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S.2.2. DESCRIPTION OF MFG. PROCESS AND PROCESS CONTROLS [BNT MAINZ AND RENTSCHLER]

This leaflet describes the manufacturing process for Process 2 drug substance manufactured in Mainz and Laupheim, Germany. The differences between Process 1 and Process 2 are outlined in [Section S.2.6 Manufacturing Process Development \(modRNA\) \(Process 2\)](#).

S.2.2.1. Batch Scale and Definition

S.2.2.2. Definition of a Production Batch – BNT162b2 Drug Substance

Commercial scale drug substance batches are executed at a scale of 37.6 L starting volume for in vitro transcription (IVT). All material produced is purified by a single two-stage ultrafiltration/diafiltration (UFDf) to produce drug substance.

S.2.2.3. Overview of Manufacturing Process

The RNA is first synthesized via an in vitro transcription (IVT) followed by DNase I and proteinase K digestion steps, which aid in purification. The crude RNA is then purified through a 2-stage ultrafiltration/diafiltration (UFDf). Lastly, the RNA undergoes a final filtration before being dispensed and stored frozen.

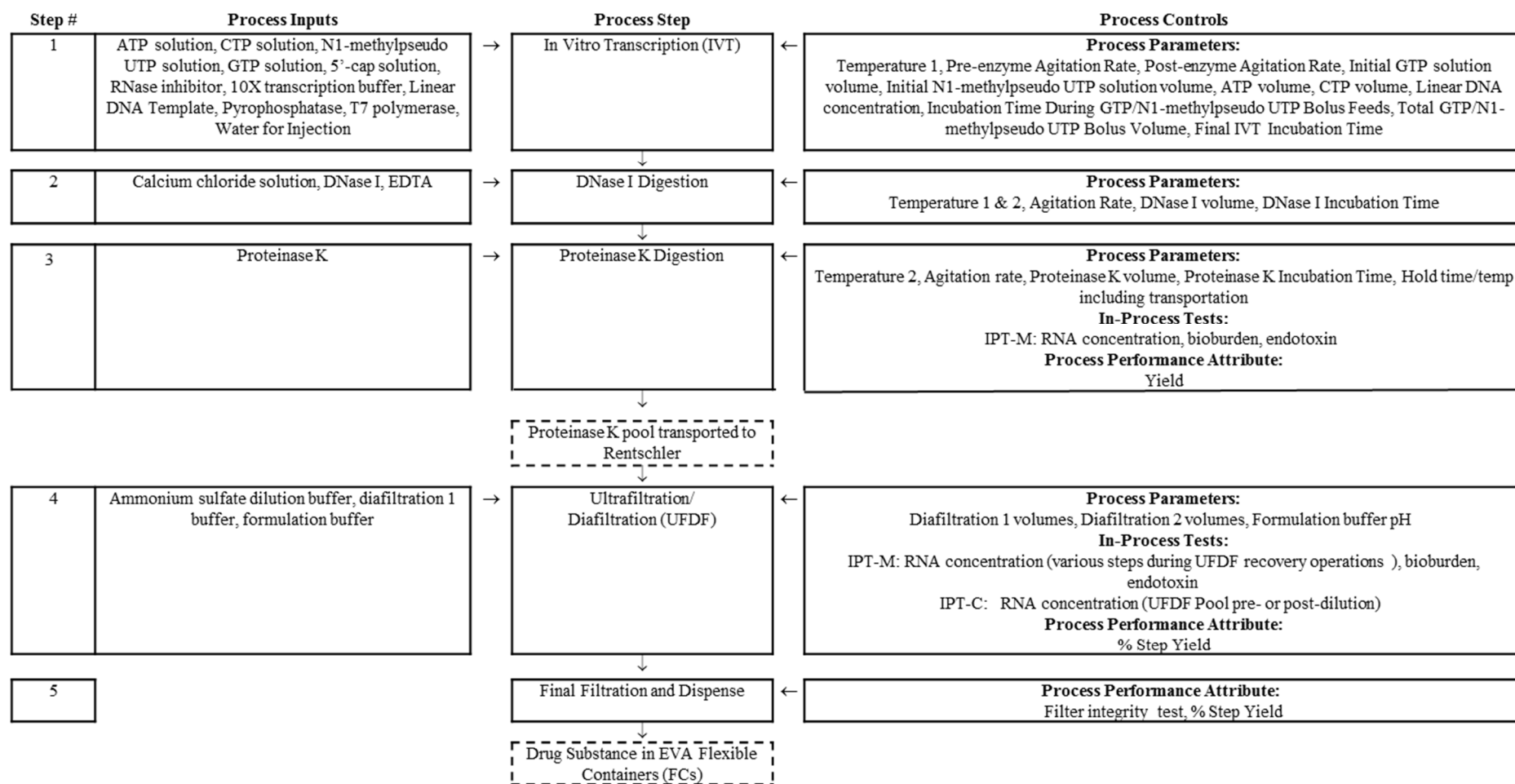
A flow diagram for the drug substance process is shown in [Figure S.2.2-1](#). For each process step, this flow diagram lists the process inputs (materials added) and the process controls (process parameters, material attributes, process performance attributes (PPA), in-process tests for control (IPT-C) and in-process tests for monitoring (IPT-M)).

IPT-Cs are in-process tests used to control a QA/CQA within a specified range so that it meets the desired DS/DP quality. The IPT-Cs have an associated acceptance criterion. These IPT-Cs are tabulated in this section with their associated acceptance criteria and also described in [Section S.2.4 Control of Critical Steps and Intermediates \[BNT Mainz and Rentschler\]](#).

In addition to IPT-Cs, in-process tests for monitoring (IPT-M) have been implemented throughout the process to ensure consistency of the manufacturing process. IPT-Ms are in-process tests used to monitor a QA/CQA to either ensure that it is consistent with respect to previous process history or for forward processing. The monitoring tests may have action limits. These IPT-Ms are described in [Section S.2.4 Control of Critical Steps and Intermediates \[BNT Mainz and Rentschler\]](#).

All process parameters are defined and controlled within the applicable ranges, detailed in the batch records and standard operating procedures.

All unit operations are performed at ambient temperature (15-25 °C), unless otherwise stated.

Figure S.2.2-1. RNA Manufacturing Process

S.2.2.4. In Vitro Transcription (IVT)

The primary objective of the IVT step is to synthesize RNA for drug substance production.

To begin the IVT step, individual components are thawed and added to the reaction vessel, including ATP solution (100 mM adenosine 5'-triphosphate), CTP solution (100 mM cytidine 5'-triphosphate), $m^1\psi$ TP solution (100 mM N1-methylpseudouridine 5'-triphosphate), GTP solution (100 mM guanosine 5'-triphosphate), 5'-cap solution (100 mM 5'-cap), and an initial volume of water for injection (WFI). The linear DNA template, 10X transcription buffer (400 mM HEPES, 400 mM magnesium acetate, 100 mM DTT, 20 mM spermidine, pH 8.3) and RNase inhibitor are added to the reaction vessel with the remaining volume of WFI. During the reagent additions, pre-enzyme agitation and temperature control are initiated at target ranges. Finally, pyrophosphatase and T7 polymerase are added to the reaction vessel and agitation is increased to the post-enzyme agitation rate. The above volume additions total to the IVT starting target volume of 37.6 L. After these enzyme additions, the incubation time during GTP/ $m^1\psi$ TP bolus feeds begins. The parameter ranges can be seen in Table S.2.2-1.

During this incubation period, an equal mix of $m^1\psi$ TP and GTP is delivered as 11 bolus feeds. After all the feeds are completed, a final IVT incubation time is initiated. Upon completion of the final IVT incubation time, the process immediately proceeds to the DNase I digestion operation.

The IVT step is controlled using the process parameters shown in Table S.2.2-1.

Table S.2.2-1. In Vitro Transcription Process Parameters

Parameter	Acceptable Range
Temperature 1 (°C)	34.0 – 40.0
Initial CTP solution volume (mL/L starting IVT volume)	85.4 – 143.8
Initial ATP solution volume (mL/L starting IVT volume)	85.4 – 135.1
Initial GTP solution volume (mL/L starting IVT volume)	4.75 – 5.25
Initial N1-methylpseudo UTP solution volume (mL/L starting IVT volume)	4.75 – 5.25
Pre-enzyme agitation rate (rpm)	40 – 80
Post-enzyme agitation rate (RPM)	90 – 110
Linear DNA concentration (g/L)	0.09 – 0.11
Incubation time during GTP/N1-methylpseudo UTP bolus feeds (min)	67 – 70
Total GTP/N1-methylpseudo UTP bolus volume (mL/L starting IVT volume)	153.2 – 187.3
Final IVT incubation time (min)	25 – 35

S.2.2.5. DNase I Digestion

The primary objective of the DNase I digestion step is to reduce the size of linear DNA template and enable subsequent removal across the ultrafiltration/diafiltration step.

A calcium chloride solution (50 mM calcium chloride) and a DNase I solution are added at the end of the final IVT incubation. Temperature (Temperature 1) and agitation rate are maintained during this step. Upon completion of the DNase I incubation time, EDTA (500

mM EDTA) is added, the temperature setpoint is updated (Temperature 2). After the EDTA addition, the process proceeds with the proteinase K digestion.

The DNase I digestion step is controlled using the process parameters shown in Table S.2.2-2.

Table S.2.2-2.DNase I Digestion Process Parameters

Parameter	Acceptable Range
Temperature 1 (°C)	34.0 – 40.0
Temperature 2 (°C)	32.0 – 38.0
Agitation rate (RPM)	90 – 110
DNase I volume (mL/L starting IVT volume)	7.20 – 8.81
DNase I Incubation time (min)	29 – 35

S.2.2.6. Proteinase K Digestion

The primary objective of the proteinase K digestion step is to reduce the size of proteins in the reaction mixture for subsequent removal across the ultrafiltration/diafiltration step.

Proteinase K solution is added to the reaction vessel and incubated for a predetermined amount of time. Temperature and agitation rate are maintained during this step. At the completion of proteinase K incubation time, the pool is cooled to ambient temperature and filled into five 10L Flexsafe Bags. The IVT solution can be maintained at ambient for less than 24 hours or held at 2 – 8 °C for up to 96 hours before moving to ultrafiltration/diafiltration.

The proteinase K digestion step is controlled using the process parameters shown in Table S.2.2-3.

Table S.2.2-3.Proteinase K Digestion Parameters

Parameter	Acceptable Range
Temperature 2 (°C)	32.0 – 38.0
Agitation rate (RPM)	90 – 110
Proteinase K volume (mL/L starting IVT volume)	1.00 – 1.22
Proteinase K incubation time (min)	10 – 15

The Proteinase K Pool can be maintained at ambient for less than 24 hours or held at 2 – 8 °C for up to 96 hours before moving to ultrafiltration/diafiltration. During that time the Pool is transported to Rentschler, Laupheim for further processing at 2 – 8 °C.

S.2.2.7. Transportation

The five 10L Bags with IVT solution are packed into qualified shells for transportation and cooled to 2 – 8 °C. The shipment from BNT Manufacturing to Rentschler Biopharma SE is performed at 2 – 8 °C using an insulated shipper.

The transportation step is controlled using the process parameters shown in Table S.2.2-4.

Table S.2.2-4. Transportation Parameters

Parameter	Acceptable Range
Temperature (°C)	2.0 – 8.0
Hold time	≤ 96.0

S.2.2.8. Ultrafiltration/Diafiltration (UFDF)

The UFDF step reduces small process-related impurities and concentrates and buffer exchanges the RNA into the final DS formulation (10 mM HEPES, 0.1 mM EDTA, pH 7.0).

To prepare for the UFDF step, the sanitized UFDF membranes are equilibrated with diafiltration 1 buffer (200 mM ammonium sulfate, 10 mM HEPES, 0.1 mM EDTA, pH 7.0). The pH and conductivity of the equilibrated membranes are verified.

Prior to UFDF, the post-proteinase K pool is diluted 2-fold with an ammonium sulfate dilution buffer (400 mM ammonium sulfate, 10 mM HEPES, 0.1 mM EDTA, pH 7.0). The diluted proteinase K pool then undergoes a 2-stage diafiltration; first with a minimum of 5 diavolumes (DV) using diafiltration 1 buffer followed by a minimum of 10 diavolumes using formulation buffer (10 mM HEPES, 0.1 mM EDTA, pH 7.0).

Based on the retentate RNA concentration determined after diafiltration 2, the diafiltered retentate is then concentrated, if needed, and recovered through a 0.45/0.2 µm dual-layer filter in a flexible container. Formulation buffer may be added to target approximately 2.25 mg/mL. An in-process test for control (IPT-C), with established acceptance criteria, is then performed for RNA concentration (as described in [Section S.2.4 Control of Critical Steps and Intermediates \[BNT Mainz and Rentschler\]](#)). The final pool is then filtered through a second 0.45/0.2 µm dual-layer filter into a flexible container (UFDF pool).

After use, the UFDF membranes are cleaned with 1 N sodium hydroxide solution and stored in 0.1 N sodium hydroxide solution.

The UFDF is controlled using the following process parameters.

Table S.2.2-5. UFDF and Formulation Process Parameters

Parameter	Acceptable Range
Diafiltration 1 volumes (DV)	≥5.0
Diafiltration 2 volumes (DV)	≥10.0
Formulation buffer pH	6.90 – 7.10

The following in-process tests for control (IPT-C) are conducted for the UFDF step.

Table S.2.2-6. In-Process Tests (Control) for UFDF

Test	Acceptance Criteria
RNA concentration (mg/mL)	≥2.00

S.2.2.9. Final Filtration and Dispense

The UFDF pool undergoes a bulk final 0.45/0.2 µm filtration into a flexible container. Final drug substance release testing is performed at this stage. The drug substance (DS) is then dispensed into 12 L ethylene vinyl acetate (EVA) flexible containers (FC) as described in [Section S.6 Container Closure](#).

The DS pool can be maintained at ambient temperature for less than 24 hours after final filtration and prior to start of drug substance dispensing.

S.2.2.10. Drug Substance Storage

The DS FFT Bags are frozen and stored between -15 °C and -25 °C. See [Section 3.2.S.7.1 Stability Summary and Conclusions](#) for stability information.

S.2.2.11. Transportation

DS FCs shipments using an insulated shipper are qualified for a shipping time of up to 89 hours at temperatures between -25 and -15 °C.

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S.2.6. MANUFACTURING PROCESS DEVELOPMENT

This section discusses the development history for the drug substance Process 2.

Table S.2.6-1 lists the DS batches produced using Process 2 at manufacturing scale.

Table S.2.6-2 shows a high-level summary of the differences between Process 1 and Process 2, and Table S.2.6-3 shows the implementation of the changes by batch.

S.2.6.1. Development History

S.2.6.1.1. Manufacturing Process History

A chronological summary for the drug substance batches manufactured at the Pfizer and BioNTech sites for Process 2 is provided in Table S.2.6-1 All batches described in the table are BNT162b2.

Table S.2.6-1. Drug Substance Batches (Process 2)

Batch Number	Batch Scale ^a (L)	Manufacture Site	DOM (DD MMM YYYY)	Batch Use
20Y513C101	37.6	Pfizer, Andover, MA, US	29 JUL 2020	Emergency Use, Stability, Clinical
20Y513C201	37.6	Pfizer, Andover, MA, US	13 AUG 2020	Emergency Use, Stability
20Y513C301	37.6	Pfizer, Andover, MA, US	20 AUG 2020	Emergency Use, Process Performance Qualification, Stability
20Y513C401	37.6	Pfizer, Andover, MA, US	27 AUG 2020	Process Performance Qualification, Stability
20Y513C501	37.6	Pfizer, Andover, MA, US	10 SEP 2020	Process Performance Qualification, Stability
20Y513C601	37.6	Pfizer, Andover, MA, US	17 SEP 2020	Process Performance Qualification, Stability
20Y513C701	37.6	Pfizer, Andover, MA, US	24 SEP 2020	Process Performance Qualification, Stability
20E162001-01	37.6	BioNTech Manufacturing GmbH, Mainz, Germany	24 SEP 2020	Emergency Use, Process Performance Qualification, Stability
20E162002-01	37.6	BioNTech Manufacturing GmbH, Mainz, Germany	30 SEP 2020	Emergency Use, Process Performance Qualification, Stability
20E162003-01	37.6	BioNTech Manufacturing GmbH, Mainz, Germany	06 OCT 2020	Emergency Use, Process Performance Qualification, Stability

a. In vitro transcription reaction starting volume

Abbreviations: DOM = date of manufacture

S.2.6.2. Process Development Overview

Table S.2.6-2 provides a high-level summary of the process changes made from early development to commercial scale. Process 1 was used for production of Nonclinical, Toxicology and Phase 1/2/3 clinical trial material (CTM). Changes were implemented to improve drug substance manufacturing robustness and to develop the commercial process (Process 2). The commercial process was then used to produce CTM for Phase 2/3 clinical trials at 37.6 L scale and initial commercial supply at 37.6 L scale.

Table S.2.6-2.Process Comparison

Step	Nonclinical Toxicology and Phase 1/2/3 Clinical (Process 1)	Phase 2/3 Clinical/Initial Commercial Supply/Process Performance Qualification/Commercial (Process 2)
1	In Vitro Transcription (IVT)	In Vitro Transcription (IVT)
2	DNase I Digestion	DNase I Digestion
3	NA	Proteinase K Digestion
4	Magnetic Bead Purification	Ultrafiltration/Diafiltration (UFDF)
5	Final Filtration and Dispense	Final Filtration and Dispense

S.2.6.3. Overview of Process Development Changes

Table S.2.6-3 summarizes the development changes from Process 1 to Process 2.

Table S.2.6-3.Overview of Process Development Changes

Step	Parameter	Process 1	Process 2
Not applicable	Batch number	R427-P020.2-DS R438-P020.2-DS R443-P020.2-DS R445-P020.2-DS	20Y513C101 20Y513C201 20Y513C301 20Y513C401 20Y513C501 20Y513C601 20Y513C701 20E162001 20E162002 20E162003
Not applicable	Site	BioNTech IMFS, Idar-Oberstein, Germany	Pfizer, Andover, MA, US BioNTech Manufacturing GmbH, Mainz, Germany (step 1, 2 and 3) and Rentschler Biopharma SE Laupheim, Germany (step 4 and 5)
1. In Vitro Transcription	Scale ^a	0.140-0.720 L	37.6 L
	DNA template	PCR-amplified	Linearized plasmid DNA
3. Proteinase K Digestion	Unit operation	N/A	Proteinase K digestion
4. Purification Method	Unit operation	Magnetic beads	Ultrafiltration/diafiltration

a. IVT starting volume

S.2.6.3.1. Changes from Process 1 to Process 2

The changes implemented between Process 1 and Process 2 and the batches produced with each process are outlined in [Table S.2.6-2](#). First, there was an increase in the scale of the drug substance process, and a change to the manufacturing site. The increase in scale was required to make sufficient material for clinical trials and emergency supply. This process was based on BioNTech platform knowledge from other mRNA therapeutic programs. The manufacturing site change to Pfizer was made based on process capacity and capability.

The next change outlined in [Table S.2.6-3](#) is the transition from PCR-amplified DNA template to the use of linearized plasmid DNA in order to meet emergency and commercial demands.

Finally, two additional process changes were made to improve scalability, robustness, and productivity. Process 1 utilizes a magnetic bead purification step for removal of small molecule impurities (e.g. spermidine, DTT), residual DNA, and enzyme impurities (e.g. T7 polymerase, DNase I). As this step was not scalable, Process 2 includes a proteinase K digestion step to reduce the size of the enzyme impurities, followed by an ultrafiltration/diafiltration purification step to remove the small molecule impurities, residual DNA, and the enzyme impurity fragments.

Details of commercial scale manufacturing processes are provided in Section S.2.2 Description of Manufacturing Process and Process Controls.

S.2.6.4. Process Parameters and Attributes

For Drug Substance manufacturing process 2, critical process parameters (CPPs) have been defined and in-process test for monitoring (IPT-M) and for control (IPT-C) of the process are being used to ensure consistent manufacturing.

The acceptable ranges defined for CPPs and IPTs are identical for process 2 at the two sites whereas; for process 1, minor differences are observed (comparisons are shown in [Table S.2.6-4](#) to [Table S.2.6-8](#)). These differences pertain to difference in equipment used as well as scale and can only be applied for steps 1 and 2 of the current manufacturing process as the purification process has been changed for process 2.

The following tables show the process control for each step of the manufacturing process, comparing parameters, controls and acceptable range for process 1 and process 2 at two commercial manufacturing sites.

Table S.2.6-4. Process Controls Step 1: In Vitro Transcription

Parameter	Category	Acceptable Range		
		Process 1: BNT IMFS ²	Process 2*: Pfizer, Andover	Process 2: BNT/REN
Temperature 1 [°C]	CPP	34 – 40 (incubator)	34.0 – 40.0	
Post-enzyme agitation rate [rpm]	CPP	200 – 250 (Dependent on volume ³)	60 – 110 ³	90 - 110 ³

Table S.2.6-4. Process Controls Step 1: In Vitro Transcription

Parameter	Category	Acceptable Range		
		Process 1: BNT IMFS ²	Process 2*: Pfizer, Andover	Process 2: BNT/REN
Initial GTP solution volume ¹	CPP	5	4.75 – 5.25	
Initial N1-methylpseudo UTP solution volume ¹	CPP	5	4.75 – 5.25	
Initial CTP solution volume ¹	CPP	90	85.4 – 143.8	
Initial ATP solution volume ¹	CPP	90	85.4 – 135.1	
Incubation time during GTP/N1-methylpseudo UTP bolus feeds [min]	CPP	75	67 – 70	
Total GTP/N1-methylpseudo UTP Bolus Volume ¹	CPP	170	153.2 – 187.3	
Final IVT incubation time [min]	CPP	30	25 – 35	

¹ Unit is mL/L starting IVT volume

² For this process, set point were set instead if acceptable ranges, except for Temperature and agitation rate.

³ differences in equipment see [Table S.2.6-9](#). Process 2 post-enzyme agitation rate difference is further discussed in [Section S.2.6. Process Development and Characterization](#)

* Initial emergency supply is covered by process 2: Pfizer, Andover

Table S.2.6-5. Process Controls Step 2: DNase I Digestion

Parameter	Category	Acceptable Range		
		Process 1: Clinical supply	Process 2*: Pfizer, Andover	Process 2: BNT/REN
Temperature 1 [°C]	CPP	34 – 40 (incubator)	34.0 – 40.0	
Temperature 2 [°C]	CPP	N/A	32.0 – 38.0	
DNase I volume [mL/L starting IVT volume]	Non-CPP	3.43 – 11.66	7.20 – 8.81	
DNase I Incubation time [min]	CPP	30 - 40	29 – 35	

* Initial emergency supply is covered by process 2: Pfizer, Andover

Table S.2.6-6. Process Controls Step 3: Proteinase K Digestion

Parameter	Category	Acceptable Range		
		Process 1: Clinical supply ¹	Process 2*: Pfizer, Andover	Process 2: BNT/REN
Temperature 2 [°C]	CPP	N/A	32.0 – 38.0	
Proteinase K volume [mL/L starting IVT volume]	Non-CPP	N/A	1.00 – 1.22	
Proteinase K incubation time [min]	Non-CPP	N/A	10 – 15	
RNA concentration [mg/mL]	IPT-M	N/A	N/A	
Bioburden [CFU/mL]	IPT-M	N/A	≤100 / 10	

Table S.2.6-6.Process Controls Step 3: Proteinase K Digestion

Parameter	Category	Acceptable Range		
		Process 1: Clinical supply ¹	Process 2*: Pfizer, Andover	Process 2: BNT/REN
Endotoxin [EU/mL]	IPT-M	N/A	≤ 12.5	

¹ The clinical supply process includes purification of mRNA by magnetic beads hence, the process is different and does not include this process step; ² Control limits.; ³ No acceptable range defined for this IPT-M; N/A – Not applicable, just for monitoring.

* Initial emergency supply is covered by process 2: Pfizer, Andover

Table S.2.6-7.Process Controls Step 4: UFDF and Formulation Process Parameters

Parameter	Category	Acceptable Range		
		Process 1: Clinical supply ¹	Process 2*: Pfizer, Andover	Process 2: BNT/REN
Diafiltration 1 volumes [DV]	CPP	N/A	≥ 5.0	
Diafiltration 2 volumes [DV]	CPP	N/A	≥ 10.0	
Formulation buffer pH	CPP	N/A	6.90 – 7.10	
Bioburden [CFU/mL]	IPT-M	N/A	≤100 / 10	
Endotoxin [EU/mL]	IPT-M	N/A	≤ 12.5	
RNA concentration ² [mg/mL]	IPT-M	N/A	N/A	
RNA concentration [mg/mL]	IPT-C	N/A	≥ 2.00	

¹ The clinical supply process includes purification of mRNA by magnetic beads hence, the process is different and does not include this process step; ² Various steps during UFDF recovery operations; N/A – Not applicable, just for monitoring.

* Initial emergency supply is covered by process 2: Pfizer, Andover

Table S.2.6-8.Process Yields

Process Step	Acceptable Range		
	Process 1: Clinical supply ¹	Process 2*: Pfizer, Andover	Process 2: BNT/REN
IVT [g mRNA/L IVT starting volume]	5.36 – 11.11	≥ 3.38	
UFDF [%]	N/A	≥ 68	
Final filtration and dispense [%]	N/A	≥ 80	

¹ The clinical supply process includes purification of mRNA by magnetic beads hence, the process is different and does not include UFDF.

* Initial emergency supply is covered by process 2: Pfizer, Andover

S.2.6.4.1. Equipment

A tabulated comparison of the equipment used in process 1 and process 2 at both manufacturing sites is shown in Table S.2.6-9.

Table S.2.6-9. Comparison of equipment used in process 1 and process 2

Process step	Process 1: BNT IMFS ¹	Process 2*: Pfizer, Andover	Process 2: BNT/REN
Step 1: In Vitro Transcription	<ul style="list-style-type: none"> Incubator (Thermo BBD 6220) Magnetic stirrer (2mag MIX 1 XL) Syringe pump (KD Scientific Legato 210 P) Centrifuge Balance (Sartorius Lab Instruments) 	50L Jacketed Single Use Mixer (SUM)	Biostat STR 50L Reactor (SUM)
Step 2: DNase I Digestion	Incubator (Thermo BBD 6220) Magnetic stirrer (2mag MIX 1 XL) Balance (Sartorius Lab Instruments)	50L Jacketed Single Use Mixer (SUM)	Biostat STR 50L Reactor (SUM)
Step 3: Proteinase K Digestion	-	50L Jacketed Single Use Mixer (SUM)	Biostat STR 50L Reactor (SUM)
Step 4: UFDF	-	200L Jacketed Single Use Mixer (SUM) <ul style="list-style-type: none"> SS Ultrafiltration system with 7m² 300kD membrane 200L SS retentate tank.	200L Jacketed Single Use Mixer (SUM) <ul style="list-style-type: none"> SS Ultrafiltration system with 7m² 300kD membrane 200L SS retentate tank.
Step 5: Final Filtration	0.2um filtration Filter integrity tester (Pall palltronic Flowstar IV)	200L Jacketed Single Use Mixer (SUM), 0.2um filtration.	200L Jacketed Single Use Mixer (SUM), 0.2um filtration.

¹ The clinical supply process includes purification of mRNA by magnetic beads hence, the process is different and Step 4 and 5 are not equal.

* Initial emergency supply is covered by process 2, in Pfizer, Andover.

S.2.6.4.2. Primary Packaging Materials

Drug substance is filled and stored in ethylene vinyl acetate (EVA) containers. EVA containers are sterile, single-use, flexible, disposable bags specifically designed for freezing, thawing and frozen storage of biopharmaceuticals. At the two commercial manufacturing sites, a slightly different nominal size of EVA containers is used for filling of drug substance 12 L and 16.6 L at Rentschler and Andover, respectively. The container closure system is equivalent and the product contact layer is identical for both EVA containers.

Table S.2.6-10. Comparison of containers used in process 1 and process 2

	Process 1: BNT, IMFS	Process 2*: Pfizer, Andover	Process 2: BNT/REN
DS Container	1L and 3L EVA (Ethyl Vinyl Acetate) Flexboy Bags (Sartorius)	16.6 L EVA (Ethyl Vinyl Acetate) Bag CFT (controlled Freeze Thaw)	12 L EVA (Ethyl Vinyl Acetate) Bag FFT (Flexible Freeze Thaw)

* Initial emergency supply is covered by process 2, in Pfizer, Andover.

S.2.6.4.3. Composition

The composition of BNT162b2 drug substance remained unchanged with respect to the formulation buffer (10 M HEPES, 0.1 mM EDTA, pH 7.0) during clinical testing and between process 1 and 2. Only the RNA target concentration was increased from 1.7 mg/mL to 2.25 mg/mL already for process 1 to accommodate larger scale LNP manufacturing. No further changes are planned for commercial material.

Table S.2.6-11. Comparison of composition used in process 1 and process 2

Attribute	Process 1: BNT IMFS	Process 2*: Pfizer, Andover	Process 2: BNT/REN
Formulation buffer	10 M HEPES, 0.1 mM EDTA, pH 7.0		
pH	7.0 ± 1.0	7.0 ± 0.5	
Content (RNA Concentration)	1.70 ± 0.17 mg/mL 2.25 ± 0.25 mg/mL	2.25 ± 0.25 mg/mL	

* Initial emergency supply is covered by process 2, in Pfizer, Andover.

S.2.6.5. Comparability Assessment

S.2.6.5.1. Product Quality Comparability Overview

Comparability before and after process changes is demonstrated through a comparison of the release test results for all available batches, as well as side-by-side testing and heightened characterization for a subset of representative DS batches relevant to process changes and process performance qualification ([Table S.2.6-12](#)). The side-by-side assessment of product quality comparability was performed in a stepwise approach. In a first study, analytical comparability of the BNT162b2 drug substance critical quality attributes was evaluated for three Process 1 batches manufactured at BNT and one Process 2 batch manufactured at Pfizer, Andover. The second study focused on an assessment of BNT162b2 drug substance from the commercial process (Process 2) at two sites and additionally includes two representative clinical supply batches (Process 1) as reference to the previous comparability study.

Table S.2.6-12. BNT162b2 Clinical and Emergency Supply^a Drug Substance Batches

Batch	Date of Manufacture	Purpose of Material	Comparability Data			DS Process
			Release	Side-by-Side Characterization		
				Study #1	Study #2	
R427-P020.2-DS	17 APR 2020	Clinical, Stability	X	X	X	Process 1
R438-P020.2-DS	29 MAY 2020	Clinical, Stability	X	X		
R443-P020.2-DS	30 JUN 2020	Clinical, Stability	X	X		
R445-P020.2-DS	24 JUL 2020	Clinical Inventory, Stability	X		X	
20Y513C101	29 JUL 2020	Emergency supply ^a Clinical Inventory, Stability	X	X	X	Process 2
20Y513C201	13 AUG 2020	Emergency supply ^a Stability, Interim Reference Material	X		X	
20Y513C301	20 AUG 2020	Emergency supply ^a Process performance qualification, Stability	X			
20Y513C401	27 AUG 2020	Process performance qualification, Stability	X			
20Y513C501	10 SEP 2020	Process performance qualification, Stability	X		X	
20Y513C601	17 SEP 2020	Process Performance Qualification, Stability	X		X	
20Y513C701	24 SEP 2020	Process Performance Qualification, Stability	X		X	
20E162001	24 SEP 2020	Emergency supply ^a Process Performance Qualification, Stability	X		X	
20E162002	30 SEP 2020	Emergency supply ^a Process Performance Qualification, Stability	X		X	
20E162003	06 OCT 2020	Emergency supply ^a Process Performance Qualification, Stability	X		X	

a. Emergency supply designation applies to U.S. market.

The analytical comparability assessments evaluated a combination of release and heightened characterization testing (Table S.2.6-13). The tests executed to support the comparability evaluation are described in Section S.4.2 Analytical Procedures. Additional heightened characterization using mass spectrometry and spectroscopic characterization methods was performed to compare drug substance primary and higher order structure elements.

Taken together, the results of this analysis support the conclusion that the commercial scale Process 2 DS batches manufactured at Pfizer, Andover and BioNTech Manufacturing GmbH are comparable to each other and comparable to Process 1 batches manufactured for clinical supply. Supportive profiles, data and detailed discussions of each comparative characterization analysis are provided in the sections below. The data demonstrate that all the DS batches are comparable and no site- or scale-specific trends were noted for any of the attributes tested.

Table S.2.6-13. Summary of Analytical Comparability Assessment Between Process 1 and Process 2 Drug Substance

Quality Attribute	Analytical Procedure	Release / Heightened Characterization
Appearance (Clarity)	Appearance (Visual)	Release
Appearance (Coloration)	Appearance (Visual)	Release
pH	Potentiometry	Release
Identity of encoded RNA sequence ^a	RT-PCR	Release
Content (RNA concentration)	UV absorption spectrophotometry	Release
RNA Integrity ^a	Capillary gel electrophoresis	Release
Residual DNA Template	qPCR	Release
Double stranded RNA (dsRNA)	Immunoblot	Release
Osmolality	Osmometry	Heightened Characterization
5'-Cap ^a	LC-UV	Heightened Characterization
Capped-Intact RNA ^a	Capillary gel electrophoresis and RP-HPLC	Heightened Characterization
Poly(A) Tail ^a	ddPCR	Heightened Characterization
Poly(A) Tail: Length and Distribution ^a	RP-HPLC	Heightened Characterization
Identity (RNA length) ^a	Denaturing agarose gel electrophoresis	Heightened Characterization
Identity (as RNA) ^a	Enzymatic degradation followed by gel electrophoresis	Heightened Characterization
5'-Cap ^a	LC-UV/MS	Heightened Characterization
Poly(A) Tail ^a	LC-UV/MS	Heightened Characterization
Oligonucleotide Mapping ^a	LC-MS/MS	Heightened Characterization
Higher order structure ^a	Circular dichroism	Heightened Characterization
Expressed Protein ^a	Western blot	Heightened Characterization

a. Tested side-by-side

S.2.6.5.2. Comparative Assessment of BNT162b2 Release and Additional Test Data

Release and additional test data ranges for all available drug substance batches are provided in [Table S.2.6-14](#). All release data met the acceptance criteria established at the time of release testing. Release data for each batch, as well as acceptance criteria at the time of testing, are provided in [Section S.4.4 Batch Analyses \(modRNA\)](#). Differences observed in content (RNA concentration) were expected and reflect changes during clinical development

in the RNA concentration target and specification acceptance criterion (from 1.70 ± 0.17 mg/mL to 2.25 ± 0.25 mg/mL; [Section S.4.4 Batch Analyses \(modRNA\)](#)). Differences in osmolality are consistent with downstream process changes, where the use of ethanol and NaCl in Process 1 resulted in higher osmolality as compared to Process 2 batches. Process 1 and Process 2 DS batches show consistent removal of residual DNA template and dsRNA. Residual DNA template in Process 1 batches was cleared to levels ≤ 200 ng/mg RNA and Process 2 batches have < 220 ng/mg RNA. Additionally, all clinical BNT162b2 Process 1 and Process 2 batches were within specification acceptance criterion for dsRNA and comparably low (≤ 240 pg / mg RNA).

A small decrease in RNA integrity was observed for the initial Process 2 batches, and the variability of this attribute continues to be monitored. To further optimize process robustness, CTP volume and ATP volume process parameters were adjusted prior to the manufacture of batch 20Y513C501. Release testing revealed batch 20Y513C501 and subsequent batch RNA integrity levels more consistent with the Process 1 batches. Additionally, the relative abundance of 5'-capped RNA is slightly higher in the Process 2 batches. As the RNA integrity and 5'-cap attributes are critical to translation of the protein antigen in vivo, the proportion of capped-intact RNA is used to compare the Process 1 and Process 2 materials that were tested side-by-side (see data and discussion in [Sections S.2.6.5.3 and S.2.6.5.4](#) below). The proportion of poly-adenylated RNA is $\geq 85\%$ for all Process 1 and Process 2 materials. Small differences in poly(A) content, including some values reported over 100%, are attributed to method variability, as it is not practically feasible to achieve greater than 100% poly-adenylated RNA content.

Overall, small differences observed in the release and additional test ranges are not expected to impact safety or efficacy.

Table S.2.6-14. BNT162b2 Drug Substance Release and Additional Testing Result Ranges

Method	Clinical (Process 1)	Emergency Supply (Process 2)	ACMF Process Performance Qualification (Process 2)	BNT-REN Process Performance Qualification (Process 2)
	R427-P020.2-DS R438-P020.2-DS R443-P020.2-DS R445-P020.2-DS	20Y513C101 20Y513C201	20Y513C301 20Y513C401 20Y513C501 20Y513C601 20Y513C701	20E162001 20E162002 20E162003
Appearance (Clarity)	Clear (≤ 3 NTU)	Clear (0 – 1 NTU)	Clear (0 – 1 NTU)	Clear (0 NTU)
Appearance (Coloration)	Colorless	Colorless (≤ B9)	Colorless (≤ B9)	Colorless
pH	7.0 – 7.2	6.9	6.9	6.9
Identity of encoded RNA sequence by RT-PCR	Complies ^a	Identity confirmed	Identity confirmed	Identity confirmed
Content (RNA concentration) by UV spectrometry (mg/mL)	1.64 – 2.26 ^b	2.19 – 2.27	2.19 – 2.27	2.18 – 2.20
RNA Integrity by capillary gel electrophoresis (%)	77 – 86 ^c	62 – 69	65 – 75	70 – 72
5'-Cap by LC-UV (%) ^d	56 – 69	82 – 84	84 – 88	89 – 91
Poly(A) Tail by ddPCR (%) ^d	116 – 131	88 – 104	91 – 106	85 – 106
Residual DNA Template by qPCR (ng/mg RNA)	< 200	17 – 29	10 – 211	11 – 34
dsRNA by immunoblot (pg/μg RNA)	< 120	≤ 240	≤ 240	< 40
Osmolality	52 – 143	18	17	17

a. Identity of Process 1 batches determined from the starting material (DNA template) by sequencing

b. Acceptance criterion for Content (RNA concentration) changed from 1.7 mg/mL ± 10% to 2.25 ± 10% during clinical development.

c. Value is result of a revised integration of electropherograms, consistent with the integration used for Process 2 batches. Side-by-side test results shown in [Table S.2.6-15](#) and [Table S.2.6-21](#).

d. Process 1 data taken from side-by-side assessment ([Table S.2.6-15](#) and [Table S.2.6-21](#)). 5'-Cap and Poly(A) tail data were collected for Process 2 batches as an additional characterization test.

Abbreviations: ddPCR = Droplet digital polymerase chain reaction; dsRNA = Double stranded RNA; NT = Not Tested; NTU = Nephelometric turbidity unit; qPCR = Quantitative PCR; RP-HPLC = Reversed phase high performance liquid chromatography; RT-PCR = Reverse transcription PCR

S.2.6.5.3. Side-by-Side Comparability Study #1 - Evaluation of Drug Substance Used to Manufacture Clinical and Emergency Supply Lots

In addition to release and additional test data described above, two side-by-side assessments were performed. As a first step to demonstrate comparability, three batches manufactured using Process 1 (used for clinical supply of Phase 1/2/3) were evaluated side-by-side with a batch manufactured using Process 2 at one commercial site (Pfizer, Andover). Side-by-side comparability testing results for this first study are shown in [Table S.2.6-15](#) and confirm that Process 2 batch 20Y513C101 is comparable to the Process 1 batches.

Consistent with the release test data, a small decrease in RNA integrity and a slight increase in 5'-capped RNA were observed for the Process 2 material included in this study. As the RNA integrity and 5'-cap attributes are critical to translation of the protein antigen in vivo, the proportion of capped-intact RNA is used to compare the Process 1 and Process 2 materials that were tested side-by-side ([Table S.2.6-15](#)). Leveraging side-by-side testing results minimizes analytical variability associated with RNA integrity and 5'-cap measurements and enables a more controlled comparison of capped-intact RNA. The range of Process 1 capped-intact RNA is 46 to 52% as compared to 50% for Process 2.

Supportive data profiles are provided below for side-by-side testing of identity (RNA length), identity (as RNA), poly(A) tail length and distribution, and expressed protein size.

Additionally, a specifically selected series of state-of-the-art characterization analyses was performed to assess additional aspects and demonstrate comparability of the BNT162b2 DS structure. Side-by-side comparability studies were performed using mass spectrometry to characterize the 5'-cap, poly(A) tail, and oligonucleotide mapping. Circular dichroism was performed to characterize higher order structure.

Table S.2.6-15. BNT162b2 Drug Substance Side-by-Side Comparability Testing Results

Attribute and Method	Clinical (Process 1)			Emergency Supply ^a (Process 2)
	R427-P020.2- DS	R438-P020.2- DS	R443-P020.2- DS	20Y513C101
Identity of encoded RNA sequence by RT-PCR	Confirmed	Confirmed	Confirmed	Confirmed
RNA Integrity by capillary gel electrophoresis (%)	78.3	78.1	82.8	59.7
5'-Cap by LC-UV (%)	67	61	56	83
Capped-Intact RNA (%) ^b	52	48	46	50
Poly(A) Tail by ddPCR (%)	116	123	127	93
Poly(A) Tail: Length and Distribution by RP-HPLC	A30: 26.4% L70: 62.3% Other: 11.3%	A30: 27.6% L70: 61.3% Other: 11.1%	A30: 25.6% L70: 63.4% Other: 11.0%	A30: 23.8% L70: 64.5% Other: 11.7%
Identity (RNA length) by denaturing agarose gel electrophoresis	Single distinct band migrating at the expected location as compared to a reference RNA	Single distinct band migrating at the expected location as compared to a reference RNA	Single distinct band migrating at the expected location as compared to a reference RNA	Single distinct band migrating at the expected location as compared to a reference RNA
Identity (as RNA) by enzymatic degradation followed by gel electrophoresis	No RNase-resistant band detectable by gel electrophoresis	No RNase-resistant band detectable by gel electrophoresis	No RNase-resistant band detectable by gel electrophoresis	No RNase-resistant band detectable by gel electrophoresis

a. Emergency supply designation applies to U.S. market.

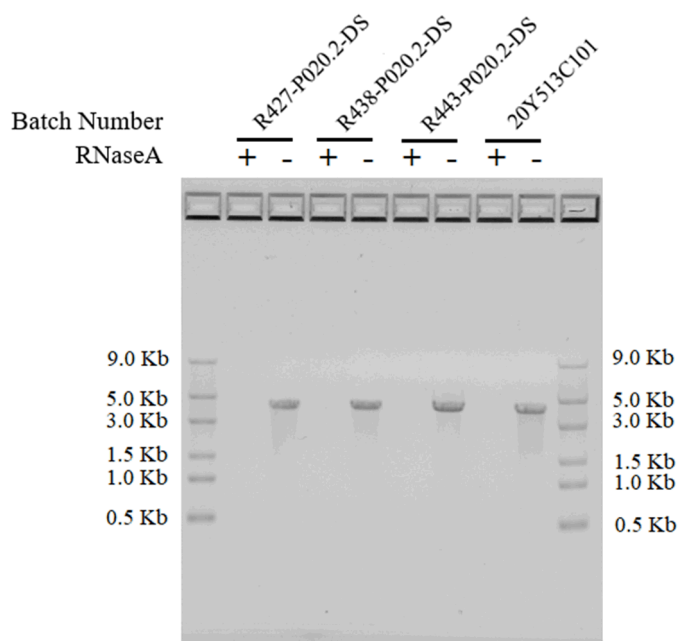
b. Capped Intact RNA (%) = RNA Integrity (%) x 5'-Cap (%)

Abbreviations: ddPCR = Droplet digital polymerase chain reaction; RP-HPLC = Reversed phase high performance liquid chromatography; RT-PCR = Reverse transcription PCR

S.2.6.5.3.1. Identity as determined by agarose gel electrophoresis (AGE)

The identity of BNT162b2 drug substance was determined using agarose gel electrophoresis (AGE). Identity (RNA length) was confirmed for three Process 1 batches and the Process 2 batch. Figure S.2.6-1 shows that the RNA size is consistent with the expected size of BNT162b2 drug substance and comparable across all tested batches. In addition, identity (as RNA) is demonstrated for all batches, as no RNase A-resistant bands are detectable after enzymatic degradation of the RNA drug substance (Figure S.2.6-1).

Figure S.2.6-1. Identity by Agarose Gel Electrophoresis



S.2.6.5.3.2. Comparative LC-UV/MS Analysis of the 5'-Cap in BNT162b2

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 260 nm 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 S.4.2 RP – HPLC \(modRNA\)](#). The identification of capped and un-capped species by mass spectrometry are presented in [Section S.3.1.3 5'-Cap Characterization by LC-UV/MS \(in Section S.3.1 Elucidation of Structure and Other Characteristics \(modRNA\)\)](#).

Using LC-UV and peak integration, the 5'-cap levels for all DS batches exceed 50% ([Table S.2.6-15](#)), with slightly higher levels of 5'-Cap observed in Process 2 DS ([Figure S.2.6-2](#)).

The mass spectra representing the main UV chromatographic peak, labeled as 5'-Cap, shows a single, predominant species in all Process 1 and 2 DS materials, corresponding to the expected 5'-capped species (Figure S.2.6-3). The observed monoisotopic masses from each material for this UV chromatographic peak match the theoretical mass of the 26 nucleotide RNase cleaved fragment with the 5' cap confirming the intended 5' capped structure (Section S.3.1 Elucidation of Structure and Other Characteristics (modRNA)). For the uncapped region, the same 5'-ppp and 5'-pp species are observed at similar relative abundances in the Process 1 and Process 2 DS (Figure S.2.6-4). For all materials, the observed monoisotopic masses for each of these un-capped RNase cleaved fragments are in agreement with the respective theoretical masses, confirming 5' uncapped structures (Table S.2.6-16).

Characterization by LC-UV/MS confirms that the 5'-capped and uncapped structures are the same in both Process 1 and 2 with a redistribution toward higher 5'-capping levels in Process 2. It is anticipated that a higher 5'-Cap level may enable a more stable and efficacious BNT162b2 DS. Therefore, Process 1 and Process 2 DS batches are considered comparable with respect to 5'-Cap.

Figure S.2.6-2. 5'-Cap Assay UV Chromatograms of BNT162b2 DS RNase Cleaved Fragments

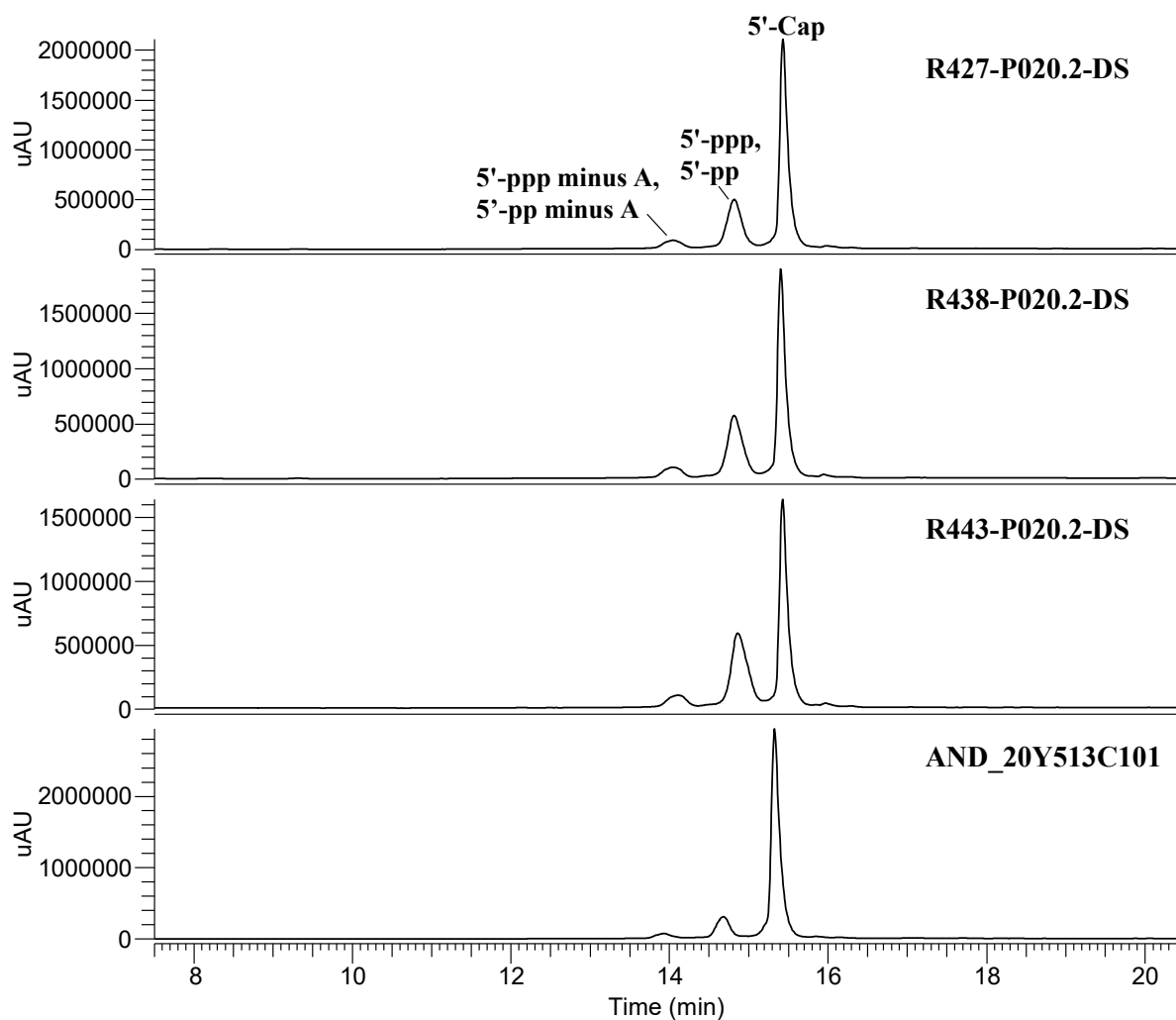


Figure S.2.6-2. The 5'-Cap assay UV 260 nm absorbance chromatograms of four BNT162b2 mRNA DS batches. The 12-18 min region of the chromatogram comprises the region where the RNase cleaved fragments elute. Peaks were identified via online mass spectrometry. Due to the shallow gradient used in this separation, minor shifts in resolution and retention time can occur between samples and are not considered significant.

Figure S.2.6-3. Mass Spectra of 5'-Cap RNase Cleaved Fragments from BNT162b2 DS Batches

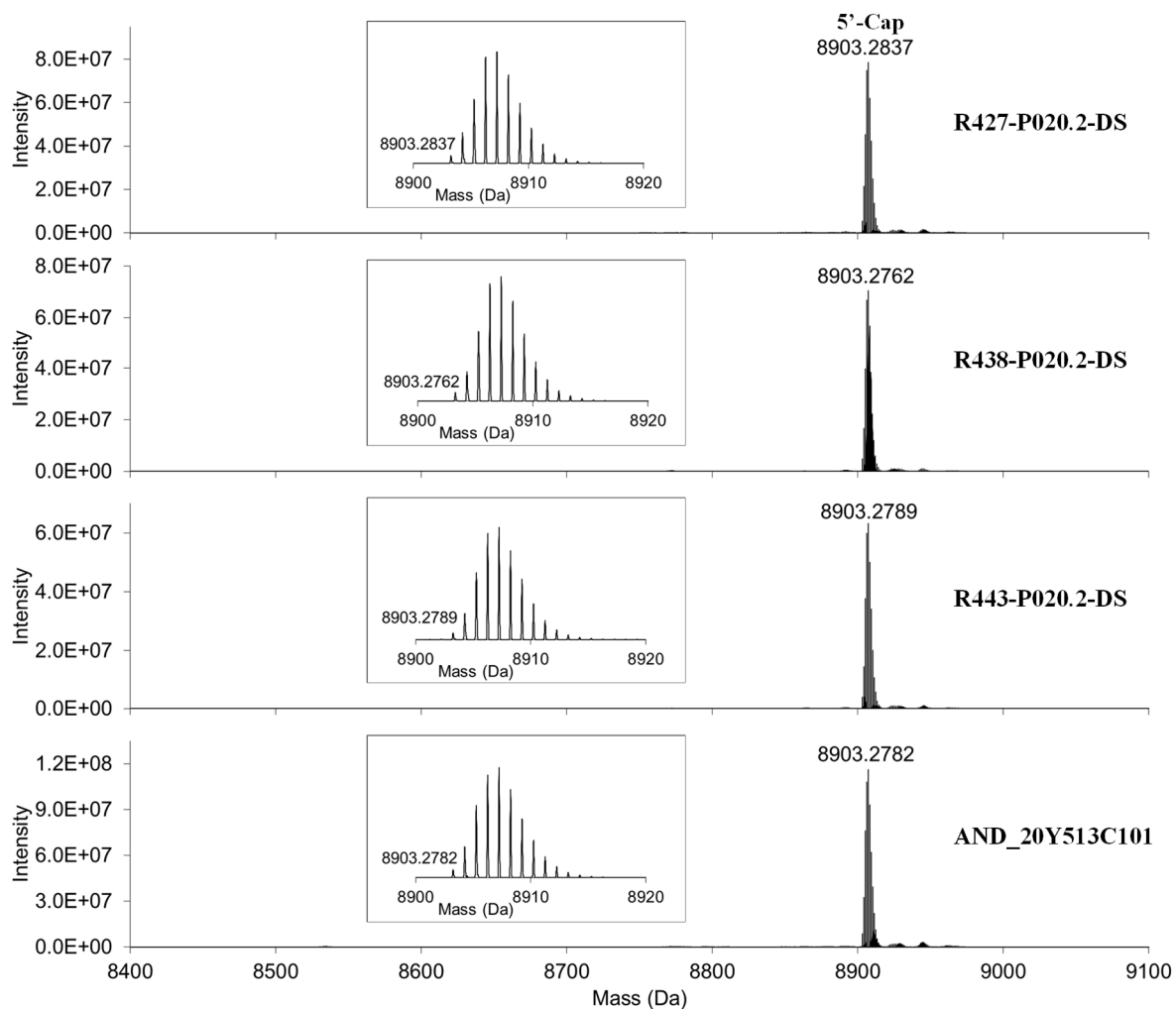


Figure S.2.6-3. Deconvoluted, zero-charge mass spectra of the 5'-capped 26 nucleotide RNase cleaved fragment of four BNT162b2 DS batches. The spectra are deconvoluted from the summation of scans across the 5'-cap elution region. The insets provide zoomed-spectra views of the 5'-cap isotopic envelopes and monoisotopic peak assignments.

Figure S.2.6-4. Mass Spectra of 5'-ppp and 5'-pp RNase Cleaved Fragments from BNT162b2 DS Batches

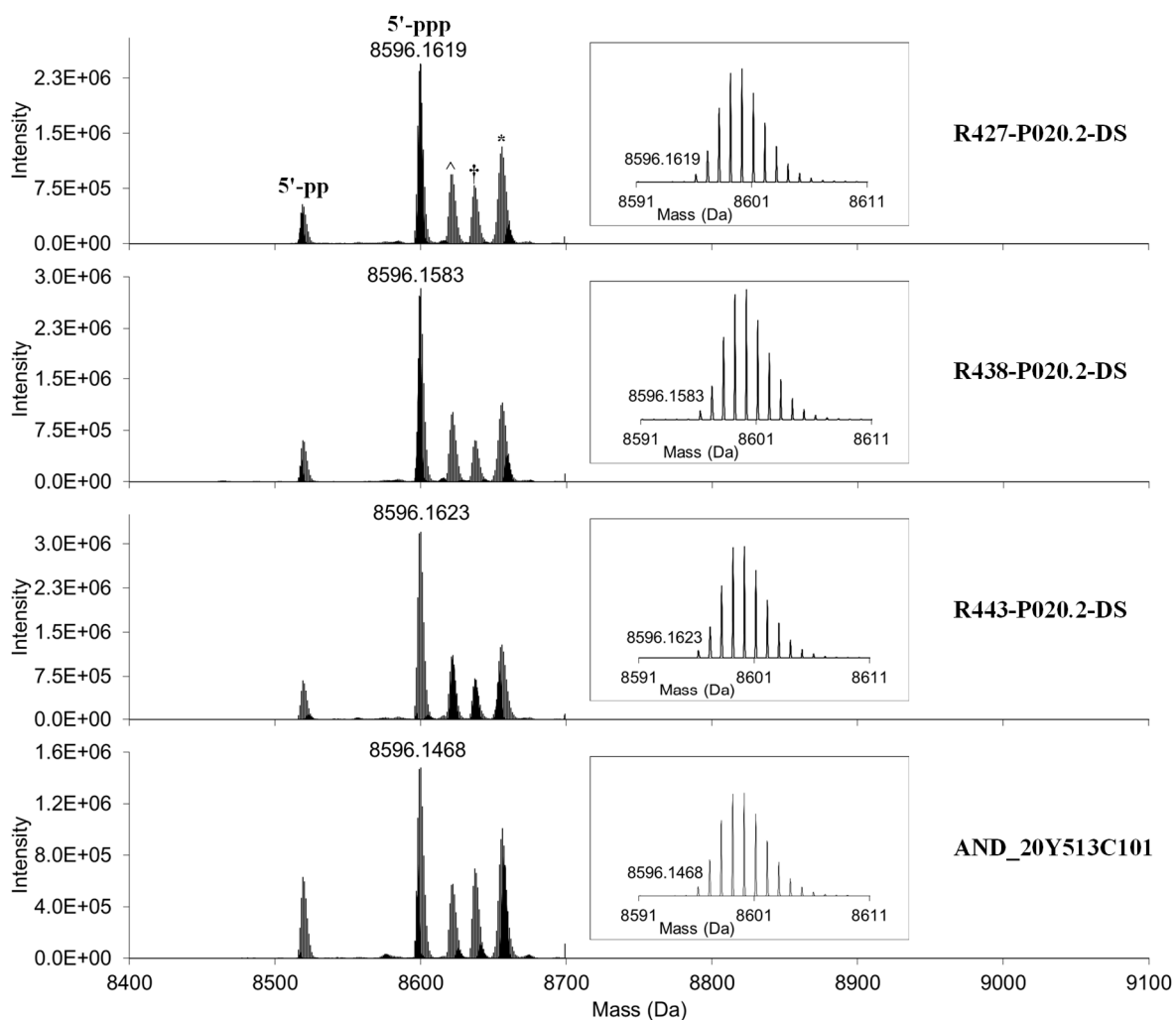


Figure S.2.6-4. Deconvoluted, zero-charge mass spectra of the 5' end non-capped 26 nucleotide RNase cleaved fragment of four BNT162b2 DS batches. The spectra are deconvoluted from the summation of scans across the 5'-cap elution region. The “ppp” represents the 5'-triphosphate species, and “pp” represents the 5'-diphosphate species. The “^” marks the sodium adduct of the 5'-ppp species. The “+” marks the potassium adduct of the 5'-ppp species. The “*” marks an approximately equimolar mixture of Fe(III) and Ni(II) adducts of the 5'-ppp species. The insets provide zoomed-spectra views of the 5'-ppp isotopic envelopes and monoisotopic peak assignments.

Table S.2.6-16. Accurate Mass Assignments for BNT162b2 5'-Cap and non-Cap RNase Cleaved Fragments

Species	Theoretical Mass (Da)	R427-P020.2-DS Observed Mass (Da) ^a	R438-P020.2-DS Observed Mass (Da) ^a	R443-P020.2-DS Observed Mass (Da) ^a	AND_20Y513C101 Observed Mass (Da) ^a
5'-Cap	8903.2562	8903.2837	8903.2762	8903.2789	8903.2782
5'-ppp	8596.1281	8596.1619	8596.1583	8596.1623	8596.1468
5'-pp	8516.1618	8516.1825	8516.1766	8516.1840	8516.1749
5'-ppp minus A	8267.0756	8267.0615	8267.0589	8267.0608	8267.0588
5'-pp minus A	8187.1093	8187.0942	8187.0900	8187.0918	8187.0924

a. Observed masses (monoisotopic) were determined from the zero-charge mass spectra after deconvolution of the multiply-charged data. Observed masses for major and minor species agree with theoretical masses to within 4 ppm, which is consistent with the accuracy and precision of contemporary mass spectrometers.

S.2.6.5.3.3. Poly(A) Tail: Length and Distribution by RP-HPLC

To evaluate the distribution of the poly(A) tail segments of ~30 adenosine nucleotides (A30) and ~70 adenosine nucleotides (L70), BNT162b2 drug substance batches were digested with RNase T1 and RNase A, and subsequently analyzed by ion-pair reversed-phase HPLC (RP-HPLC). [Figure S.2.6-5](#) shows the chromatographic profiles of the three Process 1 DS batches and the Process 2 DS batch. Visual assessment of the chromatograms demonstrates comparable overall distributions of A30 and L70 species, with a slight broadening of the L70 peak in batch 20Y513C101. Further elucidation of this species using mass spectrometry is provided [below](#) in Section [S.2.6.5.3.4](#). Quantitative assessment ([Table S.2.6-15](#)) further demonstrates comparable relative A30, L70, and Other species content.

Figure S.2.6-5. BNT162b2 Poly(A) Tail Analyzed by RP-HPLC-UV

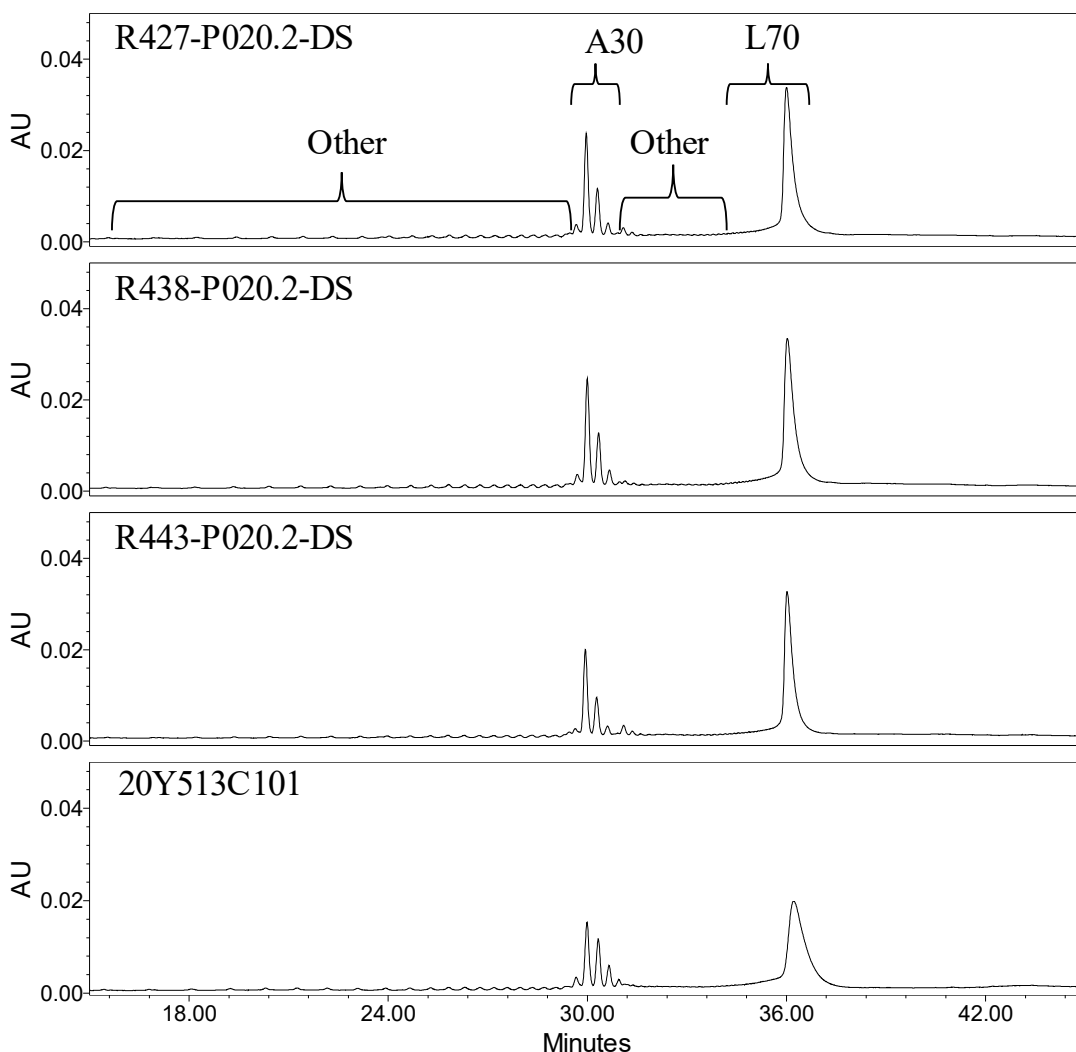


Figure S.2.6-5 RP-HPLC-UV chromatograms (260 nm) of poly(A) tail following RNase T1+RNase A digestion of BNT162b2 DS. Numerical peak labels indicate the approximate number of adenosine residues in the A30 and L70 poly(A) segments.

S.2.6.5.3.4. Comparative Mass Analysis of BNT162b2 Poly(A) Tail in Process 1 and Process 2 Batches

To facilitate in-depth characterization of the BNT162b2 poly(A) tail using state-of-the-art mass spectrometry techniques, drug substance samples were digested using only RNase T1. In contrast to the double digest method described in [S.2.6.5.3.3](#), the greater specificity of the singly-digested material, in combination with affinity purification enables improved mass spectrometric determination of the A30 and L70 distribution and sequence confirmation.

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

260 nm 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 by ribonuclease T1 (RNase T1) followed by isolation via oligo(dT)₂₅ affinity purification. This characterization method monitors two poly(A) tail segments: A30 and L70. Transcriptional slippage gives rise to a distribution of species with more or fewer adenosine nucleotides in each segment. More detail is provided in Section 3.2.S.3.1.4 3' Poly(A) tail Characterization by LC-UV/MS (in [Section S.3.1 Elucidation of Structure and Other Characteristics \(modRNA\)](#)).

Both expected short (A30) and long (L70) segments of the poly(A) tail were observed in the RP-HPLC/UV chromatograms for all four DS batches at comparable relative abundances ([Figure S.2.6-6](#)). Using LC-UV/MS, the most abundant A30 segment species for all batches was the 30-adenosine species, with a range from 28 – 34 adenosines observed in all batches ([Figure S.2.6-7](#)). A minor redistribution in the relative abundance of these species was observed, with increased abundance for the longer adenosine forms in the Process 2 DS compared to the Process 1 DS ([Table S.2.6-17](#)).

Unlike the poly(A) A30 segment, where the respective species are resolved both chromatographically and mass spectrometrically, the poly(A) L70 distribution is only resolved by MS ([Table S.2.6-18](#)). By LC-UV/MS, the mass spectral distribution of poly(A) L70 segment species was narrower for the Process 1 batches: R427-P020.2-DS, R438-P020.2-DS and R443-P020.2-DS, as compared to the Process 2 batch, 20Y513C101 ([Figure S.2.6-8](#)). This observation coincides with the narrower chromatographic elution profile of the L70 region for the Process 1 batches and the slightly later elution position of the Process 2 batch, as shown in [Figure S.2.6-6](#). Additionally, the identification of predominant poly(A) L70 segment species differed between the two processes. For Process 1 R427-P020.2-DS, R438-P020.2-DS and R443-P020.2-DS batches, the 71A1C and 72A1C species formed the apex of the distribution. Here, the 71A and 72A represent the number of adenosine residues in the sequence and the 1C denotes the inclusion of one cytidine monophosphate. Furthermore, the 71A and 72A species, representing the intended sequence with minimal transcriptional slippage, formed the apex of a secondary distribution for Process 1 DS batches. The predominant species for the 20Y513C101 Process 2 DS batch were 75A, 76A, 77A and 78A, as would be expected for the poly(A) L70 segment with some transcriptional slippage. In addition, the 75A1C, 76A1C and 77A1C species observed in Process 1 formed the apex of a secondary distribution in Process 2, signifying that the same poly(A) L70 species are present in both Process 1 and Process 2. The incorporation of cytidine monophosphate into BNT162b2 is still under investigation, see [Section S.3.1 Elucidation of Structure and Other Characteristics \(modRNA\)](#). Despite these differences, the overarching observation for Process 1 and Process 2 DS batches is that the poly(A) L70 segment is comprised of mRNA with at least 70 adenosine nucleotides and both Process 1 and Process 2 exhibit the same types of Poly(A) L70 species with the expected transcriptional slippage mechanism, albeit to different extents.

Characterization by LC-UV/MS confirms that the poly(A) A30 segments are comparable between Process 1 and 2 given the same species are detected with a minor redistribution in relative abundance. The Poly(A) L70 segments also are considered comparable given the L70 species from Process 1 and 2 are the same and exhibit at least 70 adenosine residues as

intended. Differences in the extent of cytidine monophosphate incorporation and transcriptional slippage remain under investigation. Taken together, the quantitative measurement of the poly(A) tail species by LC-UV ([Table S.2.6-13](#) and [Figure S.2.6-5](#)) and heightened characterization by LC-UV/MS demonstrate that both Process 1 and Process 2 derived DS batches contain predominantly 30 adenosine in the A30 species and at least 70 adenosine nucleotides in the L70 species, with observed distribution differences not expected to impact safety or efficacy.

Figure S.2.6-6. RP-HPLC-UV Profile (260 nm) of Extracted BNT162b2 Poly(A) tail

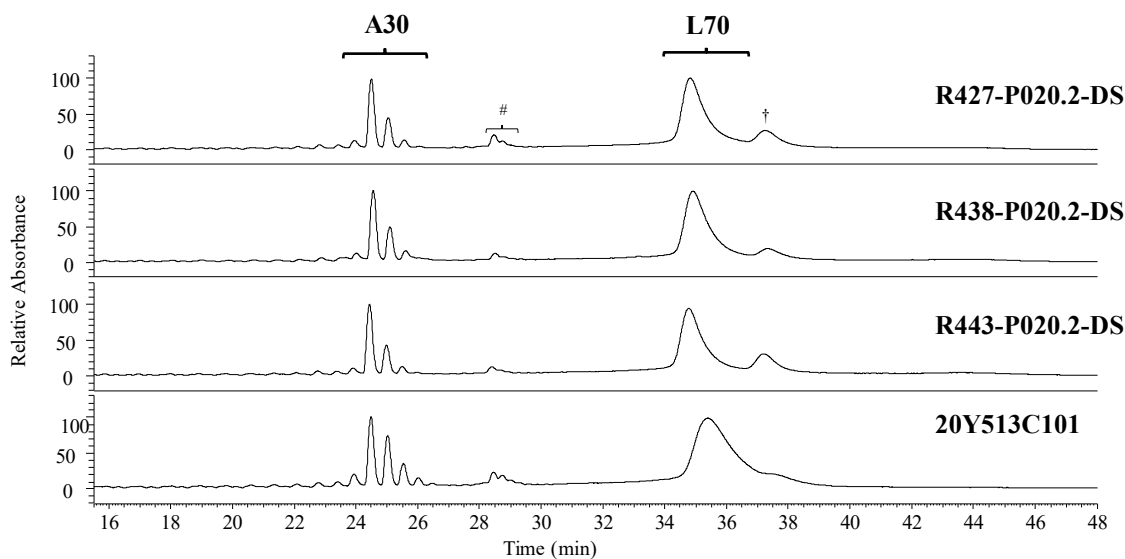


Figure S.2.6-6. RP-HPLC-UV chromatograms (260 nm) of extracted poly(A) tail following RNase T1 digestion, of four BNT162b2 DS batches. Abbreviations: numerical peak labels indicate the number of adenosine residues in the A30 and L70 poly(A) segments, “#” denotes RNase T1 miscleaved Poly(A) containing species, “†” denotes heterogeneous under-digested species.

Figure S.2.6-7. Mass Spectra of A30 Poly(A) Segment

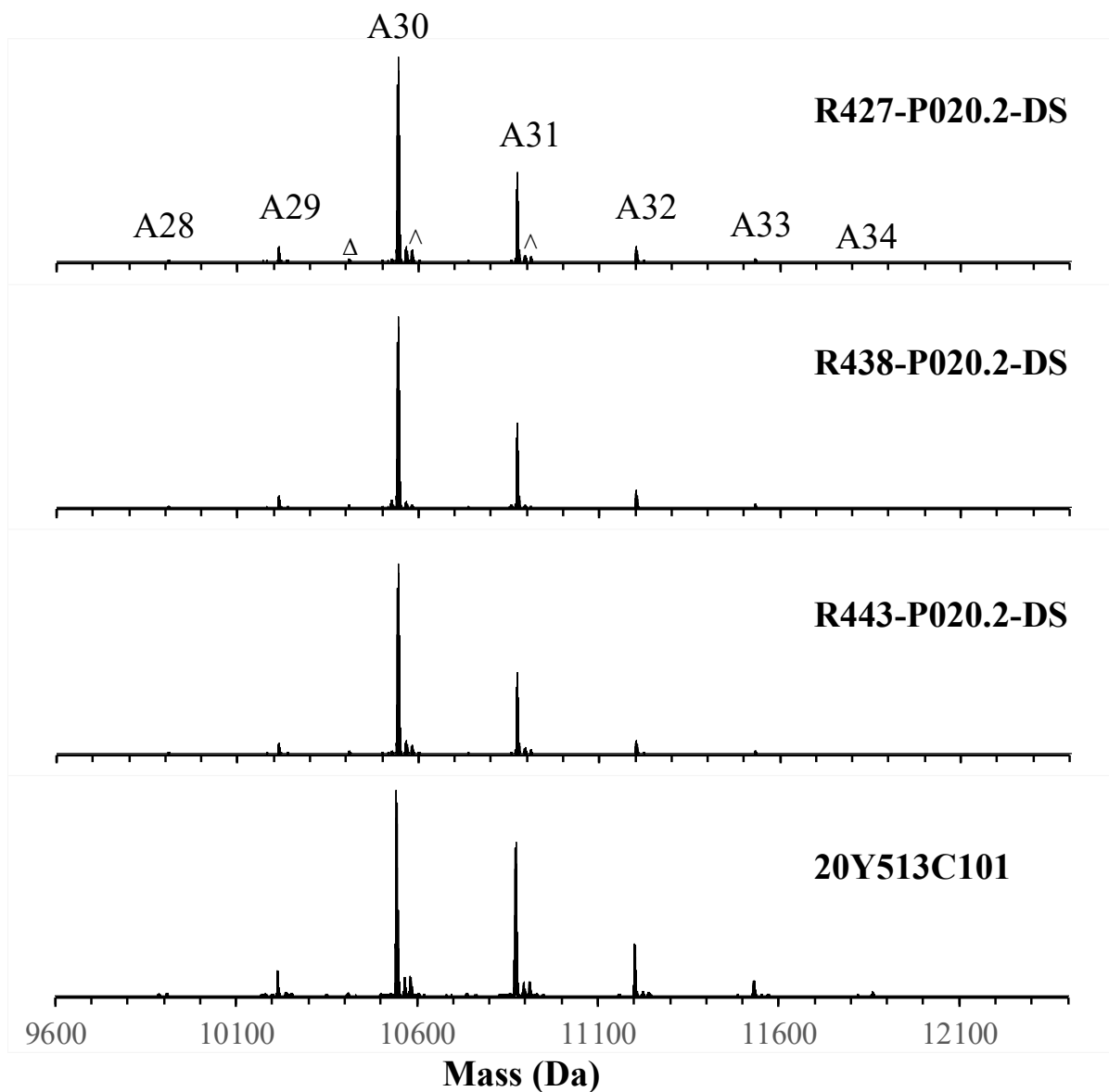


Figure S.2.6-7. Deconvoluted, zero-charge mass spectra of A30 poly(A) segment of four BNT162b2 DS batches. Spectra were constructed from the sum of the mass spectra of the A30 poly(A) elution region denoted by the bracket in [Figure S.2.6-6](#). Numerical peak labels indicate the number of adenosine residues in the detected A30 poly(A) segments. “Δ” denotes method-induced artifact of poly(A) from either digestion or mass spectral in-source fragmentation, “^” denotes +Na and +K adducts. The observed monoisotopic mass assignments for the A30 poly(A) segments are found in [Table S.2.6-17](#).

Table S.2.6-17. Accurate Mass Assignments for BNT162b2 Poly(A) tail A30 Segment

# of Adenosines (n) ^a	Theoretical Masses (Da) ^b	Observed Masses (Da) ^c				Relative Abundance ^d			
		R427-P020.2-DS	R438-P020.2-DS	R443-P020.2-DS	20Y513C101	R427-P020.2-DS	R438-P020.2-DS	R443-P020.2-DS	20Y513C101
28	9881.5698	9881.5322	9881.5326	9881.5244	9881.5335	Trace	Trace	Trace	Trace
29	10210.6224	10210.6073	10210.6059	10210.6086	10210.6065	Trace	Trace	Trace	Minor
30	10539.6749	10539.6602	10539.6618	10539.6559	10539.6668	Major	Major	Major	Major
31	10868.7274	10868.7049	10868.7106	10868.7094	10868.7131	Major	Major	Minor	Major
32	11197.7799	11197.7566	11197.7595	11197.7551	11197.7572	Trace	Trace	Trace	Minor
33	11526.8325	11526.7906	11526.7963	11526.7942	11526.8052	Trace	Trace	Trace	Trace
34	11855.8850	11855.8321	11855.8355	11855.8287	11855.8416	Trace	Trace	Trace	Trace

a. The 'n' indicates the observed number of adenosine residues in the species $^{4173}\text{C}-[\text{A}]_n-\text{G}^{4204}$, with the residues and superscripted positional values are based on plasmid-encoded linear sequence.

b. Theoretical masses (monoisotopic) take into account that the 3' end of poly(A) distribution is monophosphorylated for A30 poly(A) segment as a result of RNase T1 cleavage.

c. Observed masses (monoisotopic) were determined from the zero-charge mass spectra after deconvolution and de-isotoping of the multiply-charged data. Observed masses for major and minor species agree with theoretical masses to within 5 ppm, which is consistent with the accuracy and precision of contemporary mass spectrometers.

d. Relative abundance is categorized based on relative peak height to the base peak (representing 100%) where major, minor, and trace represent: > 45%, 45 to 10%, and <10% of relative peak height, respectively.

Figure S.2.6-8. Mass Spectra of L70 Poly(A) Segment

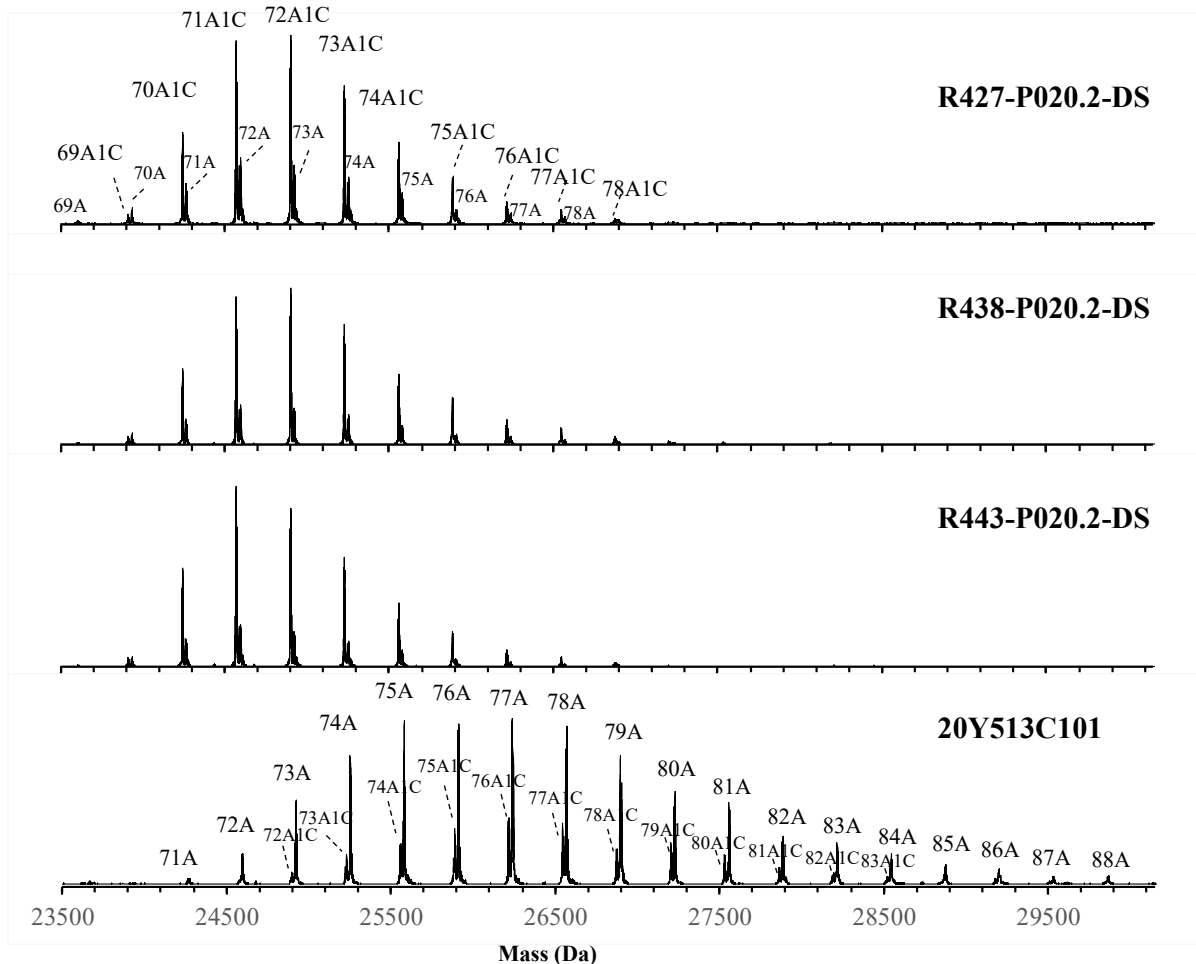


Figure S.2.6-8. Deconvoluted, zero-charge mass spectra of L70 poly(A) segment of four BNT162b2 DS batches. Spectra were constructed from the sum of the mass spectra of the L70 poly(A) elution region denoted by the bracket in [Figure S.2.6-6](#). Numerical peak labels indicate the number of adenosine residues in the detected L70 poly(A) segments. The “1C” suffix denotes a second distribution with mass spacing of adenosine; the mass difference between this distribution and the predominant poly(A) distribution is consistent with the mass of a cytosine addition. The observed monoisotopic mass assignments for the L70 poly(A) segment are found in [Table S.2.6-18](#).

Table S.2.6-18. Accurate Mass Assignments for BNT162b2 Poly(A) tail L70 Segment

Sequence ^a	n	Theoretical Masses (Da) ^b	Observed Masses (Da) ^c				Relative Abundance ^d			
			R427-P020.2-DS	R438-P020.2-DS	R443-P020.2-DS	20Y513C101	R427-P020.2-DS	R438-P020.2-DS	R443-P020.2-DS	20Y513C101
⁴²¹¹ ACT-[A] _n -[C]	70	23901.844	23901.874	23901.873	23901.873	ND	Trace	Trace	Trace	ND
	71	24230.897	24230.870	24230.863	24230.873	ND	Major	Major	Major	ND
	72	24559.949	24559.876	24559.873	24559.872	ND	Major	Major	Major	ND
	73	24889.002	24888.970	24888.969	24888.968	<i>24886.883</i>	Major	Major	Major	Trace
	74	25218.054	25217.969	25217.978	<i>25218.973</i>	<i>25218.976</i>	Major	Major	Major	Minor
	75	25547.107	25547.071	25547.066	25547.075	<i>25548.070</i>	Minor	Minor	Minor	Minor
	76	25876.159	<i>25877.080</i>	25876.082	<i>25877.083</i>	<i>25877.079</i>	Minor	Minor	Minor	Minor
	77	26205.212	26205.167	26205.162	26205.169	<i>26206.169</i>	Minor	Minor	Trace	Minor
	78	26534.264	26534.171	26534.175	26534.176	26534.171	Trace	Minor	Trace	Minor
	79	26863.317	26864.262	<i>26864.269</i>	<i>26865.264</i>	<i>26864.267</i>	Trace	Trace	Trace	Minor
	80	27192.369	27192.359	27192.362	27192.362	<i>27193.277</i>	Trace	Trace	Trace	Minor
	81	27521.422	27521.366	27521.370	27520.367	<i>27521.371</i>	Trace	Trace	Trace	Minor
	82	27850.474	ND	ND	ND	<i>27852.461</i>	ND	ND	ND	Minor
	83	28179.527	ND	ND	ND	28179.464	ND	ND	ND	Trace
	84	28508.580	ND	ND	ND	<i>28510.464</i>	ND	ND	ND	Trace
⁴²¹¹ ACT-[A] _n	69	23596.803	23596.772	<i>23597.770</i>	23596.779	ND	Trace	Trace	Trace	ND
	70	23925.855	23925.867	23925.869	23925.868	ND	Minor	Minor	Minor	ND
	71	24254.908	24254.868	24254.871	24254.874	<i>24257.879</i>	Minor	Minor	Minor	Trace
	72	24583.960	<i>24582.872</i>	24583.876	<i>24582.879</i>	<i>24584.971</i>	Minor	Minor	Minor	Minor
	73	24913.013	<i>24911.961</i>	24912.968	<i>24911.967</i>	24912.969	Minor	Minor	Minor	Major
	74	25242.065	<i>25240.972</i>	<i>25240.971</i>	<i>25240.970</i>	25241.975	Minor	Minor	Minor	Major
	75	25571.118	<i>25570.067</i>	<i>25570.069</i>	<i>25570.072</i>	25571.066	Minor	Minor	Minor	Major
	76	25900.171	<i>25898.076</i>	<i>25898.077</i>	<i>25897.162</i>	25900.080	Trace	Trace	Trace	Major
	77	26229.223	26229.162	26229.160	26229.167	26229.164	Trace	Trace	Trace	Major
	78	26558.276	26558.170	<i>26557.169</i>	<i>26557.170</i>	26558.178	Trace	Trace	Trace	Major
	79	26887.328	ND	ND	ND	26887.263	ND	ND	ND	Major
	80	27216.381	ND	ND	ND	27216.275	ND	ND	ND	Major
	81	27545.433	ND	ND	ND	27545.368	ND	ND	ND	Major
	82	27874.486	ND	ND	ND	27874.461	ND	ND	ND	Minor
	83	28203.538	ND	ND	ND	28203.464	ND	ND	ND	Minor
	84	28532.591	ND	ND	ND	28532.555	ND	ND	ND	Minor
	85	28861.643	ND	ND	ND	28862.560	ND	ND	ND	Minor
	86	29190.696	ND	ND	ND	29190.657	ND	ND	ND	Minor
	87	29519.748	ND	ND	ND	29518.761	ND	ND	ND	Trace

Table S.2.6-18. Accurate Mass Assignments for BNT162b2 Poly(A) tail L70 Segment

Sequence ^a	n	Theoretical Masses (Da) ^b	Observed Masses (Da) ^c				Relative Abundance ^d			
			R427-P020.2-DS	R438-P020.2-DS	R443-P020.2-DS	20Y513C101	R427-P020.2-DS	R438-P020.2-DS	R443-P020.2-DS	20Y513C101
	88	29848.801	ND	ND	ND	29850.660	ND	ND	ND	Trace

a. The residues and superscripted positional values are based on plasmid-encoded linear sequence. The subscripted 'n' indicates the observed number of adenosine residues. A = Adenine; C = Cytosine; G = Guanine; T = N1-methylpseudouridine

b. Theoretical masses (monoisotopic) take into account that the 3' end of the BNT162b2 mRNA is a hydroxyl group.

c. Observed masses (monoisotopic) were determined from the zero-charge mass spectra after deconvolution and de-isotoping of the multiply-charged data. Most observed masses for major and minor species agree with theoretical masses to within 5 ppm, which is consistent with the accuracy and precision of contemporary mass spectrometers. Italicized masses signify a deconvolution monoisotopic mass assignment error of ≥ 1 Da, which arises when isotopic distributions of more than one species significantly overlap.

d. Relative abundance is categorized based on relative peak height to the base peak (representing 100%) where major, minor, and trace represent: > 45%, 45 to 10%, and <10% of relative peak height, respectively.

ND = Not Detected

S.2.6.5.3.5. Comparative LC/MS/MS – Oligonucleotide Mapping of BNT162b2 Process 1 and Process 2 DS Batches

The primary sequence of BNT162b2 drug substance was analyzed by LC/MS/MS - oligonucleotide mapping. BNT162b2 DS batches were 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 260 nm (Figure S.2.6-9 through Figure S.2.6-13). All major and minor peaks in the oligonucleotide map were identified by on-line electrospray ionization mass spectrometry with tandem mass spectrometry (MS/MS) by higher-collision dissociation (IP-RP-HPLC/ESI MS/MS or LC/MS/MS) as shown in Figure S.2.6-9 through Figure S.2.6-13. The observed chromatographic profiles for the Process 1 and Process 2 substance batches are highly similar in terms of peak position, shape and abundance. The observed masses and MS/MS fragmentation patterns of the oligonucleotides in each peak observed in each of the four drug substance batches were consistent with the expected RNase T1 fragments of BNT162b RNA. Given the possibility of sequence isomers after RNase T1 digestion, the oligonucleotide map also was assigned via software using decoy sequences of BNT162b2 (randomized and reversed) in the presence of the BNT162b2 target sequence to confirm correct peak assignments. The vast majority of the oligonucleotide isomers were assigned to the target BNT162b2 RNA sequence rather than to any of the decoy RNA sequences, demonstrating the specificity of the method. The detected enzymatic fragments account for 90.5% of the overall BNT162b2 RNA sequence for all four drug substance batches (See Table S.2.6-19).

The 3' terminal sequence region of BNT162b2 RNA was detected by LC/MS/MS – oligonucleotide mapping in both the Process 1 and Process 2 DS batches, and the observed species are consistent with those observed by the Poly(A) tail LC/MS methods described in Section S.2.6.5.3.3. The 5' terminal sequence region was not recoverable after RNase T1 digestion as the oligonucleotide generated was too hydrophilic. The 5' terminal region including the 5'-Cap was characterized separately by LC/MS as described in Section S.2.6.5.3.2.

The LC/MS/MS – oligonucleotide mapping results are summarized in Table S.2.6-19. In conclusion, the highly comparable chromatographic profiles and consistent LC/MS/MS peak identifications between materials demonstrate that the Process 1 and Process 2 BNT162b2 RNA DS batches are comparable and contain the correct, identical sequence as predicted from the linear DNA template (Section S.2.3 Control of Materials Source, History and Generation of Plasmids (modRNA)).

Figure S.2.6-9. LC/MS/MS – Oligonucleotide Mapping of BNT162b2 DS Batches (3-15 min)

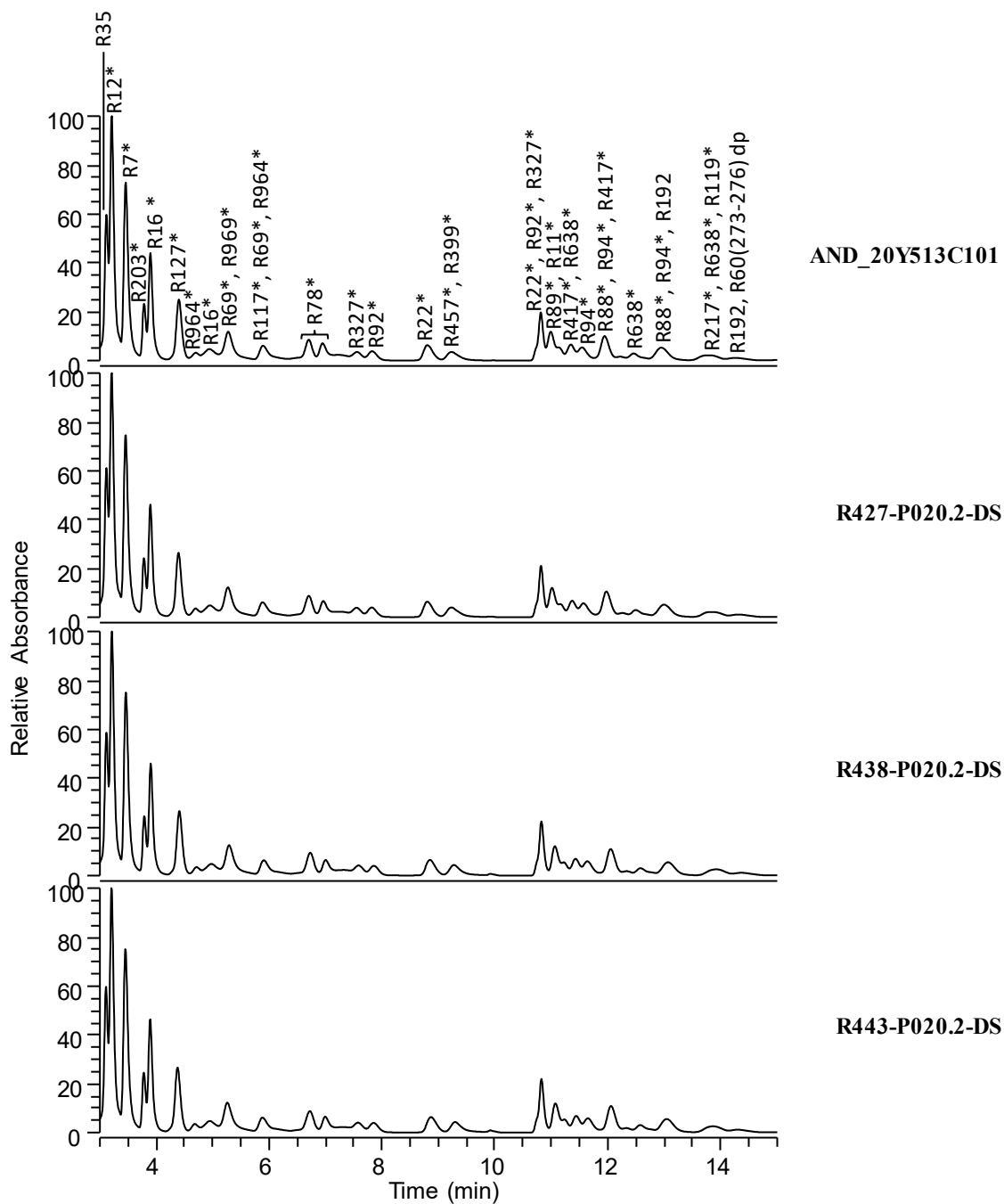


Figure S.2.6-9 RNase T1 oligonucleotide map of BNT162b2 DS batches. Abbreviations: “R” represents BNT162b2 oligonucleotide fragment resulting from RNase T1 digestion, “dp” denotes dephosphorylation, and “*” denotes a non-unique oligonucleotide (i.e., the sequence is repeated in BNT162b2) where these single assignments represent all oligonucleotides with the same sequence.

Figure S.2.6-10. LC/MS/MS – Oligonucleotide Mapping of BNT162b2 DS Batches (15-67.6 min)

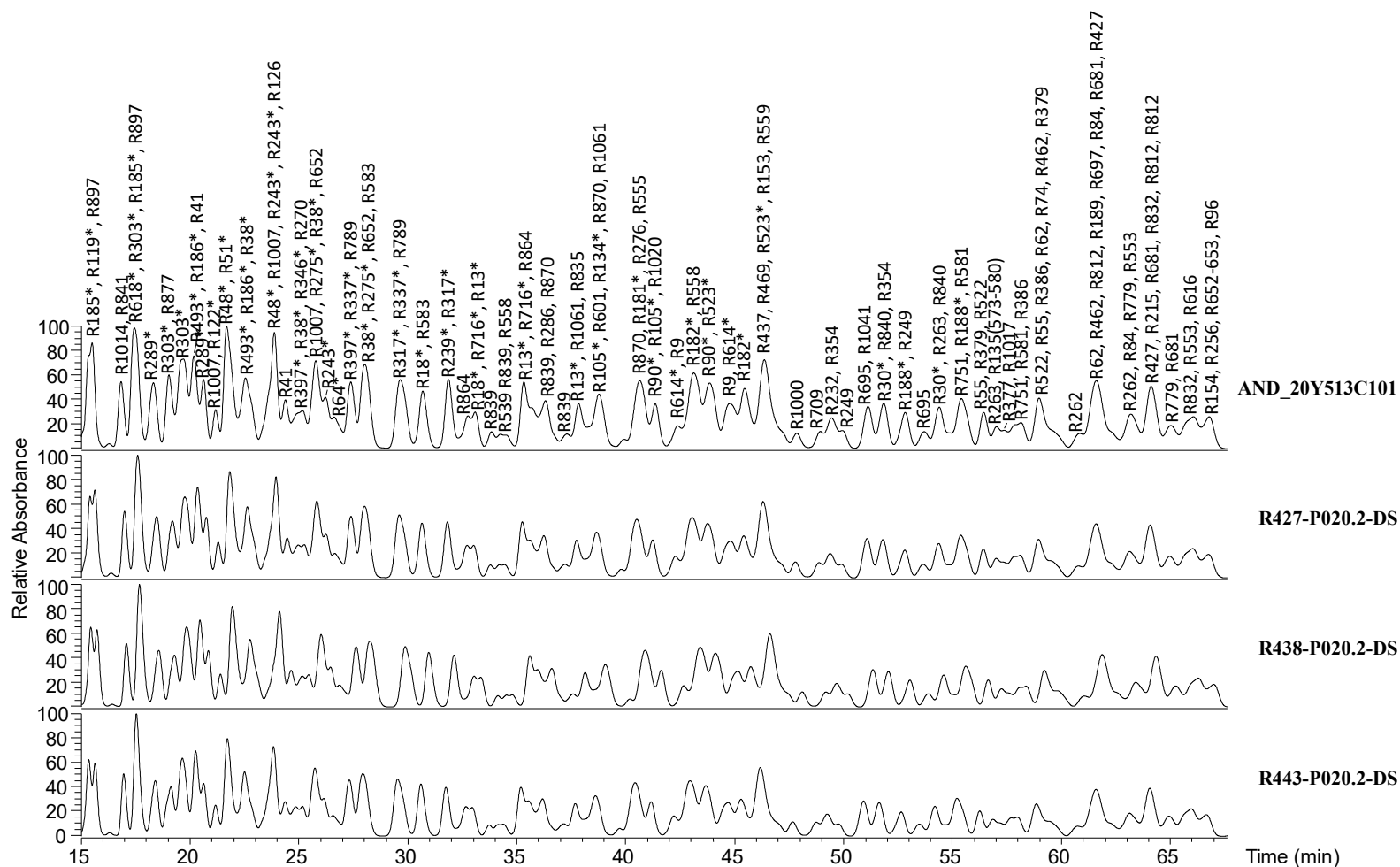


Figure S.2.6-10 RNase T1 oligonucleotide map of BNT162b2 DS batches. Abbreviations: “R” represents BNT162b2 oligonucleotide fragment resulting from RNase T1 digestion, “dp” denotes dephosphorylation, and “*” denotes a non-unique oligonucleotide (i.e., the sequence is repeated in BNT162b2) where these single assignments represent all oligonucleotides with the same sequence.

Figure S.2.6-11. LC/MS/MS – Oligonucleotide Mapping of BNT162b2 DS Batches (67.6-142 min)

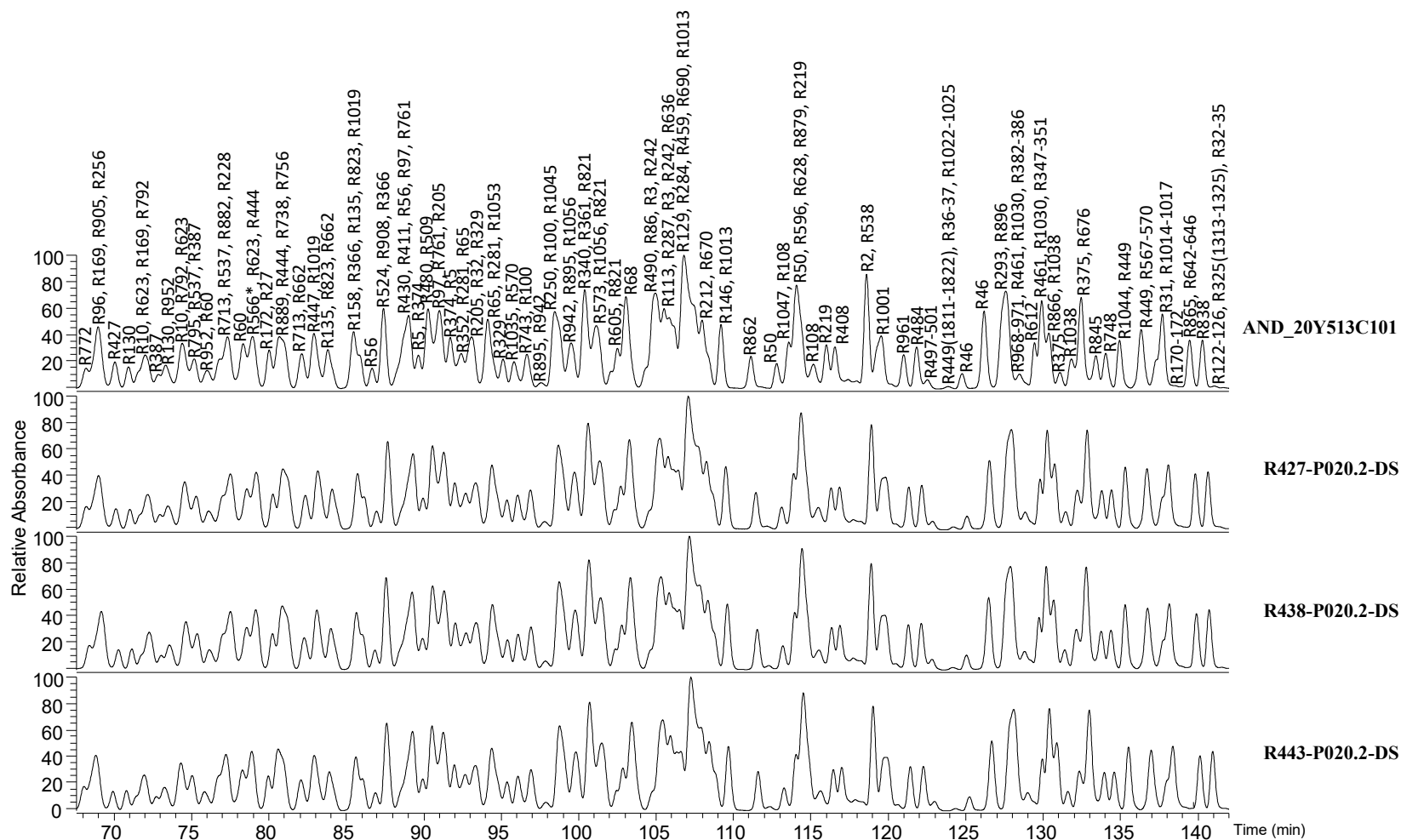


Figure S.2.6-11 RNase T1 oligonucleotide map of BNT162b2 DS batches. Abbreviations: “R” represents BNT162b2 oligonucleotide fragment resulting from RNase T1 digestion, “dp” denotes dephosphorylation, and “*” denotes a non-unique oligonucleotide (i.e., the sequence is repeated in BNT162b2) where these single assignments represent all oligonucleotides with the same sequence.

Figure S.2.6-12. LC/MS/MS – Oligonucleotide Mapping of BNT162b2 DS Batches (142-210 min)

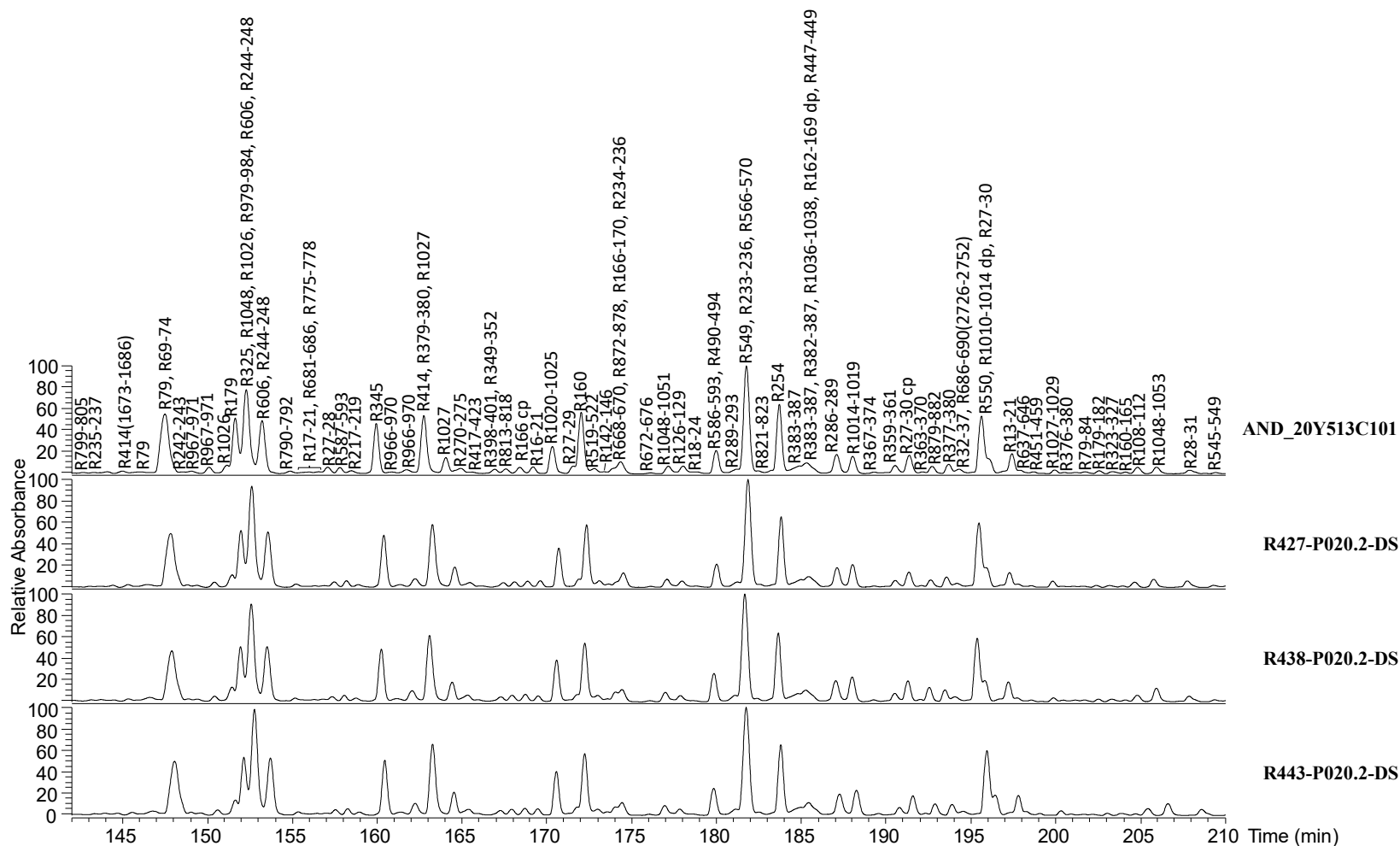


Figure S.2.6-12 RNase T1 oligonucleotide map of BNT162b2 DS batches. Abbreviations: “R” represents BNT162b2 oligonucleotide fragment resulting from RNase T1 digestion, “dp” denotes dephosphorylation, and “*” denotes a non-unique oligonucleotide (i.e., the sequence is repeated in BNT162b2) where these single assignments represent all oligonucleotides with the same sequence.

Figure S.2.6-13. LC/MS/MS – Oligonucleotide Mapping of BNT162b2 DS Batches (210-260 min)

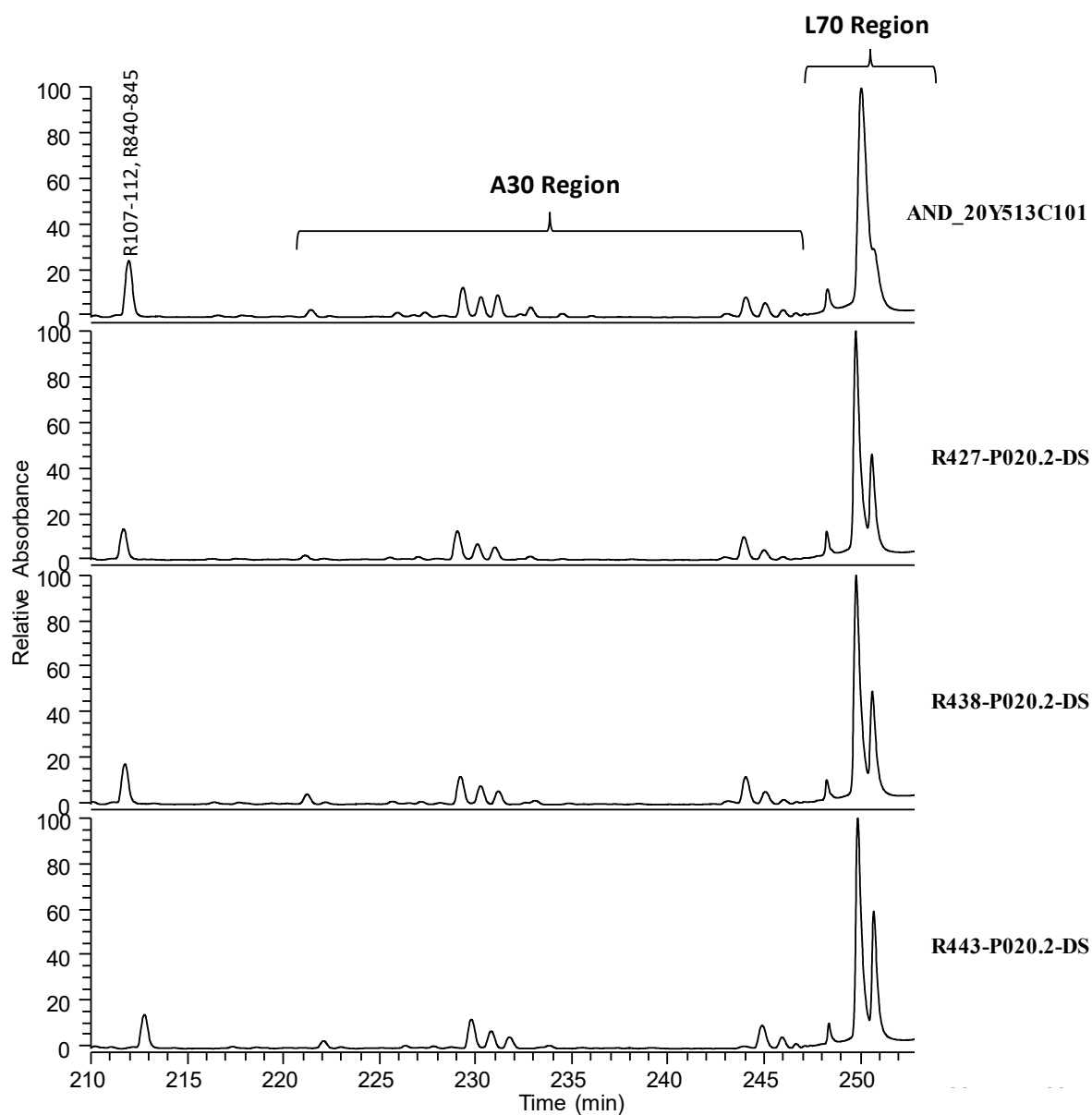


Figure S.2.6-13 RNase T1 oligonucleotide map of BNT162b2 DS batches. Abbreviations: “R” represents BNT162b2 oligonucleotides, and the A30 and L70 regions represent the polyA-tail region of BNT162b2 (see [S.2.6.5.3.3](#))

Table S.2.6-19. LC/MS/MS – Oligonucleotide Mapping Summary of BNT162b2 DS Batches

Characteristics	AND_20Y513C101	R427-P020.2-DS	R438-P020.2-DS	R443-P020.2-DS
RNA sequence confirmation	<p>BNT162b2 RNA sequence coverage:</p> <p>Detected nucleotides represent 3874 out of 4283 residues or 90.5%</p>	<p>BNT162b2 RNA sequence coverage:</p> <p>Detected nucleotides represent 3874 out of 4283 residues or 90.5%</p>	<p>BNT162b2 RNA sequence coverage:</p> <p>Detected nucleotides represent 3874 out of 4283 residues or 90.5%</p>	<p>BNT162b2 RNA sequence coverage:</p> <p>Detected nucleotides represent 3874 out of 4283 residues or 90.5%</p>
RNA termini	<p>5'-terminus not recovered by RNase T1 RNA map – see S.2.6.5.3.2 for detailed analysis of 5' terminus</p> <p>3'-terminus detected: A30 and L70 polyA tail regions detected – (see S.2.6.5.3.3 for detailed analysis of PolyA tail)</p>	<p>5'-terminus not recovered by RNase T1 RNA map – see S.2.6.5.3.2 for detailed analysis of 5' terminus</p> <p>3'-terminus detected: A30 and L70 polyA tail regions detected – (see S.2.6.5.3.3 for detailed analysis of PolyA tail)</p>	<p>5'-terminus not recovered by RNase T1 RNA map – see S.2.6.5.3.2 for detailed analysis of 5' terminus</p> <p>3'-terminus detected: A30 and L70 polyA tail regions detected – (see S.2.6.5.3.3 for detailed analysis of PolyA tail)</p>	<p>5'-terminus not recovered by RNase T1 RNA map – see S.2.6.5.3.2 for detailed analysis of 5' terminus</p> <p>3'-terminus detected: A30 and L70 polyA tail regions detected – (see S.2.6.5.3.3 for detailed analysis of PolyA tail)</p>

S.2.6.5.3.6. Comparative Higher Order Structure Characterization of BNT162b2 DS Batches

Circular dichroism (CD) spectroscopy was used to assess the higher-order structure of BNT162b2 in solution for Process 1 and Process 2 DS batches. CD spectra were recorded in triplicate for each BNT162b2 DS batch, and all samples from all batches were analyzed side-by-side from a 1xPBS solution. The averaged CD spectra were overlaid for visual inspection, and for each DS batch, the CD spectra exhibit the same alternating peaks and troughs and match each other closely at all wavelengths from 200 nm to 330 nm (Figure S.2.6-14). The averaged CD spectra were further analyzed by a chemometric classification method to evaluate spectral similarity ([Table S.2.6-20](#)).

Figure S.2.6-14. CD Spectral Overlay BNT162b2 DS batches

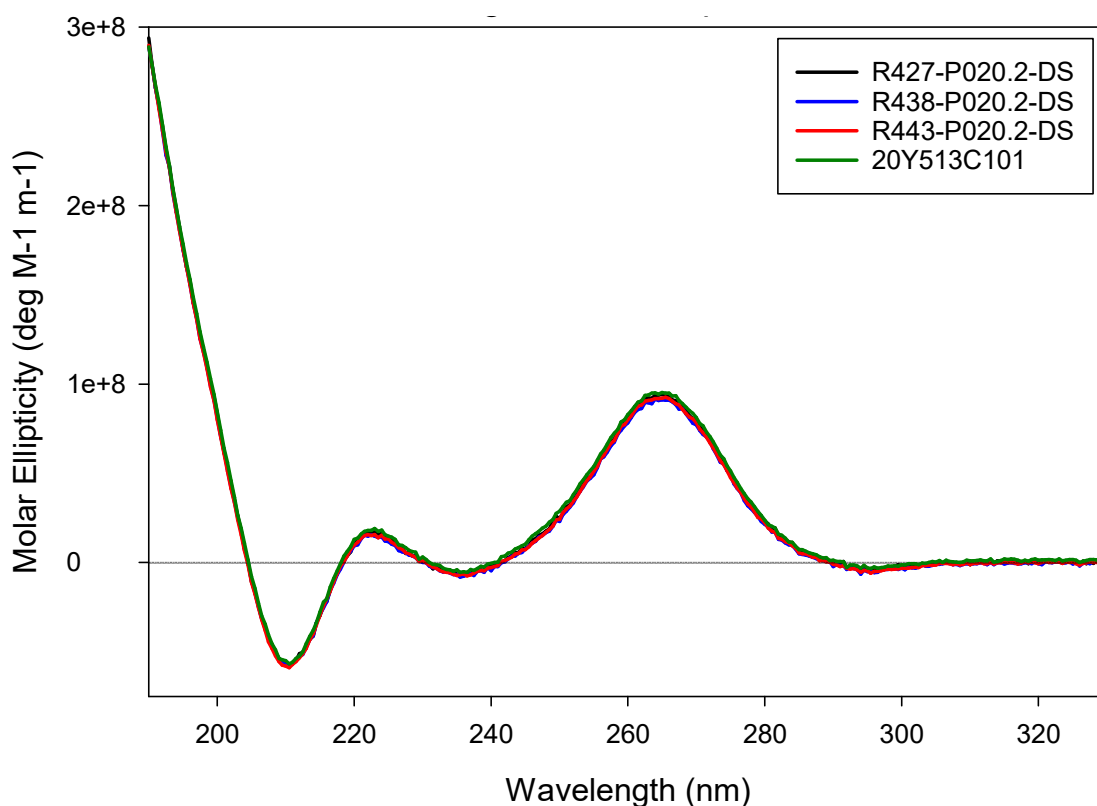


Figure S.2.6-14 Overlaid CD spectra of BNT162b2 DS in 1xPBS buffer solution. For each DS batch, the spectra were acquired in triplicate using a CD spectrometer with 0.01 cm pathlength rectangular cuvettes at 20 °C. The triplicate spectra were averaged at each data point and the average values were plotted. All four DS batches were analyzed by CD in a side-by-side manner.

Table S.2.6-20. Spectral Similarity Scores between BNT162b2 Drug Substance Batches

	20Y513C101	R427-P020.2-DS	R438-P020.2-DS	R443-P020.2-DS
20Y513C101 ^a	100%	99.3%	98.4%	98.6%

a. all lots are compared against batch 20Y513C101: 100% being identical

The close match of all CD spectra in the superimposed views and respective similarity scores confirm that BNT162b2 Process 1 and Process 2 DS batches are comparable to each other with respect to higher order structure.

S.2.6.5.3.7. Expressed protein size by Western blot

The protein size after in-vitro expression of BNT162b2 drug substance was determined using Western blot. Expressed protein size was confirmed to be comparable for three Process 1 batches and the Process 2 batch. Figure S.2.6-15 shows that the expressed protein size is consistent with the expected size of the translated BNT162b2 drug substance and comparable across all tested batches. In addition, relative expression levels are comparable for all batches, as evidenced by comparable band intensity at each load level across all batches.

Figure S.2.6-15. BNT162b2 Expressed Protein Size by Western Blot

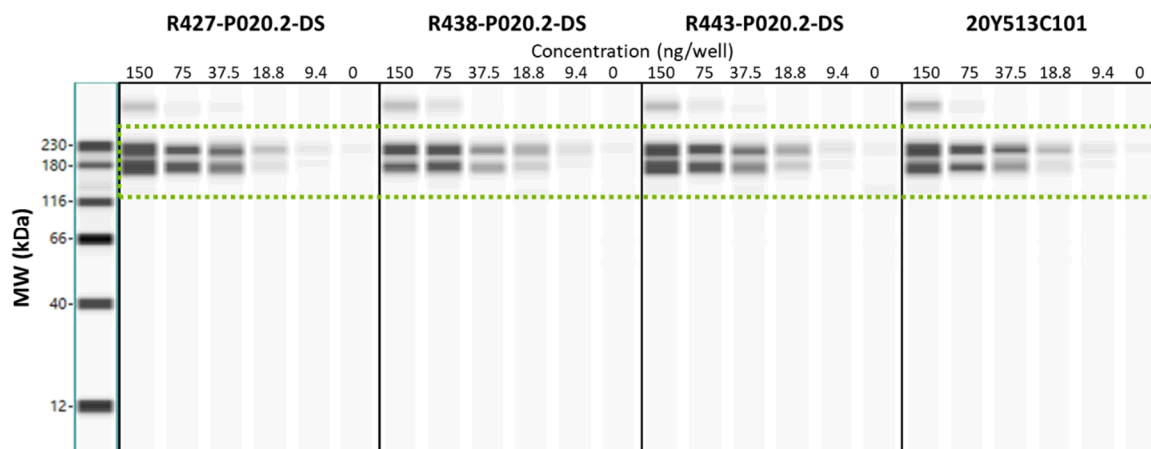


Figure S.2.6-15. To evaluate expressed protein size, BNT162b2 DS was mixed with Lipofectamine and then transfected into HEK-293 cells. Following incubation, cell lysates were evaluated for the expressed protein antigen by Western blot using an antibody specific for the SARS-CoV-2 spike protein. The first lane shows a molecular weight (MW) marker. The concentrations shown for each DS batch correspond to the amounts of DS transfected per well of HEK-293 cells.

S.2.6.5.4. Side-by-Side Comparability Study #2 - Evaluation of Clinical through PPQ Drug Substance Batches

To assess comparability across Process 2 manufacturing facilities, a second study was conducted and included three Process Performance Qualification (PPQ) batches each from Pfizer, Andover and BioNTech Manufacturing GmbH. Each of the selected PPQ batches is

representative of the commercial manufacturing process at the respective site. Two additional Process 2 batches, planned for clinical supply and for emergency supply in the US market, were included. Lastly, two representative batches from Process 1 were included in the comparison to allow bridging to the previous comparability study, described above. These Process 1 batches were selected applying a bracketing approach with respect to manufacturing scale and date. [Table S.2.6-12](#) provides an overview of the ten batches included in comparability study #2, and the comparability testing strategy is described in [Table S.2.6-13](#).

[Table S.2.6-14](#) provides a summary of the release test results for DS batches included in each comparability study. In addition to the release and additional test data described above, side-by-side comparability testing results for the second study shown in [Table S.2.6-21](#) demonstrate that that Process 2 PPQ batches manufactured at both sites are comparable to each other and are comparable to the Process 1 batches. Consistent with the release test data, a small decrease in RNA integrity was observed for the Process 2 emergency supply batches, and all Process 2 batches show increased 5'-capped RNA levels. As the RNA integrity and 5'-cap attributes are critical to translation of the protein antigen in vivo, the proportion of capped-intact RNA is used to compare the Process 1 and Process 2 materials that were tested side-by-side ([Table S.2.6-21](#)). Leveraging side-by-side testing results minimizes analytical variability associated with RNA integrity and 5'-cap measurements and enables a more controlled comparison of capped-intact RNA. The capped-intact RNA content in the Process 1 batches included in study #2 range from 55 to 56%. Emergency supply batches range from 50 to 59% capped-intact RNA, and the Process 2 PPQ batches range from 64 to 70%. The slight increase in capped-intact RNA content at the PPQ manufacturing sites is not expected to have a meaningful impact to product safety or efficacy. Supportive data profiles are provided below for side-by-side testing of identity (RNA length), identity (as RNA), poly(A) tail length and distribution, and expressed protein size.

Additionally, a specifically selected series of state-of-the-art characterization analyses was performed to assess additional aspects and demonstrate comparability of the BNT162b2 DS structure. Side-by-side comparability studies were performed using mass spectrometry to characterize the 5'-cap, poly(A) tail, and oligonucleotide mapping. Circular dichroism was performed to characterize higher order structure.

Table S.2.6-21. BNT162b2 Drug Substance Side-by-Side Comparability Testing Results – Study #2

Attribute and Method	Clinical (Process 1)		Emergency Supply ^a (Process 2)		Process Performance Qualification – Pfizer, Andover (Process 2)			Process Performance Qualification - BioNTech Mainz / Rentschler (Process 2)		
	R427-P020.2-DS	R445-P020.2-DS	20Y513C1 01	20Y513C2 01	20Y513C5 01	20Y513C6 01	20Y513C7 01	20E162001	20E162002	20E162003
Identity of encoded RNA sequence by RT-PCR	Confirmed	Confirmed	Confirmed	Confirmed	Confirmed	Confirmed	Confirmed	Confirmed	Confirmed	Confirmed
RNA Integrity by capillary gel electrophoresis (%)	80	83	60	68	73	72	72	75	76	76
5'-Cap by LC-UV (%)	69	67	84	87	88	89	90	92	92	91
Capped-Intact RNA ^b (%)	55	56	50	59	64	64	65	69	70	69
Poly(A) Tail by ddPCR (%)	128	131	98	112	115	121	111	117	118	103
Poly(A) Tail: Length and Distribution by RP-HPLC (%)	A30: 30.0 L70: 50.3 Other: 19.8	A30: 30.4 L70: 48.8 Other: 20.9	A30: 27.4 L70: 53.2 Other: 19.4	A30: 27.2 L70: 55.0 Other: 17.7	A30: 27.5 L70: 50.8 Other: 21.8	A30: 27.4 L70: 53.1 Other: 19.5	A30: 27.2 L70: 54.1 Other: 18.7	A30: 27.3 L70: 54.1 Other: 18.6	A30: 26.9 L70: 52.6 Other: 20.5	A30: 27.3 L70: 54.7 Other: 18.0

Table S.2.6-21. BNT162b2 Drug Substance Side-by-Side Comparability Testing Results – Study #2

Attribute and Method	Clinical (Process 1)		Emergency Supply ^a (Process 2)		Process Performance Qualification – Pfizer, Andover (Process 2)			Process Performance Qualification - BioNTech Mainz / Rentschler (Process 2)		
	R427-P020.2-DS	R445-P020.2-DS	20Y513C1 01	20Y513C2 01	20Y513C5 01	20Y513C6 01	20Y513C7 01	20E162001	20E162002	20E162003
Identity (RNA length) by denaturing agarose gel electrophoresis	Single distinct band migrating at the expected location as compared to a reference RNA	Single distinct band migrating at the expected location as compared to a reference RNA	Single distinct band migrating at the expected location as compared to a reference RNA	Single distinct band migrating at the expected location as compared to a reference RNA	Single distinct band migrating at the expected location as compared to a reference RNA	Single distinct band migrating at the expected location as compared to a reference RNA	Single distinct band migrating at the expected location as compared to a reference RNA	Single distinct band migrating at the expected location as compared to a reference RNA	Single distinct band migrating at the expected location as compared to a reference RNA	Single distinct band migrating at the expected location as compared to a reference RNA
Identity (as RNA) by enzymatic degradation followed by gel electrophoresis	No RNase-resistant band detectable by gel electrophoresis	No RNase-resistant band detectable by gel electrophoresis	No RNase-resistant band detectable by gel electrophoresis	No RNase-resistant band detectable by gel electrophoresis	No RNase-resistant band detectable by gel electrophoresis	No RNase-resistant band detectable by gel electrophoresis	No RNase-resistant band detectable by gel electrophoresis	No RNase-resistant band detectable by gel electrophoresis	No RNase-resistant band detectable by gel electrophoresis	No RNase-resistant band detectable by gel electrophoresis

- a. Emergency supply designation applies to U.S. market.
b. Capped Intact RNA (%) = RNA Integrity (%) x 5'-Cap (%)

Abbreviations: ddPCR = Droplet digital polymerase chain reaction; RP-HPLC = Reversed phase high performance liquid chromatography; RT-PCR = Reverse transcription PCR

S.2.6.5.4.1. Identity as determined by agarose gel electrophoresis (AGE)

The identity of BNT162b2 drug substance was determined using agarose gel electrophoresis (AGE). Identity (RNA length) was confirmed all batches. [Figure S.2.6-1](#) shows that the RNA size is consistent with the expected size of BNT162b2 drug substance and comparable across all tested batches. In addition, identity (as RNA) is demonstrated for all batches, as no RNase A-resistant bands are detectable after enzymatic degradation of the RNA drug substance ([Figure S.2.6-1](#)).

Figure S.2.6-16. Identity by Agarose Gel Electrophoresis

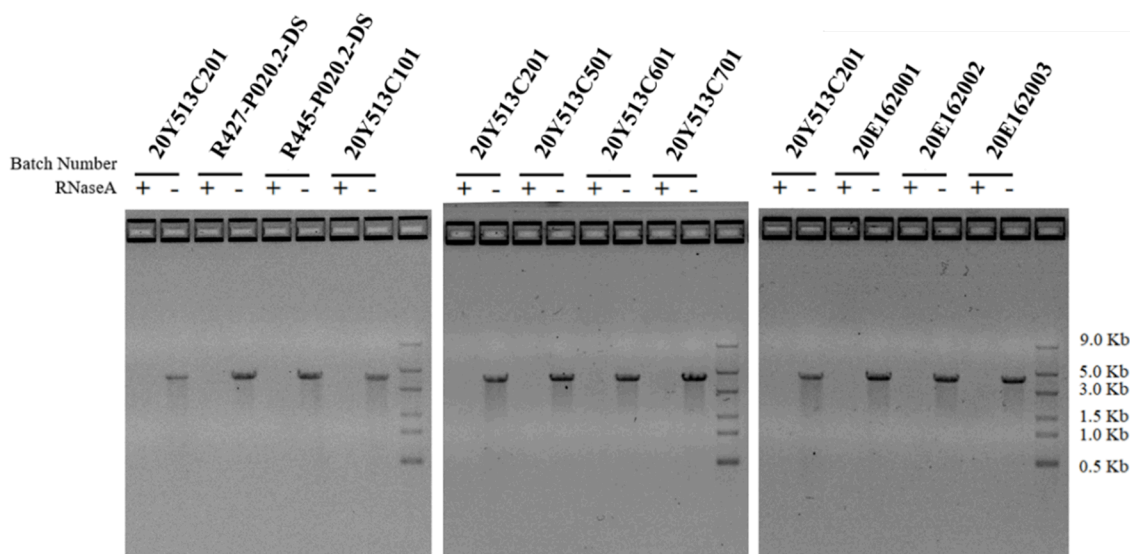


Figure S.2.6-16 – Identity as determined by agarose gel electrophoresis was evaluated side-by-side for the designated batches. Based on the number of samples, three gels were run, with interim reference material batch 20Y513C201 included as a reference across all gels. The final lane in each gel includes in RNA ladder, with the corresponding band sizes noted on the far right.

S.2.6.5.4.2. Comparative LC-UV/MS Analysis of the 5'-Cap in BNT162b2

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 260 nm 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 S.4.2 RP-HPLC \(modRNA\)](#). The identification of capped and un-capped species by mass spectrometry are presented in [Section 3.2.S.3.1.3 5'-Cap Characterization by LC-UV/MS \(in Section S.3.1 Elucidation of Structure and Other Characteristics \(modRNA\)\)](#).

Using LC-UV and peak integration, the 5'-cap levels for all DS batches exceed 60% ([Table S.2.6-21](#)) with slightly higher levels of 5'-Cap observed in Process 2 DS (

Figure S.2.6-17 and [Figure S.2.6-18](#)).

The mass spectra representing the main UV chromatographic peak, labeled as 5'-Cap, show a single, predominant species in all Process 1 and 2 DS materials, corresponding to the expected 5'-capped species ([Figure S.2.6-19](#) and [Figure S.2.6-20](#)). The observed monoisotopic masses from each material for this UV chromatographic peak match the theoretical mass of the 26 nucleotide RNase cleaved fragment with the 5' cap, confirming the intended 5' capped structure ([Section S.3.1 Elucidation of Structure and Other Characteristics \(modRNA\)](#)). For the uncapped region, the same 5'-ppp and 5'-pp species are observed at similar relative abundances in the Process 1 and Process 2 DS ([Figure S.2.6-21](#) and [Figure S.2.6-22](#)). For all materials, the observed monoisotopic masses for each of these un-capped RNase cleaved fragments are in agreement with the respective theoretical masses, confirming 5' uncapped structures [Table S.2.6-22](#)).

Characterization by LC-UV/MS confirms that the 5'-capped and uncapped structures are the same in both Process 1 and 2 with a redistribution toward higher 5'-capping levels in Process 2. It is anticipated that a higher 5'-Cap level may enable a more stable and efficacious BNT162b2 DS. Therefore, Process 1 and Process 2 DS batches are considered comparable with respect to 5'-Cap.

Figure S.2.6-17. 5'-Cap Assay UV Chromatograms of BNT162b2 DS RNase Cleaved Fragments, Sample Set 1

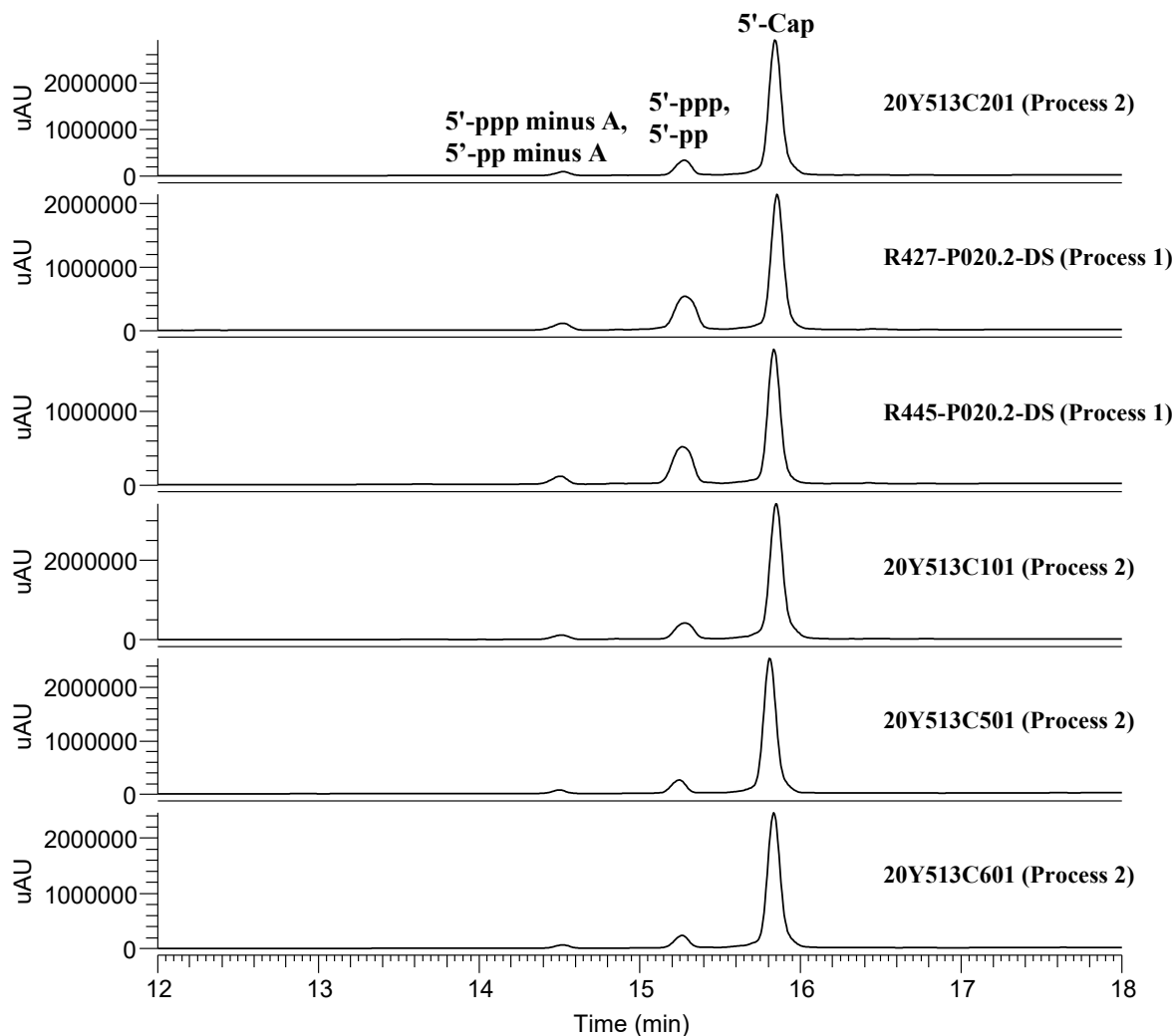


Figure S.2.6-17. The 5'-Cap assay UV 260 nm absorbance chromatograms of six BNT162b2 mRNA DS batches. Batch 20Y513C201 serves as an interim reference to bridge sample set figures. The 12-18 min region of the chromatogram comprises the region where the RNase cleaved fragments elute. Peaks were identified via online mass spectrometry. Due to the shallow gradient used in this separation, minor shifts in resolution and retention time can occur between samples and are not considered significant.

Figure S.2.6-18. 5'-Cap Assay UV Chromatograms of BNT162b2 DS RNase Cleaved Fragments, Sample Set 2

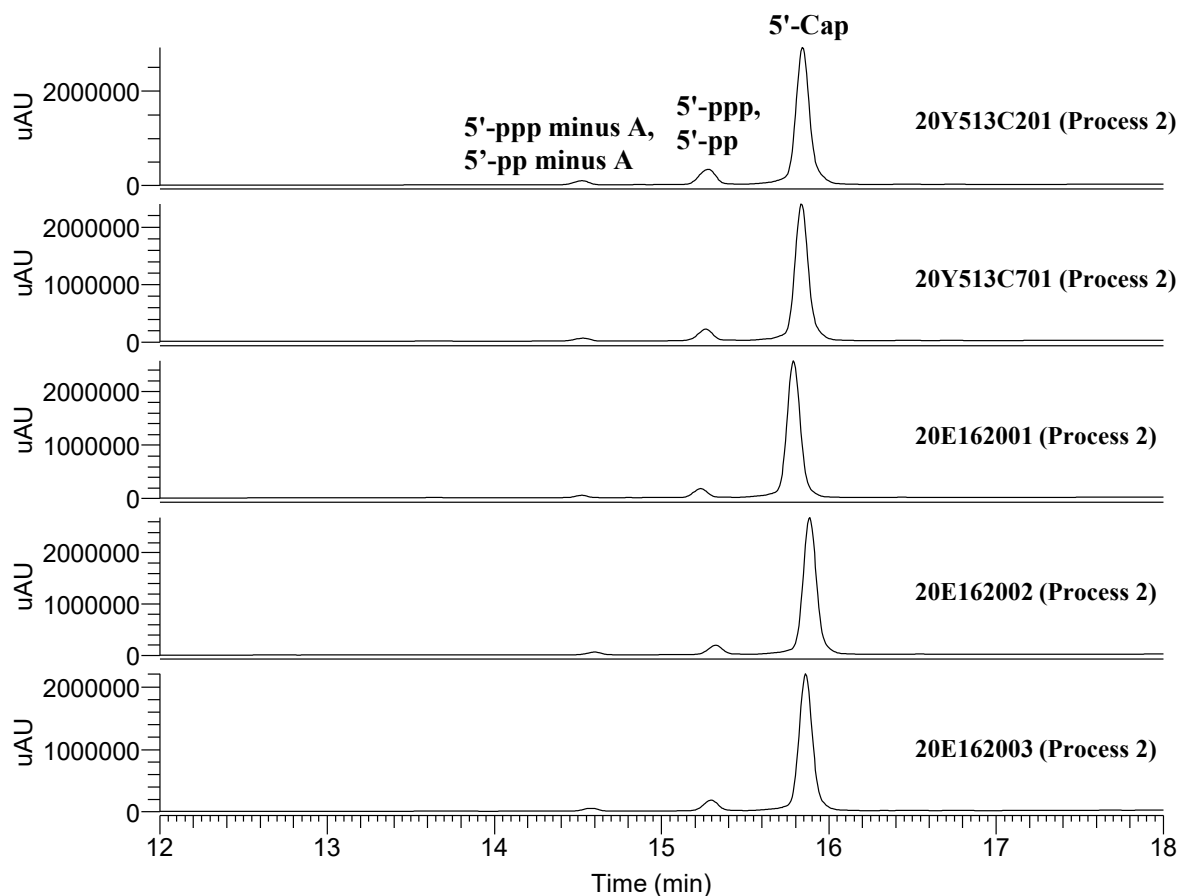


Figure S.2.6-18. The 5'-Cap assay UV 260 nm absorbance chromatograms of five BNT162b2 mRNA DS batches. Batch 20Y513C201 serves as an interim reference to bridge sample set figures. The 12-18 min region of the chromatogram comprises the region where the RNase cleaved fragments elute. Peaks were identified via online mass spectrometry. Due to the shallow gradient used in this separation, minor shifts in resolution and retention time can occur between samples and are not considered significant.

Figure S.2.6-19. Mass Spectra of 5'-Cap RNase Cleaved Fragments from BNT162b2 DS Batches, Sample Set 1

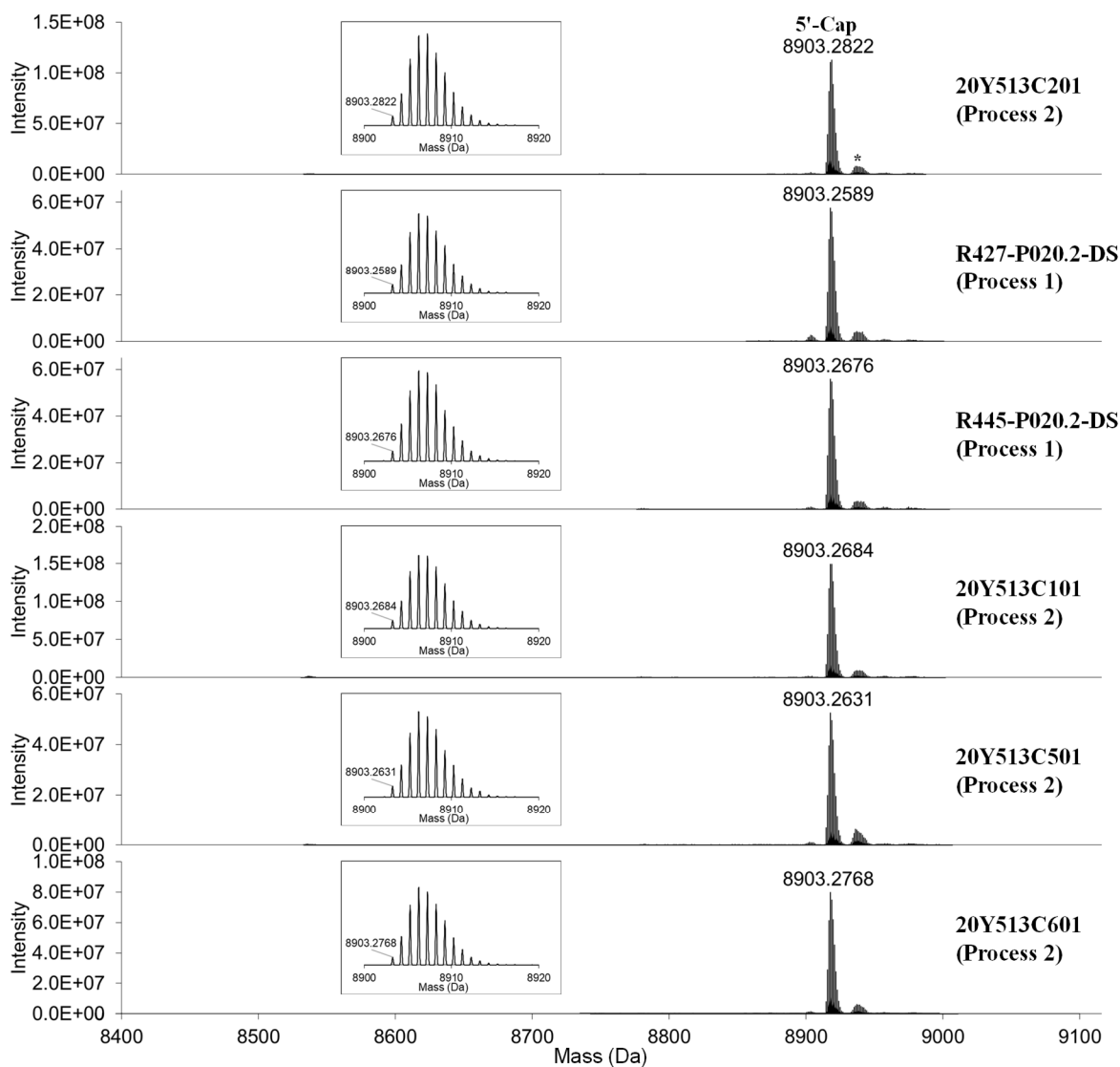


Figure S.2.6-19. Deconvoluted, zero-charge mass spectra of the 5'-capped 26 nucleotide RNase cleaved fragment of six BNT162b2 DS batches. Batch 20Y513C201 serves as an interim reference to bridge sample set figures. The spectra are deconvoluted from the summation of scans across the 5'-cap elution region. The “*” marks water and sodium adducts of the 5'-Cap species. The insets provide zoomed-spectra views of the 5'-cap isotopic envelopes and monoisotopic peak assignments.

Figure S.2.6-20. Mass Spectra of 5'-Cap RNase Cleaved Fragments from BNT162b2 DS Batches, Sample Set 2

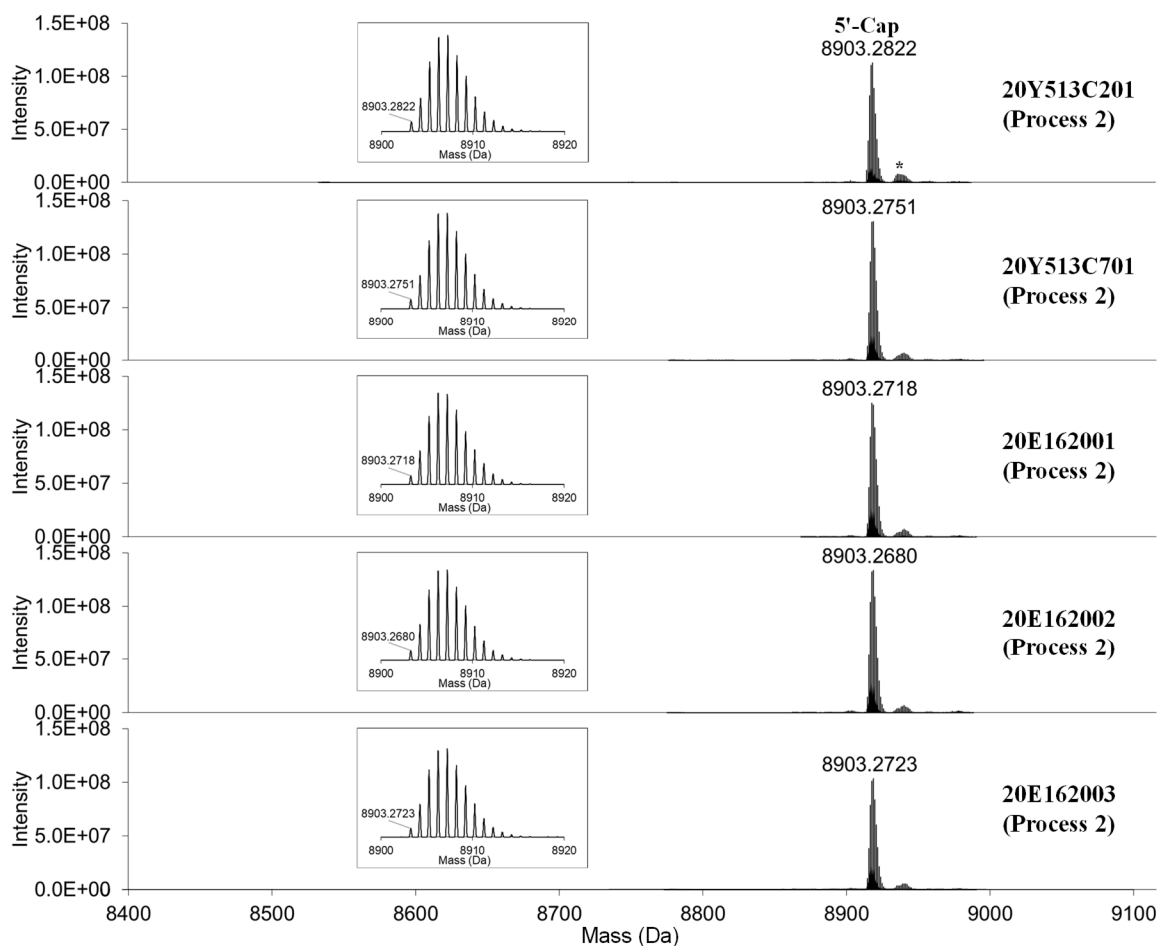


Figure S.2.6-20. Deconvoluted, zero-charge mass spectra of the 5'-capped 26 nucleotide RNase cleaved fragment of five BNT162b2 DS batches. Batch 20Y513C201 serves as an interim reference to bridge sample set figures. The spectra are deconvoluted from the summation of scans across the 5'-cap elution region. The “*” marks water and sodium adducts of the 5'-Cap species. The insets provide zoomed-spectra views of the 5'-cap isotopic envelopes and monoisotopic peak assignments.

Figure S.2.6-21. Mass Spectra of 5'-ppp and 5'-pp RNase Cleaved Fragments from BNT162b2 DS Batches, Sample Set 1

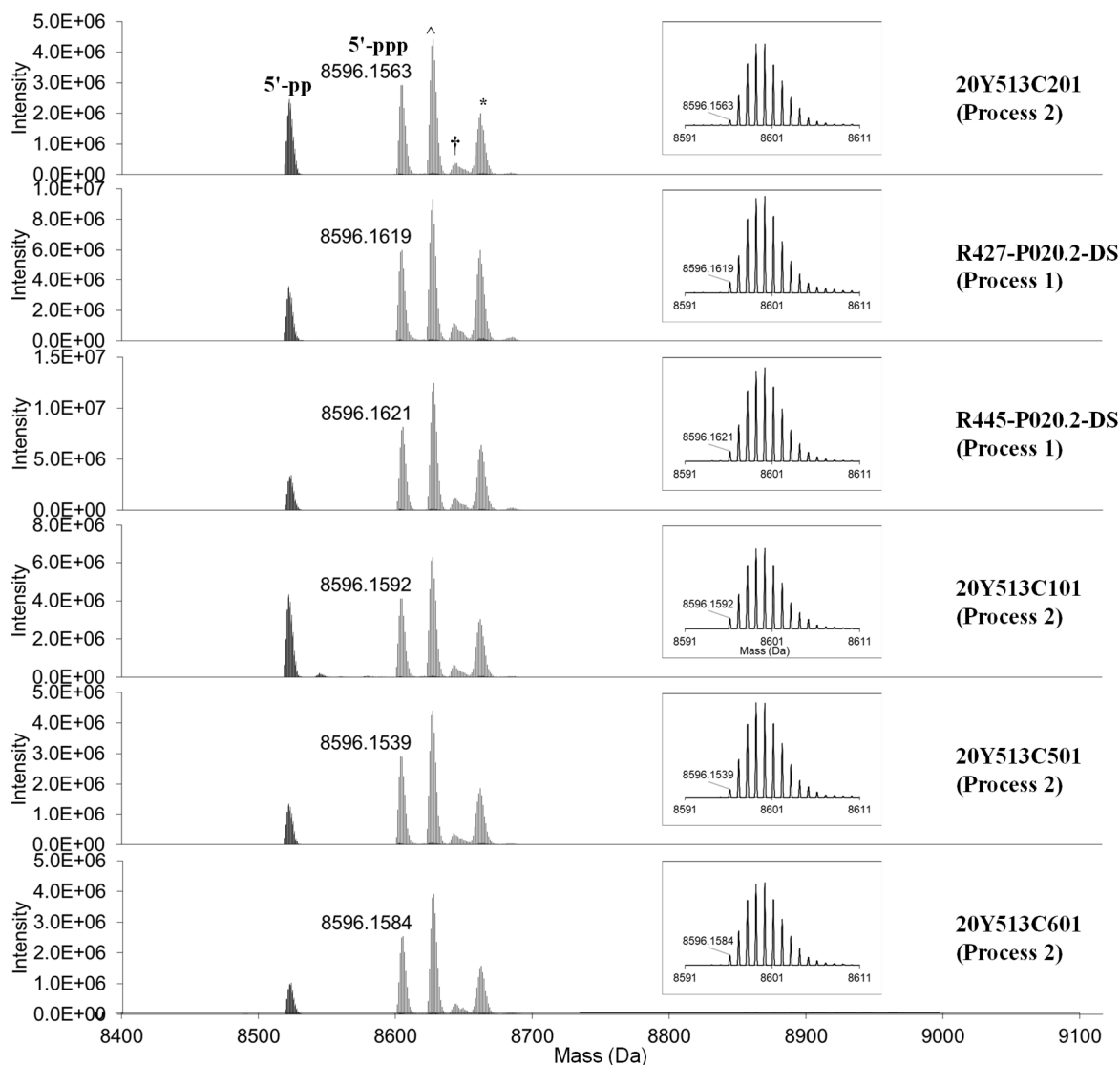


Figure S.2.6-21. Deconvoluted, zero-charge mass spectra of the 5' end non-capped 26 nucleotide RNase cleaved fragment of six BNT162b2 DS batches. Batch 20Y513C201 serves as an interim reference to bridge sample set figures. The spectra are deconvoluted from the summation of scans across the 5'-cap elution region. The “ppp” represents the 5'-triphosphate species, and “pp” represents the 5'-diphosphate species. The “^” marks the sodium adduct of the 5'-ppp species. The “+” marks the potassium and double sodium adducts of the 5'-ppp species. The “*” marks an approximately equimolar mixture of Fe(III) and Ni(II) adducts of the 5'-ppp species. The insets provide zoomed-spectra views of the 5'-ppp isotopic envelopes and monoisotopic peak assignments.

Figure S.2.6-22. Mass Spectra of 5'-ppp and 5'-pp RNase Cleaved Fragments from BNT162b2 DS Batches, Sample Set 2

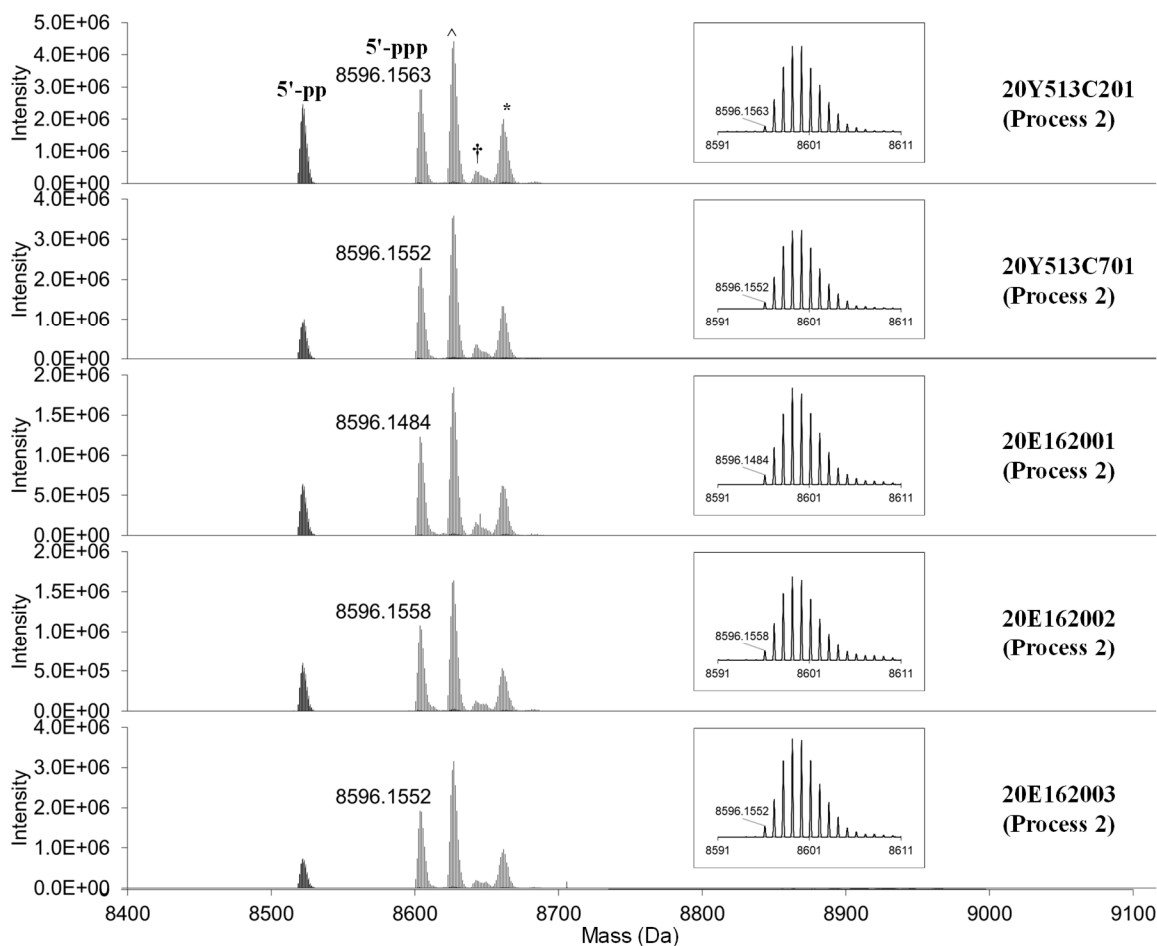


Figure S.2.6-22. Deconvoluted, zero-charge mass spectra of the 5' end non-capped 26 nucleotide RNase cleaved fragment of six BNT162b2 DS batches. Batch 20Y513C201 serves as an interim reference to bridge sample set figures. The spectra are deconvoluted from the summation of scans across the 5'-cap elution region. The “ppp” represents the 5'-triphosphate species, and “pp” represents the 5'-diphosphate species. The “^” marks the sodium adduct of the 5'-ppp species. The “†” marks potassium and double sodium adducts of the 5'-ppp species. The “*” marks an approximately equimolar mixture of Fe(III) and Ni(II) adducts of the 5'-ppp species. The insets provide zoomed-spectra views of the 5'-ppp isotopic envelopes and monoisotopic peak assignments.

Table S.2.6-22. Accurate Mass Assignments for BNT162b2 5'-Cap and non-Cap RNase Cleaved Fragments

Species	Theoretical Mass	Clinical (Process 1)		Emergency Supply (Process 2)		Process Performance Qualification – Pfizer, Andover (Process 2)			Process Performance Qualification - BioNTech Mainz / Rentschler (Process 2)		
		R427-P020.2-DS ^a	R445-P020.2-DS ^a	20Y513C101 ^a	20Y513C201 ^a	20Y513C501 ^a	20Y513C601 ^a	20Y513C701 ^a	20E162001 ^a	20E162002 ^a	20E162003 ^a
5'-Cap	8903.2562	8903.2589	8903.2676	8903.2684	8903.2822	8903.2631	8903.2768	8903.2751	8903.2718	8903.2680	8903.2723
5'-ppp	8596.1281	8596.1619	8596.1621	8596.1592	8596.1563	8596.1539	8596.1584	8596.1552	8596.1484	8596.1558	8596.1552
5'-pp	8516.1618	8516.1582	8516.1582	8516.1660	8516.1641	8516.1602	8516.1582	8516.1631	8516.1602	8516.1592	8516.1621
5'-ppp minus A	8267.0756	8267.1074	8267.1104	8267.1084	8267.1211	8267.1133	8267.1133	8267.1084	8267.1064	8267.1016	8267.1025
5'-pp minus A	8187.1093	8187.1162	8187.1157	8187.1172	8187.1250	8187.1187	8187.1157	8187.1206	8187.1099	8187.1128	8187.1152

a. Observed masses (monoisotopic) were determined from the zero-charge mass spectra after deconvolution of the multiply-charged data. Observed masses for major and minor species agree with theoretical masses to within 6 ppm, which is consistent with the accuracy and precision of contemporary mass spectrometers.

S.2.6.5.4.3. Poly(A) Tail: Length and Distribution by RP-HPLC

To evaluate the distribution of the poly(A) tail segments of ~30 adenosine nucleotides (A30) and ~70 adenosine nucleotides (L70), BNT162b2 drug substance batches were digested with RNase T1 and RNase A, and subsequently analyzed by ion-pair reversed-phase HPLC (RP-HPLC). [Figure S.2.6-23](#) and [Figure S.2.6-24](#) show the chromatographic profiles of the two Process 1 DS batches, two emergency supply batches, and the three Process 2 PPQ batches from each manufacturing site. Visual assessment of the chromatograms demonstrates comparable overall distributions of A30 and L70 species, with a slight broadening of the L70 peak consistently observed in Process 2 batches, compared to Process 1 batches. Further elucidation of these species using mass spectrometry is provided [below](#) in Section [S.2.6.5.4.4](#). Quantitative assessment ([Table S.2.6-21](#)) further demonstrates comparable relative A30, L70, and Other species content.

Figure S.2.6-23. BNT162b2 Poly(A) Tail Analyzed by RP-HPLC-UV, Sample Set 1

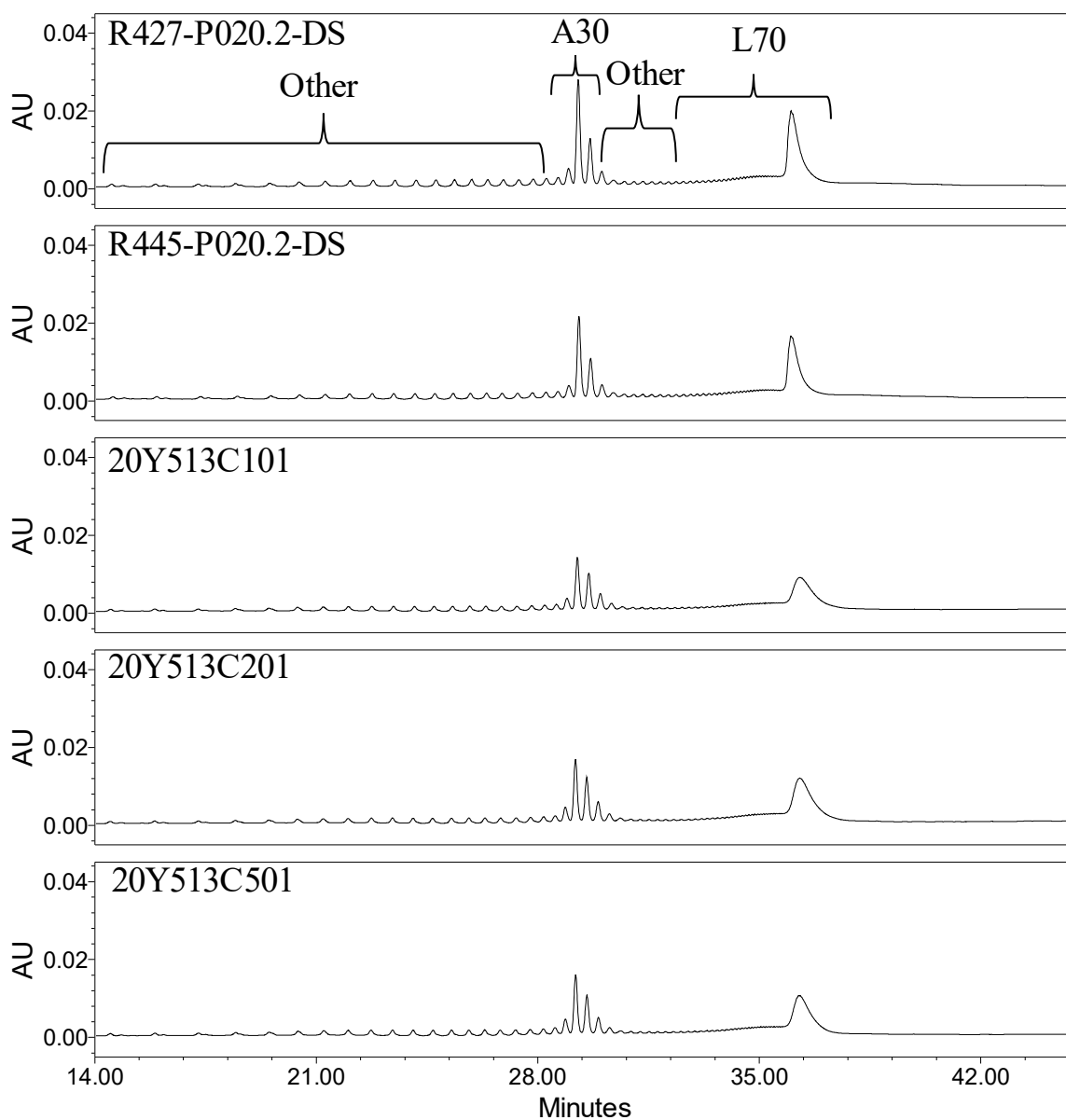


Figure S.2.6-23. RP-HPLC-UV chromatograms (260 nm) of poly(A) tail following RNase T1+RNase A digestion of BNT162b2 DS. Numerical peak labels indicate the approximate number of adenosine residues in the A30 and L70 poly(A) segments.

Figure S.2.6-24. BNT162b2 Poly(A) Tail Analyzed by RP-HPLC-UV, Sample Set 2

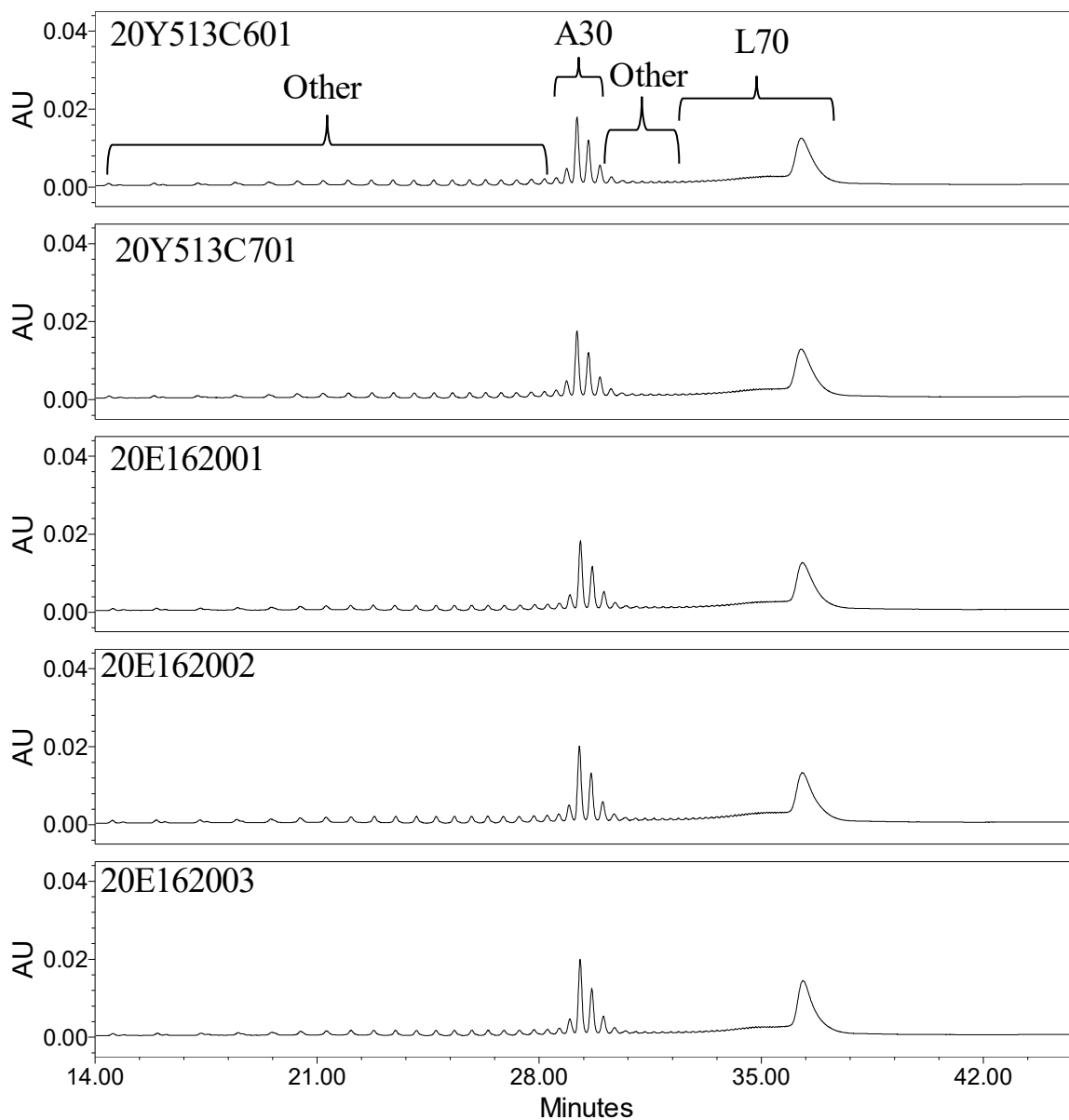


Figure S.2.6-24. RP-HPLC-UV chromatograms (260 nm) of poly(A) tail following RNase T1+RNase A digestion of BNT162b2 DS. Numerical peak labels indicate the approximate number of adenosine residues in the A30 and L70 poly(A) segments.

S.2.6.5.4.4. Comparative Mass Analysis of BNT162b2 Poly(A) Tail in Process 1 and Process 2 Batches

To facilitate in-depth characterization of the BNT162b2 poly(A) tail using state-of-the-art mass spectrometry techniques, drug substance samples were digested using only RNase T1. In contrast to the double digest method described in [S.2.6.5.3.3](#), the greater specificity of the singly-digested material, in combination with affinity purification enables improved mass spectrometric determination of the A30 and L70 distribution and sequence confirmation.

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 260 nm 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 by ribonuclease T1 (RNase T1) followed by isolation via oligo(dT)₂₅ affinity purification. This characterization method monitors two poly(A) tail segments: A30 and L70. Transcriptional slippage gives rise to a distribution of species with more or fewer adenosine nucleotides in each segment. More detail is provided in [Section S.3.1 Elucidation of Structure and Other Characteristics \(modRNA\)](#).

Both expected short (A30) and long (L70) segments of the poly(A) tail were observed in the RP-HPLC/UV chromatograms for all ten DS batches at comparable relative abundances ([Figure S.2.6-25](#) and [Figure S.2.6-26](#)). Using LC-UV/MS, the most abundant A30 segment species for all batches was the 30-adenosine species, with a range from 28 – 34 adenosines observed in all batches ([Figure S.2.6-27](#) and [Figure S.2.6-28](#)). A minor redistribution in the relative abundance of these species was observed, with increased abundance for the longer adenosine forms in the Process 2 DS compared to the Process 1 DS ([Table S.2.6-23](#) and [Table S.2.6-24](#)).

Unlike the poly(A) A30 segment, where the respective species are resolved both chromatographically and mass spectrometrically, the poly(A) L70 distribution is only resolved by MS ([Table S.2.6-25](#) and [Table S.2.6-26](#)). By LC-UV/MS, the mass spectral distribution of poly(A) L70 segment species was narrower for the Process 1 batches compared to the Process 2 batches [Figure S.2.6-29](#) and [Figure S.2.6-30](#)). This observation coincides with the narrower chromatographic elution profile of the L70 region for the Process 1 batches and the slightly later elution position of the Process 2 batches, as shown in [Figure S.2.6-29](#) and [Figure S.2.6-30](#). Additionally, the identification of predominant poly(A) L70 segment species differed between the two processes. For Process 1 batches, the 71A1C and 72A1C species formed the apex of the distribution. Here, the 71A and 72A represent the number of adenosine residues in the sequence and the 1C denotes the inclusion of one cytidine monophosphate. Furthermore, the 71A and 72A species, representing the intended sequence with minimal transcriptional slippage, formed the apex of a secondary distribution for Process 1 DS batches. The predominant species for the Process 2 DS batches were 74A, 75A, 76A, 77A and 78A, as would be expected for the poly(A) L70 segment with some transcriptional slippage. In addition, the 75A1C, 76A1C and 77A1C species observed in Process 1 formed the apex of a secondary distribution in Process 2, signifying that the same poly(A) L70 species are present in both Process 1 and Process 2. The incorporation of cytidine monophosphate into BNT162b2 is described in [Section S.3.1 Elucidation of](#)

Structure and Other Characteristics (modRNA). Despite these differences, the overarching observation for Process 1 and Process 2 DS batches is that the poly(A) L70 segment is comprised of mRNA with at least 70 adenosine nucleotides and both Process 1 and Process 2 exhibit the same types of Poly(A) L70 species with the expected transcriptional slippage mechanism, albeit to different extents.

Characterization by LC-UV/MS confirms that the poly(A) A30 segments are comparable between Process 1 and 2 given the same species are detected with a minor redistribution in relative abundance. The Poly(A) L70 segments also are considered comparable given the L70 species from Process 1 and 2 are the same and exhibit at least 70 adenosine residues as intended. Taken together, the quantitative measurement of the poly(A) tail species by LC-UV (Table S.2.6-21 and Figure S.2.6-23) and heightened characterization by LC-UV/MS demonstrate that both Process 1 and Process 2 derived DS batches contain predominantly 30 adenosine in the A30 species and at least 70 adenosine nucleotides in the L70 species, with observed distribution differences not expected to impact safety or efficacy.

Figure S.2.6-25. RP-HPLC-UV Profile (260 nm) of Extracted BNT162b2 Poly(A) tail, Sample Set 1

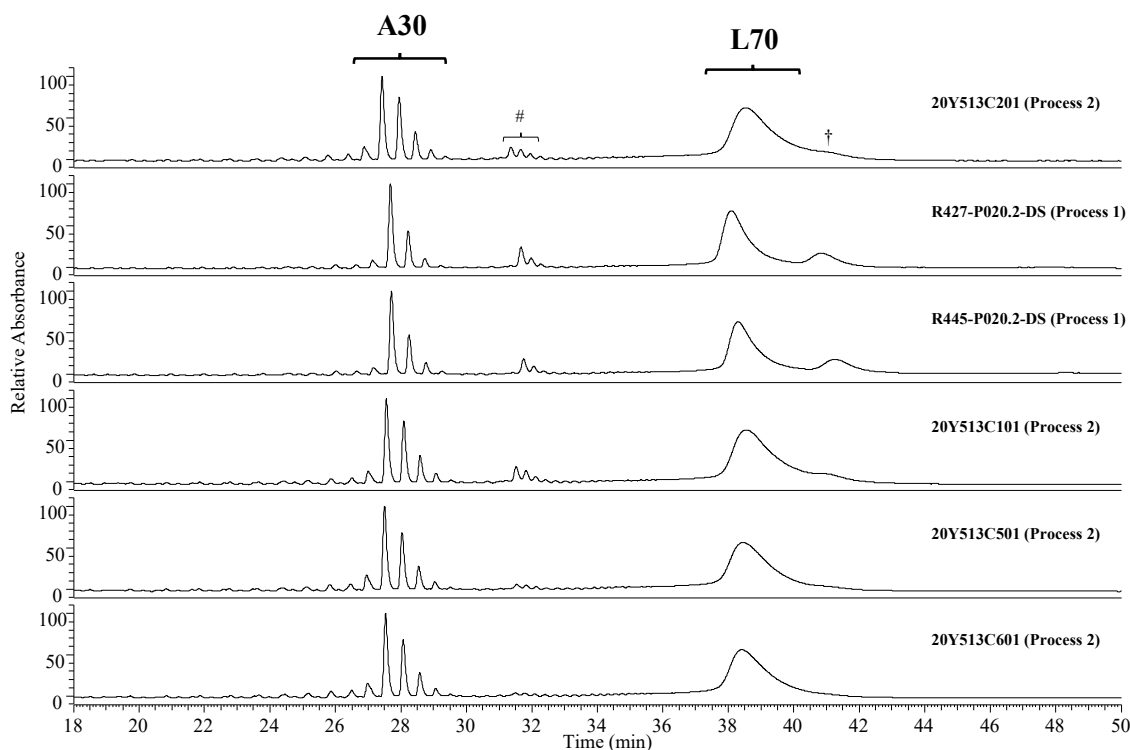


Figure S.2.6-25. RP-HPLC-UV chromatograms (260 nm) of extracted poly(A) tail following RNase T1 digestion, of six BNT162b2 DS batches. Batch 20Y513C201 serves as an interim reference to bridge sample set figures. Abbreviations: numerical peak labels indicate the number of adenosine residues in the A30 and L70 poly(A) segments, “#” denotes RNase T1 miscleaved Poly(A) containing species, “†” denotes heterogeneous under-digested species.

Figure S.2.6-26. RP-HPLC-UV Profile (260 nm) of Extracted BNT162b2 Poly(A) tail, Sample Set 2

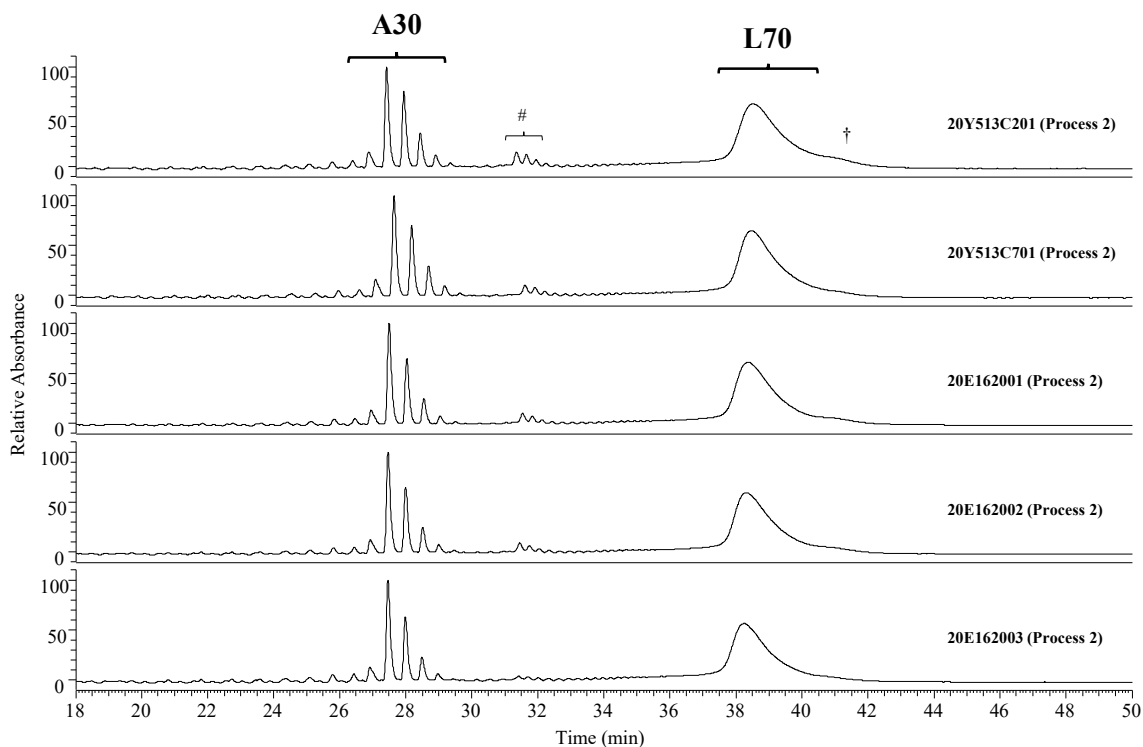


Figure S.2.6-26. RP-HPLC-UV chromatograms (260 nm) of extracted poly(A) tail following RNase T1 digestion, of five BNT162b2 DS batches. Batch 20Y513C201 serves as an interim reference to bridge sample set figures. Abbreviations: numerical peak labels indicate the number of adenosine residues in the A30 and L70 poly(A) segments, “#” denotes RNase T1 miscleaved Poly(A) containing species, “†” denotes heterogeneous under-digested species.

Figure S.2.6-27. Mass Spectra of A30 Poly(A) Segment, Sample Set 1

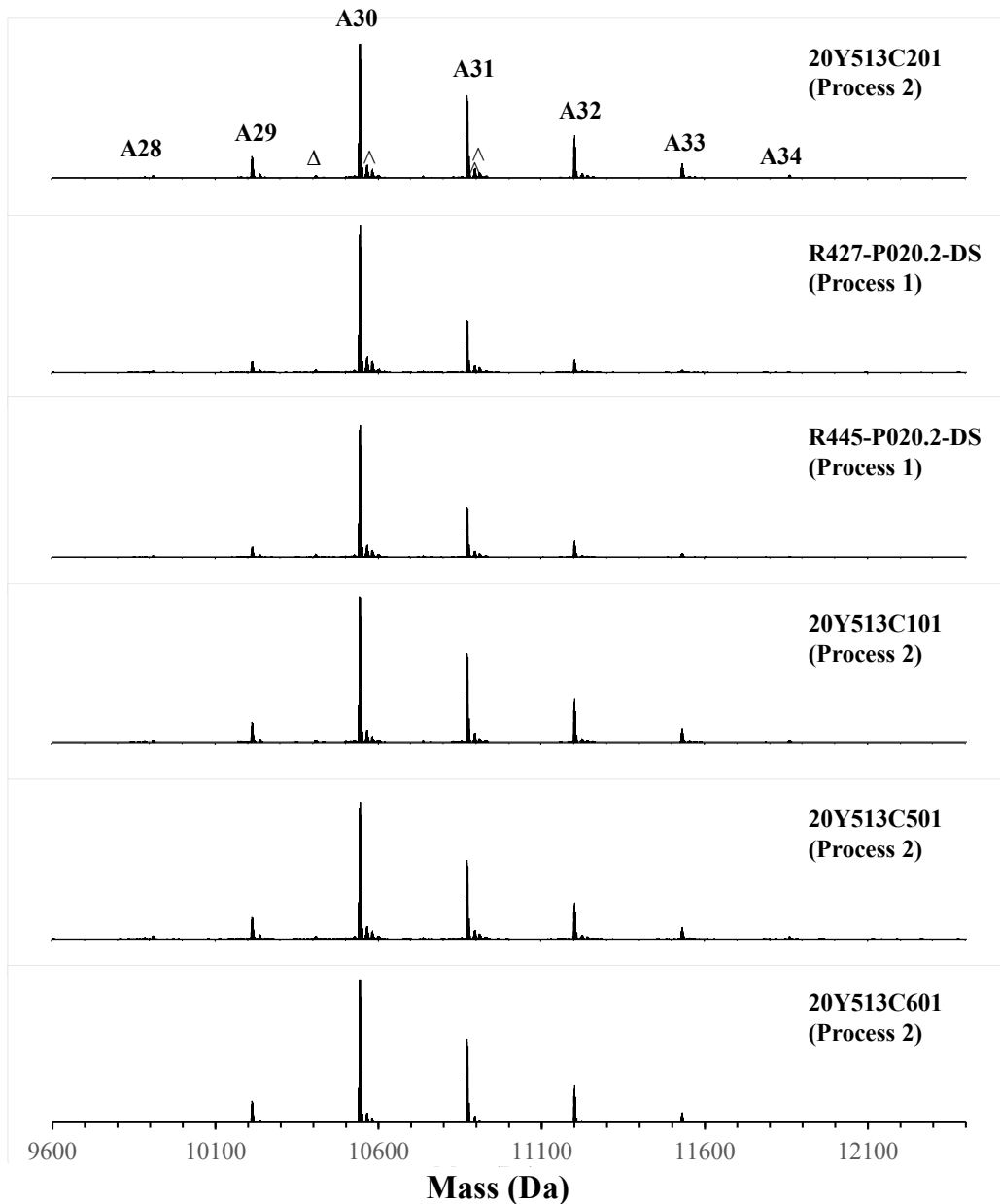


Figure S.2.6-27. Deconvoluted, zero-charge mass spectra of A30 poly(A) segment of six BNT162b2 DS batches. Batch 20Y513C201 serves as an interim reference to bridge sample set figures. Spectra were constructed from the sum of the mass spectra of the A30 poly(A) elution region denoted by the bracket in [Figure S.2.6-25](#). Numerical peak labels indicate the number of adenosine residues in the detected A30 poly(A) segments. “Δ” denotes method-induced artifact of poly(A) from either digestion or mass spectral in-source fragmentation, “^” denotes +Na and +K adducts. The observed monoisotopic mass assignments for the A30 poly(A) segments are found in [Table S.2.6-23](#).

Figure S.2.6-28. Mass Spectra of A30 Poly(A) Segment, Sample Set 2

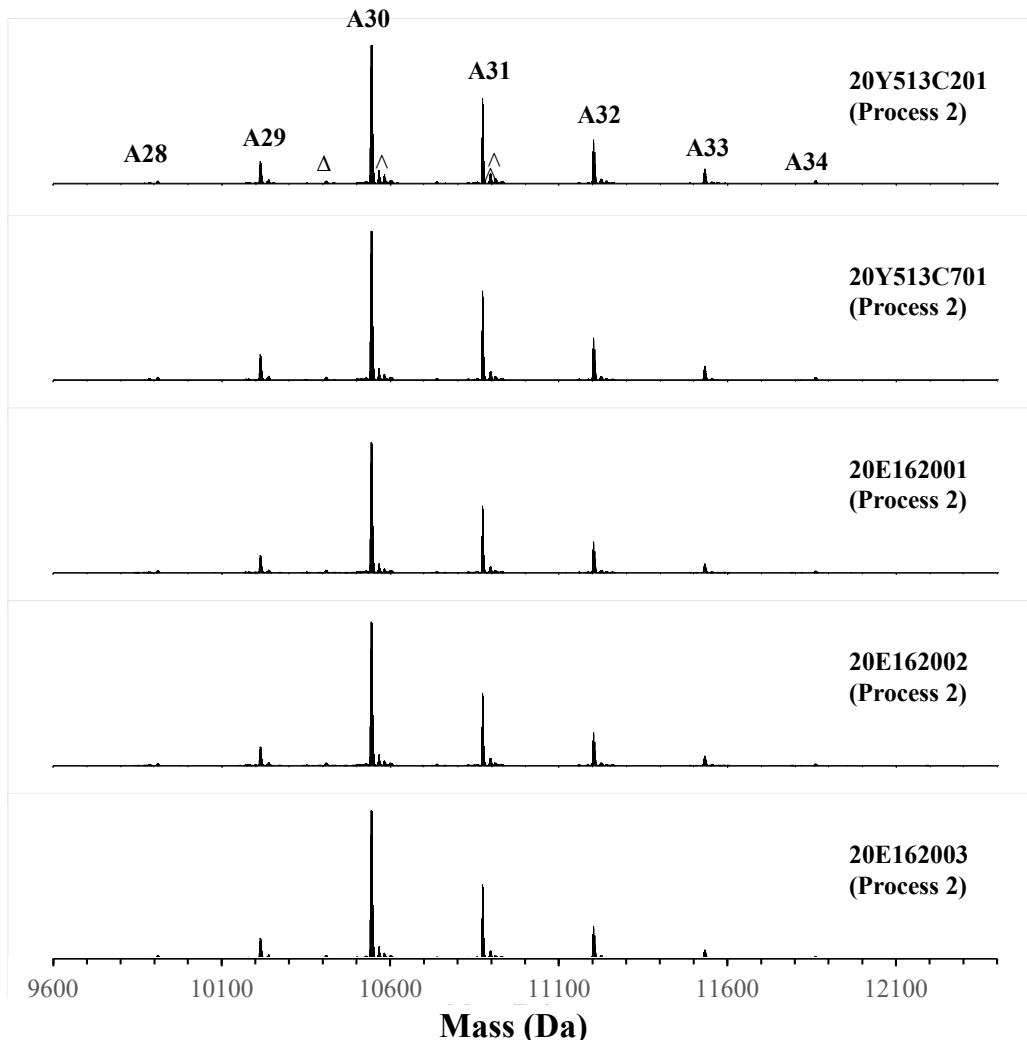


Figure S.2.6-28. Deconvoluted, zero-charge mass spectra of A30 poly(A) segment of five BNT162b2 DS batches. Batch 20Y513C201 serves as an interim reference to bridge sample set figures. Spectra were constructed from the sum of the mass spectra of the A30 poly(A) elution region denoted by the bracket in [Figure S.2.6-26](#). Numerical peak labels indicate the number of adenosine residues in the detected A30 poly(A) segments. “Δ” denotes method-induced artifact of poly(A) from either digestion or mass spectral in-source fragmentation, “^” denotes +Na and +K adducts. The observed monoisotopic mass assignments for the A30 poly(A) segments are found in [Table S.2.6-23](#).

Table S.2.6-23. Accurate Mass Assignments for BNT162b2 Poly(A) tail A30 Segment

Sequence	n ^a	Theoretical Masses (Da) ^b	Observed Masses (Da) ^c									
			Clinical (Process 1)		Emergency Supply (Process 2)		Process Performance Qualification – Pfizer, Andover (Process 2)			Process Performance Qualification - BioNTech Mainz / Rentschler (Process 2)		
			R427-P020.2-DS	R445-P020.2-DS	20Y513C101	20Y513C201	20Y513C501	20Y513C601	20Y513C701	20E162001	20E162002	20E162003
⁴¹⁷³ C- [A] _n - G ⁴²⁰⁴	28	9881.5698	9881.5697	9881.5870	9881.5901	9881.5911	9881.5912	9881.5942	9881.6010	9881.5953	9881.5968	9881.5886
	29	10210.6224	10210.6256	10210.6249	10210.6508	10210.6504	10210.6551	10210.6460	10210.6440	10210.6458	10210.6490	10210.6435
	30	10539.6749	10539.6807	10539.6797	10539.6982	10539.7048	10539.7030	10539.7023	10539.7024	10539.7044	10539.6931	10539.7028
	31	10868.7274	10868.7381	10868.7278	10868.7584	10868.7727	10868.7673	10868.7625	10868.7715	10868.7653	10868.7659	10868.7598
	32	11197.7799	11197.7888	11197.7853	11197.8008	11197.8080	11197.8010	11197.8103	11197.8017	11197.8050	11197.7977	11197.8011
	33	11526.8325	11526.8552	11526.8582	11526.8767	11526.8819	11526.8852	11526.8776	11526.8802	11526.8758	11526.8679	11526.8737
	34	11855.8850	<i>11854.8542</i>	11855.8677	11855.9021	11855.9089	11855.9060	11855.9062	11855.9090	11855.9049	11855.9045	11855.9045

a. The 'n' indicates the observed number of adenosine residues in the species ⁴¹⁷³C-[A]_n-G⁴²⁰⁴, with the residues and superscripted positional values are based on plasmid-encoded linear sequence.

b. Theoretical masses (monoisotopic) take into account that the 3' end of poly(A) distribution is monophosphorylated for A30 poly(A) segment as a result of RNase T1 cleavage.

c. Observed masses (monoisotopic) were determined from the zero-charge mass spectra after deconvolution and de-isotoping of the multiply-charged data. Observed masses for most major and minor species agree with theoretical masses to within 5 ppm, which is consistent with the accuracy and precision of contemporary mass spectrometers. Italicized masses signify a deconvolution monoisotopic mass assignment error of 1 Da, which arises when isotopic distributions of more than one species significantly overlap or insufficient signal-to-noise leading to non-statistical isotope distributions.

Table S.2.6-24. Relative Abundance of BNT162b2 Poly(A) tail A30 Species

Sequence	n ^a	Clinical (Process 1)		Emergency Supply (Process 2)		Process Performance Qualification – Pfizer, Andover (Process 2)			Process Performance Qualification - BioNTech Mainz / Rentschler (Process 2)		
		R427-P020.2-DS	R445-P020.2-DS	20Y513C101	20Y513C201	20Y513C501	20Y513C601	20Y513C701	20E162001	20E162002	20E162003
⁴¹⁷³ C- [A] _n - G ⁴²⁰⁴	28	Trace	Trace	Trace	Trace	Trace	Trace	Trace	Trace	Trace	Trace
	29	Trace	Trace	Minor	Minor	Minor	Minor	Minor	Minor	Minor	Minor
	30	Major	Major	Major	Major	Major	Major	Major	Major	Major	Major
	31	Minor	Minor	Major	Major	Major	Major	Major	Major	Major	Major
	32	Trace	Minor	Minor	Minor	Minor	Minor	Minor	Minor	Minor	Minor
	33	Trace	Trace	Trace	Minor	Trace	Trace	Trace	Trace	Trace	Trace
	34	Trace	Trace	Trace	Trace	Trace	Trace	Trace	Trace	Trace	Trace

Relative abundance is categorized based on relative peak height to the base peak (representing 100%) where major, minor, and trace represent: ≥ 45%, 45 to 10%, and <10% of relative peak height, respectively.

Figure S.2.6-29. Mass Spectra of L70 Poly(A) Segment, Sample Set 1

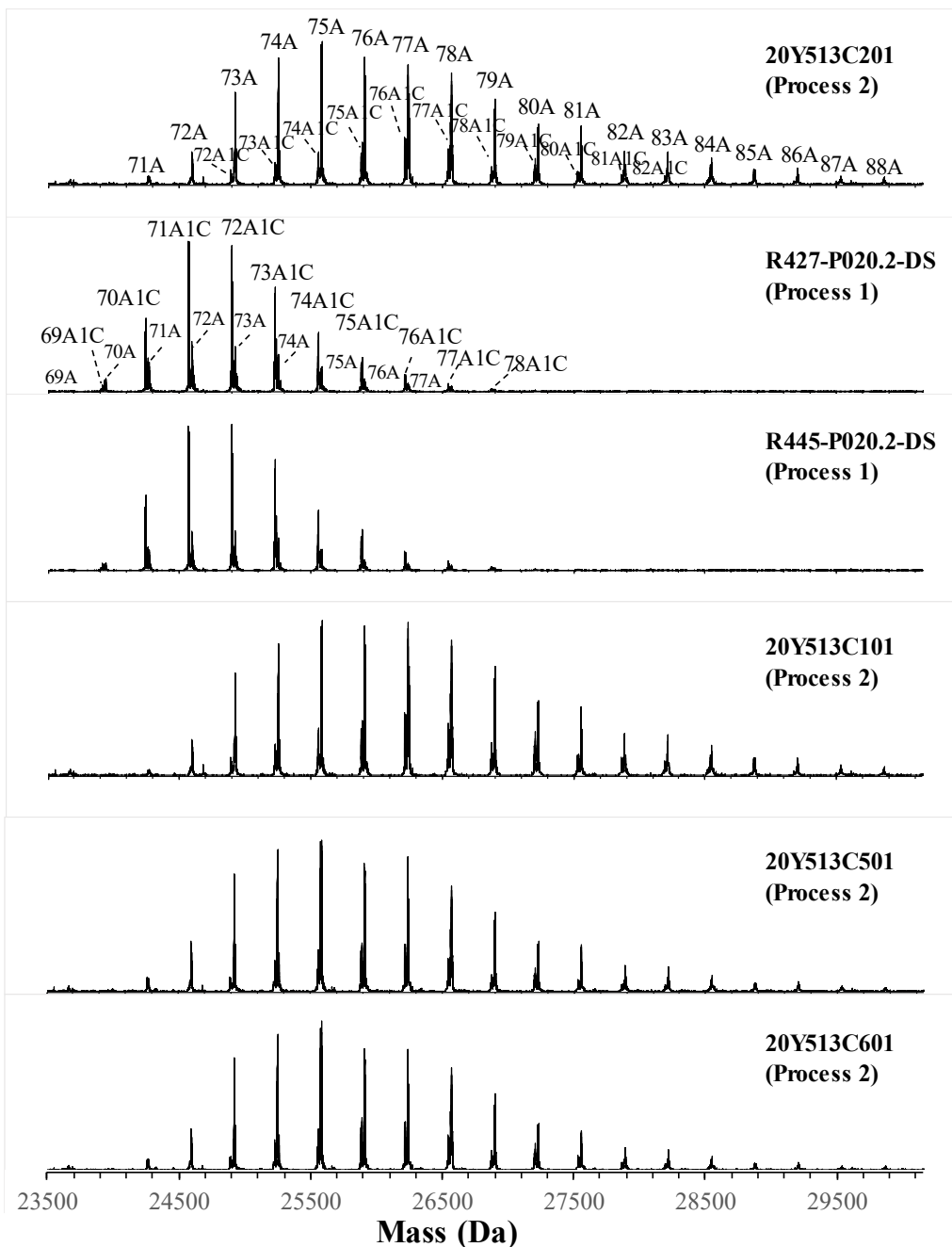


Figure S.2.6-29. Deconvoluted, zero-charge mass spectra of L70 poly(A) segment of six BNT162b2 DS batches. Batch 20Y513C201 serves as an interim reference to bridge sample set figures. Spectra were constructed from the sum of the mass spectra of the L70 poly(A) elution region denoted by the bracket in [Figure S.2.6-25](#). Numerical peak labels indicate the number of adenosine residues in the detected L70 poly(A) segments. The “1C” suffix denotes a second distribution with mass spacing of adenosine; the mass difference between this distribution and the predominant poly(A) distribution is consistent with the mass of a cytidine addition. The observed monoisotopic mass assignments for the L70 poly(A) segment are found in [Table S.2.6-25](#).

Figure S.2.6-30. Mass Spectra of L70 Poly(A) Segment, Sample Set 2

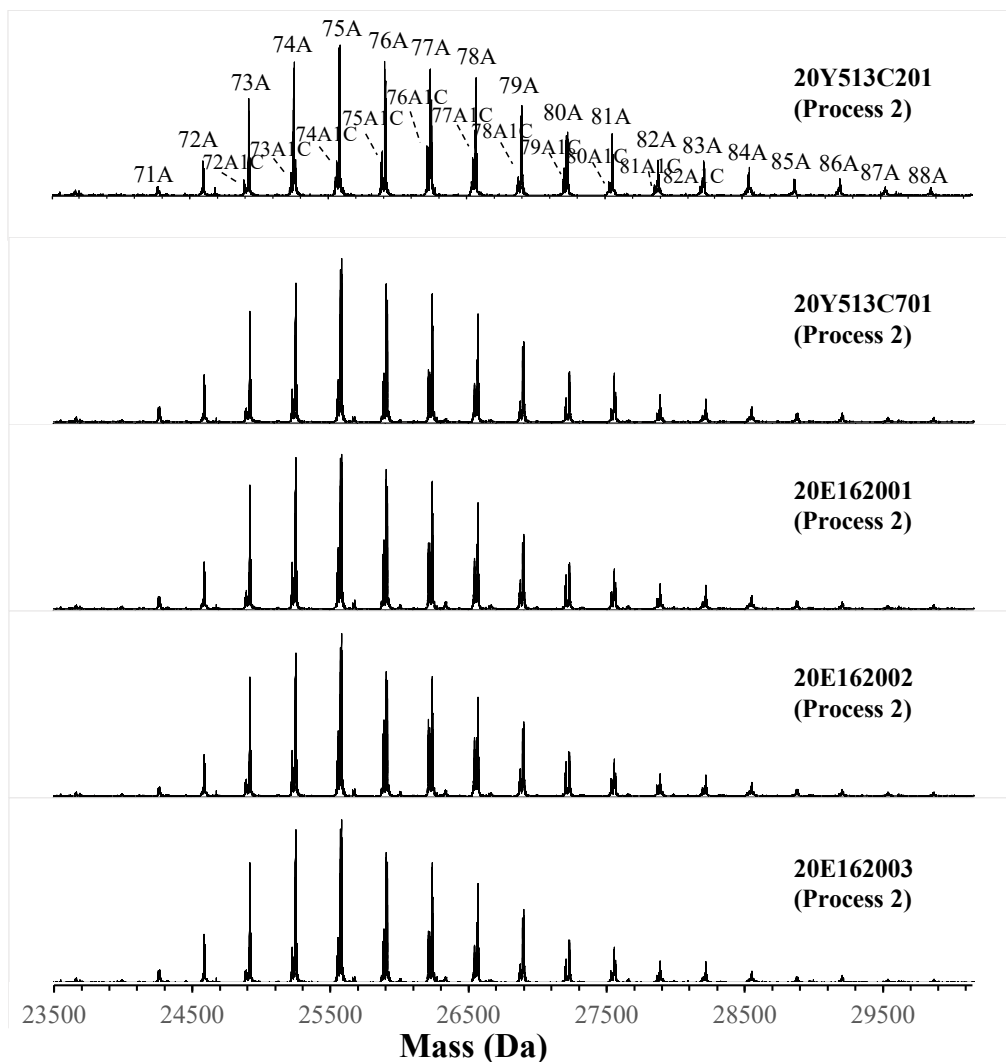


Figure S.2.6-30. Deconvoluted, zero-charge mass spectra of L70 poly(A) segment of five BNT162b2 DS batches. Batch 20Y513C201 serves as an interim reference to bridge sample set figures. Spectra were constructed from the sum of the mass spectra of the L70 poly(A) elution region denoted by the bracket in [Figure S.2.6-26](#). Numerical peak labels indicate the number of adenosine residues in the detected L70 poly(A) segments. The “1C” suffix denotes a second distribution with mass spacing of adenosine; the mass difference between this distribution and the predominant poly(A) distribution is consistent with the mass of a cytidine addition. The observed monoisotopic mass assignments for the L70 poly(A) segment are found in [Table S.2.6-25](#).

Table S.2.6-25. Accurate Mass Assignments for BNT162b2 Poly(A) tail L70 Segment

Sequence ^a	n	Theoretical Masses (Da) ^b	Observed Masses (Da) ^c									
			Clinical (Process 1)		Emergency Supply (Process 2)		Process Performance Qualification – Pfizer, Andover (Process 2)			Process Performance Qualification - BioNTech Mainz / Rentschler (Process 2)		
			R427-P020.2-DS	R445-P020.2-DS	20Y513C101	20Y513C201	20Y513C501	20Y513C601	20Y513C701	20E162001	20E162002	20E162003
4211ACT-[A] _n -i[C]	70	23901.844	23901.872	23901.871	ND	ND	ND	ND	ND	ND	ND	ND
	71	24230.897	24230.874	24230.972	ND	ND	ND	ND	ND	ND	ND	ND
	72	24559.949	24559.880	24559.973	ND	ND	ND	ND	ND	ND	ND	ND
	73	24889.002	24888.971	24889.065	24887.072	24887.074	24888.069	24888.071	24888.066	24888.060	24888.067	24888.060
	74	25218.054	25218.065	25218.073	25219.073	25219.080	25219.070	25219.073	25219.072	25219.072	25219.075	25219.078
	75	25547.107	25547.071	25548.166	25548.167	25549.175	25548.169	25548.169	25548.173	25548.168	25548.165	25548.171
	76	25876.159	25877.167	25876.175	25877.177	25877.171	25877.172	25877.175	25877.170	25877.174	25877.170	25877.170
	77	26205.212	26205.170	26205.263	26206.271	26206.266	26206.265	26206.263	26206.270	26206.264	26205.262	26205.262
	78	26534.264	26534.262	26534.274	26534.358	26534.357	26534.271	26534.275	26534.359	26534.266	26534.276	26534.272
	79	26863.317	26865.271	26864.369	26864.361	26864.368	26864.365	26864.359	26864.367	26864.362	26864.360	26864.361
	80	27192.369	27193.370	27193.371	27193.378	27193.373	27193.375	27193.370	27193.370	27193.369	27193.367	27193.366
	81	27521.422	27521.367	27521.462	27521.469	27521.468	27521.465	27521.471	27521.466	27521.467	27521.470	27521.472
	82	27850.474	ND	ND	27852.469	27852.473	27852.471	27852.472	27852.479	27852.469	27852.469	27852.475
	83	28179.527	ND	ND	28180.566	28181.569	28183.566	28181.575	28181.572	28182.570	28181.570	28183.570
4211ACT-[A] _n	69	23596.803	23596.769	23597.780	ND	ND	ND	ND	ND	ND	ND	ND
	70	23925.855	23925.860	23925.873	ND	ND	ND	ND	ND	ND	ND	ND
	71	24254.908	24254.873	24254.961	24255.974	24255.974	24255.970	24255.971	24255.970	24255.975	24255.967	24255.972
	72	24583.960	24582.882	24582.973	24584.056	24584.061	24583.985	24584.061	24584.057	24583.978	24583.977	24583.973
	73	24913.013	24911.970	24912.064	24913.073	24913.068	24913.063	24913.072	24913.072	24913.066	24913.064	24913.064
	74	25242.066	25241.061	25241.074	25242.075	25242.074	25242.072	25242.075	25242.083	25242.074	25242.076	25242.075
	75	25571.118	25570.079	25570.167	25571.171	25571.169	25571.166	25571.165	25571.170	25571.170	25571.169	25571.163
	76	25900.171	25899.173	25898.174	25900.185	25900.179	25900.171	25900.177	25900.182	25900.166	25900.168	25900.172
	77	26229.223	26229.166	26228.267	26229.272	26229.265	26229.264	26229.271	26229.274	26229.263	26229.264	26229.262
	78	26558.276	26557.260	26557.263	26558.359	26558.359	26558.278	26558.272	26558.365	26558.272	26558.270	26558.274
	79	26887.328	26886.273	26886.371	26887.368	26887.368	26887.371	26887.364	26887.369	26887.366	26887.365	26887.366
	80	27216.381	27218.270	27218.357	27217.453	27217.461	27217.465	27217.459	27217.456	27217.376	27217.374	27217.373
	81	27545.433	ND	ND	27545.466	27545.465	27545.464	27545.472	27545.472	27545.470	27545.470	27545.468
	82	27874.486	ND	ND	27874.469	27874.556	27874.476	27874.554	27874.480	27874.469	27874.475	27874.469
	83	28203.538	ND	ND	28203.568	28203.569	28203.565	28203.572	28204.575	28203.565	28203.568	28203.571
	84	28532.591	ND	ND	28532.574	28532.665	28532.572	28532.664	28532.663	28532.576	28532.573	28533.570
	85	28861.643	ND	ND	28862.668	28862.670	28862.668	28861.665	28862.660	28862.660	28862.665	28862.669
	86	29190.696	ND	ND	29190.762	29190.762	29190.762	29189.761	29190.761	29190.766	29190.761	29190.761
	87	29519.748	ND	ND	29519.768	29519.775	29519.775	29519.775	29519.775	29519.778	29519.772	29518.769

Table S.2.6-25. Accurate Mass Assignments for BNT162b2 Poly(A) tail L70 Segment

Sequ ce ^a	n	Theoretical Masses (Da) ^b	Observed Masses (Da) ^c									
			Clinical (Process 1)		Emergency Supply (Process 2)		Process Performance Qualification – Pfizer, Andover (Process 2)			Process Performance Qualification - BioNTech Mainz / Rentschler (Process 2)		
			R427-P020.2- DS	R445-P020.2- DS	20Y513C101	20Y513C201	20Y513C501	20Y513C601	20Y513C701	20E162001	20E162002	20E162003
	88	29848.801	ND	ND	29849.760	29849.770	29849.677	29849.750	29849.678	29849.750	29848.671	29849.668

- a. The residues and superscripted positional values are based on plasmid-encoded linear sequence. The subscripted 'n' indicates the observed number of adenosine residues. A = Adenine; C = Cytosine; G = Guanine; T = N1-methylpseudouridine
- b. Theoretical masses (monoisotopic) take into account that the 3' end of the BNT162b2 mRNA is a hydroxyl group.
- c. Observed masses (monoisotopic) were determined from the zero-charge mass spectra after deconvolution and de-isotoping of the multiply-charged data. Most observed masses for major and minor species agree with theoretical masses to within 5 ppm, which is consistent with the accuracy and precision of contemporary mass spectrometers. Italicized masses signify a deconvolution monoisotopic mass assignment error of ≥ 1 Da, which arises when isotopic distributions of more than one species significantly overlap. ND = Not Detected

Table S.2.6-26. Relative Abundance of BNT162b2 Poly(A) tail L70 Species

Sequence	n	Relative Abundance ^a									
		Clinical (Process 1)		Emergency Supply (Process 2)		Process Performance Qualification – Pfizer, Andover (Process 2)			Process Performance Qualification - BioNTech Mainz / Rentschler (Process 2)		
		R427-P020.2-DS	R445-P020.2-DS	20Y513C101	20Y513C201	20Y513C501	20Y513C601	20Y513C701	20E162001	20E162002	20E162003
4211ACT-[A] _{n-1} [C]	70	Trace	Trace	ND	ND	ND	ND	ND	ND	ND	ND
	71	Major	Major	ND	ND	ND	ND	ND	ND	ND	ND
	72	Major	Major	ND	ND	ND	ND	ND	ND	ND	ND
	73	Major	Major	Trace	Trace	Trace	Trace	Trace	Minor	Minor	Trace
	74	Major	Major	Minor	Minor	Minor	Minor	Minor	Minor	Minor	Minor
	75	Minor	Minor	Minor	Minor	Minor	Minor	Minor	Minor	Minor	Minor
	76	Minor	Minor	Minor	Minor	Minor	Minor	Minor	Minor	Major	Minor
	77	Minor	Minor	Minor	Minor	Minor	Minor	Minor	Minor	Major	Minor
	78	Trace	Trace	Minor	Minor	Minor	Minor	Minor	Minor	Minor	Minor
	79	Trace	Trace	Minor	Minor	Minor	Minor	Minor	Minor	Minor	Minor
	80	Trace	Trace	Minor	Minor	Minor	Minor	Minor	Minor	Minor	Minor
	81	Trace	Trace	Minor	Trace	Trace	Trace	Trace	Minor	Minor	Trace
	82	ND	ND	Minor	Trace	Trace	Trace	Trace	Trace	Trace	Trace
	83	ND	ND	Trace	Trace	Trace	Trace	Trace	Trace	Trace	Trace
4211ACT-[A] _n	69	Trace	Trace	ND	ND	ND	ND	ND	ND	ND	ND
	70	Trace	Trace	ND	ND	ND	ND	ND	ND	ND	ND

Table S.2.6-26. Relative Abundance of BNT162b2 Poly(A) tail L70 Species

Sequence	n	Relative Abundance ^a									
		Clinical (Process 1)		Emergency Supply (Process 2)		Process Performance Qualification – Pfizer, Andover (Process 2)			Process Performance Qualification - BioNTech Mainz / Rentschler (Process 2)		
		R427-P020.2-DS	R445-P020.2-DS	20Y513C101	20Y513C201	20Y513C501	20Y513C601	20Y513C701	20E162001	20E162002	20E162003
	71	Minor	Minor	Trace	Trace	Trace	Trace	Trace	Trace	Trace	Trace
	72	Minor	Minor	Minor	Minor	Minor	Minor	Minor	Minor	Minor	Minor
	73	Minor	Minor	Major	Major	Major	Major	Major	Major	Major	Major
	74	Minor	Minor	Major	Major	Major	Major	Major	Major	Major	Major
	75	Minor	Minor	Major	Major	Major	Major	Major	Major	Major	Major
	76	Trace	Trace	Major	Major	Major	Major	Major	Major	Major	Major
	77	Trace	Trace	Major	Major	Major	Major	Major	Major	Major	Major
	78	Trace	Trace	Major	Major	Major	Major	Major	Major	Major	Major
	79	Trace	Trace	Major	Major	Major	Major	Major	Major	Major	Major
	80	Trace	Trace	Major	Minor	Minor	Minor	Minor	Minor	Minor	Minor
	81	ND	ND	Minor	Minor	Minor	Minor	Minor	Minor	Minor	Minor
	82	ND	ND	Minor	Minor	Minor	Minor	Minor	Minor	Minor	Minor
	83	ND	ND	Minor	Minor	Minor	Minor	Minor	Minor	Minor	Minor
	84	ND	ND	Minor	Minor	Minor	Trace	Minor	Trace	Trace	Trace
	85	ND	ND	Minor	Minor	Trace	Trace	Trace	Trace	Trace	Trace
	86	ND	ND	Minor	Minor	Trace	Trace	Trace	Trace	Trace	Trace
	87	ND	ND	Trace	Trace	Trace	Trace	Trace	Trace	Trace	Trace
	88	ND	ND	Trace	Trace	Trace	Trace	Trace	Trace	Trace	Trace

a. Relative abundance is categorized based on relative peak height to the base peak (representing 100%) where major, minor, and trace represent: $\geq 45\%$, 45 to 10%, and $<10\%$ of relative peak height, respectively.

S.2.6.5.4.5. Comparative LC/MS/MS – Oligonucleotide Mapping of BNT162b2 Process 1 and Process 2 DS Batches

The primary sequence of BNT162b2 drug substance was analyzed by LC/MS/MS - oligonucleotide mapping. BNT162b2 DS batches were 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 260 nm (Figure S.2.6-31 through Figure S.2.6-40). All major and minor peaks in the oligonucleotide map were identified by on-line electrospray ionization mass spectrometry with tandem mass spectrometry (MS/MS) by higher-collision dissociation (IP-RP-HPLC/ESI MS/MS or LC/MS/MS) as shown in Figure S.2.6-31 through Figure S.2.6-40. The observed chromatographic profiles for the Process 1 and Process 2 substance batches are highly similar in terms of peak position, shape and abundance. The observed masses and MS/MS fragmentation patterns of the oligonucleotides in each peak observed in each of the ten drug substance batches were consistent with the expected RNase T1 fragments of BNT162b RNA. The detected enzymatic fragments account for 94.7% of the overall BNT162b2 RNA sequence for all ten drug substance batches (Table S.2.6-27).

The 3' terminal sequence region of BNT162b2 RNA was detected by LC/MS/MS – oligonucleotide mapping in both the Process 1 and Process 2 DS batches, and the observed species are consistent with those observed by the Poly(A) tail LC/MS methods described in Section S.2.6.5.3.3. The 5' terminal sequence region was not recoverable after RNase T1 digestion as the oligonucleotide generated was too hydrophilic. The 5' terminal region including the 5'-Cap was characterized separately by LC/MS as described in Section S.2.6.5.3.2.

The LC/MS/MS – oligonucleotide mapping results are summarized in Table S.2.6-27. In conclusion, the highly comparable chromatographic profiles and consistent LC/MS/MS peak identifications between materials demonstrate that the Process 1 and Process 2 BNT162b2 RNA DS batches are comparable and contain the correct, identical sequence as predicted from the linear DNA template (Section S.2.3 Control of Materials Source, History and Generation of Plasmids (modRNA)).

Figure S.2.6-31. LC/MS/MS – Oligonucleotide Mapping of BNT162b2 DS Batches (2.6-15.5 min) , Sample Set 1

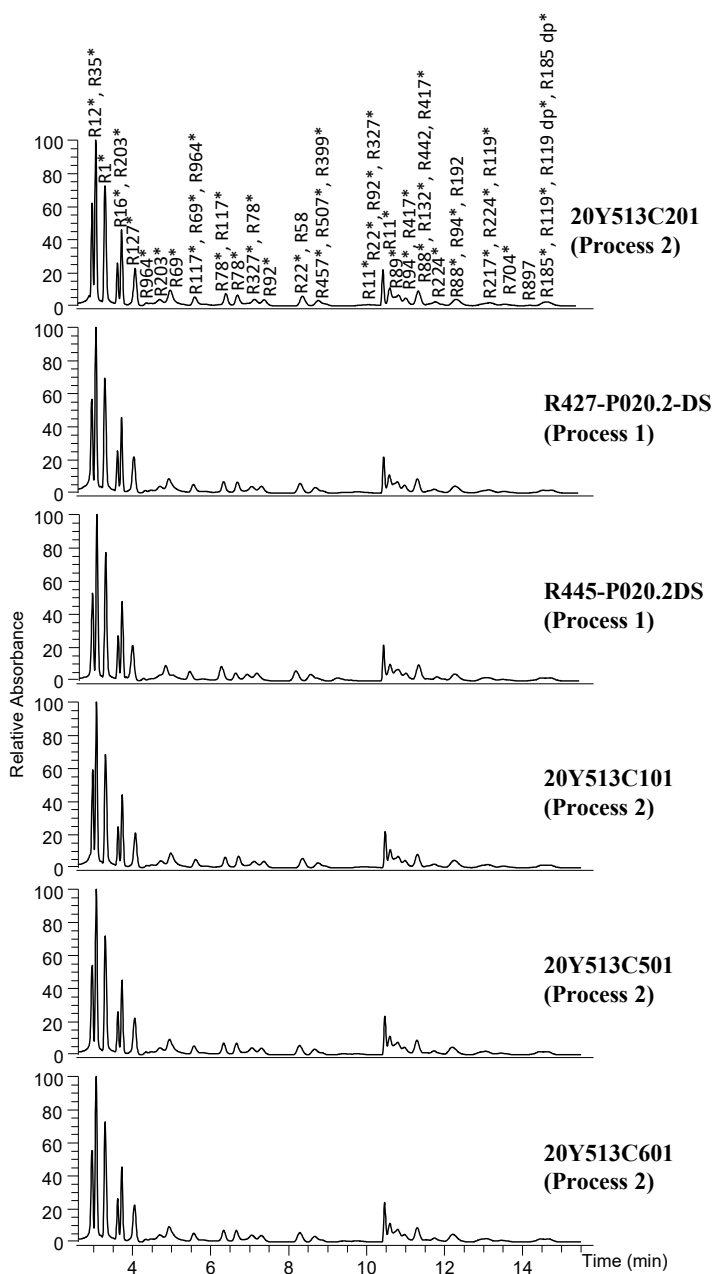


Figure S.2.6-31. RNase T1 oligonucleotide map of six BNT162b2 DS batches. Batch 20Y513C201 serves as an interim reference to bridge sample set figures. Abbreviations: “R[n]” represents BNT162b2 oligonucleotide fragment resulting from RNase T1 digestion, counting *n* fragments from the 5’ end, “dp” denotes dephosphorylation, and “*” denotes a non-unique oligonucleotide (i.e., the sequence is repeated in BNT162b2) where these single assignments represent all oligonucleotides with the same sequence. Missed-cleavage oligonucleotide fragments are listed as “R[n]-[n+m]”, with *m* representing the number of additional RNase T1 fragments. Fragments beginning or ending with a non-RNase T1 cleavage site are signified by their nucleotide start and end numbers in parentheses.

Figure S.2.6-32. LC/MS/MS – Oligonucleotide Mapping of BNT162b2 DS Batches (2.6-15.5 min) , Sample Set 2

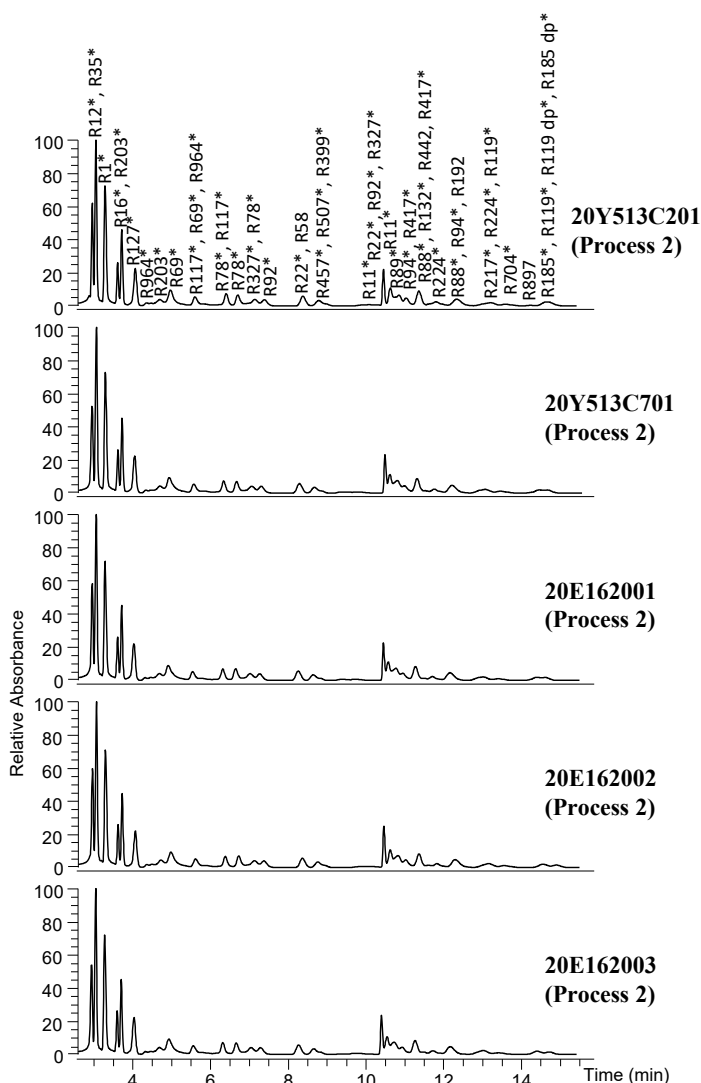


Figure S.2.6-32. RNase T1 oligonucleotide map of five BNT162b2 DS batches. Batch 20Y513C201 serves as an interim reference to bridge sample set figures. Abbreviations: “R[n]” represents BNT162b2 oligonucleotide fragment resulting from RNase T1 digestion, counting *n* fragments from the 5’ end, “dp” denotes dephosphorylation, and “*” denotes a non-unique oligonucleotide (i.e., the sequence is repeated in BNT162b2) where these single assignments represent all oligonucleotides with the same sequence. Missed-cleavage oligonucleotide fragments are listed as “R[n]-[n+m]”, with *m* representing the number of additional RNase T1 fragments. Fragments beginning or ending with a non-RNase T1 cleavage site are signified by their nucleotide start and end numbers in parentheses.

Figure S.2.6-33. LC/MS/MS – Oligonucleotide Mapping of BNT162b2 DS Batches (15-66 min), Sample Set 1

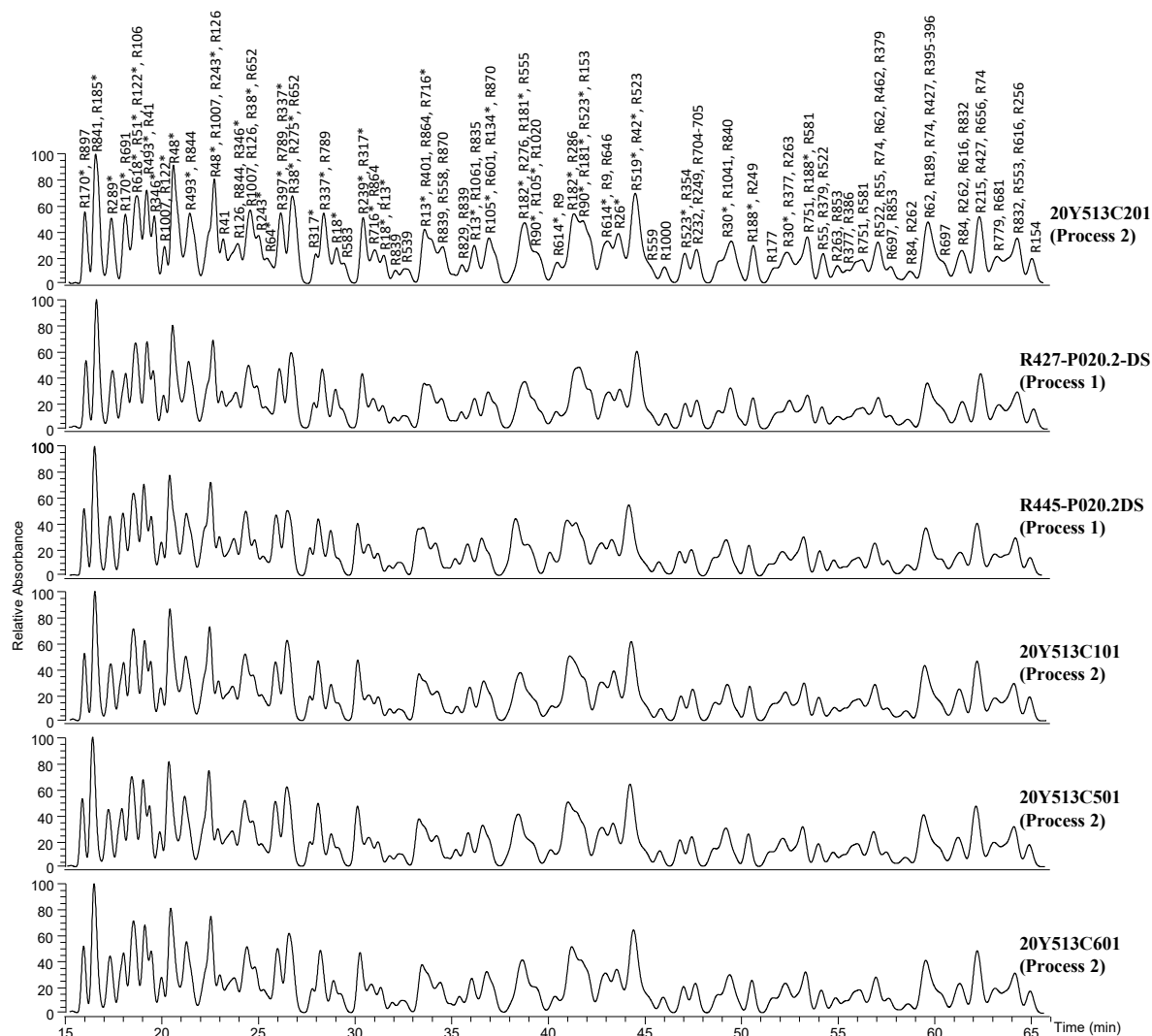


Figure S.2.6-33. RNase T1 oligonucleotide map of BNT162b2 DS batches. Batch 20Y513C201 serves as an interim reference to bridge sample set figures. Abbreviations: “R[n]” represents BNT162b2 oligonucleotide fragment resulting from RNase T1 digestion, counting n fragments from the 5’ end, “dp” denotes dephosphorylation, and “*” denotes a non-unique oligonucleotide (i.e., the sequence is repeated in BNT162b2) where these single assignments represent all oligonucleotides with the same sequence. Missed-cleavage oligonucleotide fragments are listed as “R[n]-[n+m]”, with m representing the number of additional RNase T1 fragments. Fragments beginning or ending with a non-RNase T1 cleavage site are signified by their nucleotide start and end numbers in parentheses.

Figure S.2.6-34. LC/MS/MS – Oligonucleotide Mapping of BNT162b2 DS Batches (15-66 min), Sample Set 2

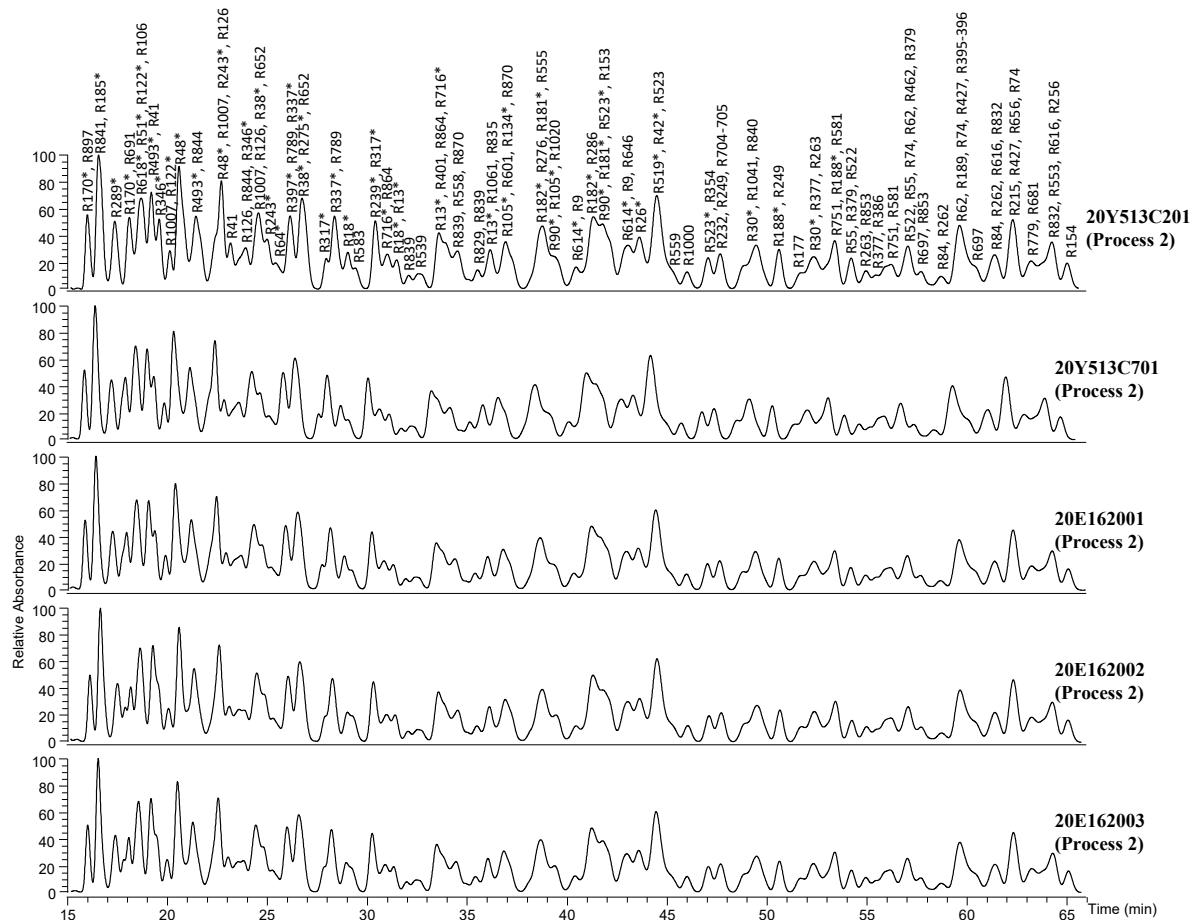


Figure S.2.6-34. RNase T1 oligonucleotide map of BNT162b2 DS batches. Batch 20Y513C201 serves as an interim reference to bridge sample set figures. Abbreviations: “R[n]” represents BNT162b2 oligonucleotide fragment resulting from RNase T1 digestion, counting *n* fragments from the 5’ end, “dp” denotes dephosphorylation, and “*” denotes a non-unique oligonucleotide (i.e., the sequence is repeated in BNT162b2) where these single assignments represent all oligonucleotides with the same sequence. Missed-cleavage oligonucleotide fragments are listed as “R[n]-[n+m]”, with *m* representing the number of additional RNase T1 fragments. Fragments beginning or ending with a non-RNase T1 cleavage site are signified by their nucleotide start and end numbers in parentheses.

Figure S.2.6-35. LC/MS/MS – Oligonucleotide Mapping of BNT162b2 DS Batches (65-140 min), Sample Set 1

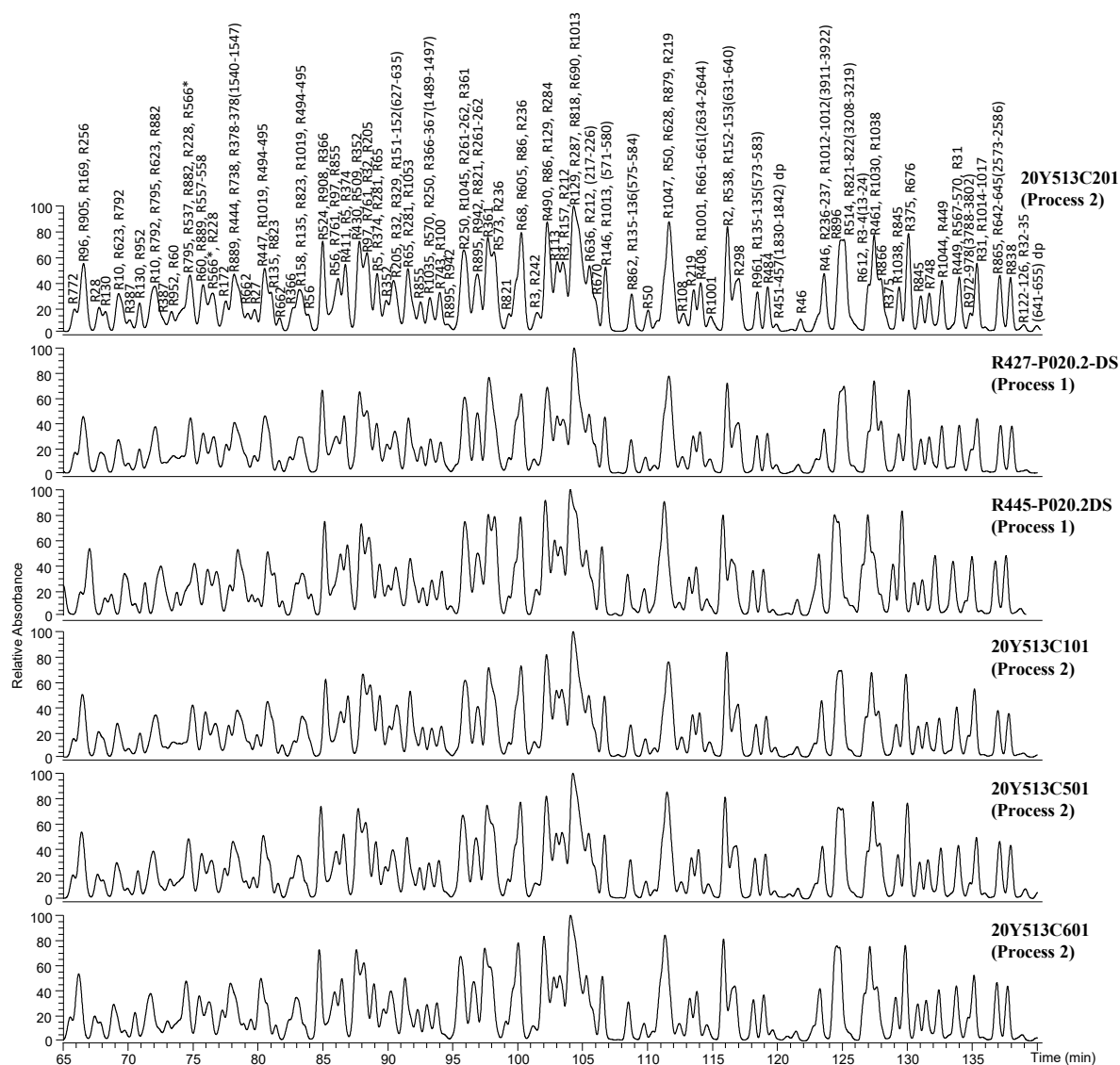


Figure S.2.6-35. RNase T1 oligonucleotide map of BNT162b2 DS batches. Batch 20Y513C201 serves as an interim reference to bridge sample set figures. Abbreviations: “R[n]” represents BNT162b2 oligonucleotide fragment resulting from RNase T1 digestion, counting *n* fragments from the 5’ end, “dp” denotes dephosphorylation, and “*” denotes a non-unique oligonucleotide (i.e., the sequence is repeated in BNT162b2) where these single assignments represent all oligonucleotides with the same sequence. Missed-cleavage oligonucleotide fragments are listed as “R[n]-[n+m]”, with *m* representing the number of additional RNase T1 fragments. Fragments beginning or ending with a non-RNase T1 cleavage site are signified by their nucleotide start and end numbers in parentheses.

Figure S.2.6-36. LC/MS/MS – Oligonucleotide Mapping of BNT162b2 DS Batches (65-140 min), Sample Set 2

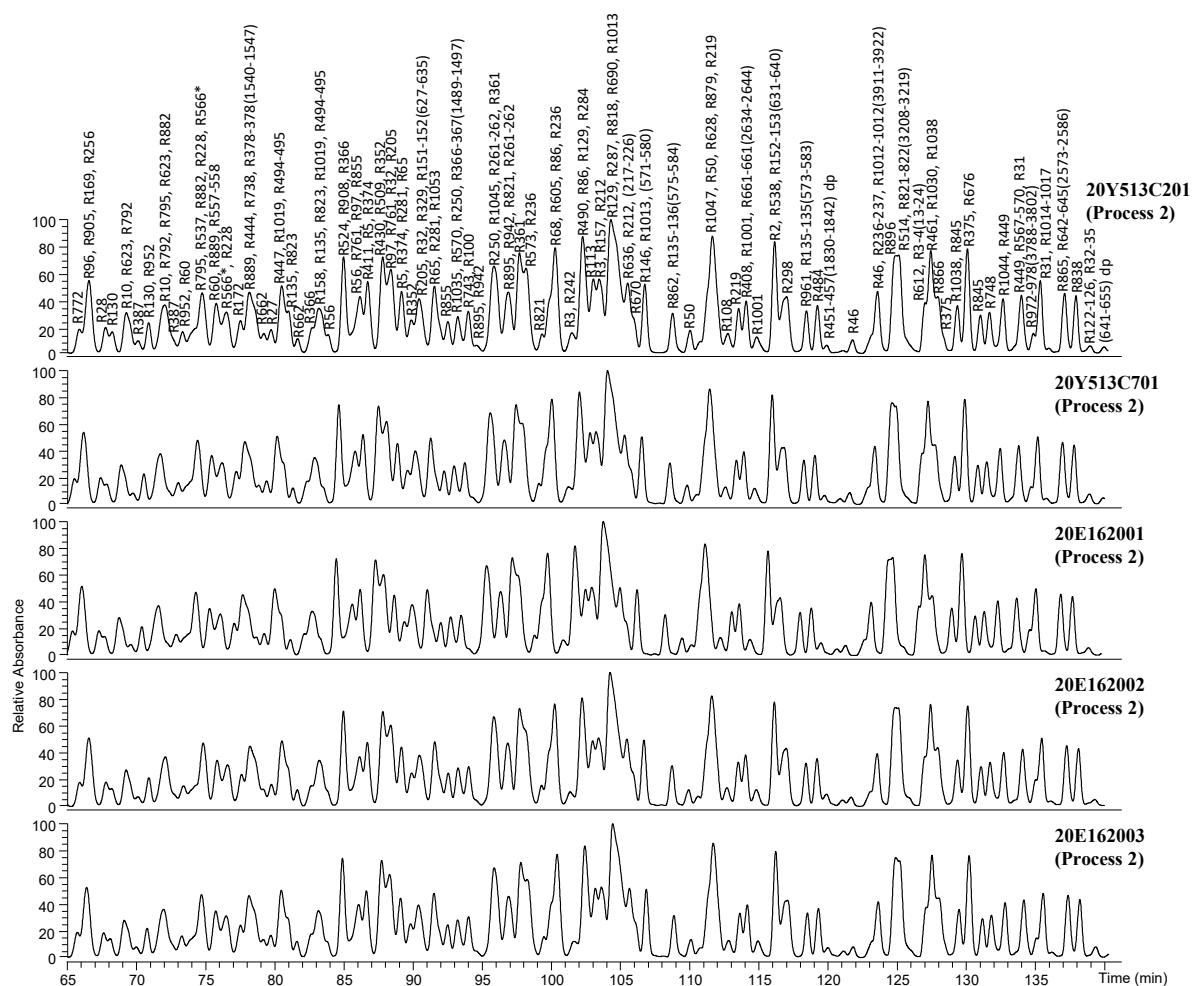


Figure S.2.6-36. RNase T1 oligonucleotide map of BNT162b2 DS batches. Batch 20Y513C201 serves as an interim reference to bridge sample set figures. Abbreviations: “R[n]” represents BNT162b2 oligonucleotide fragment resulting from RNase T1 digestion, counting *n* fragments from the 5’ end, “dp” denotes dephosphorylation, and “*” denotes a non-unique oligonucleotide (i.e., the sequence is repeated in BNT162b2) where these single assignments represent all oligonucleotides with the same sequence. Missed-cleavage oligonucleotide fragments are listed as “R[n]-[n+m]”, with *m* representing the number of additional RNase T1 fragments. Fragments beginning or ending with a non-RNase T1 cleavage site are signified by their nucleotide start and end numbers in parentheses.

Figure S.2.6-37. LC/MS/MS – Oligonucleotide Mapping of BNT162b2 DS Batches (140-208 min), Sample Set 1

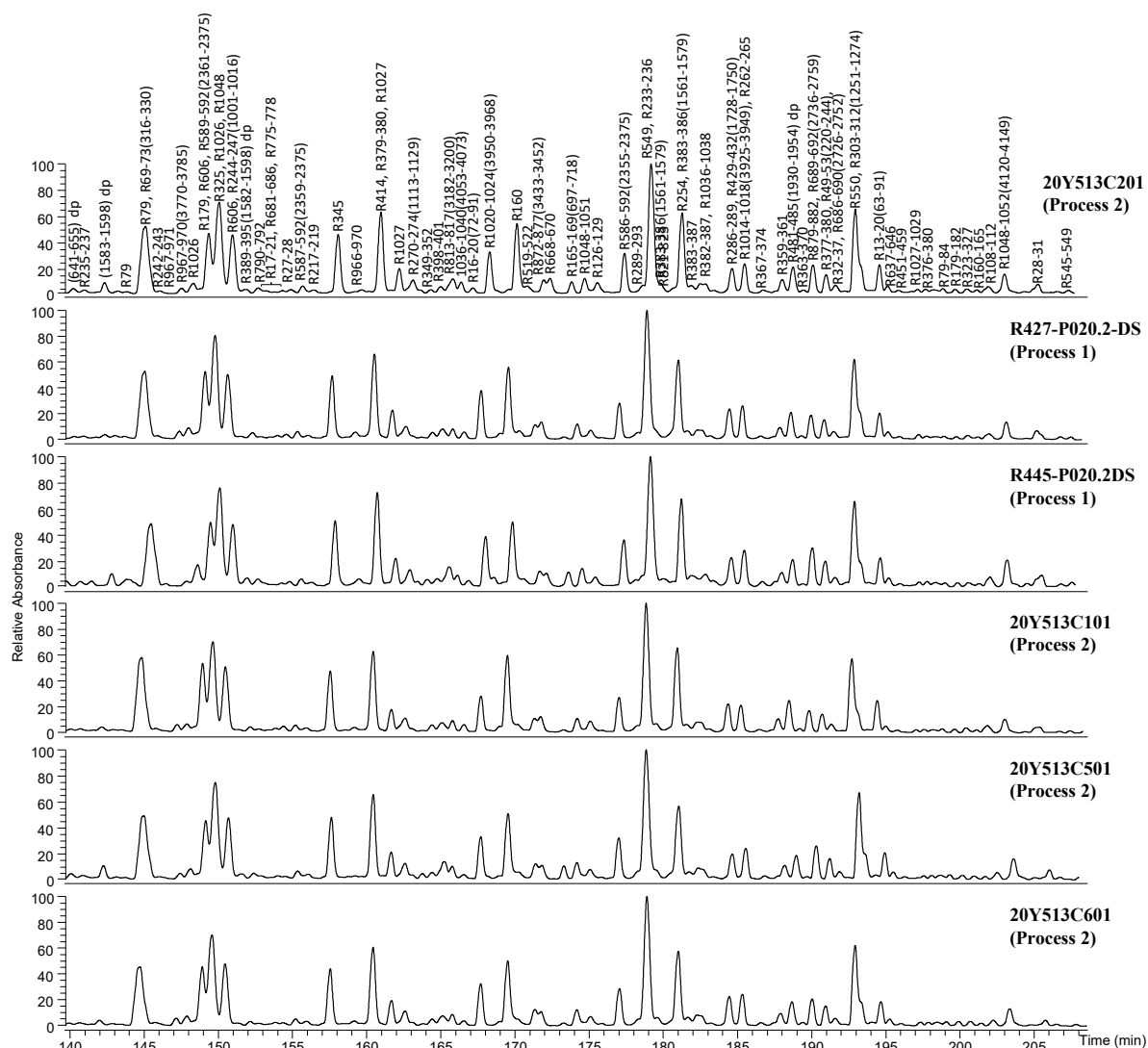


Figure S.2.6-37. RNase T1 oligonucleotide map of BNT162b2 DS batches. Batch 20Y513C201 serves as an interim reference to bridge sample set figures. Abbreviations: “R[n]” represents BNT162b2 oligonucleotide fragment resulting from RNase T1 digestion counting *n* fragments from the 5’ end, and “dp” denotes dephosphorylation. Missed-cleavage oligonucleotide fragments are listed as “R[n]-[n+m]”, with *m* representing the number of additional RNase T1 fragments. Fragments beginning or ending with a non-RNase T1 cleavage site are signified by their nucleotide start and end numbers in parentheses.

Figure S.2.6-38. LC/MS/MS – Oligonucleotide Mapping of BNT162b2 DS Batches (140-208 min), Sample Set 2

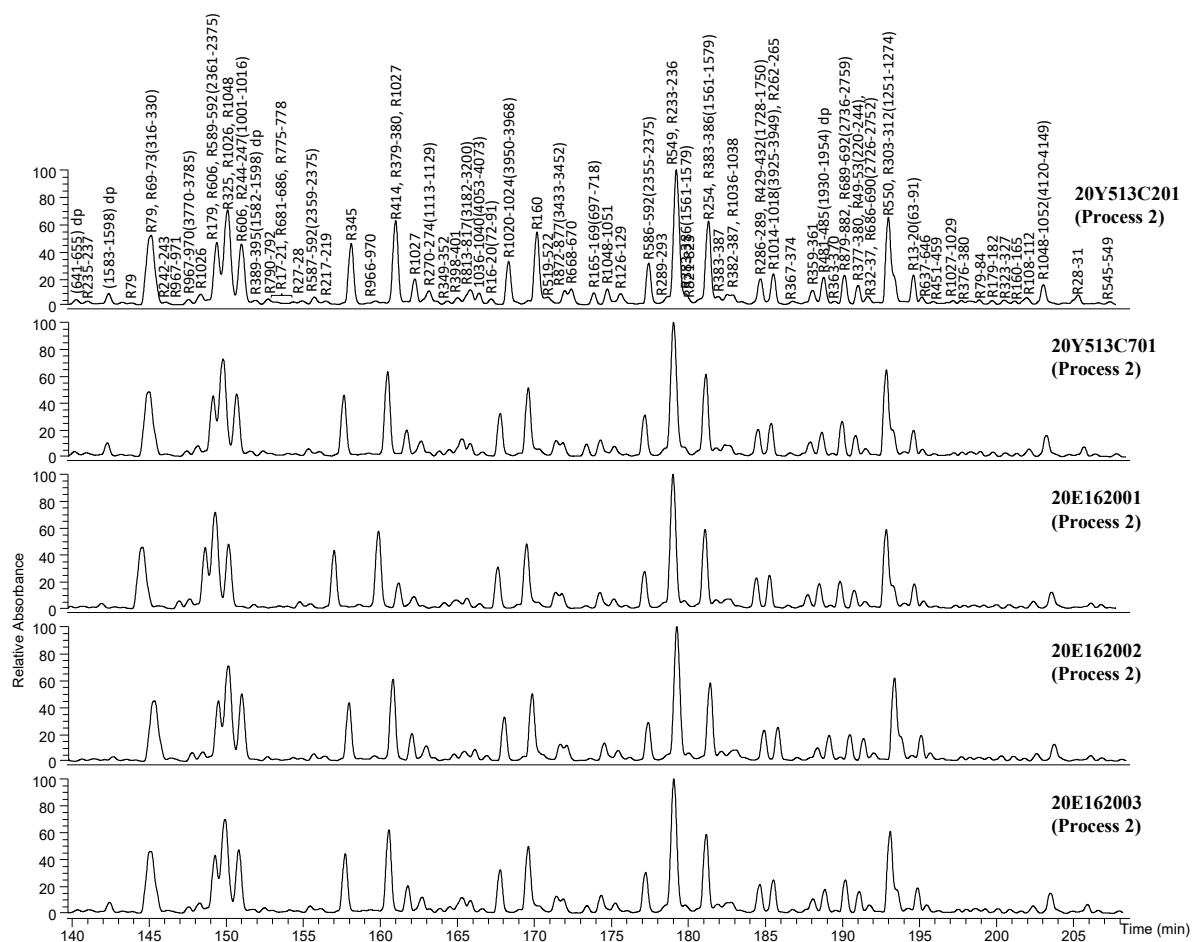


Figure S.2.6-38. RNase T1 oligonucleotide map of BNT162b2 DS batches. Batch 20Y513C201 serves as an interim reference to bridge sample set figures. Abbreviations: “R[n]” represents BNT162b2 oligonucleotide fragment resulting from RNase T1 digestion counting *n* fragments from the 5’ end, and “dp” denotes dephosphorylation. Missed-cleavage oligonucleotide fragments are listed as “R[n]-[n+m]”, with *m* representing the number of additional RNase T1 fragments. Fragments beginning or ending with a non-RNase T1 cleavage site are signified by their nucleotide start and end numbers in parentheses.

Figure S.2.6-39. LC/MS/MS – Oligonucleotide Mapping of BNT162b2 DS Batches (208-260 min), Sample Set 1

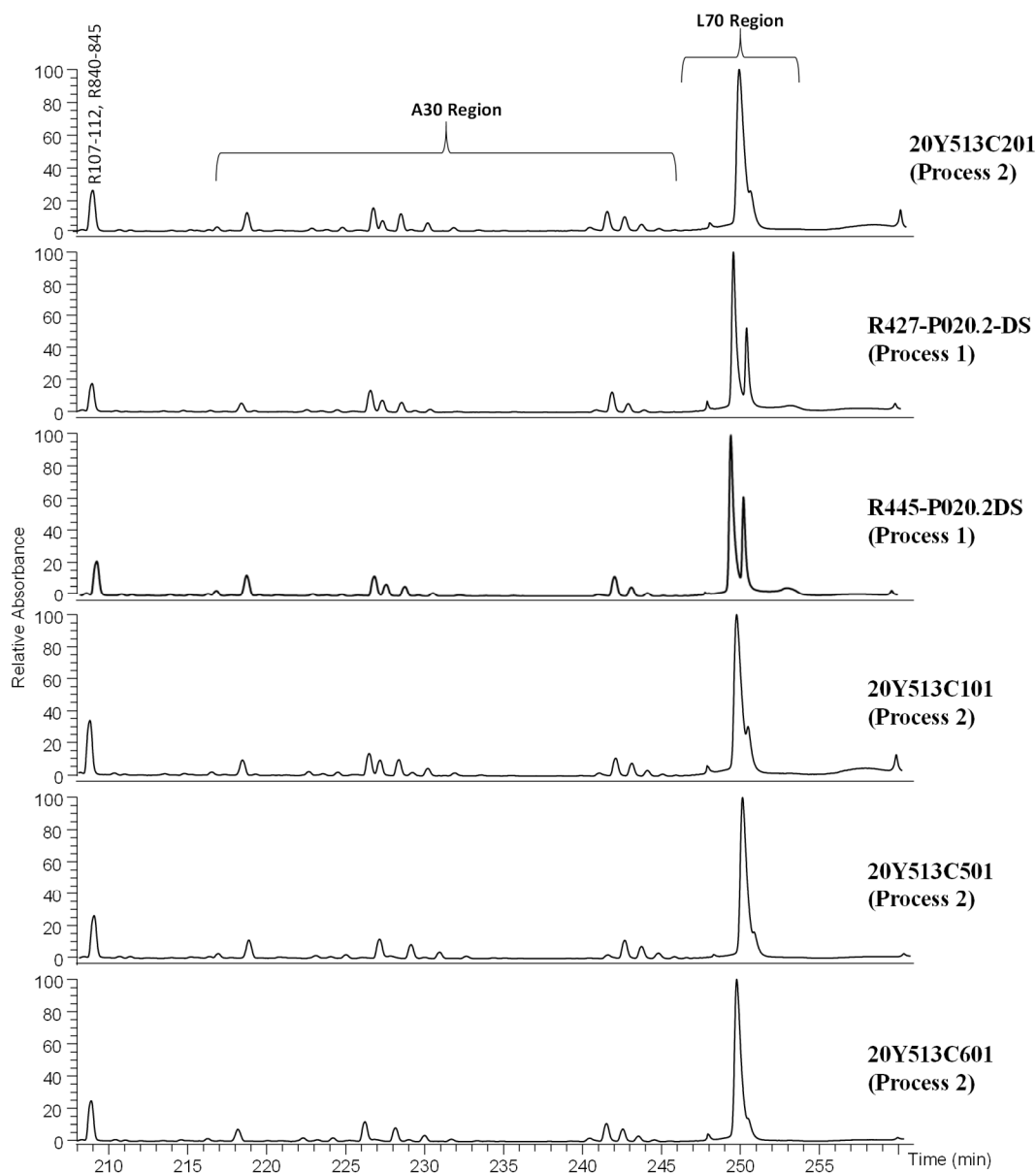


Figure S.2.6-39. RNase T1 oligonucleotide map of BNT162b2 DS batches. Batch 20Y513C201 serves as an interim reference to bridge sample set figures. Abbreviations: “R[n]” represents BNT162b2 oligonucleotide fragment resulting from RNase T1 digestion, counting n fragments from the 5’ end, missed-cleavage oligonucleotide fragments are listed as “R[n]-[n+m]”, with m representing the number of additional RNase T1 fragments, and the A30 and L70 regions represent the polyA-tail region of BNT162b2 (see [S.2.6.5.4.3](#)).

Figure S.2.6-40. LC/MS/MS – Oligonucleotide Mapping of BNT162b2 DS Batches (208-260 min), Sample Set 2

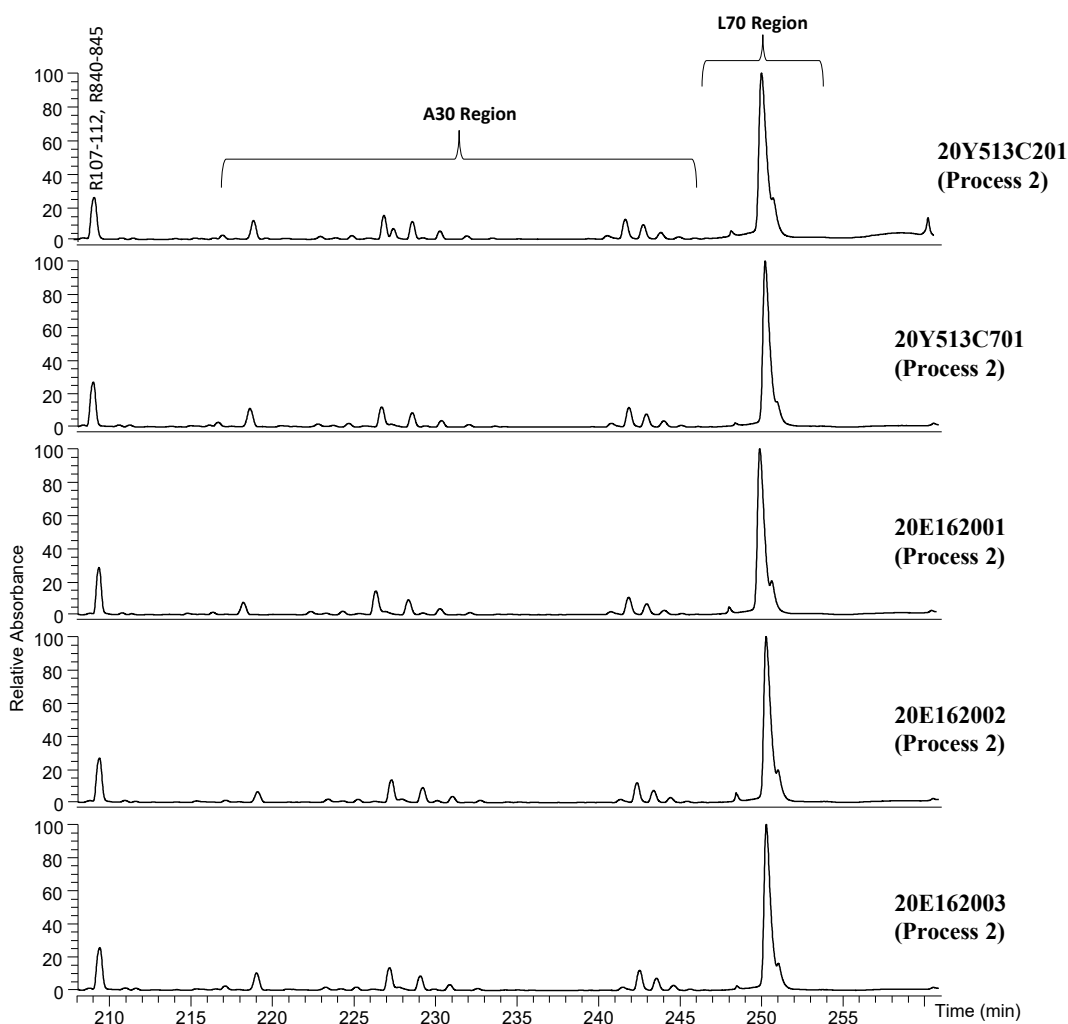


Figure S.2.6-40. RNase T1 oligonucleotide map of BNT162b2 DS batches. Batch 20Y513C201 serves as an interim reference to bridge sample set figures. Abbreviations: “R[n]” represents BNT162b2 oligonucleotide fragment resulting from RNase T1 digestion, counting n fragments from the 5’ end, missed-cleavage oligonucleotide fragments are listed as “R[n]-[n+m]”, with m representing the number of additional RNase T1 fragments, and the A30 and L70 regions represent the polyA-tail region of BNT162b2 (see [S.2.6.5.4.3](#)).

Table S.2.6-27. LC/MS/MS – Oligonucleotide Mapping Summary of BNT162b2 DS Batches

Characteristics	Clinical (Process 1)		Emergency Supply (Process 2)		Process Performance Qualification – Pfizer, Andover (Process 2)			Process Performance Qualification - BioNTech Mainz / Rentschler (Process 2)		
	R427-P020.2-DS	R445-P020.2-DS	20Y513C101	20Y513C201	20Y513C501	20Y513C601	20Y513C701	20E162001	20E162002	20E162003
RNA sequence confirmation	BNT162b2 RNA sequence coverage: Detected nucleotides represent 4054 out of 4283 residues or 94.7%	BNT162b2 RNA sequence coverage: Detected nucleotides represent 4054 out of 4283 residues or 94.7%	BNT162b2 RNA sequence coverage: Detected nucleotides represent 4054 out of 4283 residues or 94.7%	BNT162b2 RNA sequence coverage: Detected nucleotides represent 4054 out of 4283 residues or 94.7%	BNT162b2 RNA sequence coverage: Detected nucleotides represent 4054 out of 4283 residues or 94.7%	BNT162b2 RNA sequence coverage: Detected nucleotides represent 4054 out of 4283 residues or 94.7%	BNT162b2 RNA sequence coverage: Detected nucleotides represent 4054 out of 4283 residues or 94.7%	BNT162b2 RNA sequence coverage: Detected nucleotides represent 4054 out of 4283 residues or 94.7%	BNT162b2 RNA sequence coverage: Detected nucleotides represent 4054 out of 4283 residues or 94.7%	BNT162b2 RNA sequence coverage: Detected nucleotides represent 4054 out of 4283 residues or 94.7%
RNA termini	5'-terminus not recovered by RNase T1 RNA map – see S.2.6.5.4.2 for detailed analysis of 5' terminus 3'-terminus detected: A30 and L70 polyA tail regions detected – (see S.2.6.5.4.4 for detailed analysis of PolyA tail)	5'-terminus not recovered by RNase T1 RNA map – see S.2.6.5.4.2 for detailed analysis of 5' terminus 3'-terminus detected: A30 and L70 polyA tail regions detected – (see S.2.6.5.4.4 for detailed analysis of PolyA tail)	5'-terminus not recovered by RNase T1 RNA map – see S.2.6.5.4.2 for detailed analysis of 5' terminus 3'-terminus detected: A30 and L70 polyA tail regions detected – (see S.2.6.5.4.4 for detailed analysis of PolyA tail)	5'-terminus not recovered by RNase T1 RNA map – see S.2.6.5.4.2 for detailed analysis of 5' terminus 3'-terminus detected: A30 and L70 polyA tail regions detected – (see S.2.6.5.4.4 for detailed analysis of PolyA tail)	5'-terminus not recovered by RNase T1 RNA map – see S.2.6.5.4.2 for detailed analysis of 5' terminus 3'-terminus detected: A30 and L70 polyA tail regions detected – (see S.2.6.5.4.4 for detailed analysis of PolyA tail)	5'-terminus not recovered by RNase T1 RNA map – see S.2.6.5.4.2 for detailed analysis of 5' terminus 3'-terminus detected: A30 and L70 polyA tail regions detected – (see S.2.6.5.4.4 for detailed analysis of PolyA tail)	5'-terminus not recovered by RNase T1 RNA map – see S.2.6.5.4.2 for detailed analysis of 5' terminus 3'-terminus detected: A30 and L70 polyA tail regions detected – (see S.2.6.5.4.4 for detailed analysis of PolyA tail)	5'-terminus not recovered by RNase T1 RNA map – see S.2.6.5.4.2 for detailed analysis of 5' terminus 3'-terminus detected: A30 and L70 polyA tail regions detected – (see S.2.6.5.4.4 for detailed analysis of PolyA tail)	5'-terminus not recovered by RNase T1 RNA map – see S.2.6.5.4.2 for detailed analysis of 5' terminus 3'-terminus detected: A30 and L70 polyA tail regions detected – (see S.2.6.5.4.4 for detailed analysis of PolyA tail)	5'-terminus not recovered by RNase T1 RNA map – see S.2.6.5.4.2 for detailed analysis of 5' terminus 3'-terminus detected: A30 and L70 polyA tail regions detected – (see S.2.6.5.4.4 for detailed analysis of PolyA tail)

S.2.6.5.4.6. Comparative Higher Order Structure Characterization of BNT162b2 DS Batches

Circular dichroism (CD) spectroscopy was used to assess the higher-order structure of BNT162b2 in solution for clinical, emergency use, and PPQ DS batches. CD spectra were recorded in triplicate for each BNT162b2 DS batch, and all samples from all batches were analyzed side-by-side from a 1xPBS solution. The averaged CD spectra were overlaid for visual inspection, and for each DS batch, the CD spectra exhibit the same alternating peaks and troughs and match each other closely at all wavelengths from 200 nm to 330 nm (Figure S.2.6-41). The averaged CD spectra were further analyzed by a chemometric classification method to evaluate spectral similarity ([Table S.2.6-28](#)).

Figure S.2.6-41. CD Spectral Overlay BNT162b2 DS batches

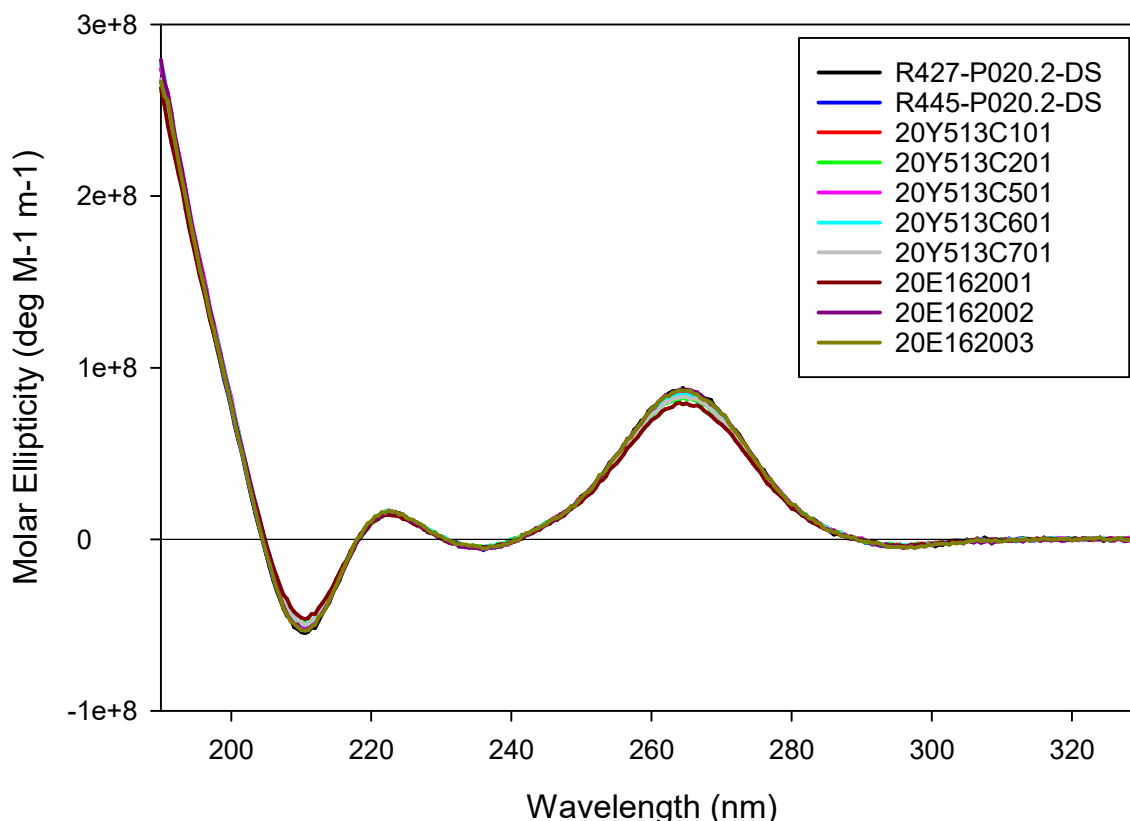


Figure S.2.6-41 Overlaid CD spectra of BNT162b2 DS in 1xPBS buffer solution. For each DS batch, the spectra were acquired in triplicate using a CD spectrometer with 0.01 cm pathlength rectangular cuvettes at 20 °C. The triplicate spectra were averaged at each data point and the average values were plotted. All DS batches were analyzed by CD in a side-by-side manner.

Table S.2.6-28. Spectral Similarity Scores between BNT162b2 Drug Substance Batches

	R427 - P020. 2-DS	R445 - P020. 2-DS	20Y513C 101	20Y513C 201	20Y513C 501	20Y513C 601	20Y513C 701	20E162 001	20E162 002	20E162 003
20Y513C 101^a	98.0 %	98.2 %	100.0%	99.8%	99.6%	99.4%	99.5%	99.1%	99.0%	98.2%

a. All lots are compared against batch 20Y513C101: 100% being identical

The close match of all CD spectra in the superimposed views and respective similarity scores confirm that BNT162b2 clinical, emergency use, and PPQ DS batches are comparable to each other with respect to higher order structure.

S.2.6.5.4.7. Expressed protein size by Western blot

The protein size after in-vitro expression of BNT162b2 drug substance was determined using Western blot. Expressed protein size was confirmed to be comparable across clinical, emergency use, and PPQ DS batches. [Figure S.2.6-42](#) shows that the expressed protein size is consistent with the expected size of the translated BNT162b2 drug substance and comparable across all tested batches. In addition, relative expression levels are comparable for all batches, as evidenced by comparable band intensity at each load level across all batches.

Figure S.2.6-42. BNT162b2 Expressed Protein Size by Western Blot

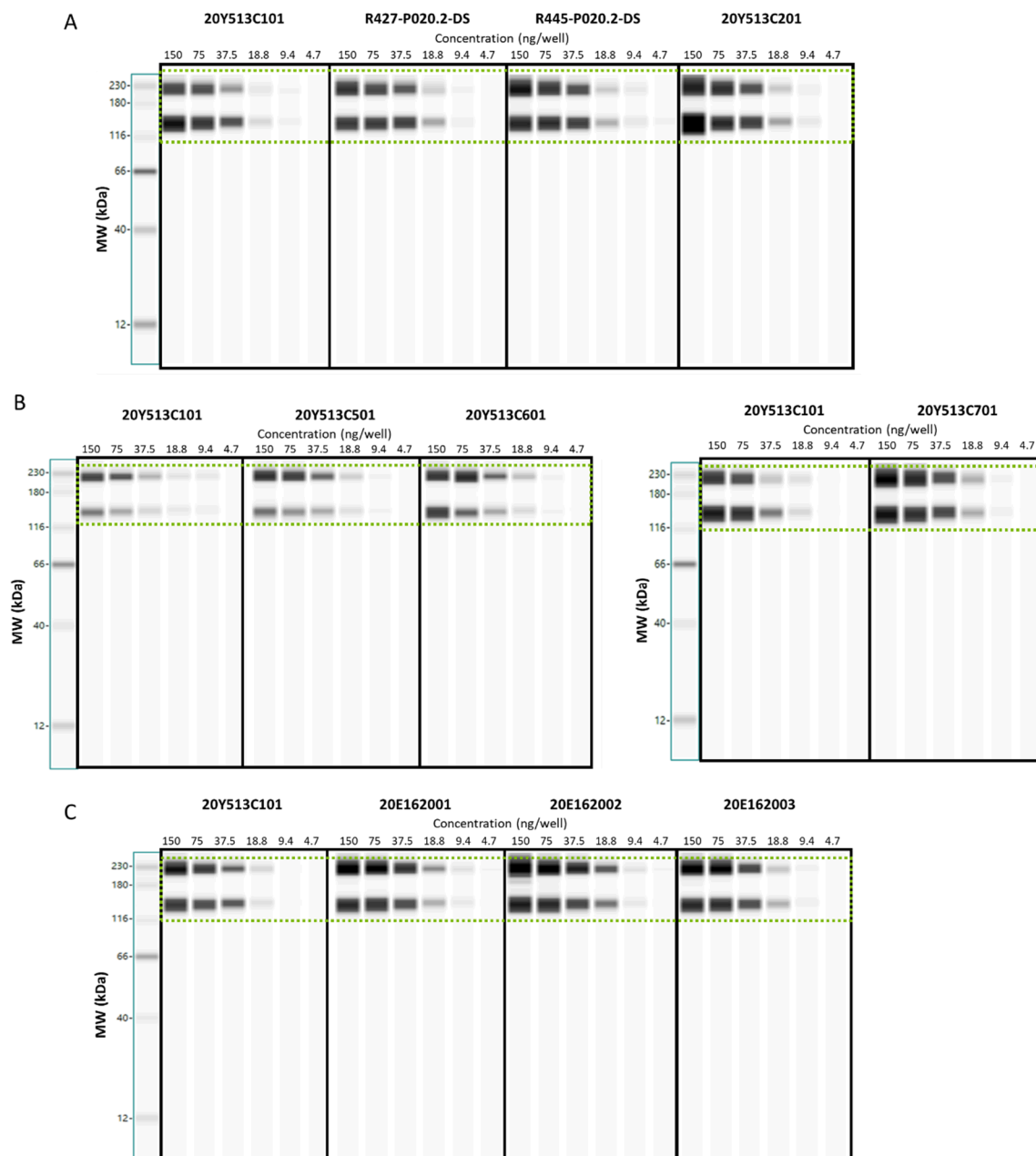


Figure S.2.6-42. To evaluate expressed protein size, BNT162b2 DS was mixed with Lipofectamine and then transfected into HEK-293 cells. Following incubation, cell lysates were evaluated for the expressed protein antigen by Western blot using an antibody specific for the SARS-CoV-2 spike protein. Samples were analyzed across four gels, which included clinical and emergency supply batches (panel A), PPQ batches from Pfizer, Andover (Panel B), and PPQ batches from BioNTech Manufacturing (Panel C). The first lane in each gel shows a molecular weight (MW) marker, and each gel included batch 20Y513C101 as an internal reference to enable comparison across gels. The concentrations shown for each DS batch correspond to the amounts of DS transfected per well of HEK-293 cells.

S.2.6.5.4.8. Overall Conclusions for Comparability

The comparability assessment presented here focused on an assessment of the BNT162b2 drug substance critical quality attributes for Process 1 batches manufactured for clinical supply and Process 2 batches manufactured for emergency supply at Pfizer, Andover and PPQ at Pfizer, Andover and BioNTech Mainz and Rentschler, Laupheim. The heightened characterization and comparability study for the BNT162b2 drug substance employed several state-of-the-art techniques and methodologies to evaluate primary structure and higher order structure. All results demonstrate that the Process 1 drug substance batches manufactured at BioNTech and the Process 2 drug substance batches manufactured at Pfizer, Andover and BioNTech Mainz and Rentschler, Laupheim are comparable.

Table R.1-1. BNT162b2 Drug Product Comparability of Release Test Results

Manufacturing Information								
Lot #	Clinical ^a	Clinical ^b	EE8492	EE8493 ^d	EJ0553	EJ1685	EJ1686	EK1768
Drug Substance	BioNTech	BioNTech	Pfizer, Andover	Pfizer, Andover	Pfizer, Andover	BioNTech; Rentschler	BioNTech; Rentschler	Pfizer, Andover
Manufacturing Site								
DS Batch Scale	0.035 L	0.035L	37.6 L	37.6 L	37.6 L	37.6 L	37.6 L	37.6 L
DS Process	Process 1	Process 1	Process 2	Process 2	Process 2	Process 2	Process 2	Process 2
LNP Manufacturing Site	Polymun	Polymun	Polymun	Polymun	Polymun	Polymun	Polymun	Polymun
DP Manufacturing Site	Polymun	Pfizer, Puurs	Pfizer, Puurs	Pfizer, Puurs	Pfizer, Puurs	Pfizer, Puurs	Pfizer, Puurs	Pfizer, Puurs
DP Fill/Finish DOM	Apr -Jul 2020	Jul 2020	05-Aug-2020	05-Aug-2020	25-Sep-2020	05-Oct-2020	07-Oct-2020	16-Oct-2020
Drug Product Analytical Information								
Release Test	Acceptance Criteria	Clinical Range	Results					
Appearance	White to off-white suspension	White to off-white suspension	White to off-white suspension	White to off-white suspension	White to off-white suspension	White to off-white suspension	White to off-white suspension	White to off-white suspension
Appearance (Visible Particles)	May contain white to off-white opaque, amorphous particles	Free from observable particles	Essentially free from visible particulates	Essentially free from visible particulates	Essentially free from visible particulates	Essentially free from visible particulates	Essentially free from visible particulates	Essentially free from visible particulates
Subvisible Particles	Particles ≥10 µm: ≤6000 per container Particles ≥ 25 µm: ≤600 per container	≥25 µm: <1 to 1 ≥10 µm: <1 to 3	≥25 µm: 0 ≥10 µm: 307	≥25 µm: 10 ≥10 µm: 343	≥25µm: 7 ≥10 µm: 160	≥25 µm: 7 ≥10 µm: 103	≥25 µm: 0 ≥10 µm: 73	≥25 µm: 10 ≥10 µm: 233
pH	6.9 – 7.9	7.1-7.2	7.1	7.1	7.2	7.2	7.2	7.2
Osmolality	425 - 625 mOsmol/kg	540-575	572	569	562	570	565	566
LNP Size	40 to 180 nm	59-74	68	71	66	71	77	68
LNP Polydispersity	≤0.3	0.1-0.2	0.1	0.2	0.1	0.1	0.2	0.1
RNA Encapsulation	≥80%	92-95	94	94	93	94	94	94
RNA Content	0.50±0.13 mg/mL	0.50-0.58	0.53	0.53	0.49	0.57	0.58	0.56

Table R.1-1. BNT162b2 Drug Product Comparability of Release Test Results

Manufacturing Information								
Lot #	Clinical ^a	Clinical ^b	EE8492	EE8493 ^d	EJ0553	EJ1685	EJ1686	EK1768
Drug Substance	BioNTech	BioNTech	Pfizer, Andover	Pfizer, Andover	Pfizer, Andover	BioNTech; Rentschler	BioNTech; Rentschler	Pfizer, Andover
Manufacturing Site								
DS Batch Scale	0.035 L	0.035L	37.6 L	37.6 L	37.6 L	37.6 L	37.6 L	37.6 L
DS Process	Process 1	Process 1	Process 2	Process 2	Process 2	Process 2	Process 2	Process 2
LNP Manufacturing Site	Polymun	Polymun	Polymun	Polymun	Polymun	Polymun	Polymun	Polymun
DP Manufacturing Site	Polymun	Pfizer, Puurs	Pfizer, Puurs	Pfizer, Puurs	Pfizer, Puurs	Pfizer, Puurs	Pfizer, Puurs	Pfizer, Puurs
DP Fill/Finish DOM	Apr -Jul 2020	Jul 2020	05-Aug-2020	05-Aug-2020	25-Sep-2020	05-Oct-2020	07-Oct-2020	16-Oct-2020
Drug Product Analytical Information								
Release Test	Acceptance Criteria	Clinical Range	Results					
ALC-0315 Content	4.50 to 9.25 mg/mL	5.94-7.49	6.70	6.78	7.16	7.26	7.29	6.07
ALC-0159 Content	0.55 to 1.20 mg/mL	0.69-0.91	0.82	0.86	0.87	0.91	0.93	0.75
DSPC Content	0.90 to 2.05 mg/mL	1.23-1.65	1.42	1.43	1.49	1.55	1.55	1.28
Cholesterol Content	1.80 to 3.90 mg/mL	2.41-3.15	2.80	2.87	2.99	3.01	3.04	2.58
Container Content for Injections	Not less than the sum of the nominal values of 5 doses	NA	Not less than the sum of the nominal values of 5 doses.	Not less than the sum of the nominal values of 5 doses.	Not less than the sum of the nominal values of 5 doses.	Not less than the sum of the nominal values of 5 doses.	Not less than the sum of the nominal values of 5 doses.	Not less than the sum of the nominal values of 5 doses.
Lipid Identities	Retention times consistent with references (ALC-0315, ALC-0159, Cholesterol, DSPC)	Conforms to reference	Retention times consistent with references (ALC-0315, ALC-0159, Cholesterol, DSPC)	Retention times consistent with references (ALC-0315, ALC-0159, Cholesterol, DSPC)	Retention times consistent with references (ALC-0315, ALC-0159, Cholesterol, DSPC)	Retention times consistent with references (ALC-0315, ALC-0159, Cholesterol, DSPC)	Retention times consistent with references (ALC-0315, ALC-0159, Cholesterol, DSPC)	Retention times consistent with references (ALC-0315, ALC-0159, Cholesterol, DSPC)
Identity of Encoded RNA Sequence	Identity confirmed	NA	Confirmed	Confirmed	Confirmed	Confirmed	Confirmed	Confirmed
In Vitro Expression	≥ 30% Cells Positive	NA	63	65	62	54	63	NA ^c

Table R.1-1. BNT162b2 Drug Product Comparability of Release Test Results

Manufacturing Information								
Lot #	Clinical ^a	Clinical ^b	EE8492	EE8493 ^d	EJ0553	EJ1685	EJ1686	EK1768
Drug Substance	BioNTech	BioNTech	Pfizer, Andover	Pfizer, Andover	Pfizer, Andover	BioNTech; Rentschler	BioNTech; Rentschler	Pfizer, Andover
Manufacturing Site								
DS Batch Scale	0.035 L	0.035L	37.6 L	37.6 L	37.6 L	37.6 L	37.6 L	37.6 L
DS Process	Process 1	Process 1	Process 2	Process 2	Process 2	Process 2	Process 2	Process 2
LNP Manufacturing Site	Polymun	Polymun	Polymun	Polymun	Polymun	Polymun	Polymun	Polymun
DP Manufacturing Site	Polymun	Pfizer, Puurs	Pfizer, Puurs	Pfizer, Puurs	Pfizer, Puurs	Pfizer, Puurs	Pfizer, Puurs	Pfizer, Puurs
DP Fill/Finish DOM	Apr -Jul 2020	Jul 2020	05-Aug-2020	05-Aug-2020	25-Sep-2020	05-Oct-2020	07-Oct-2020	16-Oct-2020
Drug Product Analytical Information								
Release Test	Acceptance Criteria	Clinical Range	Results					
RNA Integrity	≥55% Intact RNA	62-86	55	55	68	66	69	60
Bacterial Endotoxins	≤12.5 EU/mL	<1	< 5.0	< 5.0	< 5.0	< 5.0	< 5.0	< 5.0
Sterility	No growth detected	Sterile	No growth detected	No growth detected	No growth detected	No growth detected	No growth detected	No growth detected

- a. Clinical lots BCV40420-A, BCV40620-A, BCV40620-B, BCV40620-C, BCV40620-D, BCV40720-A, BCV40720-B, BCV40720-C
- b. Clinical lots BCV40720-P and BCV40820-P
- c. Data not available (NA) at the time of filing.
- d. Batch EE8493 also used in clinical trials.



**BIOTHERAPEUTICS PHARMACEUTICAL SCIENCES
ANALYTICAL RESEARCH & DEVELOPMENT**

Method Qualification Report

VAL100122803 Version 2.0

Title	Qualification of TM100010380 Determination of the In-vitro Expression of PF-07302048 by Flow Cytometry
Project Code(s)	PF-07302048
Author	Rachel Dorner
Summary	<p>This report describes the results from the qualification of method TM100010380, a bioassay method used to determine the in-vitro expression of PF-07302048 drug product (DP). The method explained in this qualification report was evaluated for its linearity, specificity, precision (repeatability and intermediate precision), range, detection limit and robustness.</p> <p>The qualification results demonstrate that the test method TM100010380 is suitable for determining the in-vitro expression of PF-07302048 DP samples for release and stability testing.</p>
Keywords	COVID-19 vaccine
Acknowledgements	Juxiang Cao, Jamie Lee

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1 SUMMARY OF RESULTS

Table 1 - Summary of Qualification Results

Qualification Element			Result
Target	Repeatability (%RSD)	100 ng (S1+), n=6	34.7
		150 ng (S1+), n=6	15.1
		300 ng (S1+), n=6	6.1
Range	Precision (%RSD)	400 ng (S1+), n=6	5.6
		300 ng (S1+), n=6	9.4
		250 ng (S1+), n=6	7.1
		200 ng (S1+), n=6	14.3
		150 ng (S1+), n=6	18.3
		125 ng (S1+), n=6	21.9
		100 ng (S1+), n=6	27.8
		75 ng (S1+), n=6	21.1
		50 ng (S1+), n=6	53.8
Detection Limit		Lowest concentration S1+ expression can be reliably detected	50 ng
Linearity (optimal linearity between 100-250 ng)		R ²	0.981
		Intercept	26.8
		Slope	0.218
Specificity		Response specific to analyte	Response demonstrated specificity

2 TEST METHOD SUMMARY

The test method applies to PF-07302048 drug product (DP) release and stability testing with a concentration equal to or higher than 0.1 mg/mL.

PF-07302048 is a messenger RNA (mRNA) lipid nanoparticle (LNP) vaccine that encodes SARS-CoV-2 glycoprotein S-derived antigens.

Human embryonic kidney (HEK293T) cells are seeded on 12-well assay plates for 16-24 hours in a 37±1 °C, 5±1% CO₂ incubator. Cells are then transfected with test samples (DP) and drug product control. Phosphate buffered solution (PBS) is used as a negative control (NC). After 21-24 hours cells are harvested from the 12-well plates and transferred to 96-well assay plates. The cells are then stained with fixable Aqua viability dye before being fixed and permeabilized. After the fixative is washed from the cells, a SARS-CoV-2 Spike S1 antibody is added which binds to any surface-expressed and intracellular SARS-CoV-2 S1 antigen. The amount of bound SARS-CoV-2 antigen is detected by treatment with a secondary antibody conjugated with a phycoerythrin (PE) fluorophore.

The cells are analyzed via flow cytometer. The in-vitro expression of the SARS-CoV-2 S1 antigen is determined from the percent of viable, single cells bound with PE and the mean fluorescent intensity (MFI) of the bound PE.

3 OBJECTIVE

The objective of this report is to summarize the results from qualification experiments and, based on the results, to report an assessment of the key method performance characteristics per SOP-LAB-01249 (i.e., specificity, precision, assay range, linearity and detection limit). The qualification exercise determines if the method is suitable for the in-vitro expression of PF-07302048 drug product samples for release and stability testing.

4 QUALIFICATION MATERIALS

4.1 Samples

PF-07302048 lot 00714578-0007-M01 (stock concentration of 0.586 mg/mL) is used as Drug Product Control (DPC) and for preparation of qualification range samples. It is representative of PF-07302048 mRNA construct BNT162b2 drug product.

PF-07305885 lot 00709054-0162-M01 (stock concentration of 1.92 mg/mL) is used as Specificity Negative DS Control. The DSC material is representative of mRNA construct BNT162b2 drug substance. See [Table 2](#) for details.

PF-07252220 DP is used as a Specificity Negative DP sample. PF-07252220 DP is a mRNA LNP vaccine that encodes *Influenza* Hemagglutinin protein H1N1 A/California/07/2009.

Table 2 - Qualification Sample Information

Sample Name	Sample Concentration (ng/mL)	On Plate Transfection Amount (ng)
Drug Product Control	1500	150
Drug Substance Control	5000	500
400	4000	400
300	3000	300
250	2500	250
200	2000	200
150	1500	150
125	1250	125
100	1000	100
75	750	75
50	500	50
Specificity Negative DP Sample	1500	150

5 EQUIPMENT AND REAGENTS

All equipment, materials, and reagents utilized during the execution of the qualification work are recorded within the test method and the laboratory records.

6 DEVIATIONS AND OBSERVATIONS

The S1+ gate was initially set using the positively-stained population of a Drug Substance Control (DSC) and applying the same gate to all DPC, NC, and test samples. The decision was made to align with the VRD gating strategy for determining %S1+ population for the final TM100010380 test method. Data generated in qualification experiments was reanalyzed using the new gating strategy and is provided in this qualification report. Going forward, the S1+ gate will be set at a mean of 4% on the Negative Control and applied to all DPC and test samples. The designated 4% S1+ population in the Negative Control also serves as the baseline for the assay.

Qualification was performed with each control and test sample run in duplicate; however, intra-plate variability is a major source of method variability based on qualification data. Generating the reportable value based on >2 intra-assay replicates helps to reduce the method variability of the reportable value. For the final method, the replication strategy has been increased from 2 to 4 within a single plate based on statistical analysis.

7 EXPERIMENTAL DESIGN

In summary, three experiments were executed to assess method performance.

7.1 Experiment 1 (Repeatability)

Assay precision under the same operating conditions over a short interval of time by a single analyst. One assay plate was used to run test samples.

7.2 Experiment 2 (Precision, Linearity, Assay Range and Detection Limit)

Assay precision, detection limit, and linearity were evaluated across assay range. It involved at least six instances of the test sample concentrations (300, 150, and 100 ng) and at least six instances of the additional range sample concentrations (400, 250, 200, 125, 75 and 50 ng). The results were generated by two analysts over the course of three assay instances per analyst. An example testing plan is shown below in [Table 3](#).

Table 3 - Allocation of Range Results from Experiment #2

Sample	Analyst #1			Analyst #2			Total
	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3	
	Plate 1	Plate 2	Plate 3	Plate 5	Plate 6	Plate 7	
400	1	1	1	1	1	1	6
300	1	1	1	1	1	1	6
250	1	1	1	1	1	1	6
200	1	1	1	1	1	1	6
150	1	1	1	1	1	1	6
125	1	1	1	1	1	1	6
100	1	1	1	1	1	1	6
75	1	1	1	1	1	1	6
50	1	1	1	1	1	1	6

7.3 Experiment 3 (Specificity)

This experiment was designed to confirm test method specificity. Specificity was assessed by analyzing DP positive sample (PF-07302048) at 150 ng in parallel with specificity DP negative sample (PF-07252220) and DS negative samples (PF-07305885) at 150 ng. The testing of the positive sample is performed in the presence and absence of SARS-CoV-2 Spike S1 antibody and secondary PE labeled detection antibody. Absence of the S1 antigen is tested both by transfecting cells with DS without the transfection reagent and with a negative DP sample that does not encode SARS-CoV-2 Spike S1 antigen. The flow cytometry graphs were analyzed to assess in-vitro expression. An example plate map is shown below in [Figure 1](#).

Figure 1 - Specificity Plate Map

	1	2	3	4	5	6	7	8	9	10	11	12
A	NC		DPC		DSC		T1		R1		R2	
B	R3		R4		N/A		N/A		N/A		N/A	

T1 = Positive sample

R1 = Positive sample, No primary antibody

R2 = Positive sample, No secondary antibody

R3 = Negative DP sample

R4 = Negative DS sample

7.4 Experiments 4 and 5 (Robustness)

This series of experiments were performed to assess test method robustness. In order to test the robustness of collecting different numbers of events during acquisition on the flow cytometer, 50,000 events were collected as well as 20,000 events to assess differences in cell populations.

In a separate experiment, stability of the SARS-Cov-2 Spike S1 (S1+) cell staining was assessed by sealing and storing the stained plate at 2-8°C for varying lengths of time before collecting results on the flow cytometer.

8 QUALIFICATION RESULTS

8.1 Repeatability Results: Experiment 1

Experiment 1 consisted of sample results from one assay plate. The S1+ and MFI results of the test sample at each concentration were calculated per test method. The results of the 100, 150 and 300 ng concentrations are listed in [Table 1](#) and [Table 4](#).

Table 4 - Repeatability Results

100 ng		
Result #	S1+ (%)	MFI
1	40	247
2	58	315
3	31	221
4	28	211
5	39	249
6	22	195
Mean	36	240
Standard Deviation	12.6	42.4
%RSD	34.7	17.7
150 ng		
Result #	S1+ (%)	MFI
1	57	329
2	77	484
3	56	319
4	65	367
5	73	462
6	54	312
Mean	64	379
Standard Deviation	9.6	75.7
%RSD	15.1	20.0
300 ng		
Result #	S1+ (%)	MFI
1	75	444
2	75	463
3	73	423
4	81	545
5	67	396
6	75	474
Mean	74	458
Standard Deviation	4.5	51.2
%RSD	6.1	11.2

8.2 Intermediate Precision across Assay Range: Experiment 2

For this experiment a total of six assay plates were set up by two analysts in three runs. Nine target concentrations were tested in this experiment to examine the range, precision and detection limit of the method. Individual assay results are listed in [Table 5](#) below and the summarized sample results are given in [Table 6](#) below.

Table 5 - Reportable Assay Results from Experiment #2

	400 ng	300 ng	250 ng	200 ng	150 ng	125 ng	100 ng	75 ng	50 ng
Result #	Results for S1+ (%)								
1	78	69	76	55	45	35	24	20	14
2	89	84	82	71	52	66	46	39	15
3	79	82	88	83	74	68	53	37	6
4	82	77	75	69	72	50	63	35	19
5	86	86	77	62	65	53	44	30	6
6	88	91	87	76	64	58	48	35	26
Result #	Results for MFI								
1	473	394	455	306	265	232	200	192	175
2	805	615	570	406	313	383	286	264	191
3	738	699	728	580	464	421	334	272	169
4	433	390	389	341	346	231	277	186	151
5	629	643	510	378	404	338	290	232	153
6	687	673	615	426	339	299	267	216	192

For each concentration the mean, standard deviation, and %RSD is determined from all S1+ and MFI results using the formulas below.

$$\text{mean} = \frac{\sum_{i=1}^n X_i}{n} \text{ where } X_i \text{ is a test result, } n \text{ is the number of test results}$$

$$\text{standard deviation} = \sqrt{\frac{\sum_{i=1}^n (X_i - m)^2}{n-1}}, \text{ where } X_i \text{ is a test result, } n \text{ is the number of test results}$$

$$\%RSD = \frac{\text{standard deviation}}{\text{mean}} \times 100\%$$

Table 6 - Results of Intermediate Precision Estimation

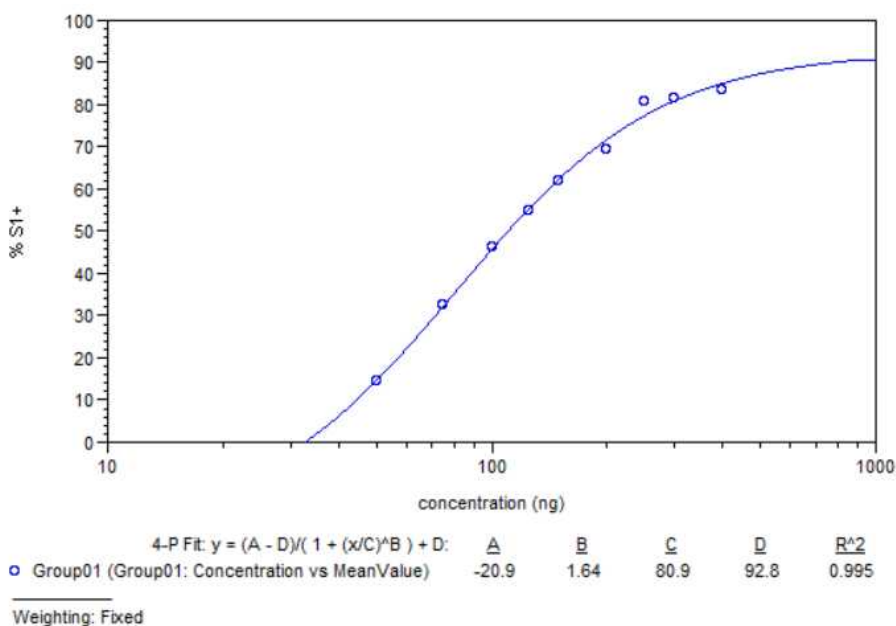
Samples	N	S1+ (%)			MFI		
		Mean	Standard Deviation	Precision (% RSD)	Mean	Standard Deviation	Precision (% RSD)
400	6	84	4.7	5.6	628	147.6	23.5
300	6	82	7.7	9.4	569	140.0	24.6
250	6	81	5.7	7.1	545	120.6	22.1
200	6	69	9.9	14.3	406	95.6	23.5
150	6	62	11.4	18.3	355	70.0	19.7
125	6	55	12.1	21.9	317	78.2	24.6
100	6	46	12.9	27.8	276	43.6	15.8
75	6	33	6.9	21.1	227	35.9	15.8
50	6	14	7.7	53.8	172	17.8	10.3

8.3 Linearity: Experiment 2

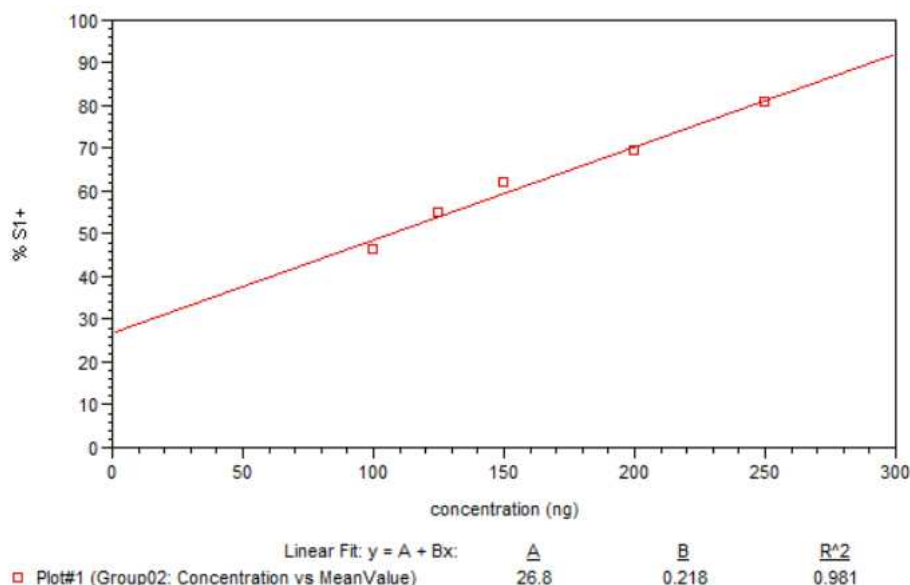
The definition of linearity is the ability to elicit test results that are directly proportional to the concentrations of analyte in samples within a given range. The full dose-response curve (by 4-parameter logistic fit) demonstrated that the curve reaches saturation at or above 250 ng. Linearity analysis was performed by plotting the mean measured S1+ (y-axis) obtained from Experiment 2 against the concentration at 100-250 ng (x-axis). The results of the full dose-response and linear regression analysis are displayed in [Figure 2](#) below.

Figure 2 - Linearity Analysis of S1+

A. Full Dose-Response Curve by 4-PL Fit



B. Linear Analysis (100 ng to 250 ng)



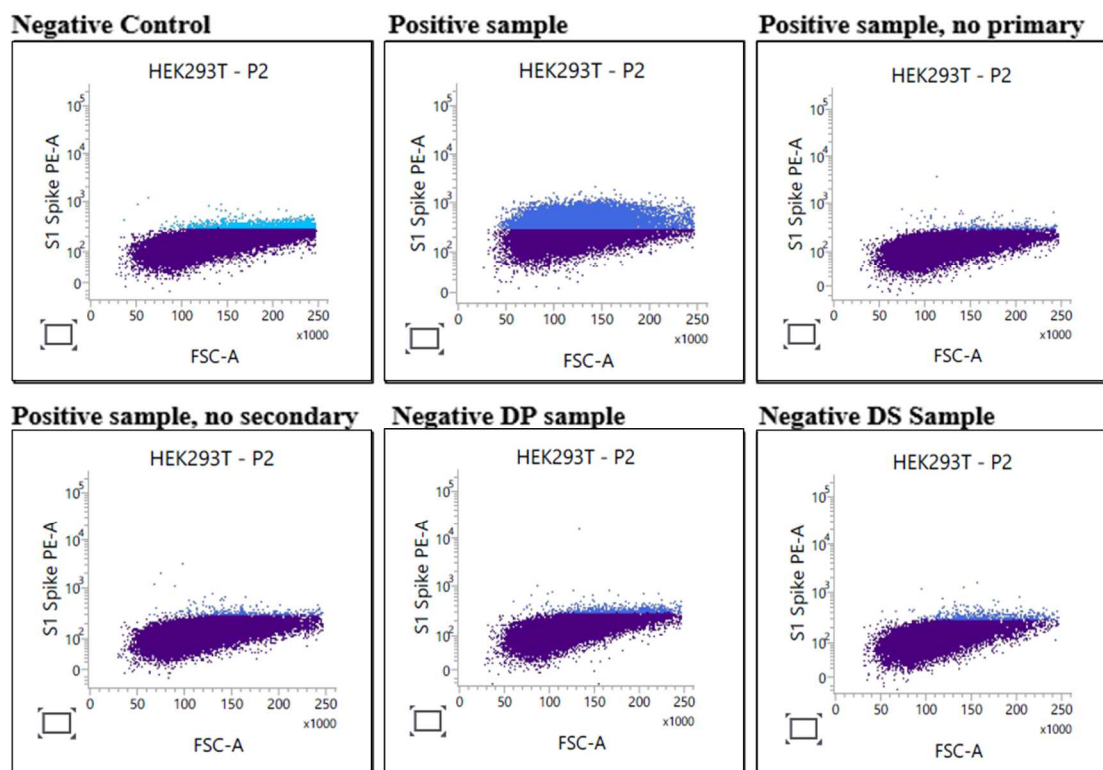
8.4 Detection Limit: Experiment 2

Estimation of detection limit is determined by 3:1 signal-to-noise ratio of S1+ expression. The baseline of S1+ expression by the Negative Control is set at 4%. Detection limit is determined to be 12%. Therefore, the minimum concentration at which the analyte can be reliably detected is 50 ng.

8.5 Specificity Results: Experiment 3

Experiment 3 consisted of one assay plate with three samples, Positive DP sample, Negative DS sample (mRNA without LNP or lipofectamine, PF-07305885), and Negative DP sample (PF-07252220). A full concentration set of the Positive Sample was run with wash buffer rather than the S1 primary antibody and the PE secondary antibody. The resulting PE dot plots for specificity are displayed in [Figure 3](#) below.

Figure 3 - Assay Specificity



Assay specificity and selectivity were confirmed. Response consistent with negative control was observed with the positive DP sample in the absence of primary antibody and in the absence of secondary antibody. The response for the Negative DP was also consistent with negative control indicating that the staining antibodies are specific for the S1 antigen. Transfection was not achieved in the presence of mRNA without an appropriate delivery mechanism such as a liquid nanoparticle or lipofectamine. This confirms the assay specificity.

8.6 Robustness Results: Experiments 4 and 5

In robustness experiment 00712294-0252, 50,000 events were collected per TM100010380 and 20,000 events were collected to determine the impact of fewer events on the overall data. The 20,000 events data was consistently 2-3% lower than the 50,000 events data in the %S1+ population. The MFI was also consistently lower in the 20,000 events data. Based on this, collected events will be kept at 50,000 for the acquisition step.

In experiment 00711015-0281, stained cells were kept at 2-8°C for up to 6 days before reading on the flow cytometer. These results were compared to stained cells that were read on the flow cytometer immediately after completion of staining. The S1+ signal was stable for up to 6 days after staining when the cells were stored at 2-8°C ([Table 7](#)).

Table 7 - Stability of Cell Staining

		00711015-0281plate1	00711015-0281plate2
Incubation time at 2-8°C after cell staining		none	6 days
Negative Control	Viability (%)	97	97
	S1+ (%)	4	4
	MFI	137	165
DP Control	Viability (%)	97	97
	S1+ (%)	74	62
	MFI	466	431
Test Sample at 300 ng	Viability (%)	96	97
	S1+ (%)	81	86
	MFI	705	728
Test Sample at 150 ng	Viability (%)	95	97
	S1+ (%)	64	63
	MFI	447	414
Test Sample at 100 ng	Viability (%)	96	98
	S1+ (%)	60	48
	MFI	344	302

9 CONCLUSIONS

The qualification results demonstrate that the test method has acceptable assay specificity, precision, linearity, range and detection limit. Test method TM100010380 is suitable for determining the in-vitro expression of PF-07302048 construct BNT162b2 drug product samples for release and stability testing. The method performance characteristics determined in the qualification are deemed suitable for the intended use as a limit test.

10 REFERENCES

- 10.1 Analytical Method: TM100010380, Determination of the In-vitro Expression of PF-07302048 by Flow Cytometry
- 10.2 Raw Data:
 - Summary eLN# 00712294-0260
 - Robustness eLN# 00712294-0252
 - Robustness eLN# 00711015-0281

11 ADDENDUM

The original qualification was performed at Pfizer Analytical R&D laboratories in Andover, MA. The Pfizer Analytical R&D laboratories in St. Louis (STL), MO, performed a subset of qualification experiments to establish STL as a suitable and qualified lab to execute TM100010380. Two analysts performed 3 instances of Experiment 2 above for linearity and intermediate precision across assay range. In addition, robustness was tested with the use of the FACSVerse flow cytometer which is in St. Louis, instead of the FACSLyric flow cytometer which is in Andover, MA.

During the course of St. Louis lab qualification experiments, the decision was made to align the gating strategy with that of Vaccine Research & Development (VRD) by setting a mean 4% of the negative control as “positive” for S1 staining and applying that gate to all samples, as stated in Section 6. Individual assay results are listed in Table 8 below and the summarized sample results are given in Table 9 below.

In experiment 00707222-0793, the 200 ng data point failed visual sample acceptance criteria so data for 200 ng data point was not included in the qualification data summary.

Table 8 - Reportable Assay Results from Experiment #2, STL

	400 ng	300 ng	250 ng	200 ng	150 ng	125 ng	100 ng	75 ng	50 ng
Result #	Results for S1+ (%)								
1	86	76	73	67	56	61	44	27	8
2	80	70	69	49	39	39	27	18	24
3	96	94	93	93	87	89	84	78	64
4	97	96	94	90	82	81	76	68	40
5	97	95	92	ND	83	82	71	61	30
6	98	98	96	94	88	89	83	71	48
Result #	Results for MFI								
1	232	188	182	170	145	152	130	106	85
2	171	142	138	105	95	92	84	72	80
3	488	440	414	402	315	287	228	175	119
4	615	585	508	439	291	266	212	173	115
5	473	432	374	ND	254	232	175	133	90
6	578	542	482	413	284	273	213	162	108

ND: No data

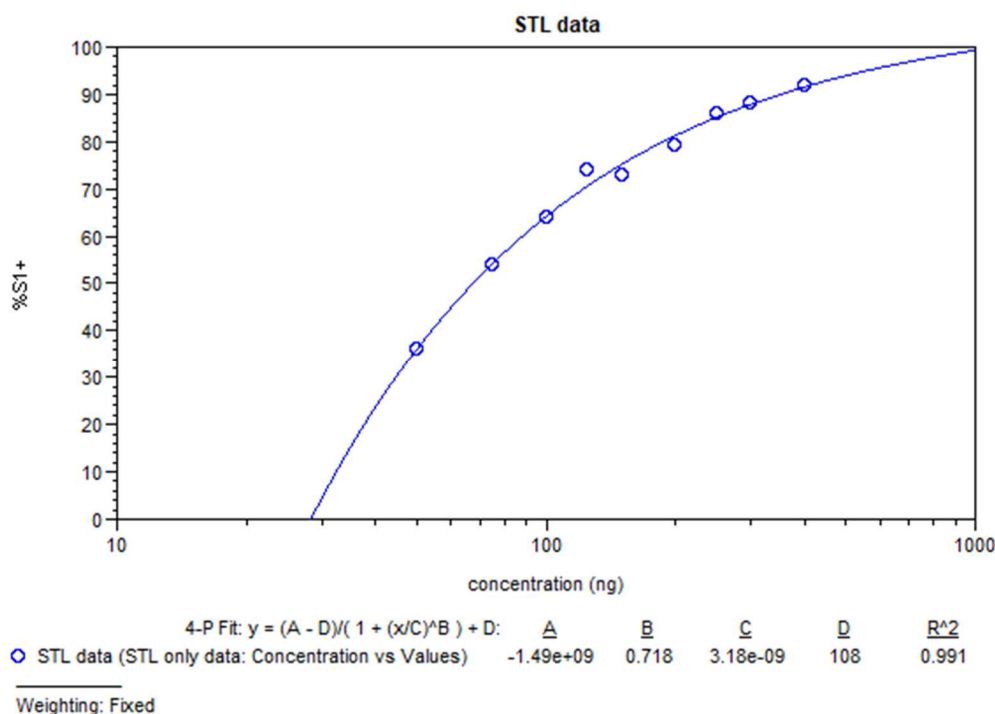
Table 9 - Results of Intermediate Precision Estimation, STL

Samples	N	S1+ (%)			MFI		
		Mean	Standard Deviation	Precision (% RSD)	Mean	Standard Deviation	Precision (% RSD)
400	6	92	7.5	8.1	426	183.1	43.0
300	6	88	12.0	13.6	388	183.1	47.2
250	6	86	11.9	13.8	350	155.1	44.3
200	6	79	19.9	25.3	306	155.9	51.0
150	6	73	20.2	27.9	231	89.3	38.7
125	6	74	19.8	26.9	217	78.1	36.0
100	6	64	23.3	23.3	174	56.4	36.4
75	6	54	25.0	25.0	137	41.4	30.3
50	6	36	19.5	19.5	100	16.6	16.7

Linearity analysis was performed by plotting the mean measured S1+ (y-axis) obtained from Experiment 2 against the concentration at 100-250 ng (x-axis). The results of the full dose-response and linear regression analysis are displayed in [Figure 4](#) below. The St. Louis site qualification data is summarized in a modified format ([Table 10](#)).

Figure 4 - Linearity Analysis of S1+, STL

A. Full Dose-Response Curve by 4-PL Fit



B. Linear Analysis (100 ng to 250 ng)

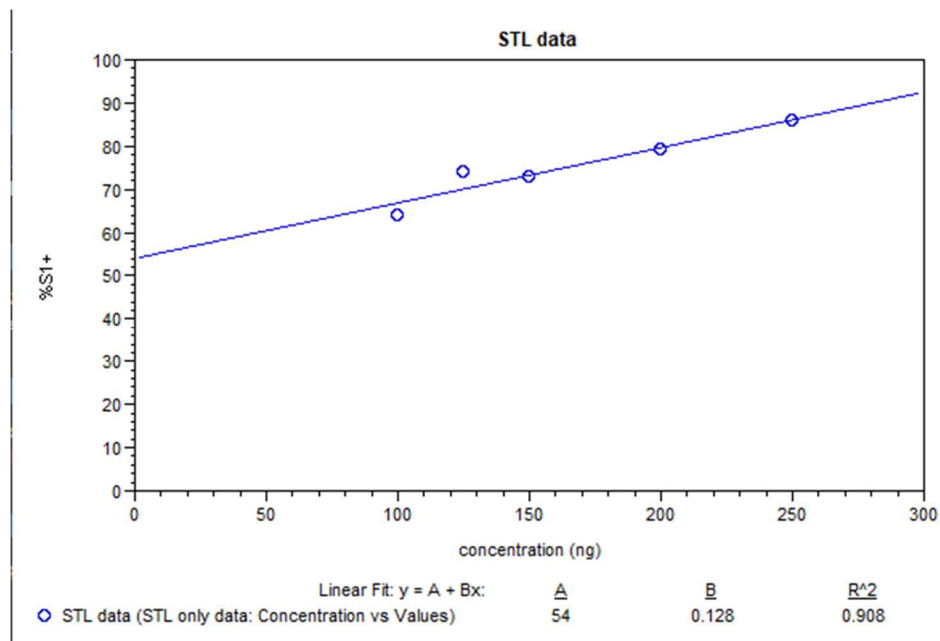


Table 10 - Summary of STL Qualification Results

Qualification Element			Result
Range	Precision (%RSD)	400 ng (S1+), n=6	8.1
		300 ng (S1+), n=6	13.6
		250 ng (S1+), n=6	13.8
		200 ng (S1+), n=6	25.3
		150 ng (S1+), n=6	27.9
		125 ng (S1+), n=6	26.9
		100 ng (S1+), n=6	23.3
		75 ng (S1+), n=6	25.0
		50 ng (S1+), n=6	19.5
Linearity (between 100 and 250 ng)		R ²	0.908
		Intercept	54
		Slope	0.128

11.1 Conclusions:

The qualification results from Analytical R&D lab in St. Louis demonstrate a dose dependent increase in S1+ expression from 50 ng to 400 ng. Although results show slightly higher variability, the overall trend of increased variability (% RSD) at lower concentration is consistent with the results obtained in

Andover. Analysts demonstrated the ability to perform this method in St. Louis labs, with modifications due to the different flow cytometer instrument model.

11.2 Raw Data:

Summary eLN# 00707380-1099

Deviation eLN# 00707222-0793

12 REVISION HISTORY

SUMMARY OF CHANGES	JUSTIFICATION OF CHANGE
1) Version 1.0	1) New Report
2) Version 2.0	2) Addendum (Section 11) added to include St. Louis Laboratories data

-END OF DOCUMENT-

Document Approval Record

Document Name:

VAL100122803

Document Title:

Qualification Report for TM100010380 for Determination of In-Vitro Expression of mRNA by Flow Cytometry

Signed By:

Date(GMT)

Signing Capacity

Shields, Kathleen M

05-Oct-2020 12:55:04

Manager Approval

Burney, Mary W

05-Oct-2020 13:40:12

Data Verification

Lee, Jamie L


05-Oct-2020 14:53:40

Author Approval

Flecke, Kimberly

05-Oct-2020 14:56:32

Quality Assurance Approval

PHARMACEUTICAL SCIENCES		ANALYTICAL METHOD	
	TITLE: Determination of the In-vitro Expression of PF-07302048 by Flow Cytometry	NUMBER: TM100010380	
		GDMS VER. 2.0	PAGE: 1 OF 29

1. PURPOSE

This method is qualified to confirm the presence and determine the in-vitro expression of PF-07302048.

2. SCOPE

This method is qualified to test PF-07302048 drug product release and stability samples with a concentration equal to or higher than 0.100 mg/mL. This test method is suitable for use with the BNT162b2 construct.

3. RESPONSIBILITIES

- 3.1 The analyst must complete all appropriate training prior to performing the method.
- 3.2 The analyst must follow this procedure as written and document all calculations appropriately.
- 3.3 The analyst must ensure all equipment is calibrated and capable of maintaining appropriate settings and conditions as specified in this method.
- 3.4 The analyst must report and properly document all deviations from the method procedure.

4. PRINCIPLE

PF-07302048 is a messenger RNA (mRNA) lipid nanoparticle (LNP) vaccine that encode SARS-CoV-2 glycoprotein S-derived antigens.

Human embryonic kidney (HEK293T) cells are seeded on 12-well assay plates for 16-24 hours in a 37 ± 1 °C, $5\pm 1\%$ CO₂ incubator. Cells are then transfected with control and drug product (DP) test samples. After 21-24 hours cells are harvested from the 12-well plates and transferred to 96-well assay plates. The cells are then stained with fixable Aqua viability dye before being fixed and permeabilized. After the fixative is washed from the cells, a SARS-CoV-2 Spike S1 antibody is added which binds to any surface-expressed and intracellular SARS-CoV-2 S1 antigen. The amount of bound SARS-CoV-2 antigen is detected by treatment with a secondary antibody conjugated with a phycoerythrin (PE) fluorophore.

The cells are analyzed via flow cytometer. The in-vitro expression of the SARS-CoV-2 S1 antigen is determined from the percent of viable, single cells bound with PE and the mean fluorescent intensity (MFI) of the bound PE.


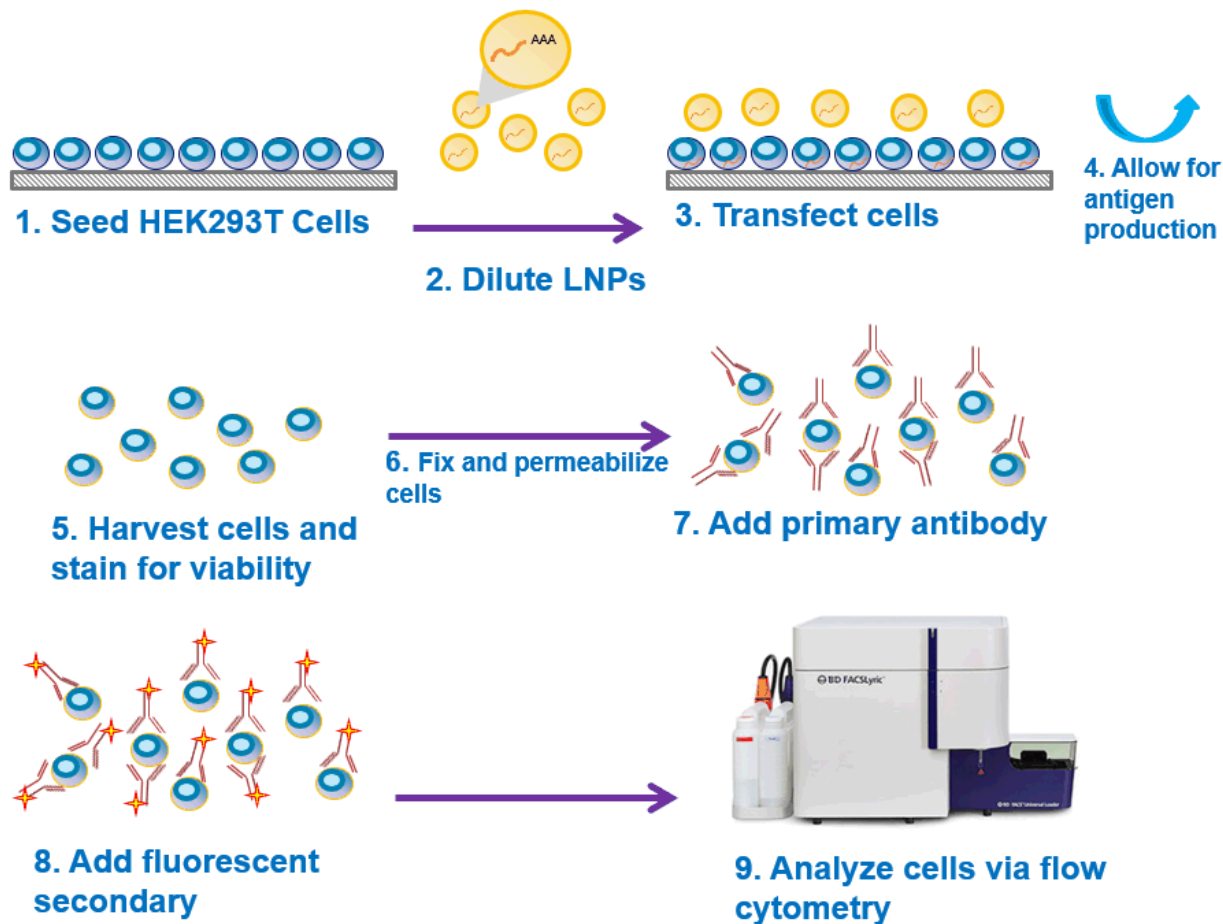
PHARMACEUTICAL SCIENCES		ANALYTICAL METHOD	
	TITLE: Determination of the In-vitro Expression of PF-07302048 by Flow Cytometry	NUMBER: TM100010380	
		GDMS VER. 2.0	PAGE: 2 OF 29

Figure 1 - Illustration of IVE Assay Design



5. SAFETY


Warning: Potent Compound! Due to their potential toxicological properties, the occupational exposure classification is B-OEB5.

Appropriate personal protective equipment must be worn when handling. Avoid ingestion, inhalation and skin contact with samples and reagents.

Instrumentation and equipment should be decontaminated in accordance with laboratory policies established for the handling of B-OEB5 compounds. Consult the laboratory manager for questions or instructions on handling, decontamination, disposal, or emergency response to exposure.

6. DEFINITIONS

- 6.1 AWCB – Analytical working cell bank
- 6.2 BSA – Bovine serum albumin
- 6.3 DMEM – Dulbecco's modified eagle medium
- 6.4 DMSO – Dimethyl sulfoxide

PHARMACEUTICAL SCIENCES		ANALYTICAL METHOD	
	TITLE: Determination of the In-vitro Expression of PF-07302048 by Flow Cytometry	NUMBER: TM100010380	
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- 6.5 DPBS – Dulbecco’s phosphate buffered saline
- 6.6 DPC – Drug product material used as Drug Product Control
- 6.7 FBS – Fetal bovine serum
- 6.8 HEK – Human embryonic kidney
- 6.9 IgG – Immunoglobulin G
- 6.10 mAb – Monoclonal antibody
- 6.11 modRNA – Nucleoside modified messenger RNA
- 6.12 mRNA – Messenger ribonucleic acid
- 6.13 MFI – Mean fluorescent intensity
- 6.14 NC – Cells only as Negative Control
- 6.15 PBS-CMF – Phosphate buffered saline, Calcium Magnesium Free
- 6.16 PE – Phycoerythrin
- 6.17 PES – Polyethersulfone
- 6.18 RCF – Relative centrifugal force (also known as “x gravity”)
- 6.19 SARS-CoV-2 – Severe acute respiratory syndrome coronavirus 2
- 6.20 TS – Test Sample

7. EQUIPMENT AND REAGENTS

7.1 Equipment


“Substitute”, where indicated, means like for like replacement or equipment from another source.

- 7.1.1 Flow Cytometer, BD FACSLyric or FACSVerse, capable of blue and violet laser acquisition
- 7.1.2 Humidified incubator capable of maintaining 37±1 °C, 5±1 %CO₂
- 7.1.3 Microscope capable of 5x to 10x magnification
- 7.1.4 Hemocytometer (for manual counting) or an automated cell counter and compatible counting chambers
- 7.1.5 Water bath capable of maintaining 37±2 °C, or substitute
- 7.1.6 Centrifuge capable of maintaining 130 - 600 RCF
- 7.1.7 Biosafety Cabinet, Class II/A2
- 7.1.8 Refrigerator capable of maintaining 5±3 °C

7.2 Materials

“Substitute”, where indicated, means like for like replacement or materials from another source.

- 7.2.1 Multi-channel pipettes capable of delivering 2-300 µL
- 7.2.2 Serological pipettes capable of delivering between 1-100 mL

PHARMACEUTICAL SCIENCES		ANALYTICAL METHOD	
	TITLE: Determination of the In-vitro Expression of PF-07302048 by Flow Cytometry	NUMBER: TM100010380	
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- 7.2.3 Laboratory ware for handling volumes of samples and reagents, including appropriate nuclease-free containers
- 7.2.4 12-well Culture Plate: 12-well, cell-culture treated, polystyrene plates. ThermoFisher, Catalog No. 150628, or substitute
- 7.2.5 96-Well Assay Plate: 96-well, round-bottomed, polystyrene plates. ThermoFisher, Catalog No. 268200; Falcon, Catalog No. 353910 or substitute
- 7.2.6 Acquisition Plate: any 96-well, round-bottomed, polystyrene plate suitable for acquisition on the flow cytometer in use
- 7.2.7 Microtiter plate sealers
- 7.2.8 Nalgene Rapid Flow Sterile Filter Unit with PES Membrane, 500 mL, 0.2 µm, Thermo, Catalog No. 566-0020 or substitute
- 7.2.9 Tissue culture treated cell culture flasks, 75 and 175 cm², Falcon, Catalog No. 353136 and 353112 or substitutes
- 7.2.10 Aluminum foil
- 7.2.11 Ster-Ahol, Veltek, Catalog No. DSTER-WFI-TR-04, or 70% Ethanol, or 70% Isopropanol
- 7.2.12 Multichannel pipette reagent reservoirs
- 7.2.13 Paper towels or substitute absorbent material

7.3 Key Reagents


Key Reagents – commercially available “off-the-shelf” reagents: new lots must be qualified prior to use.

- 7.3.1 Primary Antibody: SARS-CoV-2 (2019-nCoV) Spike S1 Antibody, Rabbit IgG mAb, Sino Biological, Catalog No. 40150-R007. Store at -20±10 °C for one year after receipt.
 - 7.3.1.1 Vials may be thawed to create aliquots and stored at -20±10 °C with the original expiry. Once an aliquot is thawed, store at 5±3 °C for one month.
- 7.3.2 Secondary Antibody: Goat anti-Rabbit IgG (H+L) Cross-Absorbed Secondary Antibody, PE conjugated, ThermoFisher, Catalog No. P-2771MP. Store at 5±3 °C for six months.
- 7.3.3 HEK-293T (H293T) Cells, ATCC, Catalog No. CRL-3216. Store at <-125 °C. Cell thaw, propagation, and freeze instructions are found in Attachment A.
- 7.3.4 Fetal Bovine Serum (FBS) certified One Shot, ThermoFisher, Catalog No. A31604. Store at -20±10 °C. Alternatively heat inactivated FBS, certified One Shot, ThermoFisher, Catalog No. A38401.

7.4 Other Reagents

“Substitute” where indicated, means like-for-like replacement (same composition) of a reagent from another source.

- 7.4.1 Fixation and Permeabilization Solution, BD Biosciences, Catalog No. 554722, or substitute.
- 7.4.2 BD Permeabilization Wash Buffer (10x), BD Biosciences, Catalog No. 554723, or substitute.


PHARMACEUTICAL SCIENCES		ANALYTICAL METHOD	
	TITLE: Determination of the In-vitro Expression of PF-07302048 by Flow Cytometry	NUMBER: TM100010380	
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- 7.4.3 LIVE/DEAD Fixable Aqua Dead Cell Stain Kit, ThermoFisher, Catalog No. L34966, or substitute. Store at -20 ± 10 °C for six months.
- 7.4.4 Phosphate Buffered Saline, calcium and magnesium free, pH 7.4, (PBS-CMF) Corning, Catalog No. 21-040-CM, or substitute.
- 7.4.5 Transfection Dulbecco's Phosphate Buffered Saline, calcium and magnesium free, pH 7.4, (pH 7.4 DPBS) Corning, Catalog No. 21-031-CM, or substitute.
- 7.4.6 Cell Culture Dulbecco's Phosphate Buffered Saline, calcium and magnesium free, pH 7.2, (pH 7.2 DPBS) Gibco, Catalog No. 14190-144, or substitute.
- 7.4.7 Purified Water, Molecular biology grade, Corning, Catalog No. 46-000-CM, or substitute.
- 7.4.8 Trypan Blue (0.4% (w/v) Sigma, Catalog No. T8154, or substitute.
- 7.4.9 Accutase, Life Technologies, Catalog No. 00-4555-56, or substitute.
- 7.4.10 Dulbecco's Minimum Essential Medium with GlutaMax, (DMEM) Gibco, Catalog No. 10569-010, or substitute.
- 7.4.11 Recovery Cell Culture Freezing Medium, Gibco, Catalog No. 12648-010, or substitute.

7.5 Prepared Solutions

All quantities may be adjusted as necessary. Visually examine media for signs of contamination, discard if contamination is apparent.

- 7.5.1 H293T Growth Media, DMEM with 10% FBS
 - 7.5.1.1 Add approximately 250 mL of DMEM to a 500 mL filter unit.
 - 7.5.1.2 Add 50 mL of FBS One Shot.
 - 7.5.1.3 Add sufficient quantity of DMEM to make 500 mL.
 - 7.5.1.4 Filter through the 0.2 μ m filter unit.
 - 7.5.1.5 Store this solution at 5 ± 3 °C for up to 1 month.
- 7.5.2 1X Permeabilization Wash Buffer
 - 7.5.2.1 Add 20 mL of 10X Permeabilization Wash Buffer to 180 mL of Purified Water. Mix thoroughly to ensure homogeneity.
 - 7.5.2.2 Store at 5 ± 3 °C for up to 4 days.
- 7.5.3 Live/Dead Fixable Aqua Dead Cell Stain
 - 7.5.3.1 Remove stock vial of anhydrous DMSO solution (green cap) and a vial of lyophilized Live/Dead Aqua dye (blue cap) from LIVE/DEAD Fixable Aqua Dead Cell Stain Kit and allow to come to room temp.
 - 7.5.3.2 Confirm that DMSO is completely liquified before proceeding.
 - 7.5.3.3 Reconstitute lyophilized Live/Dead Aqua dye by adding 50 μ L of anhydrous DMSO solution to the tube.
 - 7.5.3.4 Pipette solution up and down to ensure proper reconstitution. Keep protected from light.

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- 7.5.3.5 Manufacturer suggests using the dye within the first 2 hours post reconstitution. Any remaining reconstituted material may be stored at -20 ± 10 °C, protected from light and moisture, for up to 2 weeks.

8. PROCEDURE


Handle cells, media, and other applicable assay materials and components in an aseptic manner. Visually examine assay media and cell culture for signs of contamination, discard if contamination is apparent.

Warm the Growth Media in a 37 ± 2 °C water bath for at least 15 minutes or at room temperature for at least 30 minutes prior to use.

Cells thawed from an AWCB can be cultured continuously following [Attachment A – Cell Culture](#) and used in this assay from passage 3 through passage 15.

8.1 Day 1: Seeding of Cells in 12-Well Culture Plates.

- 8.1.1 Aspirate the culture medium from the cell culture flask.
- 8.1.2 Rinse cells with 10 mL of pH 7.2 DPBS per flask.
- 8.1.3 Aspirate the pH 7.2 DPBS from the flask.
- 8.1.4 Add 2 mL of Accutase per T75 flask or 3 mL of Accutase per T175 and rock flask to coat cell layer with Accutase.
- 8.1.5 Incubate the flask for 2-3 minutes in a 37 ± 1 °C, $5\pm 1\%$ CO₂ incubator for detachment. Gently tap cell culture flask with hand to dislodge cells.
- 8.1.6 If cells have not detached, replace flasks in the incubator for an additional 5 minutes. Check that cells have detached by visual observation.
 - 8.1.6.1 If cells have not detached, discard the cells and restart Section [8.1](#) with a new flask. If a new flask is not available, do not proceed and notify a laboratory supervisor.
- 8.1.7 Add 10 mL of H293T Growth Medium to the flask. Rinse the flask by gently sheeting media against cell surface several times with a 10 mL pipette.
- 8.1.8 Transfer the cell suspension from each flask to an appropriately sized sterile conical tube.
- 8.1.9 Centrifuge the cells at 130 RCF for 5 minutes.
- 8.1.10 Aspirate the supernatant and re-suspend the cell pellet in 5 mL of H293T Growth Medium per T75 flask or 10 mL of H293T Growth Medium per T175 flask. Pipette the cells up and down to break up clumps with a serological pipette.
- 8.1.11 Prepare an appropriate dilution of cells for counting. For example, dilute 50 µL of the cell suspension with 350 µL of 0.4% Trypan Blue for counting manually with a hemocytometer. If an automated cell counter is used prepare a 0.1% Trypan Blue or other appropriate cell solution for the counter in use. For example, add 50 µL of 0.4% Trypan Blue, 100 µL of growth medium and 50 µL of the cell suspension.
- 8.1.12 Count live and dead cells using an automated cell counter or manually on a hemocytometer.

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- 8.1.13 Record the live cell density (cells/mL), the total cell density (cells/mL), and cell viability (%) for each count.

Note: If the cell viability is <80%, the cells should be discarded. If another flask is available restart Section 8.1 with a new flask. If a new flask is not available, do not proceed and notify a laboratory supervisor

- 8.1.14 Determine the number of 12-well Culture Plates needed for the assay. Four wells are required per test sample and eight wells are required in total for the controls. Round the calculated number up to the nearest integer.

$$\text{Number of plates} = \frac{(\text{\#of Samples} \times 4) + 8}{12 \text{ wells}}$$

- 8.1.15 Dilute the cell suspension using Growth Medium to 200,000 viable cells/mL based on the mean viable cell count using the equations below. A minimum volume of 13 mL is recommended per plate.

$$\text{Volume of Cell Suspension (mL)} = \frac{2.0 \times 10^5 (\text{Viable cells/mL}) \times \text{Volume Required (mL)}}{\text{Viable Cell Harvest Density (Viable cells/mL)}}$$

$$\text{Volume of Assay Medium Needed (mL)} = \text{Total Volume (mL)} - \text{Volume of Viable Cells needed (mL)}$$

- 8.1.16 Pipette 1 ml/well of the cell suspension into all wells of the Culture Plates.

- 8.1.17 Cover the Culture Plate(s) with the lid and incubate in a 37±1 °C, 5±1% CO₂ incubator for 16-24 hours.

Day 2: Transfection.

Prepare dilutions of DP Control, Test Samples (TSs), and Negative Control (NC) in appropriate nuclease-free containers (typically polypropylene).

DP materials containing mRNA should be wiped with 70% alcohol before being brought into the BSC. All DP solutions should be mixed gently by pipette and/or swirling. Do not invert, vortex or mix vigorously.

Volumes for dilutions of reagents and samples are recommended in the corresponding preparation instructions. More than one dilution may be needed to achieve the desired final concentration.

8.2 Preparation of TSs.

- 8.2.1 Recommended volume of TS for primary dilution is 10-20 µL.

- 8.2.2 Prepare a single TS target for all replicates.

- 8.2.3 Recommended final volume of working TS solution is at least 450 µL.


- 8.2.4 Dilute TS to 1.5 µg/mL target concentration with pH 7.4 DPBS using the equation below. Mix gently approximately 5 times by pipette to ensure homogeneity.

$$\text{Volume of TS (}\mu\text{L)} = \frac{1.5 (\mu\text{g/mL}) \times \text{Total Volume (}\mu\text{L)}}{\text{TS Stock Concentration (}\mu\text{g/mL)}}$$

- 8.2.5 Label each TS appropriately, for example: TS1, TS2, TS3, etc.

8.3 Preparation of DP Control (DPC).

- 8.3.1 Recommended volume of DP for primary dilution is 10-20 µL.

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8.3.2 Prepare a single DPC for all replicates.

8.3.3 Recommended final volume of the working DPC solution is at least 450 µL.

8.3.4 Dilute DPC to 1.5 µg/mL target concentration with pH 7.4 DPBS using the equation below. Mix gently approximately 5 times by pipette to ensure homogeneity.

$$\text{Volume of DPC (}\mu\text{L)} = \frac{1.5 \text{ (}\mu\text{g/mL)} \times \text{Total Volume (}\mu\text{L)}}{\text{DPC Stock Concentration (}\mu\text{g/mL)}}$$

8.3.5 Label appropriately, for example: DPC.

8.4 Preparation of Negative Control (NC).

8.4.1 Prepare a single NC for all replicates.

8.4.2 Add 450 µL of pH 7.4 DBPS to an appropriately sized nuclease-free container.

8.4.3 Label appropriately, for example: NC.

8.5 Cell Transfection Procedure.

8.5.1 After the Culture Plates have incubated at 37±1 °C, 5±1% CO₂ incubator for 16-24 hours, remove the plates from the incubator.

8.5.2 Visually confirm that the cells have attached and are evenly distributed throughout the well. Cells should be 70% - 90% confluent before proceeding with the transfection.

8.5.2.1 If the majority of the cells appear to be floating or rounded this is an indication of poor cell health. The plate should be discarded and the method terminated.

Pipette dropwise 100 µL/well of NC, DPC and TSs into quadruplicate wells of the Culture Plate(s) following the layout shown in

8.5.3 [Figure 2](#). See [Table 1](#) for assay target concentrations.

8.5.4 Gently swirl the Culture Plate(s) in a circular motion to ensure even dispersion of transfection solutions.

8.5.5 Cover the plate(s) and centrifuge at 550 ± 50 RCF for 6 ± 1 minutes at room temperature.

8.5.6 Place the covered plate(s) in a 37±1 °C, 5±1% CO₂ incubator for 21-24 hours.

Figure 2 – Culture Plate Layouts

Plate 1					Plate 2, etc.				
	1	2	3	4		1	2	3	4
A	NC	NC	NC	NC	A	TS2	TS2	TS2	TS2
B	DPC	DPC	DPC	DPC	B	TS3	TS3	TS3	TS3
C	TS1	TS1	TS1	TS1	C	TS4	TS4	TS4	TS4


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Table 1 – Target Concentrations and Concentrations of DPC and TSs

	Prepared Concentration (ng/mL)	Culture Plate Concentration (ng)
TS	1500	150
DPC	1500	150

Day 3: In-vitro Expression Assay


8.6 Cell Harvest.

- 8.6.1 After the transfected Culture Plates have incubated at 37 ± 1 °C, $5 \pm 1\%$ CO₂ in the incubator for 21-24 hours, remove the plates from the incubator.
- 8.6.2 Visually confirm that cells are attached and appear healthy in the wells.
 - 8.6.2.1 If the majority of cells appear to be floating or rounded this is an indication of poor cell health. The plate should be discarded and the method terminated.
- 8.6.3 Aspirate the Medium from each well of the Culture Plate(s).
- 8.6.4 Wash each well by adding 1 mL of pH 7.2 DPBS down the side of the well, being careful to not disrupt the cell monolayer.
- 8.6.5 Remove the pH 7.2 DPBS with an aspirating pipet, being careful to not disrupt the cell monolayer.
- 8.6.6 Pipette 100 µL of Accutase to each well.
- 8.6.7 Incubate cells in a 37 ± 1 °C, $5 \pm 1\%$ CO₂ incubator for 10 ± 5 minutes for detachment. Gently tap each plate to dislodge cells.
- 8.6.8 Add 150 µL of cold PBS-CMF to each well.
- 8.6.9 Tilt the Culture Plate(s) and gently rinse the cell surface of each well by pipetting with a P1000. Each well should be rinsed approximately 5 times.
- 8.6.10 Transfer the entire cell solution, approximately 250 µL, to a 96-Well Assay Plate. Appropriately record the positions of each TS, DPC and NC, see [Figure 3](#) for example.
 - 8.6.10.1 It is recommended to add cells to the 96-well plate starting in A1 and continuing across the rows. Do not skip wells while transferring into the assay plate and any unused Culture plate wells may be discarded.

Figure 3 - Example Assay Plate Layout

	1	2	3	4	5	6	7	8	9	10	11	12
A	NC	NC	NC	NC	DPC	DPC	DPC	DPC	TS1	TS1	TS1	TS1
B	TS2	TS2	TS2	TS2	TS3	TS3	TS3	TS3	TS4	TS4	TS4	TS4
C	TS5	TS5	TS5	TS5	TS6	TS5	TS6	TS6	TS7	TS7	TS7	TS7
D	TS8	TS8	TS8	TS8	TS9	TS9	TS9	TS9	TS10	TS10	TS10	TS10
E	TS11	TS11	TS11	TS11	TS12	TS12	TS12	TS12	TS13	TS13	TD13	TS13
F	TS14	TS14	TS14	TS14	TS15	TS15	TS15	TS15	TS16	TS16	TS16	TS16
G	TS17	TS17	TS17	TS17	TS18	TS18	TS18	TS18	TS19	TS19	TS19	TS19
H	TS20	TS20	TS20	TS20	TS21	TS21	TS21	TS21	TS22	TS22	TS22	TS22

- 8.6.11 Centrifuge the Assay Plate at 550 ± 50 RCF for 6 ± 1 minutes at ambient temperature.

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8.6.12 Remove the supernatant either by pipette or by inverting the plate and gently flicking the supernatant into an appropriate waste container. Gently blot any residual liquid from the plate onto a piece of fresh paper towel.

8.7 Live/Dead Stain

8.7.1 Dilute Live/Dead dye 1/500 in PBS-CMF using the equation below. A minimum volume of 5 mL per assay plate is recommended. Invert several times to ensure homogeneity.

$$\text{Volume of Live/Dead Dye } (\mu\text{L}) = \frac{\text{Total Volume } (\mu\text{L})}{\text{Dilution Factor}}$$

8.7.2 Pipette 50 μL of the Live/Dead dye working stock to each of the wells used in the experiment.

8.7.3 Gently resuspend the cell pellet by pipetting up and down 5 times.

8.7.4 Wrap plate in foil and allow to incubate at room temperature for 45 ± 15 minutes.

8.7.5 After incubation pipette 200 μL of 1x Permeabilization Wash Buffer to each of the wells used in the experiment.

8.7.6 Centrifuge the 96-Well assay plate at 550 ± 50 RCF for 6 ± 1 minutes at ambient temperature.

8.7.7 Remove the supernatant either by pipette or by inverting the plate and gently flicking the supernatant into an appropriate waste container. Gently blot any residual liquid from the plate onto a piece of fresh paper towel

8.8 Cell Fixation and Permeabilization.

8.8.1 Pipette 100 μL of Fixation and Permeabilization solution to each of the wells used in the experiment.

8.8.2 Gently pipette solution up and down 5 times to resuspend cell pellet.

8.8.3 Wrap plate in foil and allow to incubate at 5 ± 3 °C for 20-25 minutes.

8.8.4 After incubation pipette 150 μL of 1x Permeabilization Wash Buffer to the wells used in the experiment.

8.8.5 Centrifuge the 96-Well assay plate at 550 ± 50 RCF for 6 ± 1 minutes at ambient temperature.


8.8.6 Remove the supernatant either by pipette or by inverting the plate and gently flicking the supernatant into an appropriate waste container. Gently blot any residual liquid from the plate onto a piece of fresh paper towel.

8.8.7 Pipette 250 μL of 1x Permeabilization Wash Buffer to the wells used in the experiment.

8.8.8 Centrifuge the 96-Well assay plate at 550 ± 50 RCF for 6 ± 1 minutes at ambient temperature.

8.8.9 Remove the supernatant either by pipette or by inverting the plate and gently flicking the supernatant into an appropriate waste container. Gently blot any residual liquid from the plate onto a piece of fresh paper towel.

8.9 Staining Protocol

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- 8.9.1 Dilute Primary Antibody 1/2,000 in 1x Permeabilization Wash Buffer using the equation below. A minimum volume of 10 mL per assay plate is recommended. Invert several times to ensure homogeneity.

$$\text{Volume of Spike S1 Primary Antibody } (\mu\text{L}) = \frac{\text{Total Volume } (\mu\text{L})}{\text{Dilution Factor}}$$

- 8.9.2 Pipette 50 μL of working Primary Antibody solution to each of the wells used in the experiment.
- 8.9.3 Gently pipette solution up and down 5 times to resuspend cell pellet. Avoid the creation of bubbles during mixing.
- 8.9.4 Wrap plate in foil and allow to incubate at 5 ± 3 °C for 45 ± 15 minutes.
- 8.9.5 After incubation pipette 200 μL of 1x Permeabilization Wash Buffer to the wells used in the experiment.
- 8.9.6 Centrifuge the 96-Well assay plate at 550 ± 50 RCF for 6 ± 1 minutes at ambient temperature.
- 8.9.7 Remove the supernatant either by pipette or by inverting the plate and gently flicking the supernatant into an appropriate waste container. Gently blot any residual liquid from the plate onto a piece of fresh paper towel.
- 8.9.8 Pipette 250 μL of 1x Permeabilization Wash Buffer to the wells used in the experiment.
- 8.9.9 Centrifuge the 96-Well assay plate at 550 ± 50 RCF for 6 ± 1 minutes at ambient temperature.
- 8.9.10 Remove the supernatant either by pipette or by inverting the plate and gently flicking the supernatant into an appropriate waste container. Gently blot any residual liquid from the plate onto a piece of fresh paper towel.
- 8.9.11 Dilute Secondary Antibody 1/1,000 in 1x Permeabilization Wash Buffer using the equation below. A minimum volume of 5 mL per assay plate is recommended. Invert several times to ensure homogeneity.

$$\text{Volume of PE conjugated Secondary Antibody } (\mu\text{L}) = \frac{\text{Total Volume } (\mu\text{L})}{\text{Dilution Factor}}$$

- 8.9.12 Pipette 50 μL of working Secondary Antibody solution to each of the wells used in the experiment.
- 8.9.13 Gently pipette solution up and down 5 times to resuspend cell pellet. Avoid the creation of bubbles during mixing.
- 8.9.14 Wrap plate in foil and allow to incubate at 5 ± 3 °C for 30-35 minutes.
- 8.9.15 After incubation pipette 200 μL of 1x Permeabilization Wash Buffer to the wells used in the experiment.
- 8.9.16 Centrifuge the 96-Well assay plate at 550 ± 50 RCF for 6 ± 1 minutes at ambient temperature.
- 8.9.17 Remove the supernatant either by pipette or by inverting the plate and gently flicking the supernatant into an appropriate waste container. Gently blot any residual liquid from the plate onto a piece of fresh paper towel.

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- 8.9.18 Pipette 250 µL of 1x Permeabilization Wash Buffer to the wells used in the experiment.
- 8.9.19 Centrifuge the 96-Well assay plate at 550 ± 50 RCF for 6 ± 1 minutes at ambient temperature.
- 8.9.20 Remove the supernatant either by pipette or by inverting the plate and gently flicking the supernatant into an appropriate waste container. Gently blot any residual liquid from the plate onto a piece of fresh paper towel.
- 8.9.21 Pipette 200 µL of 1x Permeabilization Wash Buffer into each well used in the experiment. Gently pipet solution up and down 5 times to resuspend cell pellet.
- 8.9.22 If the flow cytometer used for acquisition does not accept the assay plate, 200 µL of cell suspension may be transferred from each well to the corresponding well of a suitable acquisition 96-well plate.
- 8.9.23 Seal the assay plate with a plate sealer and wrap in foil to protect from light.
- 8.9.24 Proceed to flow cytometer acquisition or store the assay plate at 5 ± 3 °C for up to 7 days before proceeding to acquisition.

8.10 Flow Cytometer Acquisition

- 8.10.1 Power on the flow cytometry instrument and log into the FACSuite (RUO) Software.
- 8.10.2 Perform the daily clean and the performance QC (PQC) by following SOP-INS-05140.
- 8.10.3 Run or Update the Assay/Tube settings.

Note: If an Assay needs to be set up or changed, refer to [Attachment B – Flow Cytometer assay Setup](#).

 - 8.10.3.1 In the Setup & QC tab select “Assay/Tube settings” from the drop-down menu.
 - 8.10.3.2 Ensure the correct CS&T bead lot is selected.
 - 8.10.3.3 Click the “Select” button and check the box next to “Generate Reports” for the appropriate assay designated for this method.
 - 8.10.3.4 Select “Start”.
 - 8.10.3.5 If this fails or passes with warnings, contact a supervisor or method owner before continuing with the procedure.
- 8.10.4 In the Worklist tab, create a new Worklist.
 - 8.10.4.1 Change the Universal Loader type to the appropriate type. For example “96 well plate standard round bottom PS”.
 - 8.10.4.2 In the Task drop down menu, select the appropriate assay task designated for this method.
 - 8.10.4.3 Insert one entry for each control and one for each test sample. Label as appropriate:
For example: Notebook reference_identity
(00712294-0250_NC or 00712294-0250_TS1)
 - 8.10.4.4 The Acquisition Box for each entry should look like that shown in [Figure 4](#).


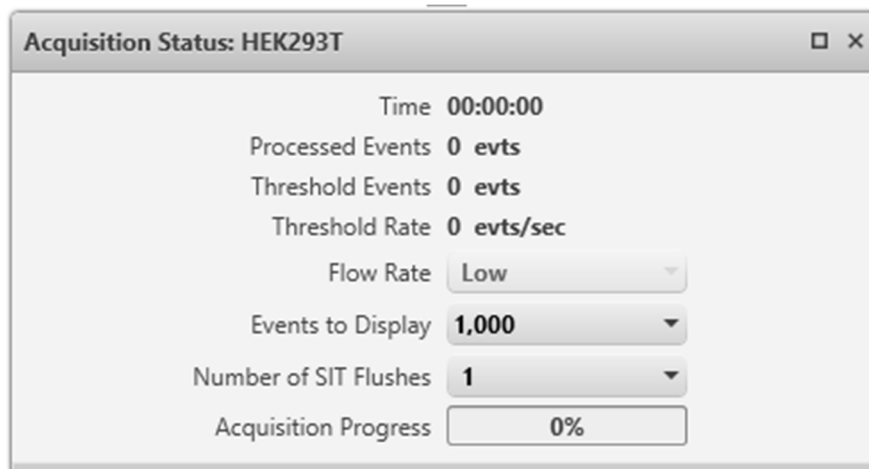
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Figure 4 – FACS Suite Acquisition Box



8.10.4.5 If working on the FACSVerse, right click on a task and select “Enable Audit Trail”. This is automatic on the FACSlyric.

8.10.4.6 Rename the Worklist (File → Rename) to include pertinent information. For example, rename to the notebook reference number.

8.10.4.7 Load the assay plate in the Universal Loader and close the lid.

8.10.4.8 From the Run drop down select “Run All”.

8.10.5 Data analysis

8.10.5.1 The exact shape and locations of gates are expected to be different between instruments. All gates should be properly shaped and sized to select for the relevant cell populations shown in the Figures.

8.10.5.2 Once the data have been collected for all wells, ensure that the P1 and P2 gates are appropriate.

8.10.5.2.1. The P1 gate should encompass the entire viable cell population as seen in [Figure 5](#).


8.10.5.2.2. The P2 gate should encompass the entire single cell population as seen in [Figure 5](#).

8.10.5.2.3. If any gate is changed, in the Audit Trail drop down select “Provide Reason for Change” and document the reason for the gate change. For example: moved P1 gate to encompass the entire population.

8.10.5.3 The P3 gate should be adjusted to define which cells are positive for the S1 antigen (S1+) in the PE channel as follows:

8.10.5.3.1. The gate should be set at average of 4% S1+ in the four NC well histograms and dot plots, like the gate seen in [Figure 5](#).

8.10.5.3.2. If any of the NC dot plots show an abnormal population of positive cells like that seen in [Figure 8](#), the assay will fail visual assessment for the NC per Section [10.3](#).

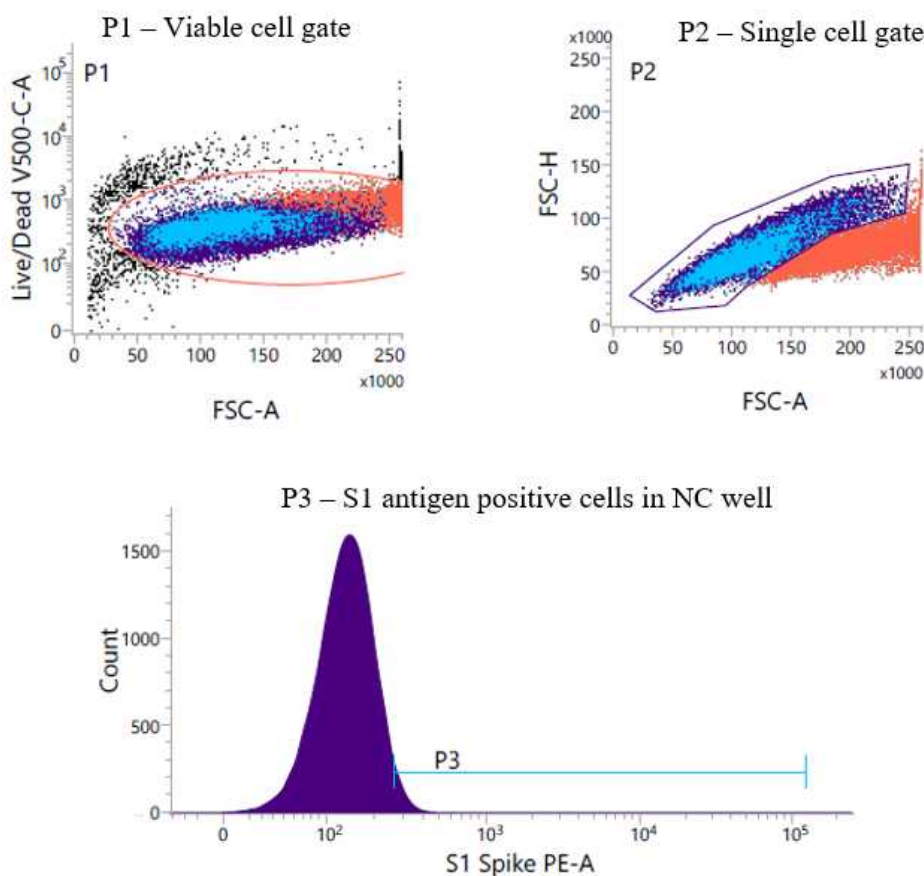
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8.10.5.3.3. When the P3 gate is finalized it should be applied to all wells of the assay and the appropriate reason documented in the audit trail.

8.10.5.3.4. If any of the DPC histograms show an abnormal double peak like that seen in [Figure 8](#), the assay will fail visual assessment for the DPC per [Section 10.3](#).

8.10.5.4 Approve each individual well by selecting “Approve”.

Figure 5 – Representative Cell Population Gates



8.11 Results

8.11.1 The data report from each well is exported as a pdf including the plots and the well statistics, see [Figure 6](#) and [Figure 7](#) for examples.


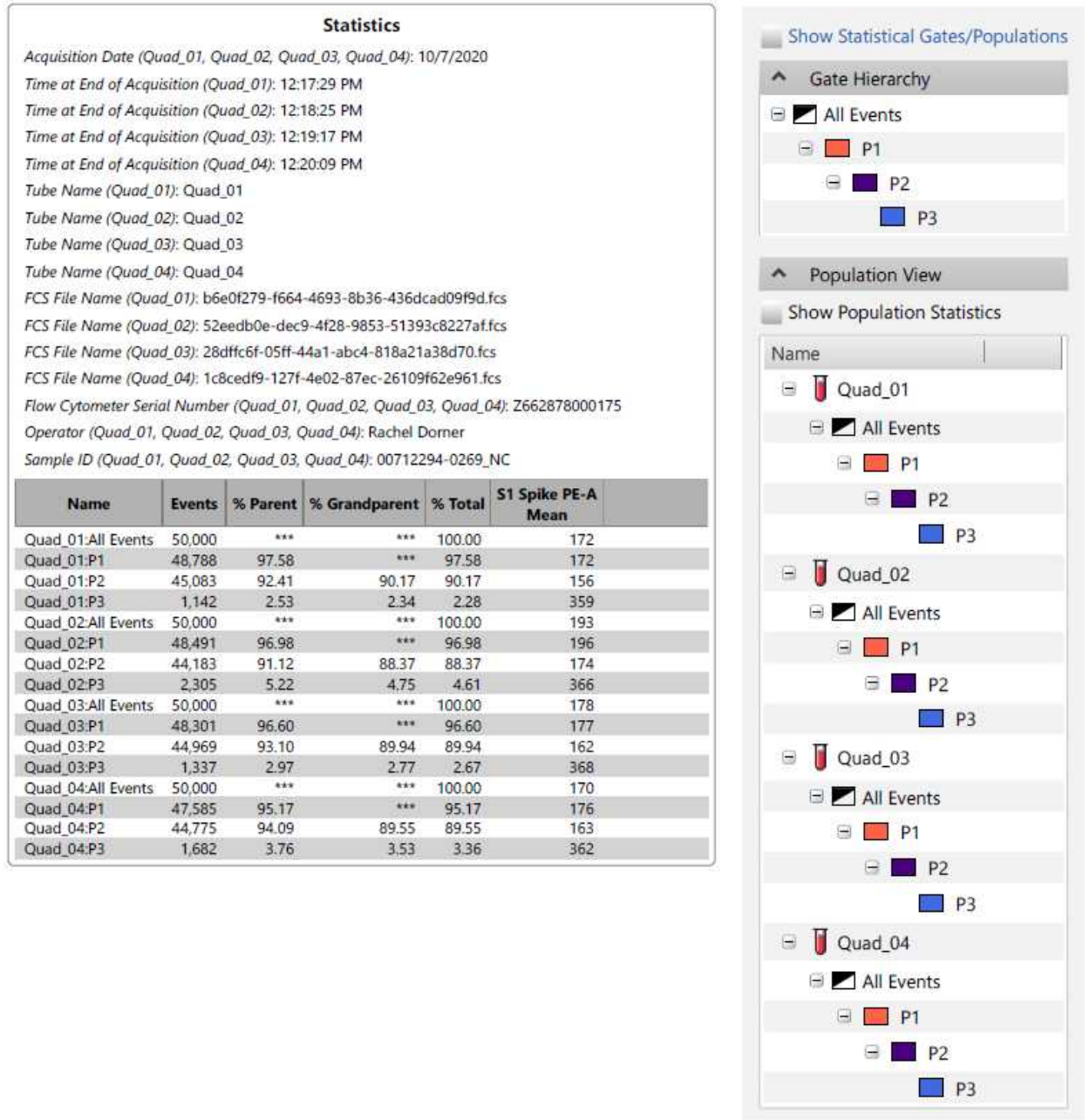
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Figure 6 – Example NC Statistics Box and Hierarchy




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Figure 7 - Representative Flow Cytometry Plots

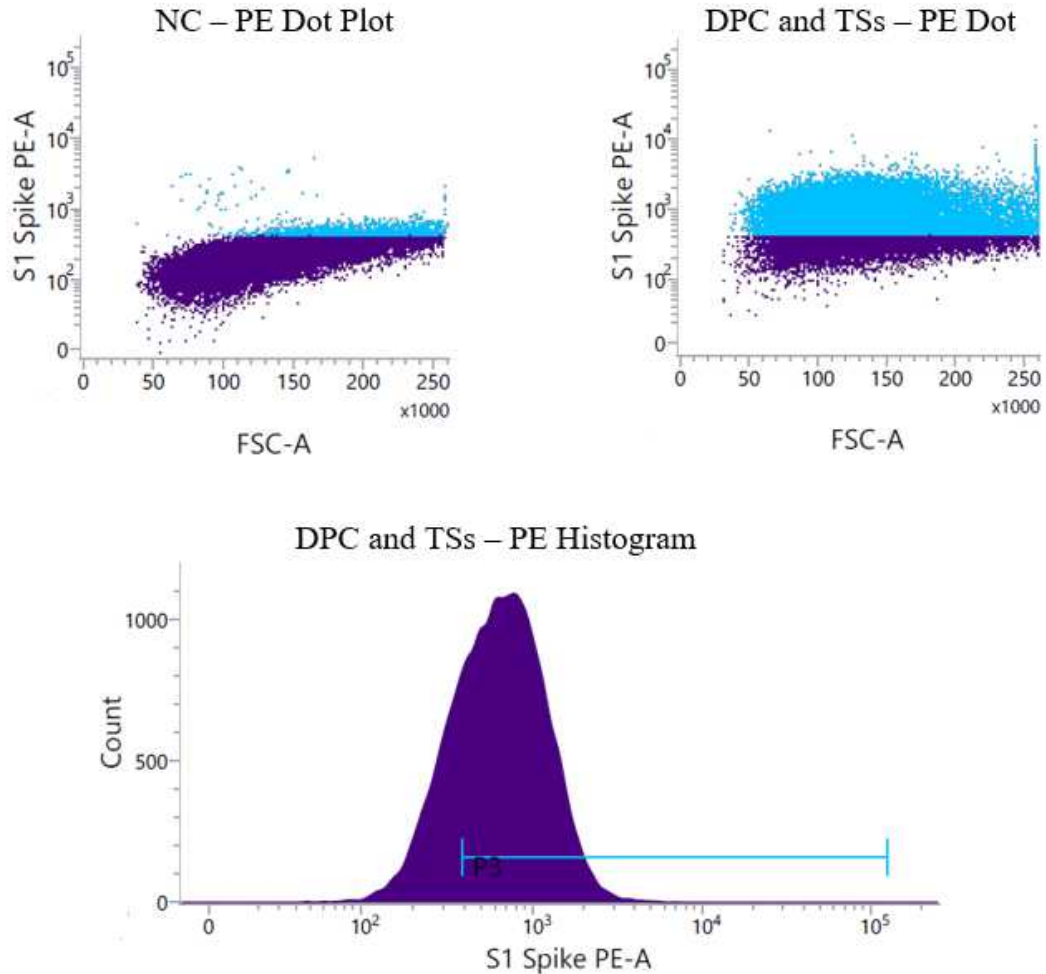
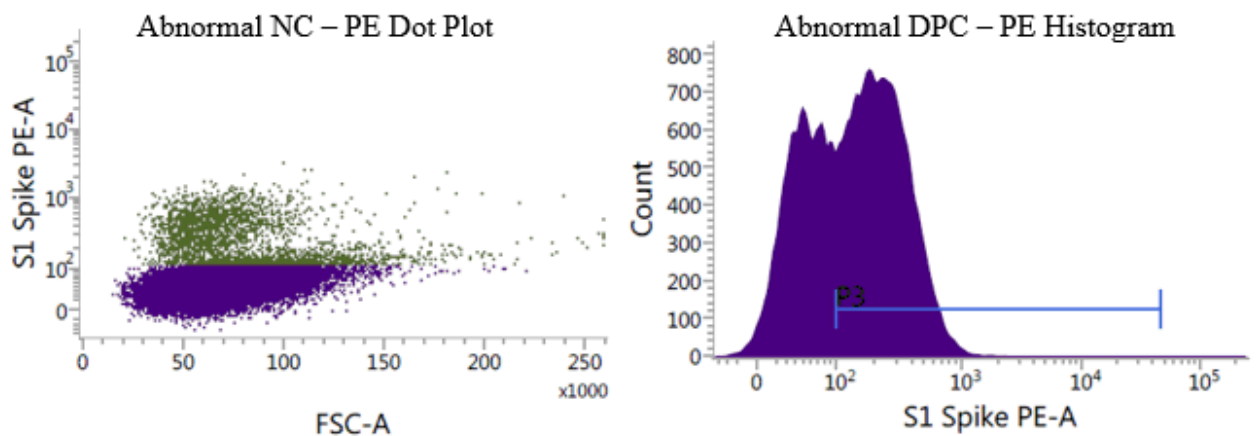



Figure 8 - Examples of Anormal Data



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9. CALCULATIONS AND FORMULAE

Data analysis may be performed manually for calculations as well as assay and sample acceptance criteria evaluations.

The calculations for the assay include the following steps in sequence. 1) Mean S1 positive cells of the replicates; 2) Mean PE channel MFI of the viable, single cell replicates.

9.1 Mean S1+ expression of the cell replicates for each concentration and each control.

9.1.1 Use the “% Parent” of the P3 population for this calculation. For example: in [Figure 6](#) the values for the quadruplicates are 2.53, 5.22, 2.97 and 3.76.

9.1.2 Calculate the mean S1+ expression (%), % of Parent as follows:

$$\text{Mean}_{S1+} = \frac{S1^{+}_{\text{Rep1}} + S1^{+}_{\text{Rep2}} + S1^{+}_{\text{Rep3}} + S1^{+}_{\text{Rep4}}}{4}$$

10. ASSAY ACCEPTANCE CRITERIA

These criteria are for evaluating that each assay has been performed correctly and that the systems (instrumentation, reagents, etc.) are performing properly as defined during method development. These criteria are not evaluating whether a test sample meets the target or specification limits. If any of the assay acceptance criteria are not met, this is evidence that the assay performance was not typical, therefore no results are generated, and the assay must be repeated.

Assay acceptance criteria evaluation includes the following steps in sequence: 1) Acquired Events Criteria; 2) %Viability Criteria.

10.1 Acquired cell count (“Events” for All Events population in [Figure 6](#)) is not less than 30,000 and not more than 50,000 total events for all NC and DPC wells. For example: in [Figure 6](#) this value is 50,000 for all quadruplicates.

10.2 Cell viability, the “% Parent” of the P1 population in [Figure 6](#), is $\geq 90\%$ for all NC and DPC wells. For example: in [Figure 6](#) these values are 97.58, 96.98, 96.60 and 95.17.

10.3 All replicates of the NC and DPC should look comparable to each other, respectively, and be visually comparable to [Figure 5](#) and [Figure 7](#).


10.4 The mean S1+ (%) for the DPC is $\geq 30\%$.

11. SAMPLE ACCEPTANCE CRITERION

This is for evaluating that each Test Sample (TS) has been prepared and tested properly. Each TS is evaluated separately against this criterion. If any sample acceptance criterion is not met for any TS, this is evidence that performance was not typical therefore no result is generated, and the sample test must be repeated.

11.1 Acquired cell count (“Events” for All Events population in [Figure 6](#)) is not less than 30,000 and not more than 50,000 total events for each well.

11.2 Cell viability, the “% Parent” of the P1 population in [Figure 6](#), is $\geq 90\%$ for each well.

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- 11.3 All replicates of each TS concentration should look comparable to each other and be visually comparable to [Figure 7](#).

12. REPORTING OF RESULTS

- 12.1 If the criteria in Section [10](#) and Section [11](#) are met and the TS meets the in-vitro expression criterion, report the mean S1+ (%) cell population for each TS to the nearest whole number.
- 12.1.1 The mean S1+ (%) for each TS is $\geq 30\%$.
- 12.2 If a test sample does not meet the in-vitro expression criteria, initiate lab investigation. If retesting is initiated because there is no assignable root cause identified during a laboratory investigation, use the following steps to assess retest results.
- 12.2.1 If any of the independent retest results are $< 30\%$, the original suspect result is confirmed. The reportable result will be the original suspect result. The retest results are treated as supportive data.
- 12.2.2 If all of the retest results are $\geq 30\%$, the original suspect result is not confirmed. The reportable result will be derived from the average of the retest results.

13. REFERENCES


- 13.1 GDMS Report VAL100122803. Qualification of PF-07302048 Bioassay: Determination of the In-vitro Expression of PF-07302048 by Flow Cytometry.
- 13.2 GDMS SOP-INS-05140. Procedure for Operation, Maintenance and Calibration of the BD FACSVerse and BD FACSLyric Flow Cytometers, BTx-ARD.
- 13.3 GDMS INX100431112. Statistical Analysis for Setting the Limit for TM10001080 the Determination of the In-vitro Expression of PF-07302048 by Flow Cytometry.

14. LIST OF ATTACHMENTS


- Attachment A Cell Culture
- Attachment B Flow Cytometer Assay Setup

15. REVISION HISTORY

SUMMARY OF CHANGES	JUSTIFICATION OF CHANGE
1) Section 7.2.5 – optional substitutional assay plate added	1) Updated for clarification
2) Section 7.3.1.1 – added for reagent aliquoting	2) Updated for clarification
3) Section 7.3.4 – catalog number corrected	3) Typographical error
4) Section 7.5.1.4 – filter size corrected	4) Typographical error
5) Section 7.5.3.4 – mixing adjusted	5) Updated for clarification
6) Section 8 – reference to Attachment A added	6) Updated for clarification
7) Section 8.1.5 – incubation time adjusted to match Attachment A	7) Updated for consistency


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SUMMARY OF CHANGES	JUSTIFICATION OF CHANGE
8) Section 8.1.11 – updated recommendation for automated cell counter counting	8) Updated for clarification
9) Sections 8.1.14, 8.2.4, 8.3.4, 8.10.4.3 – changed for single target concentration per sample and control	9) INX100431112
10) Section 8.1.15 – sentence specifying mixing removed	10) Updated for clarification
11) Sections 8.2.5, 8.3.5, Figure 2– labeling updated	11) Updated for consistency
12) Figure 2 – example plate 3 added	12) Updated for clarification
13) Section 8.6.3 – growth removed	13) Updated for clarification
14) Section 8.6.10 – transfer volume change to the entire cell suspension	14) Updated for clarification
15) Figure 3 and Section 8.6.10 – Example plate layout added as new figure with reference	15) Updated for clarification
16) Section 8.6.11 – instruction to not skip assay wells and to discard unused culture wells added	16) Updated for clarification
17) Sections 8.7.4 and 8.9.4 – incubation range rewritten	17) Updated for consistency
18) Sections 8.9.1 and 8.9.11 – per plate added to volume recommendations	18) Updated for clarification
19) Figures 4, 5, 6, 7 – numbering and cross references updated	19) Update for figure addition
20) Section 8.10.4.3 – naming updated to example	20) Updated for clarification
21) Section 8.10.5.1 – statement added to acknowledge that gate shapes and size may vary between instruments	21) Updated for clarification
22) Section 8.10.5.1.3 – i.e. changed to for example	22) Updated for consistency
23) Section 8.10.5.2.1, 8.10.5.3.2, 8.10.5.3.4 and Figure 8 – added for examples of abnormal data	23) Updated for clarification
24) Section 9.1.1, 10.1, 10.2, Figure 6, Attachment B – updated for quadruplicate assay analysis	24) Method optimization
25) Section 9.2 – removed as MFI is not reported	25) Updated for clarification
26) Sections 10.1, 10.2, 10.3 – both wells changed to all wells	26) Updated for clarification

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SUMMARY OF CHANGES	JUSTIFICATION OF CHANGE
27) Sections 10.4, 11.4, 12.1, 12.2, 12.3, 13.3 – limit added for DPC and test samples. Reporting updated for limit test.	27) Method optimization
28) Section 12 – reporting limit criteria added with instructions if the criteria are not met	28) INX100431112
29) Attachment A – statement for allowed modification removed	29) Updated for consistency

-END OF PROCEDURE-

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ATTACHMENT A – CELL CULTURE

All manipulations of cells and medium must be performed inside a suitable BSC using aseptic technique. Visually examine growth medium and cell culture for contamination and discard if contamination is apparent.

Documented modifications of the instructions outlined in this section are permitted. The suitability of the cell culture for its intended purpose is established by meeting assay acceptance criteria defined in the method.

THAWING OF CELLS

If more than one cryovial is being thawed, adjust volumes and number of tubes accordingly.

Prewarm Growth Medium in a 37 ± 2 °C water bath for at least 15 minutes or at room temperature for at least 30 minutes prior to use.

Remove cryovial(s) of cells from liquid nitrogen (LN2) storage and thaw by gently agitating the cryovial(s) in a water bath set at 37 ± 2 °C. Keep vial caps and O-rings above the water line to avoid contamination.

Thawing should be rapid and once the cells are completely thawed remove them immediately from the water bath and proceed without delay to avoid damaging cells that may be sensitive to the cryoprotective agents.

Dry and wipe exterior of cryovial with Ster-Ahol.

Transfer thawed cells to a T75 tissue culture flask containing 15 mL of Growth Medium.

Prepare an appropriate dilution of cells for counting. For example, dilute 50 µL of the cell suspension with 50 µL of 0.4% Trypan Blue for counting manually with a hemocytometer or with an automated cell counter.

Count live and dead cells using an automated cell counter or manually on a hemocytometer.

Record the live cell density (cells/mL), the total cell density (cells/mL), and cell viability (%) for each count.

Label flasks minimally with cell name, passage number, analyst initials and date. Cells placed in culture from thaw should be labeled as passage 0.

Incubate flask(s) horizontally in a humidified incubator at 37 °C, 5% CO₂ overnight.

The following day, prewarm Growth Medium in a 37 ± 2 °C water bath for at least 15 minutes prior to use.

Aspirate growth media from tissue culture flask(s).

Add 15 mL of Growth Medium to each flask. Avoid adding medium directly to cell monolayer.


Incubate flask(s) horizontally in a humidified incubator at 37 °C, 5% CO₂ for 1-2 additional days.

Once cells reach 70% - 90% confluency, harvest and passage cells as outlined in the next section.

CULTURING OF CELLS

As long as no contamination or cell viability issues arise, the cells may be maintained in continuous culture until passage 15. If at any time during culture cell growth seems atypical, cells should be discarded.

Prewarm Growth Medium in a 37 ± 2 °C water bath for at least 15 minutes or at room temperature for at least 30 minutes prior to use.

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Although cells can be grown in a variety of flasks and conditions, use the recommendations below as needed and document each harvest and passage condition for monitoring the culture.

Attachment A - Table 1 Standard Working Flask Volumes

Flask Size	Recommended DPBS Volume, (mL)	Recommended Accutase Volume, (mL)	Recommended Accutase Quench Volume, (mL)	Recommended Seeding Density (Viable Cells/cm ²)	Recommended Total Volume (mL)
T-75	10	2	10	7,000 – For 4 day culture	15
T-175		3		16,000 – For 3 day culture 36,000 – For 2 day culture	30

Cell Passage Process:

Cells are easily dislodged from the flask surface. Care should be taken to pipette gently and avoid pipetting solutions directly onto the cell monolayer.

Cells should be approximately 70% - 90% confluent at the time of harvest.

Aspirate growth media from tissue culture flask(s).

Add 10 mL of pH 7.2 DPBS and rock flask to rinse the cell monolayer.

Aspirate the pH 7.2 DPBS.

Add an appropriate amount of Accutase according to [Attachment A - Table 1](#) and rock flask to rinse the cell monolayer.

Incubate flask at 37±2 °C for approximately 2-3 minutes to allow for cell detachment.

Gently rock the flask to dislodge cells.

Add 10 mL of growth medium to dilute the Accutase harvest.

Gently pipette along the cell surface several times with a 10 mL serological pipette and then transfer cell suspension to appropriately sized conical centrifuge tube(s).

Centrifuge at 130 RCF for 5 minutes.

Aspirate the supernatant and suspend the cell pellet in 5 mL per T-75 or 10 mL per T-175 of growth medium. Pipette up and down to mix suspension prior to counting cells.


Prepare an appropriate dilution of cells for counting. For example, dilute 50 µL of the cell suspension with 350 µL of 0.4% Trypan Blue for counting manually with a hemocytometer. If an automated cell counter is used prepare a 0.1% Trypan Blue or other appropriate cell solution for the counter in use. For example, add 50 µL of 0.4% Trypan Blue, 100 µL of growth medium and 50 µL of the cell suspension.

Count live and dead cells using an automated cell counter or manually on a hemocytometer.

Record the live cell density (cells/mL), the total cell density (cells/mL), and cell viability (%) for each count.

Calculate the appropriate volume of cell suspension for each tissue culture flask to achieve a cell density that corresponds to number of days in culture before next subculture.

$$\text{Volume of Cell Suspension (mL)} = \frac{\text{Viable Cell Density of Subculture (cells/cm}^2\text{)} \times \text{Flask Size (cm}^2\text{)}}{\text{Viable Cell Harvest Density (Viable cells/mL)}}$$

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Add the appropriate volume of growth medium and cell suspension to achieve the appropriate seeding density of each flask.

Label flasks minimally with cell name, passage number, and date. Include seeding density when appropriate.

Incubate flask(s) horizontally in a humidified incubator at 37 ± 2 °C, 5 ± 1 %CO₂.

FREEZING OF CELLS

Keep thawed Recovery Cell Freezing Medium on ice or at 5 ± 3 °C until use. Prewarm Growth Medium in a 37 ± 2 °C water bath for at least 15 minutes or at room temperature for at least 30 minutes prior to use.

Culture cells as described above to expand culture to at least 25 T-175 tissue culture flasks. This should yield enough cells for approximately 150 vials based on the recommended target cell density and volume/vial.

Freezing Process:

Pre-label cryovials with the site appropriate information: cell line with bank designation and reference number, passage number, cell concentration, freezing date, and initials.

Harvest cells with pH 7.2 DPBS rinse and Accutase as described above and pool in the appropriate number of 250 mL conical tubes for counting.

Centrifuge cells at approximately 130 RCF for 5 minutes.

Aspirate supernatant and suspend the cell pellet in 7 mL of growth medium per T-175.

Count live and dead cells using an automated cell counter or manually on a hemocytometer using an appropriate dilution.

Record the live cell density (cells/mL), the total cell density (cells/mL), and cell viability (%) for each count.

Centrifuge cells at approximately 130 RCF for 5 minutes.

Aspirate supernatant and suspend the cell pellet in an appropriate volume of cold Recovery Cell Freezing Medium to achieve a cell density of approximately 1.0×10^7 cells/mL.

$$\text{Resuspension Volume (mL)} = \frac{\text{Total Cells Counted (cells)}}{1.0 \times 10^7 \text{ Target Density (Viable cells/mL)}}$$


Dispense 1.0 mL of cell suspension into each of the labeled cryovials on ice. Gently mix cell suspension periodically during the filling process to prevent settling of cells.

Transfer the vials to covered Styrofoam racks or slow freeze containers.

Place the rack(s) in a -75 ± 15 °C freezer overnight.

Within 3 days, transfer frozen cryovials to a liquid nitrogen freezer (≤ -130 °C) quickly to prevent thawing, which may cause variation in culture viability.

New Analytical Working Cell Banks (AWCBs) must be tested for mycoplasma and be bridged prior to use in a GMP assay.

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ATTACHMENT B – FLOW CYTOMETER ASSAY SETUP

If an Assay cannot be exported/imported from one instrument to another, a new Assay must be created. To create an Assay in the FACS Suite software follow the procedure below and refer to SOP-INS-05140 for additional guidance.

UPDATE THE LIBRARY

Update the library to include lots of Aqua Live/Dead Fixable Dye and of SARS-CoV-2 Spike S1 Antibody.

If the reagents are not already present in the library, select **Beads and Reagents**, then select **Reagents**.

Select **Add Reagent**.

Fill out the reagent panel for the type of reagent being added according to the below table and select **Done**.

Attachment B - Table 2 Library Reagent Entry

Entry Title	Aqua Live/Dead Fixable Dye	Spike S1 antibody
Product Name	Aqua Live/Dead Fixable Dye	Spike S1 Antibody
Single Color	checked	checked
Fluorochrome	V500-C	PE
Label	Live/Dead	S1 Spike

To add a new lot select the Aqua Live/Dead Fixable Dye reagent and select **Add Lot**.

Enter the lot ID and expiration date and select **Done**.

It is not necessary to add lots for the Spike S1 Antibody reagent as it is dependent on lots of both the S1 and the PE antibodies.

CREATE TUBE SETTINGS

In the Experiments workspace select **New**.


Rename the Experiment (File → Rename) to include pertinent information. For example: TM100010380 Settings.

Right click on **Tube_001** and open the Tube Properties box.

In the **General** tab, name the Tube Name to “Quad_01” and ensure the Tube Settings are set to “Lyse Wash”.

Set up the **Parameters**, **Acquisition** and **Reagents** tabs according to Attachment B Figure 1 below.

Ensure that the appropriate boxes are checked for the Parameters, that the current lots are selected for the Reagents and the Acquisition settings and rules match the figure.

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Attachment B - Figure 3 Tube Properties Setup

Tube Properties - HEK293T

General Parameters Spillover Values Reagents Keywords Acquisition

Threshold Operation ☐ And ☒ Or

Name	A	H	W	Voltage	Threshold
FSC	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>	<input type="checkbox"/>	101.6	<input checked="" type="checkbox"/> 10,000
SSC	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>	<input type="checkbox"/>	415.0	<input type="checkbox"/> 5,000
FITC	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>	<input type="checkbox"/>	473.1	<input type="checkbox"/> 5,000
PE	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>	<input type="checkbox"/>	366.5	<input type="checkbox"/> 5,000
PerCP-Cy5.5	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>	<input type="checkbox"/>	544.7	<input type="checkbox"/> 5,000
PE-Cy7	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>	<input type="checkbox"/>	557.2	<input type="checkbox"/> 5,000
APC	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>	<input type="checkbox"/>	625.0	<input type="checkbox"/> 5,000
APC-R700	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>	<input type="checkbox"/>	497.4	<input type="checkbox"/> 5,000
APC-Cy7	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>	<input type="checkbox"/>	565.4	<input type="checkbox"/> 5,000
V450	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>	<input type="checkbox"/>	527.6	<input type="checkbox"/> 5,000
V500-C	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>	<input type="checkbox"/>	303.7	<input type="checkbox"/> 5,000
BV605	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>	<input type="checkbox"/>	498.8	<input type="checkbox"/> 5,000

Add Remove

Close

Tube Properties - HEK293T

General Parameters Spillover Values Reagents Keywords Acquisition

Worksheet to Display during Acquisition:
Storage Gate: ☒ All Events

Stopping Rules Advanced

Time Stopping Rule
Max Time 400 Seconds

Create Gate Criteria
Gate: ☒ All Events Events: 10,000 Add Criteria

Combine Gate Criteria and Apply Rule
All Events: 50,000
And Or Apply Rule Delete

Applied Stopping Rule
[Max Time: 400] OR [All Events: 50,000]

Close

Tube Properties - HEK293T

General Parameters Spillover Values Reagents Keywords Acquisition

Name	Label	Lot ID
FITC		
PE	S1 Spike	
PerCP-Cy5.5		
PE-Cy7		
APC		
APC-R700		
APC-Cy7		
V450		
V500-C	Live/Dead	2157152 (4/21/2022)
BV605		

Close


Close the tube properties box.

In the **Acquisition Box** set the flow rate to Low and the SIT flushes to 1, refer to method [Figure 5](#).

In the **Worksheets** toolbar add at minimum the below plots to the worksheet:

Dot plot – FSC-A vs. Live/Dead V500-C-A

Dot plot – FSC-A vs. FSC-H

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Dot plot – FSC-A vs. S1 Spike PE-A

Histogram plot - S1 Spike PE-A vs. Count

Load NC cells in a sample tube onto the manual loading port.

Select **Preview** in the Data Sources panel.

Adjust the PMT Voltages of the FSC, PE, and V500-C channels so that the cell populations look visually comparable to the NC examples seen in method [Figure 5](#) and [Figure 7](#).

The voltages in the left panel of Attachment B Figure 1 can be used as a guide. It is expected that voltages will be unique to each specific instrument and do not have to exactly match the example.

Select **Stop** in the Data Sources panel.

Unload the NC tube and load a DSC sample tube onto the manual port.

Repeat the **Preview** ensuring that voltages are appropriate for the DSC.

Select **Stop** in the Data Sources panel and unload the tube.

Right click on the **Quad_01** tube and select **Create Tube Settings**.

Verify the appropriate CS&T bead lot is selected and then load the CS&T beads onto the manual port.

Select **Acquire**.

Change the **Tube Settings Name** to something appropriate. For example: TM100010380V1.0.

Select **Finish** to save the tube settings to the library.

CREATE AN ASSAY

Right click on the **Quad_01** tube and select **Copy without data**. Perform this three times.

Rename the new tubes as “Quad_02”, “Quad_03”, “Quad_04”

Load the NC sample tube onto the manual port.

Ensure the run pointer is on tube Quad_01.

Select **Preview** in the Data Sources panel and once data appears in the Worksheet select **Acquire**.

Repeat the above step to acquire data for all four tubes.

Once the acquisition is complete, remove the sample and load a tube of water onto the manual port.

In the Worksheet toolbar select **Add Report**.

Select the **Report_001** tab and rename it “Well Report”.

Add two pages to the report by selecting the **Add page** button twice.


Copy all four plots from the Worksheet to the second page of the Well Report.

Select all four graphs, right click and select **Properties**.

Unselect the ”Run from Pointer” box.

In the **Primary Data Source** space, under **Tube** select **Quad_01**.

Create an ellipse gate around the live cell population of the FSC-A vs. Live/Dead V500-C-A dot plot.

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The live cell population is lower on the Live/Dead V500-C-A scale than the dead cell population.
The gate should look similar to those seen in method [Figure 5](#) and [Figure 7](#).

Right click on the FSC-A vs. FSC-H dot plot and select **Properties**.

In the **Parent Population** space select the **P1** checkbox.

Close the plot editor.

Create a polygon gate around the single cell population of the FSC-A vs. FSC-H dot plot.

The single cell population appears on a diagonal through the dot plot space with the doublet cell population appearing further towards the lower left corner of the graph. The gate should look similar to those seen in method [Figure 5](#).

Right click on the FSC-A vs. S1 Spike PE-A dot plot and select **Properties**.

In the **Parent Population** space select the **P2** checkbox.

Close the plot editor.

Right click on the S1 Spike PE-A vs. Count histogram and select **Properties**.

In the **Parent Population** space select the **P2** checkbox.

Close the plot editor.

Create an interval gate selecting the cells expressing the S1 antigen on the S1 Spike PE-A vs. Count histogram.

The S1+ cell population is higher on the S1 Spike PE-A axis than the negative cells. The gate should look similar to the NC seen in method [Figure 5](#) and [Figure 7](#) both in the histogram and the FSC-A vs. S1 Spike PE-A dot plot.

Copy all four graphs three times onto pages two and three of the report.

Right click on the first set of copied graphs and select **Properties**.

Unselect the "Run from Pointer" box.

In the **Primary Data Source** space, under **Tube** select **Quad_02**.

Repeat these two steps for the remaining two sets of graphs and select **Quad_03** and **Quad_04**.

In the toolbar select the **Display Hierarchy** button.

Select the plus sign next to each gate in the hierarchy until all three gates are visible for all four tubes.


Move the Hierarchy window to an open space on page one of the report and select the pushpin icon to add it to the report.

In the toolbar select the **Statistics** button and select an open area on page one of the report to place the statistics box.

Right click on the Statistics box and select **Edit Keywords**.

Select the following keywords: Acquisition date, Time at end of acquisition, Tube Name, Sample ID, Carrier Location ID, FSC file name, flow cytometer serial number, and Operator.

Close the Keywords window.

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Right click on the Statistics box and select **Edit Populations**.

Select All Events, P1, P2 and P3 for all four tubes.

Close the populations window.

Right click on the Statistics box and select **Edit Statistics**.

Uncheck any FSC-A and SSC-A boxes and under S1 Spike PE-A check the **Mean** box.

Check the **Population Statistics** box.

Close the Statistics window.

Right click on the Statistics box and select **Properties**.

Check the **Include in Auto-Export** box.

Close the properties window.

The Well Report should now look similar to Attachment B Figure 2 and [Figure 6](#).

Select **Create Assay** (File → Create Assay).

Enter in an appropriate assay name. For example: TM100010380V1.0

Select **OK** to add the assay to the library.

Go to the **Library** workspace.

Under Assay select **User-Defined Assays** and then select the Assay name that was just created.

In the Assay Properties panel select **Edit**.

In the General tab uncheck the **Automatically approve results** box and select Aqua Live/Dead Fixable Dye and Spike S1 Antibody to the **Selected Reagents List**.

In the Reports tab check the **Export report to:** box both the Well Report and the Audit Trail.

The location needs to be set to the appropriate Pfizer secure drive per SOP-INS-05140.


Select **Done** to save the assay changes.

Right click on the assay from the list of User-defined assays.

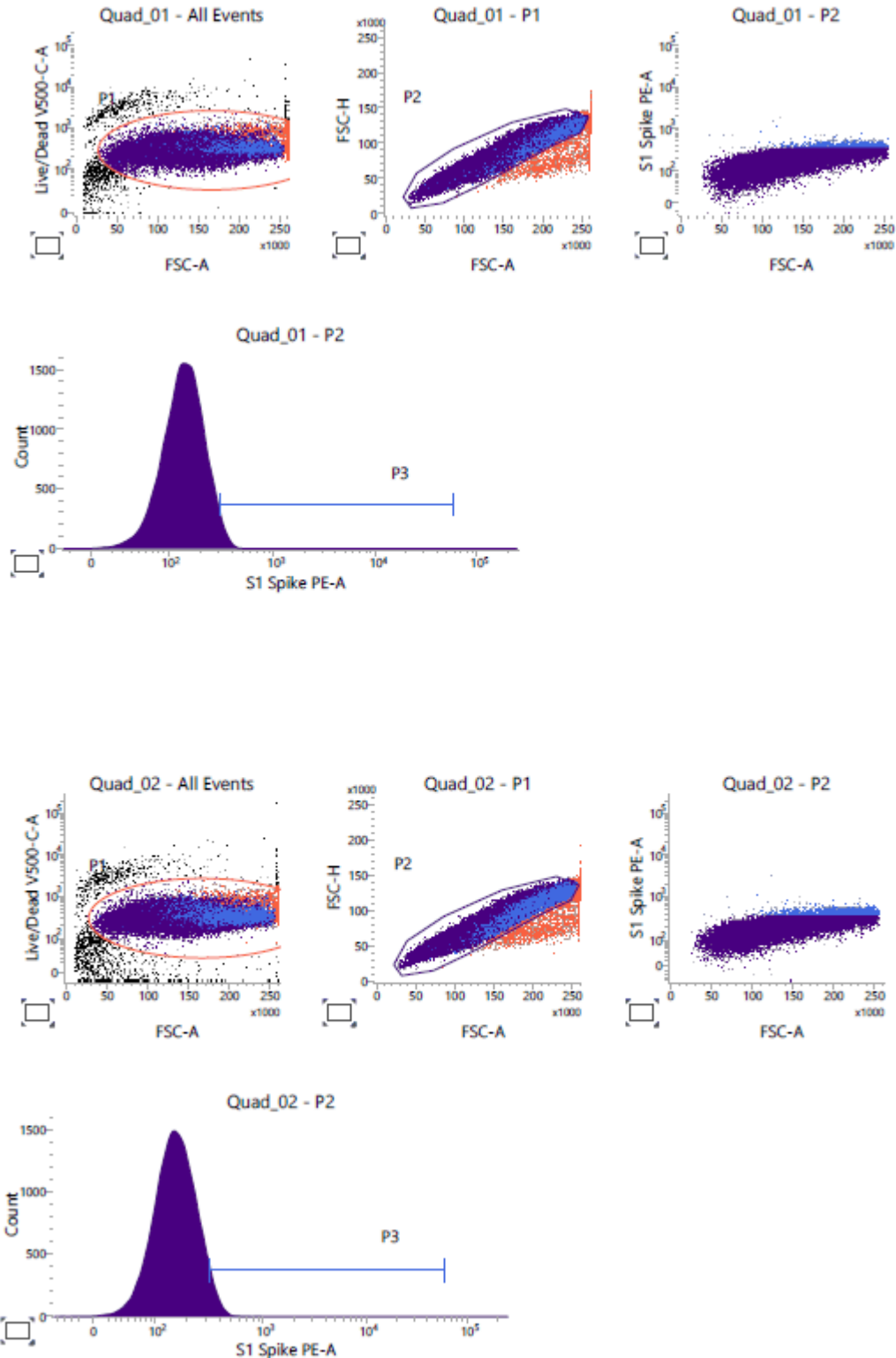
Select **Share Assay**, **Require reason for change in the audit trail** and **Require approval for auto export/print**.

Once an Assay is shared only changes in the Assay Properties are allowed.

The Assay is now ready for use.

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Attachment B - Figure 2 Example Well Report Page 2



Document Approval Record

Document Name:	TM100010380
Document Title:	Determination of In-vitro Expression of mRNA by Flow Cytometry

Signed By:	Date(GMT)	Signing Capacity
Shields, Kathleen M	13-Oct-2020 17:07:26	Manager Approval
Dorner, Rachel	13-Oct-2020 17:47:58	Author Approval
Lee, Jamie L	14-Oct-2020 02:55:25	Business Line Approver
Flecke, Kimberly	14-Oct-2020 10:59:59	Quality Assurance Approval