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3.2.S.4.2. ION PAIR-REVERSED PHASE-HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (IP-RP-HPLC)

3.2.S.4.2.1. Principle and Scope

The purpose of this analytical procedure is to measure the length of the 3' polyadenylic acid (Poly(A)) tail in BNT162b2 drug substance (DS) mRNA. DS test samples are digested using RNase T1 and RNase A followed by ion pair-reversed phase-high performance liquid chromatography (IP-RP-HPLC) with UV detection.

RNase T1 specifically cleaves single-stranded RNA on the 3'-end of guanosine (G) residues, and RNase A cleaves single-stranded RNA on the 3'-end of uridine (U) and cytidine (C) residues. The resulting products are an approximately 30 Poly (A) nucleotide tail (A30) and an approximately 70 Poly(A) nucleotide tail (L70) mixed with other shorter nucleotides. The 30 Poly(A) and the 70 Poly(A) nucleotide tails are separated by IP-RP-HPLC. The lengths of the Poly(A) tails are confirmed and reported using relative retention time (RRT) compared to a well characterized mRNA DS reference material (RM).

3.2.S.4.2.2. Apparatus and Equipment

The apparatus and equipment are provided in Table 3.2.S.4.2-1.

Table 3.2.S.4.2-1. Apparatus and Equipment

Thermal mixer
HPLC system with UV detection

Abbreviations: HPLC = high performance liquid chromatography; UV = ultraviolet

3.2.S.4.2.3. Reagents, Standards and Prepared Solutions

The reagents and standards are provided in Table 3.2.S.4.2-2 and are of sufficient quality as to be suitable for this analytical procedure.

Table 3.2.S.4.2-2. Reagents and Standards^a

Purified water
Nuclease free water
Tris (hydroxymethyl) aminomethane hydrochloride
Ethylenediaminetetraacetic acid (EDTA), 0.5 M
4.2 1st ind
4.2 1st ind
4.2 1st ind
4.2 1st ind
Methanol, LC grade
BNT162b2 DS reference material (RM)

a. Equivalent reagents may be used.

Abbreviations: HPLC = high performance liquid chromatography; LC = liquid chromatography; DS = drug substance

The prepared solutions are provided in Table 3.2.S.4.2-3.

Table 3.2.S.4.2-3. Prepared Solutions

Mobile phase A	4.2 1st ind
Mobile phase B	4.2 1st ind
EDTA	4.2 1st ind
Tris/EDTA solution	4.2 1st ind
RNase T1	4.2 1st ind
RNase A	4.2 1st ind
TEAA	4.2 1st ind

Abbreviations: EDTA = Ethylenediaminetetraacetic acid; 4.2 1st ind

3.2.S.4.2.4. Sample Preparation

A volume 4.2 1st ind of BNT162b2 DS test sample (TS) is transferred to a microfuge tube.

3.2.S.4.2.5. Standard and Control Solution Preparation

3.2.S.4.2.5.1. Reference Material Preparation

A volume 4.2 1st ind of BNT162b2 DS RM is transferred to a microfuge tube.

3.2.S.4.2.6. Procedure

3.2.S.4.2.6.1. RNase T1/RNase A Digestion

Aliquots of RNase T1, RNase A, and Tris/EDTA solution are added to each TS and RM and then brought 4.2 1st ind with nuclease free water. Samples are vortexed and incubated at 4.2 1st ind in a thermal mixer. Subsequently, the samples are cooled to ambient temperature and briefly spun in a microcentrifuge to recover the samples in the bottom of the tube.

3.2.S.4.2.6.2. IP-RP-HPLC

An aliquot of 4.2 1st ind is added to each TS and RM, followed by vortexing and a brief centrifugation. All samples are then transferred to auto-sampler vials. The IP-RP-HPLC operating parameters are provided in Table 3.2.S.4.2-4. For column equilibration, use 4.2 1st ind of mobile phase A and 4.2 1st ind of mobile phase B. After column equilibration, RM injections 4.2 1st ind are performed 4.2 1st ind, the samples are injected in the sequence shown in Table 3.2.S.4.2-5, and the A30 and L70 regions of the RM and TS are 4.2 1st ind integrated. Representative chromatograms of the blank, RM and DS are presented in Figure 3.2.S.4.2-1 through Figure 3.2.S.4.2-3.

Table 3.2.S.4.2-4. Chromatographic Conditions and System Operating Parameters

Column	4.2 1st ind	
Column temperature		
Autosampler temperature		
UV detection		
UV reference ^a		
Injection volume		
Flow rate		
Mobile phase A: 4.2 1st ind		
Mobile phase B: 4.2 1st ind		
Gradient		
Time (minutes)	Mobile Phase A (%)	Mobile Phase B (%)
4.2 1st ind		

a. instrument dependent, may not be required.

Abbreviations: UV = ultraviolet; RP = reverse phase; 4.2 1st ind

Table 3.2.S.4.2-5. Sample Sequence

Sample	Number of Injections
Mobile Phase A (Blank)	4.2 1st ind
RM (system suitability)	
TS	
RM (after every 10 TS injections)	
RM (after the last TS)	

Figure 3.2.S.4.2-1. Representative Chromatograms of Blank

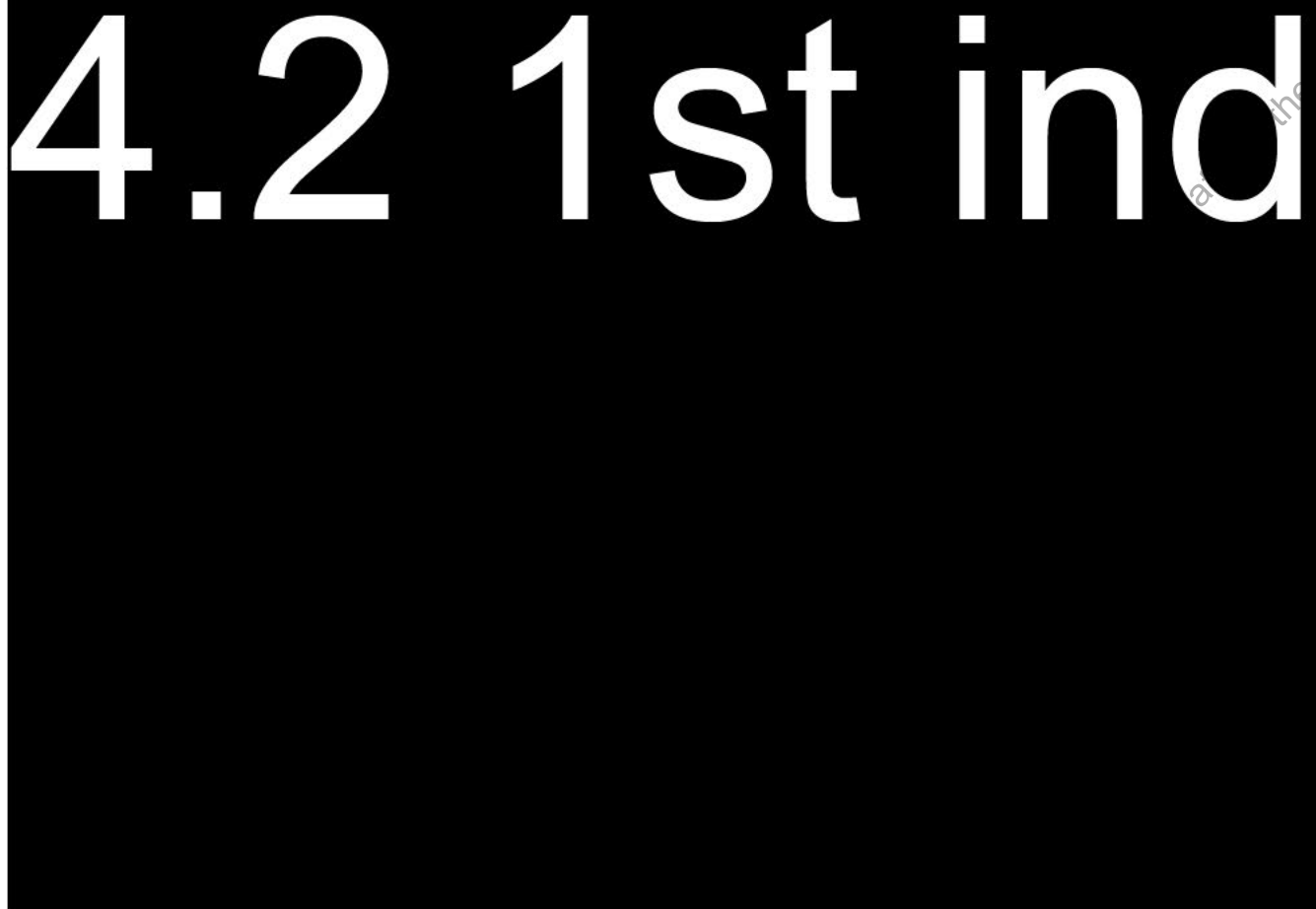


Figure 3.2.S.4.2-2. Representative Chromatograms of RM



Figure 3.2.S.4.2-3. Representative Chromatograms of BNT162b2 Drug Substance

4.2 1st ind

3.2.S.4.2.7. System Suitability, Assay, and Sample Acceptance

System suitability is assessed by analysis of the blank and RM. Assay acceptance is assessed by analysis of the RM, and sample acceptance is assessed by analysis of the TS. The criteria listed in [Table 3.2.S.4.2-6](#) must be met to demonstrate system suitability, assay, and sample acceptance.

Table 3.2.S.4.2-6. System Suitability, Assay, and Sample Acceptance

Material	Parameters Assessed	Acceptance Criteria
<i>System suitability</i>		
Blank	Chromatographic profile	Must exhibit a stable baseline, with no observable peaks (Figure 3.2.S.4.2-1)
RM (3 system suitability injections at the beginning of the assay)	Chromatographic profiles	Must be visually comparable to that shown in Figure 3.2.S.4.2-2
	RSD of the retention time of A30 and L70	
	USP resolution between the 2 highest peaks in the A30 region (H1 and H2)	
	USP tailing factor for L70	
<i>Assay acceptance</i>		
RM (all injections in the sequence)	Chromatographic profiles	
	RSD of the retention time of A30 and L70	
	USP resolution between the 2 highest peaks in the A30 region (H1 and H2)	
	USP tailing factor for L70	
<i>Sample Acceptance</i>		
TS	Chromatographic profiles	Must be visually comparable to the RM (Figure 3.2.S.4.2-3)

Abbreviations: A30 = 30 Poly (A) nucleotide tail, L70 = 70 Poly(A) nucleotide tail, RSD = relative standard deviation, USP = United States Pharmacopeia; TS = test sample, RM = Reference Material

3.2.S.4.2.8. Calculations

3.2.S.4.2.8.1. USP Resolution

The USP resolution is calculated according to the following formula:

4.2 1st ind

4.2 1st ind

3.2.S.4.2.8.2. Relative Retention Time

The relative retention time (RRT) of A30 and L70 in each TS is calculated according to the following formulas:



3.2.S.4.2.9. Data Reporting

Provided the system suitability, assay, and sample acceptance criteria are met, and the RRT of the A30 and L70 peaks are 4.2 1st ind, the TS is reported as “poly(A) tail length confirmed”.