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3.2.S.4.2. DROPLET DIGITAL POLYMERASE CHAIN REACTION (ddPCR) - [OMICRON]

3.2.S.4.2.1. Principle and Scope

The purpose of this analytical procedure is to quantitate the poly(A) tail in the messenger ribonucleic acid (mRNA) in BNT162b2 drug substance (DS) using droplet digital polymerase chain reaction (ddPCR) technology.

The ddPCR technology is a digital form of polymerase chain reaction (PCR) that uses a water-in-oil emulsion system to quantify target nucleic acids. Thousands of nanoliter sized droplets are formed from each sample, and PCR amplification is then performed on each droplet. Post amplification, fluorescence is measured in order to detect the number of positive and negative droplets.

This procedure describes a two-step reverse transcription (RT)-ddPCR assay for the quantitation of mRNA poly(A) tail.

4.2 1st. ind.

Fluorescence of the droplets is measured at the end of amplification. The poly(A) tail is quantified as a percent of the copy number that have 3' end in the total number of mRNA copies.

The rationale of this ddPCR method for the percentage poly(A) determination is based on:

- All RNA with poly(A) tail is converted into cDNA using a poly(T) primer, and each copy of this RNA generates one copy of cDNA.
- The cDNA copy number determined by the ddPCR assay using 3'-end sequence primers represents the copy number of the RNA with poly(A) tails.
- The theoretical input RNA copy number per μL is calculated based on the initial concentration of RNA in the test sample preparation and its dilution through the annealing, cDNA synthesis and RNase H treatment steps. This calculation uses the expected full-length RNA molecular weight and assumes all RNA in the test sample are full-length RNA.

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^a

4.2 1st. ind.	droplet generator,	4.2 1st. ind.
4.2 1st. ind.	droplet reader,	4.2 1st. ind.
Thermal cycler		
Mini Plate Centrifuge or Centrifuge		
PCR plate sealer		
Sterile and nuclease free reagent reservoirs		
8-strip PCR tubes with caps		
ddPCR 96 well semi-skirted plate,		4.2 1st. ind.
Pierceable foil heat seal,		4.2 1st. ind.
Peelable foil heat seal		
4.2 1st. ind.	Droplet Generator cartridges,	4.2 1st. ind.

a. Equivalent apparatus and equipment may be used unless otherwise noted.

Abbreviations: PCR = Polymerase chain reaction; ddPCR = droplet digital polymerase chain reaction.

3.2.S.4.2.3. Reagents and Standards

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

4.2 1st. ind.	Primer:	4.2 1st. ind.
3'-end forward primer:		
3'-end reverse primer:		
3'-end probe:		
TE buffer, 1X, molecular biology grade		
ddPCR supermix for Probes (no dUTP),		4.2 1st. ind.
4.2 1st. ind.	Reverse Transcriptase (RT)	4.2 1st. ind.
4.2 1st. ind.		
dNTP (10 mM)		
RNase inhibitor		
RNase H		
Nuclease-free water		
ddPCR Droplet Reader Oil,		4.2 1st. ind.
Droplet Generation Oil for Probes,		4.2 1st. ind.
BNT162b2 DS reference material or other suitable DS, used as internal control		

a. Equivalent reagents may be used unless otherwise noted.

Abbreviations: 4.2 1st. ind., dNTP = Deoxynucleotide triphosphates, ddPCR = droplet digital polymerase chain reaction; TE = Tris-HCl containing ethylenediaminetetraacetic acid; DS = Drug substance

3.2.S.4.2.4. Sample Preparation

DS test samples (TS) are diluted to a target concentration **4.2 1st. ind.** in nuclease-free water.

3.2.S.4.2.5. mRNA Internal Control (IC) Preparation

BNT162b2 DS reference material or other suitable BNT162b2 DS is used as the IC, to ensure the assay has performed correctly and the systems are performing properly. IC is prepared in the same manner as TS.

3.2.S.4.2.6. Procedure

3.2.S.4.2.6.1. mRNA Primer Annealing

4.2 1st. ind.

4.2 1st. ind.

The mRNA primer annealing reaction thermal cycling is performed as shown in Table 3.2.S.4.2-3.

Table 3.2.S.4.2-3. mRNA Primer Annealing Reaction Thermal Cycling Conditions

Temperature (°C)	Time	Cycle
4.2 1st. ind.		
Abbreviations: N/A = not applicable		

3.2.S.4.2.6.2. mRNA cDNA Synthesis

The cDNA synthesis master mix (Table 3.2.S.4.2-4) is prepared just prior to use.

Table 3.2.S.4.2-4. cDNA Synthesis Master Mix

Reagent	Target Working Concentration
4.2 1st. ind.	

4.2 1st. ind. [REDACTED]

[REDACTED]

The cDNA synthesis reaction thermal cycling is performed as shown in Table 3.2.S.4.2-5.

Table 3.2.S.4.2-5. cDNA Synthesis Reaction Thermal Cycling Conditions

Temperature (°C)	Time	Cycle
4.2 1st. ind. [REDACTED]		

Abbreviations: N/A = not applicable

3.2.S.4.2.6.3. mRNA RNase H Reaction

The RNase H master mix (Table 3.2.S.4.2-6) is prepared just prior to use.

Table 3.2.S.4.2-6. RNase H Master mix

Reagent	Target Working Concentration
4.2 1st. ind. [REDACTED]	

Abbreviations: N/A = not applicable

4.2 1st. ind. [REDACTED]

[REDACTED]

The RNase H reaction thermal cycling is performed as shown in Table 3.2.S.4.2-7.

Table 3.2.S.4.2-7. RNase H Reaction Thermal Cycling Conditions

Temperature (°C)	Time	Cycle
4.2 1st. ind. [REDACTED]		

090177e1a1a7f99d\Approved\Approved On: 18-Sep-2024 13:29 (GMT)

Table 3.2.S.4.2-7. RNase H Reaction Thermal Cycling Conditions

Temperature (°C)	Time	Cycle
Abbreviations: N/A = not applicable		

3.2.S.4.2.6.4. ddPCR Reaction and Analysis

4.2 1st. ind.

The ddPCR primer and probe working solution in nuclease-free water is prepared as shown in Table 3.2.S.4.2-8.

Table 3.2.S.4.2-8. ddPCR Primer and Probe Working Solution

Reagent	Concentration (µM)
4.2 1st. ind.	

Abbreviations: ddPCR = Digital droplet polymerase chain reaction

4.2 1st. ind.

The ddPCR reaction thermal cycling is performed as shown in [Table 3.2.S.4.2-9](#).

Table 3.2.S.4.2-9. ddPCR Reaction Thermal Cycling Conditions

Temperature (°C)	Time	Cycle	Temperature Ramp Rate (°C/seconds)
4.2 1st. ind.			

Abbreviations: ddPCR = Digital droplet polymerase chain reaction; N/A = not applicable.

The sealed ddPCR transfer plate is placed in the droplet reader. The plate is analyzed using the system software, with the instrument setup parameters listed in Table 3.2.S.4.2-10.

Table 3.2.S.4.2-10. Droplet Reader Settings

Parameter	Value / Setting
4.2 1st. ind.	

Abbreviations: 4.2 1st. ind.; NTC = Non-template control, ddPCR = Digital droplet polymerase chain reaction.

The settings apply to all wells containing IC, TS or NTC. Blank wells are not included in the droplet reader plate layout.

3.2.S.4.2.7. Assay and Sample Acceptance Criteria

Assay acceptance criteria are assessed by analysis of NTC and IC results. Sample acceptance criteria are assessed by analysis of TS results. The criteria listed in Table 3.2.S.4.2-11 must be met to demonstrate assay and sample acceptance.

Table 3.2.S.4.2-11. Assay and Sample Acceptance

Material	Parameters Assessed	Acceptance Criteria	
Assay Acceptance			
NTC	4.2 1st. ind.		
IC			
Sample acceptance			
TS			

Abbreviations: NTC = Non-template control; RSD = relative standard deviation, IC = Internal control, TS = Test sample.

3.2.S.4.2.8. Calculations

The copy number per μL values for the positive and negative droplet populations are reported by the instrument software program for each well.

The mean, standard deviation and RSD (%) are calculated for each sample from 3' copy number per μL values for triplicate wells.

Percent poly(A) tail is calculated for each sample as percent mean 3' copy number per μL relative to the input (e.g. input = 1000 copy/ μL):

$$\text{Poly(A) Tail (\%)} = \frac{\text{mean 3' } \left(\frac{\text{copy}}{\mu\text{L}} \right)}{\text{input } \left(\frac{\text{copy}}{\mu\text{L}} \right)} \times 100$$

3.2.S.4.2.9. Data Reporting

Provided the assay and sample acceptance criteria are met, the percent poly(A) tail of the TS is reported.