2 by Western blot



The additional characterization data presented here and provided previously support the following conclusions:

- Fragment species observed by Fragment Analyzer are consistent across DS Process 1 and Process 2
  - Fragment species isolated using IP-RP-HPLC (Peak 1) contain 5'-cap, but lack poly(A) tail
  - IP-RP-HPLC Peak 2, which is predominantly comprised of intact RNA, include 5'-capped and poly-adenylated species
- 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 two 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.

Response to (c):

The theoretical sizes of full-length and truncated S1S2 protein constructs (initially provided in Responses Q004 – Quality 07-Dec-2020 (seq 0004)) are shown in Table 4. As described above, the approximately 140 kDa protein expressed after in vitro expression of BNT162b2 is fully consistent with the theoretical size of the aglycosylated S1S2 protein (Figure 8). In this cell-free expression system, which lacks the cellular components required for glycosylation, the BNT162b2 expression indicates that a single, full-length or mature S1S2 protein is produced.

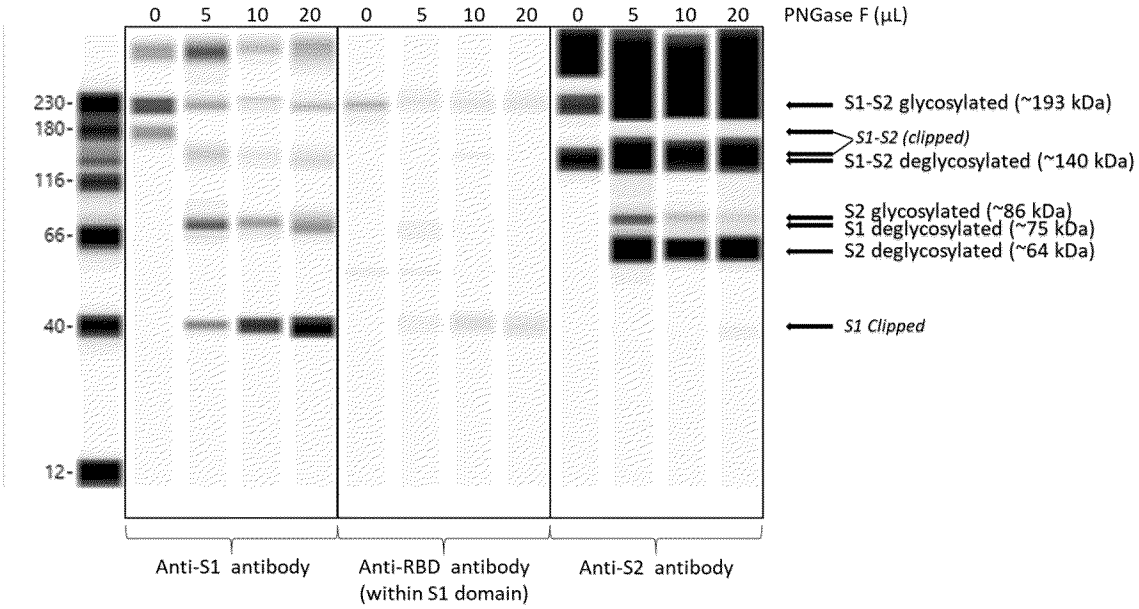
Table 4. Theoretical protein size after BNT162b2 expression

	S Protein with Signal Peptide	Mature S Protein	S1 Subdomain	S2 Subdomain
Residues	1-1273	13-1273	13-685	686-1273
# Amino Acids	1273	1261	673	588
Aglycosylated MW [Da]	141115.0	139755.3	75310.7	64462.6
Glycosylated MW[a] [Da]	192862.6	191502.9	105888.8	85632.0

Theoretical masses assume full occupancy of all 22 N-linked glycosylation sites with sialylated G2F+2NeuAc.

Additionally, the recommended enzymatic deglycosylation of the expressed proteins following BNT162b2 transfection into HEK-293 was pursued. In this study, increasing volumes of the de-glycosylating enzyme PNGase F were added to transfected cell lysates, and the resulting samples were analyzed by Western blot, with detection using antibodies specific for the S1 domain, the receptor binding domain (RBD, located within the S1 domain), and the S2 domain. The resulting Western blot is shown in Figure 9. Upon PNGase F digestion, new bands ranging from ~40 kDa to ~140 kDa are detected. The ~140 kDa protein detected by the anti-Spike protein S1 domain antibody, anti-spike protein S2 domain antibody and anti-spike protein S1 domain RBD antibody is consistent with the deglycosylated full-length spike protein (S1S2). The band of ~75 kDa detected by anti-Spike protein S1 domain antibody and anti-spike protein S1 domain RBD antibody is consistent with deglycosylated S1 domain. The band of ~64 kDa detected only by anti-Spike protein S2 domain antibody is consistent with deglycosylated S2 domain. The new ~40kDa band detected by the anti-S1 and anti-RBD antibodies and the new band of ~90 kDa detected by anti-S2 antibody are believed to be the result of further proteolysis of the S1S2 spike protein within the cell lysate during PNGase F treatment steps. High molecular weight bands in the Western blot (i.e. > 230 kDa) likely result from aggregated spike proteins that are not fully denatured during sample preparation.

Figure 9. Deglycosylated proteins after BNT162b2 transfection are consistent with the S1S2 protein



Overall Conclusions for the Fulfillment of Specific Obligation 1a, b, and c

The additional characterization data presented here fulfill the Specific Obligation 1 commitment and demonstrate that fragment species observed in BNT162b2 are not expressed as truncated or other proteins/peptides. Further, additional characterization of the expressed protein using two orthogonal cell expression systems demonstrates that the expressed protein is the expected, full-length ~140 kDa S1S2 protein. The data indicate that the S1S2 protein expressed in HEK-293 cells is post-translationally glycosylated, as confirmed by the observed removal of these glycans after addition of PNGase F to the cell lysate. Lastly, expression studies in rabbit reticulocyte lysate and in transfected cells show no evidence of truncated or other proteins expressed, including in samples degraded by elevated temperature to promote RNA fragmentation or in fragment species isolated using ion pairing RP-HPLC.

Literature References

None

SUPPORTING DOCUMENTATION

New or Replaced Supporting Documentation

None

Previously submitted supporting documentation

3.2.S.2.6 Development History and Comparability Assessment, seq 0003

Response Q002 – Quality – Major Objection 04-Dec-2020, seq 0003

Assessment of Responses to CHMP Q01 – Quality 11-Dec-2020, seq 0006

Responses Q004 – Quality 07-Dec-2020, seq 0004