

3.2.S.4.2. IMMUNOBLOT

3.2.S.4.2.1. Principle and Scope

The purpose of the immunoblot analytical procedure is to detect double stranded RNA (dsRNA) in BNT162b2 drug substance (DS). Samples are spotted onto a membrane and exposed to an anti-dsRNA antibody. A secondary antibody with a chemiluminescent readout is used to detect the primary antibody. The amount of chemiluminescence provides semi-quantitation of the amount of dsRNA when compared to limit standards.

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

Dot blot gasket
Filter paper
Nylon blotting membrane
Bioimager with chemiluminescence quantitation software

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 (molecular biology grade)
Poly(A)-RNA, polyadenylic acid potassium salt
dsRNA standard (60 ng/μL) (do not substitute)
Enhanced chemiluminescence HRP substrate
HRP-conjugated donkey anti-mouse IgG
J2 dsRNA-specific mouse mAb (do not substitute)
RNase inhibitor
1 M Tris HCl buffer, pH 7.4
5 M NaCl
Tween-20
Powdered skim milk

a. Equivalent reagents may be used unless otherwise noted.

Abbreviations: HRP = horseradish peroxidase; dsRNA = double stranded RNA; mAb = monoclonal antibody

The prepared solutions are provided in [Table 3.2.S.4.2-3](#).

Table 3.2.S.4.2-3. Prepared Solutions

Poly(A)-RNA solution	4.2 1st ind
dsRNA stock solution	4.2 1st ind in poly(A)-RNA 4.2 1st ind
Wash solution	1X Tris buffered saline-Tween (TBS-T) (20 mM Tris, 0.15 M NaCl, 0.1% Tween-20, pH 7.4)
Blocking Solution:	5% skim milk in Tris buffered saline-Tween (1X TBS-T)
Antibody Diluent:	1% skim milk in Tris buffered saline-Tween (1X TBS-T)
4.2 1st ind	mAb solution, lot-specific dilution, in antibody diluent
HRP-conjugated donkey anti-mouse IgG	in antibody diluent
HRP substrate (mix component A and component B 1:1)	

Abbreviations: HRP = horseradish peroxidase; dsRNA = double stranded RNA; mAb = monoclonal antibody

3.2.S.4.2.4. Sample Preparation

DS test samples (TS) are prepared 4.2 1st ind in purified water.

3.2.S.4.2.5. Standard and Control Solution Preparation

3.2.S.4.2.5.1. dsRNA Standards

A series of 3 dsRNA standards 4.2 1st ind are prepared by serial dilution of the 4.2 1st ind dsRNA stock solution with 4.2 1st ind Poly (A)-RNA solution.

3.2.S.4.2.5.2. Poly (A)-RNA Negative Control

The poly (A)-RNA solution 4.2 1st ind is used as the negative control.

3.2.S.4.2.6. Procedure

3.2.S.4.2.6.1. Dot Blot Assembly

The rubber seal gasket from a dot blot apparatus is rinsed with RNase inhibitor followed by purified water and allowed to dry. To assemble the dot blot, a nylon membrane is placed on top of the filter paper, and the gasket is placed on top of the membrane, flat side facing down. The membrane must stick to the gasket with no air bubbles present.

3.2.S.4.2.6.2. Sample Loading

A 4.2 1st ind aliquot of TS, dsRNA standards, and the poly (A)-RNA negative control are loaded into the appropriate sample wells of the sealing gasket in quadruplicate. To ensure complete saturation of the membrane, this process is repeated, and the membrane held at ambient temperature 4.2 1st ind

The sealing gasket is removed from the membrane, and the membrane is held at ambient temperature for 4.2 1st ind to allow complete drying.

3.2.S.4.2.6.3. Membrane Blocking

The membrane is transferred to a suitable tube with the sampling area facing inward and blocking solution is pipetted into the tube. The tube is incubated at ambient temperature for 4.2 1st ind

3.2.S.4.2.6.4. Primary Antibody Incubation

The anti-dsRNA-specific 4.2 1st ind antibody is diluted to the target concentration with antibody diluent followed by mixing. The blocking solution is removed, and the anti-dsRNA specific 4.2 1st ind antibody solution is pipetted into the tube, followed by a 4.2 1st ind incubation 4.2 1st ind on a tube rotator.

3.2.S.4.2.6.5. Secondary Antibody Incubation

The primary antibody solution is removed, and the membrane is rinsed twice with wash solution. An additional aliquot of wash solution is added to the tube, followed by incubation at ambient temperature for 4.2 1st ind on a tube rotator. The 4.2 1st ind minute wash step is performed a total of 3 times. Subsequently, horseradish peroxidase (HRP)-conjugated anti-mouse IgG antibody is prepared at the target concentration in antibody diluent. After removing the third wash, the HRP-conjugated anti mouse IgG solution is pipetted into the tube and incubated at ambient temperature for 4.2 1st ind on a tube rotator.

3.2.S.4.2.6.6. Development with Substrate and Dot Blot Image Acquisition

The secondary antibody solution is removed, and the membrane is immediately rinsed twice with wash solution and then washed 3 times for 4.2 1st ind, as described in Section 3.2.S.4.2.6.5. The HRP substrate solution is prepared and transferred to the membrane. After a 4.2 1st ind incubation at ambient temperature, the HRP substrate solution is discarded, the membrane placed into the imager, and an image is obtained within the dynamic range of the densitometer. To determine the intensity of each dot, a region of interest (ROI) is defined by adding an identical ring-shaped area around each dot, local background is subtracted, and the data exported. [Figure 3.2.S.4.2-1](#) shows a representative dot blot.

Figure 3.2.S.4.2-1. Representative Dot Blot^a



3.2.S.4.2.7. Assay Acceptance

Assay acceptance is assessed by analysis of the dsRNA standards and TS. The criteria listed in Table 3.2.S.4.2-4 must be met to demonstrate assay acceptance.

Table 3.2.S.4.2-4. Assay and Sample Acceptance

Material	Parameter Assessed	Acceptance Criteria
Dot blot image	Visual appearance	Must be free of smears or streaks
	Dot intensity	4.2 1st ind [REDACTED]

Table 3.2.S.4.2-4. Assay and Sample Acceptance

Material	Parameter Assessed	Acceptance Criteria
dsRNA standards	Mean signal intensity	4.2 1st ind
dsRNA standards and TS	Intensity RSD ^a	4.2 1st ind

a. If any TS or dsRNA standard does not meet the RSD acceptance criteria, one data point furthest from the mean may be dropped and the RSD value re-calculated. If any TS has a mean signal intensity [REDACTED] the mean intensity of the negative control, the RSD criterion of [REDACTED] does not apply.

b. Range may be updated as needed for specific instruments.

Abbreviations: dsRNA = double stranded RNA; RSD = relative standard deviation, std = standard; TS = test sample

3.2.S.4.2.8. Calculations

The mean adjusted intensity value for each standard level and TS is calculated.

3.2.S.4.2.9. Data Reporting

Provided the assay acceptance criteria are met, the dsRNA content of the TS is reported as pass/fail 4.2 1st ind