BioNTech SE
An der Goldgrube 12
55131 Mainz, Germany
Phone: +49 (0)6131 9084-0
Telefax: +49 (0)6131 9084-390

# R&D DATA REPORT No. R-20-0211

# *In Vitro* Expression of BNT162b2 Drug Substance and Drug Product

Version 02
Date: 17 SEP 2020

Reported by (b) (6)

Test item: BNT162b2
Key words: COVID19, modRNA, ATM material, Western blot, immunofluorescence, FACS

This R&D report consists of 22 pages.

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# LIST OF ABBREVIATIONS

| | |
|---|---|
| ATM | Animal trial material |
| BNT162b2 | Investigational vaccine in this study (DP) |
| BNT162b2-RNA | Investigational vaccine in this study (DS) |
| COVID-19 | Coronavirus disease emerged in 2019 |
| DNA | Deoxyribonucleic acid |
| DP | Drug product |
| DS | Drug substance |
| ER | Endoplasmic reticulum |
| FACS | Fluorescence-activated cell sorting |
| GFP | Green fluorescent protein |
| HEK | Human embryonic kidney |
| modRNA | modified RNA |
| PAGE | Polyacrylamide gel electrophoresis |
| RBD | Receptor binding domain |
| RNA | Ribonucleic acid |
| S protein | Spike protein |
| S1 | Subdomain 1 of the S protein |
| SARS-CoV-2 | Severe acute respiratory syndrome-Coronavirus-2 |
| saRNA | self-amplifying RNA |
| SDS | Sodium dodecyl sulfate |
| SOP | Standard operating procedure |
| uRNA | unmodified RNA |
| V9 | Antigen variant of the generated variants of the S protein |

## RESPONSIBILITIES

| | | |
|---|---|---|
| Person responsible for the study: | (b) (6) (b) (6) BioNTech RNA Pharmaceuticals GmbH | 18 Sep 2020 Date |
| Author: | (b) (6) (b) (6) BioNTech RNA Pharmaceuticals GmbH | 18 Sep 2020 Date |
| Reviewer: | (b) (6) (b) (6) BioNTech RNA Pharmaceuticals GmbH | 18 Sep 2020 Date |
| QA representative: | (b) (6) BioNTech SE | 22 Sep 2020 Date |

Meaning of the signatures:

Person responsible for the study: I am responsible for the content of the R&D report and confirm that it represents an accurate record of the results. This study was performed according to the SOPs and methods as well as the rules and regulations described in the report.

Author: I am the author of this document.

Reviewer: I reviewed the R&D report and confirm that this document complies with the scientific and technical standards and requirements.

QA representative: I confirm that this document complies with the relevant quality assurance requirements.

# 1 SUMMARY

BioNTech is developing RNA-based vaccines designed to protect against the novel coronavirus disease that emerged in 2019 (COVID-19). The project involves testing of three RNA platforms, namely non-modified uridine containing mRNA (uRNA), nucleoside modified mRNA (modRNA) and self-amplifying RNA (saRNA), which are under development at BioNTech, with the spike protein (S protein) of the novel Coronavirus (SARS-CoV-2) as the viral antigen.

In the present study, antigen expression was evaluated of the current clinical lead candidate, BNT162b2. BNT162b2 is a nucleoside-modified mRNA (modRNA) encoding the antigen variant 9 (V9) of the S protein. BNT162b2 was analyzed both as drug substance (DS; BNT162b2-RNA), and as LNP-formulated drug product (DP; BNT162b2) in HEK293T cells. DS was transfected into cells using a commercial transfection reagent.

Antigen expression was investigated and confirmed by Western blot analysis of HEK293T cell lysates of cells that had been transfected with DS BNT162b2-RNA.

Transfection frequencies and expression of the antigen in both DS and DP, BNT162b2-RNA and BNT162b2, were evaluated by FACS analysis of transfected HEK293T cells. Both, BNT162b2-RNA and BNT162b2 revealed high frequencies of transfected cells, with BNT162b2 cells showing slightly higher transfection frequencies compared to BNT162b2-RNA transfection. There were no differences in cell viability after transfection with BNT162b2-RNA or BNT162b2 when comparing to non-transfected cells.

In addition, fluorescence co-staining was performed with an endoplasmic reticulum (ER) marker and an antibody recognizing the S1 protein subunit to evaluate the correct antigen localization using DS BNT162b2-RNA-transfected HEK293T cells. Co-localization of the antigen expressed by DS encoding the full length S protein with an ER marker was confirmed.

| | | |
|---|---|---|
| (b) (6) | | 18Sep2020 |
| GmbH | BioNTech RNA Pharmaceuticals | Date |

# 2 GENERAL INFORMATION

## 2.1 Sponsor and Test Facilities

**Sponsor**

BioNTech RNA Pharmaceuticals GmbH
An der Goldgrube 12
55131 Mainz
Germany

**Test Facilities**

BioNTech RNA Pharmaceuticals GmbH
An der Goldgrube 12
55131 Mainz
Germany

(b) (4)

## 2.2 Participating Personnel

| | |
|---|---|
| **Responsible person:** (as defined in SOP-100-024) | (b) (6)<br>BioNTech RNA Pharmaceuticals GmbH<br>An der Goldgrube 12<br>55131 Mainz |
| **Author:** | (b) (6)<br>BioNTech RNA Pharmaceuticals GmbH |
| **Experimenter:** Western blot, FACS | (b) (6)<br>BioNTech RNA Pharmaceuticals GmbH |
| **Experimenter:** Immunofluorescence | (b) (6), (b) (4) |

## 2.3 Study Dates

Start of experiments: 22 JUN 2020

Completion of experiments: 14 AUG 2020

## 2.4 Guidelines and Regulations

All experiments are executed in accordance with the existing standard operating procedures and described processes from BioNTech SE. Applicable documents are listed below.

- SOP-020-009 Ansetzen von Medien und Zusätzen für die Zellkultur
- SOP-030-038 Standardisierte Kultivierung von Zellen
- SOP-030-039 Zellzahlbestimmung mittels Neubauer Zählkammer
- SOP-030-117 Durchführung einer SDS Polyacrylamid Gelelektrophorese (SDS-PAGE)
- LA-50-255-000 Direkte / Indirekte Immunfluoreszenzfärbung

## 2.5 Changes and Deviations

Not applicable. There is no formal R&D plan available.

## 2.6 Documentation and Archive

Study plans and reports are stored and archived according to SOP-100-003 Archiving of Paper-Based Documents.

Raw data and evaluated data are saved at

- P:\BioNTechRNA\RN9391R00_CoV-VAC\04_Preclinic\04_in vitro\CorVac_IDV.invitro#050_b1_b2_b3c_DS_and_DP_IVE
- P:\BioNTechRNA\RN9391R00_CoV-VAC\04_Preclinic\04_in vitro\CorVac_IDV.invitro#059_HEK_IF_microscopy_at(b) (4)
- P:\BioNTechRNA\RN9391R00_CoV-VAC\04_Preclinic\04_in vitro\CorVac_IDV.invitro#064_mod9-WB_IVE
- Lab book MeGl, No. 2034
- Lab book MeGl, No. 2035

# 3 MATERIALS AND METHODS

## 3.1 Test Item

BNT162b2-RNA (ATM RNA): For CoA see Appendix 1: Certificates of Analysis.

BNT162b2 (ATM LNP): For CoA see Appendix 1: Certificates of Analysis.

- RNA batch: RNA-RF200321-06, 97.5% integrity
- Polymun batch RBP020.2 LNP with the lot: CoVVAC/270320

## 3.2 Control Item

- Modified RNA GFP, p4.AGA_eGFP, RNA_RF200309_01c, 96% integrity

## 3.3 Test System

- *In vitro* test system, cell culture, HEK293T cells

## 3.4 Materials

**Table 1: Equipment**

| Product name | Provider |
|---|---|
| Microscope IX53 | Olympus Life Science |
| Microscope SP8 | Leica |
| HeraCell 150i incubator | Thermo Fisher Scientific |
| Vortexer | Neolab |
| Biological Safety Cabinet HeraSafe2020 | Thermo Fisher Scientific |
| Trans-Blot Turbo Transfer System | Bio-Rad |
| Gel Dokumentation System ChemiDoc MP Imaging system, Detektor Supercooled CCD -30 °C, Pixel (Graustufen) 65535 | Bio-Rad |
| Vacuum pump BVC-vacuu-control | Vacuubrand |
| Pipette Eppendorf Research, 10-100 µL | Eppendorf |
| Pipette Eppendorf Research, 100-1,000 µL | Eppendorf |
| Pipette Eppendorf Research, 20-200 µL | Eppendorf |
| Centrifuge 5810R | Eppendorf |
| FACSCanto II | BD |
| Leica Application Suite LAS-X Version 3.1.5.16308 | Leica |
| FlowJo software version 10.6.2 | FlowJo LLC, BD Biosciences |
| Image Lab software version 5.0. | Bio-Rad |

**Table 2: Consumables**

| Product name | Application/specification | Article no. | Provider |
|---|---|---|---|
| 12-well plates | Tissue culture | 665180 | Greiner-bio-one |
| 96-well plates | Tissue culture | 650101 | Greiner-bio-one |
| Safe-lock tubes | 1.5 mL | 0030120.086 | Eppendorf |
| Tubes | 15 mL | 188271 | Greiner-bio-one |
| Filter tips | 20-200 µL | 30077555 | Eppendorf |
| Filter tips | 100-1,000 µL | 10212393 | Eppendorf |
| Filter tips | 0.1-10 µL | 30077512 | Eppendorf |
| Aspirating pipets | 2 mL w/o plug | 710183 | Greiner-bio-one |
| Serological pipets | 10 mL | 607160 | Greiner-bio-one |

**Table 3: Reagents**

| Product name | Application/specification | Article no. | Provider |
|---|---|---|---|
| RiboJuice | Transfection reagents | TR-1013 | Merck Millipore |
| FBS superior | Fetal bovine serum | 81D2925 | Biochrom GmbH |
| DPBS | No calcium, no magnesium | 14190-094 | Thermo Fisher Scientific |
| StemPro™ Accutase™ | Cell Dissociation Reagent | A11105-01 | Thermo Fisher Scientific |
| Opti-MEM GlutaMAX | Reduced serum medium | 51985034 | Thermo Fisher Scientific |
| EDTA | 0.5 M | 03690-100ML | Sigma-Aldrich |
| DMEM, high glucose, GlutaMAX™ Supplement, pyruvate | Cell culture | 31966047 | Thermo Fisher Scientific |
| BSA | Bovine serum albumin | GAUBSA01-64 | Eurobio |
| Triton X-100 | Immunofluorescence | X100-100ML | Sigma Aldrich |
| Hoechst | 1:5,000 | H3570 | Life Technologies |
| PFA, 32% | Fixation | 15714-5 | Electron Microscopy |
| ImmoMount media | Mounting media | 9990402 | Life Technologies |
| 4–15% Mini-PROTEAN® TGX™ Precast Protein Gels | Polyacrylamide gel | 4561083 | Bio-Rad |
| cOmplete™ ULTRA Tablets, Mini, EASYpack | Protease Inhibitor Cocktail | 5892970001 | Roche |
| Transfermembran Amersham™ Protran® NC | Nitrocellulose membrane | 4675.1 | Carl Roth |
| Color Prestained Protein Standard, Broad Range (11–245 kDa) | Molecular marker | P7712 | New England BioLabs |
| 10x Tris/Glycine/SDS | Running Buffer | 1610772 | Bio-Rad |
| 4 x Laemmli Sample Buffer | Western blot | 1610747 | Bio-Rad |
| DTT | Western blot | A2948,0025 | PanReac AppliChem |

| Product name | Application/specification | Article no. | Provider |
|---|---|---|---|
| Fixable Viability Dye eFluor™ 450 | FACS | 65-0863-14 | eBioscience |
| Fixation Buffer | FACS | 420801 | Biolegend |
| Permeabilization Buffer (10X) | FACS | 00-8333-56 | eBioscience |
| Ethanol | Western blot | 5054.4 | Carl Roth |
| Nonfat dried milk powder | Western blot | A0830,1000 | AppliChem |
| Tween-20 | Western blot | 9127.1 | Carl Roth |
| Pierce™ ECL Western Blotting Substrate | Chemiluminescent substrate | 32209 | Thermo Fisher Scientific |
| Tris | Western blot | 3170.2 | Carl Roth |
| Glycin | Western blot | 3790.2 | Carl Roth |
| SDS | Western blot | 0183.1 | Carl Roth |
| NaCl | Western blot | 9265.2 | Carl Roth |
| Sodium deoxycholate | Western blot | S1827-100G | Sigma Aldrich |
| EDTA | Western blot | 8040.3 | Carl Roth |

**Table 4: Antibodies and recombinant protein controls**

| Product name | Dilution | Article no. | Provider |
|---|---|---|---|
| SARS-CoV Spike S1 Subunit Protein Antibody, Rabbit PAb | 1:100 (IF); 1:1,000 (WB) | 40150-RP01 | Sino Biological |
| SARS-CoV-2 (2019-nCoV) Spike Antibody, Rabbit Mab | 1:400 (FACS) | 40150-R007 | Sino Biological |
| Alexa Fluor™ 647 Antibody Labeling Kit | N/A | A20186 | ThermoFisher Scientific |
| Concanavalin A, Alexa Fluor™ 594 Conjugate | 1:100 (IF) | C11253 | Invitrogen |
| Lectin GS-II From Griffonia simplicifolia, Alexa Fluor™ 594 Conjugate | 1:100 | L21416 | Invitrogen |
| Alexa Fluor® 488 AffiniPure Goat Anti-Rabbit IgG (H+L) | 1:400 (IF) | 111-545-003 | Jackson ImmunoResearch |
| Anti-Rabbit IgG (whole molecule)–Peroxidase antibody produced in goat | 1:2,000 (WB) | A0545 | Sigma Aldrich |
| NCP-CoV(2019-nCoV) Spike Protein (S1 Subunit, His Tag) | N/A | 40591-V08H | Sino Biological |

## 3.5 Methods

Western blot and FACS assays were performed to analyze antigen expression; immunofluorescence experiments were performed to assess correct localization in cells.

### 3.5.1 Study Design

The aim of this study was to analyze the expression of BNT162b2 DS and DP in HEK293T cells as a surrogate for a mammalian cell culture system. HEK293T cells were transfected with DS BNT162b2-RNA using a commercial transfection reagent or with DP BNT162b2 to express the S protein of SARS-CoV-2 as the viral antigen. Transfected HEK293T cells were allowed to express the S protein for 18 h before analysis.

Expression of DS BNT162b2-RNA was analyzed using Western blot to confirm expression.

FACS analysis was performed in triplicates in permeabilized cells to assess transfection frequencies and viability of cells transfected with either the DS BNT162b2-RNA, or with the DP BNT162b2.

Finally, immunofluorescence microscopic analysis was used on cells transfected with the DS BNT162b2-RNA to assess processing of the expressed protein in the endoplasmic reticulum (ER). Since the S protein contains a transmembrane domain it is expressed on the cell surface, and therefore processing in the ER was presumed.

### 3.5.2 Transfection of RNA Constructs in HEK293T Cells

HEK293T cells were seeded in 12-well plates with a cell number of $2 \times 10^5$ per well one day before transfection or with a cell number of $4 \times 10^5$ per well 6 h before transfection in DMEM with 10% FBS. For immunofluorescence experiments, HEK293T cells were seeded in 12-well plates with cover slips previously coated in collagen with a cell number of $2 \times 10^5$ per well one day before transfection in DMEM with 10% FBS.

Cells were transfected with DS BNT162b2-RNA using Ribojuice according to the manufacturer's protocol. As a transfection control, a modRNA construct encoding GFP was used. Briefly, 1 µg of RNA was diluted in Opti-MEM and mixed with transfection reagents. After an incubation of 4 min, 100 µL of the mixture was applied to the cells, mixed gently, and incubated at 37°C/5% $CO_2$ for 18 h. For FACS analysis DP BNT162b2 was transfected by diluting 1 µg of DP in 100 µl OptiMEM. The mixture was applied to the cells, mixed gently and centrifuged by 500×g for 5 min at room temperature before incubating at 37°C/5% $CO_2$ for 18 h.

Before proceeding with subsequent analyses, cells transfected with GFP were examined microscopically for successful transfection. Cells transfected with DS or DP encoding for the antigen were either harvested for Western blot analysis or prepared for subsequent immunofluorescence or FACS analysis

### 3.5.3 Western Blot Analysis

Western blot analyses were used to evaluate whether the designed constructs were expressed in HEK293T cells.

HEK293T cells were washed with PBS and detached from the well plate. Cells were collected in a 1.5 ml Eppendorf tube, centrifuged for 5 min at 300×g/4°C. Supernatants were discarded and the cell pellet was dissolved in 40 µL RIPA buffer (20 mM Tris, 0 15 M NaCl, 1% Triton X 100, 1% odium deo ycholate, 0 1% SDS, 10 mM EDTA) with protease inhibitors and incubated for 30 min on ice. 13.3 µL 4x Laemmli sample buffer with 10% DTT was added to the Eppendorf tubes and samples were heated for 5 min at 95°C. Recombinant protein controls were treated equally with 4x Laemmli sample buffer/10% DTT diluted in PBS to achieve a 1x dilution. Afterwards, gels were loaded with 25 µL of the samples and 3 µL of a marker. Gel electrophoresis was performed with Tris/glycine/SDS running buffer at 120 V. Proteins were transferred on a nitrocellulose membrane for 30 min at 25 V (max. 1 A) using transfer buffer (43 mM Tris, 35 mM glycine, 10% ethanol). The membranes were subsequently washed with PBS/0.1% Tween-20, blocked for 1 h with blocking buffer (PBS, 0.1% Tween-20, 5% nonfat dried milk powder) and incubated with the primary antibody in blocking buffer overnight at 4°C.

After incubation, membranes were washed with PBS/0.1% Tween-20 before incubation with the secondary antibody for 1 h at room temperature, and washed again before developing with chemiluminescent substrate and subsequent analysis on a BioRad ChemiDoc system.

### 3.5.4 FACS Analysis

To assess transfection frequencies of DS BNT162b2-RNA transfected cells using a commercial transfection reagent or DP BNT162b2 transfected cells, FACS analysis was performed. Cells were transferred to a 96-well plate format and stained with 50 µl fixable viability dye eFluor™ 450 diluted 1:500 for 15 min at room temperature. To remove residual dye, cells were washed with FACS Buffer (1xDPBS, 1% BSA, 1% 0.5M EDTA), centrifuged at 300xg for 5 min at 4°C and fixed with 100 µl Fixation Buffer (Biolegend) for 12 minutes at room temperature. Cells were washed with 1x Permeabilization Buffer (eBioscience) by centrifuging at 500xg for 5 min at 4°C and stained with 50 µl anti-S1 antibody labelled with AF647 diluted 1:400 in 1x permeabilization buffer for 30 min on ice. Afterwards, cells were washed twice with 1x permeabilization buffer, centrifuging at 500xg for 5 min at 4°C. Cells were resuspended in 100 µl FACS buffer before acquisition with a BD FACSCanto II.

### 3.5.5 Immunofluorescence

Immunofluorescence staining of transfected cells was used to test whether the construct was processed within the endoplasmic reticulum (ER) towards the cell membrane leading to secretion or surface expression.

HEK293T cells were washed twice in PBS and fixed in 4% PFA for 10 min. Afterwards, cells were washed three times for 5 min in PBS, permeabilized in PBS/0.2% Triton X-100 for 5 min and blocked in PBS with 2% BSA and 5% goat sera for 30 min. Cells were then incubated for 1.5 h with the primary antibody in PBS/2% BSA (anti-S1 antibody), washed three times in PBS for 5 min, and incubated with secondary antibodies and conjugated Concanavalin A and Lectin GS-II in PBS/2%BSA for 2 h. Afterwards, cells were stained with Hoechst for 3 min, washed three times in PBS for 5 min and were then mounted on slides and stored at 4°C until analysis. Cells were analyzed with a Leica SP8 confocal microscope.

# 4 TABLES AND FIGURES

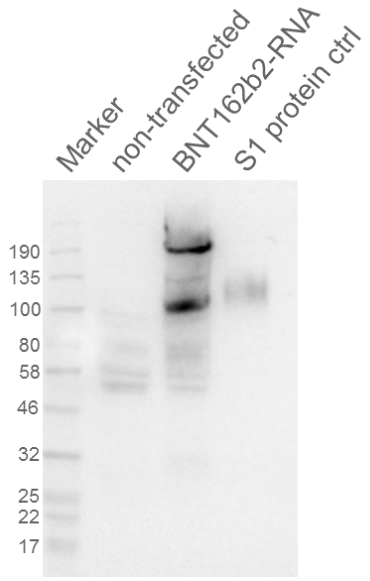


**Figure 1: Western blot analysis for detection of BNT162b2 antigen expression**

Cells were transfected with DS BNT162b2-RNA, and harvested after 18 h to allow antigen expression. Cells were lysed and lysates were subjected to a sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE) system using a 4–15% gradient polyacrylamide gel followed by Western blot analysis. BNT162b2 has a predicted size of 141.14 kDa. A recombinant SARS-CoV-2 S1 Subunit protein (76.5 kDa) was used as a positive control.

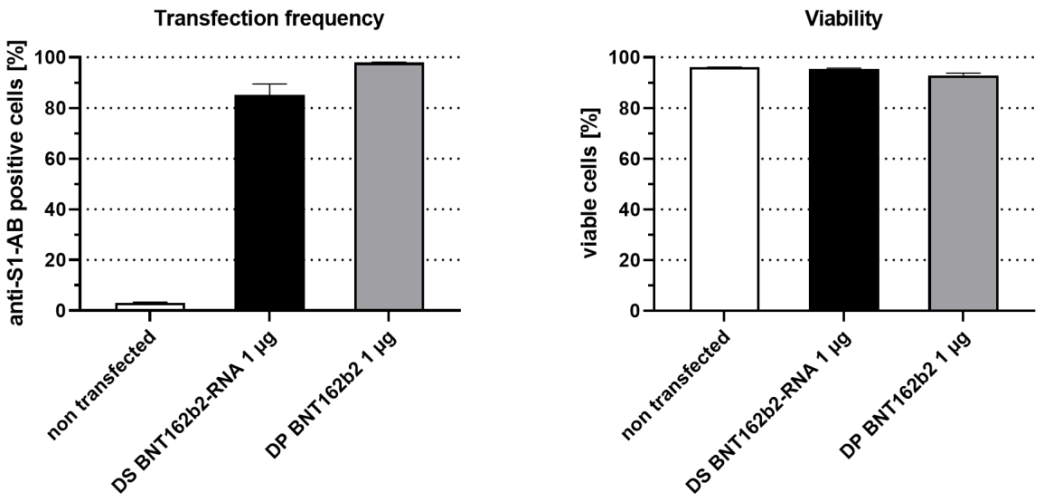


**Figure 2: FACS analysis of transfection frequency and cell viability**

Cells were transfected with BNT162b2-RNA or BNT162b2. After 18 h in culture, cells were stained with a viability dye, fixed, permeabilized and stained with a monoclonal rabbit antibody recognizing the S1 protein subdomain labelled with AF647. Non-transfected cells were used as a control.

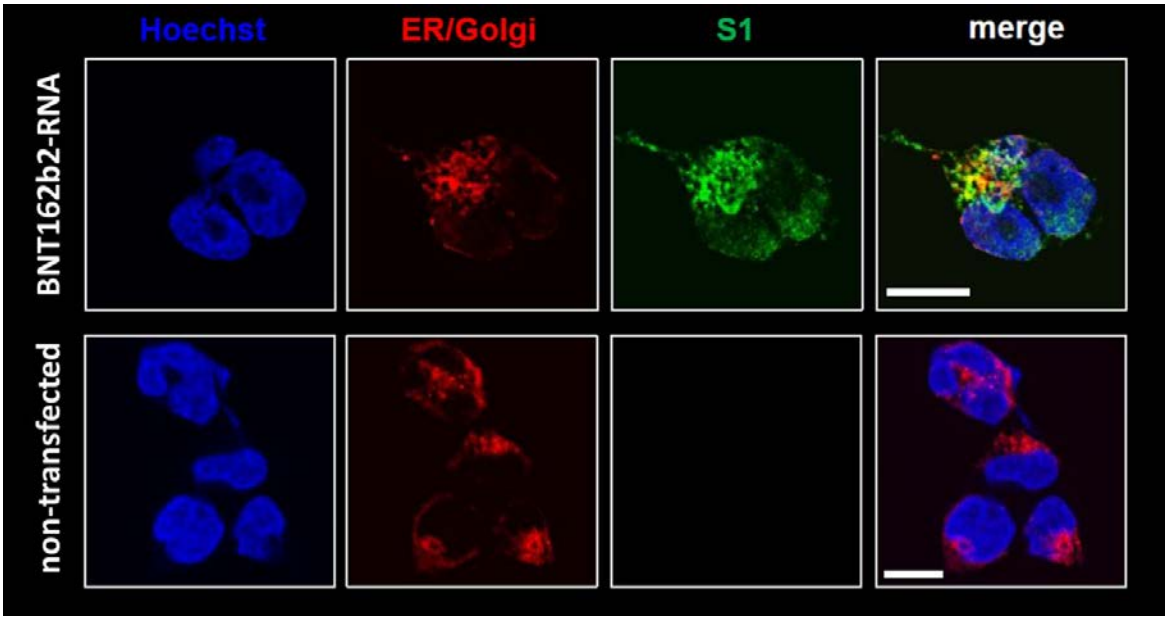


**Figure 3: Immunofluorescence staining of transfected cells**

Cells were transfected with BNT162b2. After 18 h in culture, cells were fixed and stained for: the ER (Concanavalin A, Alexa Fluor™ 594 conjugate and Lectin GS-II from Griffonia simplicifolia, Alexa Fluor™ 594 conjugate, red), the S1 protein subdomain using a polyclonal antibody (anti-S1 antibody and Alexa Fluor® 488, green) and deoxyr bonucleic acid (DNA) to define the nucleus (Hoechst, blue). The merged colored picture shows the co-localization of the two candidates within the ER (scale: 10 µm). A control, using non-transfected cells, is shown in the bottom row.

# 5 CONCLUSION

Western blot analysis confirmed the expression and size of the BNT162b2 antigen in cell lysates of HEK293T cells as a surrogate for correct expression in a eukaryotic system.

FACS analysis was performed to assess transfection frequencies of HEK293T cells transfected with either BNT162b2 drug substance (BNT162b2-RNA) or drug product (BNT162b2). Both, BNT162b2-RNA and BNT162b2 led to high frequencies of cells being transfected, with BNT162b2-transfected cells showing slightly higher transfection frequencies compared to BNT162b2-RNA transfected cells using a commercial transfection reagent. There were no differences in cell viability after transfection with BNT162b2-RNA or BNT162b2 when comparing to non-transfected cells.

Furthermore, co-localization of the S protein antigen with an ER marker was detected by immunofluorescence experiments in HEK293T cells expressing BNT162b2-RNA. These results show that the S protein is processed within the ER for surface expression.

# 6 DOCUMENT HISTORY

Reasons for changes compared to previous version:

| Section | Version | Version | Reason for change |
|---|---|---|---|
| 2.6 | 01 | 02 | Specification of lab book numbers |
| 3.4 | 01 | 02 | Equipment was added |
| 3.4 | 01 | 02 | Correction of IF staining |
| 3.5.5 | 01 | 02 | Correction of IF staining |
| 4 | 01 | 02 | Correction of IF staining |

# 7 REFERENCES

Not applicable.

# 8 APPENDIX

## Appendix 1: Certificates of Analysis

### BNT162b2-RNA

BioNTech RNA Pharmaceuticals GmbH

An der Goldgrube 12, 55131 Mainz, Germany
Tel.: +49 (0) 6131-90 84-0, Fax: +49 (0) 6131-90 84-390,
info@biontech.de

BIONTECH
RNA Pharmaceuticals

## Report of Results
## *In vitro* transcribed mRNA

| | |
|---|---|
| **Product:** | ***In vitro* transcribed mRNA RBP020.2 (ATM batch modRNAv09)** |
| Lot/Batch No.: | RNA-RF200321-06 |
| RNA length: | 4283 nt |
| Media and additives: | 10 mM HEPES/0.10 mM EDTA (pH 7.0) |
| Production date: | 19 Mar 2020 (produced by BioNTech RNA Pharmaceuticals GmbH) |
| Storage: | -30 °C to -15 °C |

| Test | Result |
|---|---|
| **Content (RNA concentration)**<br>Ultraviolet Absorption Spectrophotometry; $A_{260}$ | (b) (4) |
| **Identity (RNA length)**<br>Denaturing Agarose Gel Electrophoresis | |
| **RNA integrity**<br>Capillary Electrophoresis (Fragment Analyzer, Advanced Analytical) | |
| **Potency**<br>*In vitro* translation followed by gel electrophoresis | |
| **pH**<br>Potentiometric Determination of pH | |
| **Bacterial Endotoxins**<br>LAL-test (Ph. Eur. 2.6.14) | |
| **Residual DNA template**<br>Quantitative PCR | |
| **Residual dsRNA**<br>Antibody-based limit test | |
| **Osmolality**<br>Measurement of depression of freezing point | |
| **Bioburden**<br>Microbial examination of non sterile products (Ph. Eur. 2.6.12) | |

**Remarks:**

None.

**BNT162b2**

POLYMUN SCIENTIFIC
Immunologische Forschung GmbH

Donaustraße 99
A-3400 Klosterneuburg, Austria
Tel.: +43-2243-25060-300
Fax: +43-2243-25060-399
E-Mail: office@polymun.com
http://www.polymun.com

**Non-GMP CoA**
Material not for human use
Version 3

**Product:** **CoVVAC**
Batch: RBP020.2LNP
Lot: CoVVAC/270320

| Test | Method |
|---|---|
| Appearance | Visual Inspection (224/SOP/011) |
| RNA identity | CE (223/SOP/016) |
| RNA integrity | CE (223/SOP/016) |
| RNA content | Ribogreen Assay (221/SOP/018) |
| RNA encapsulation | Ribogreen assay +/- LNP disruption (221/SOP/018) |
| ALC-0315 content | HPLC-CAD (222/SOP/044) |
| ALC-0159 content | HPLC-CAD (222/SOP/044) |
| DSPC content | HPLC-CAD (222/SOP/044) |
| Cholesterol content | HPLC-CAD (222/SOP/044) |
| Particle size ($Z_{ave}$) | Dynamic light scattering (224/SOP/002) |
| Polydispersity index (PDI) | Dynamic light scattering (224/SOP/002) |
| pH | pH (224/SOP/016) |
| Osmolality | Freezing point depression (224/SOP/009) |
| Endotoxins/Pyrogens | Turbidimetric, kinetic LAL assay (Ph.Eur. 2.6.14/ USP<85>) |
| Bioburden | Membrane filtration method 225/SOP/001 |

(b) (4)

**Store at: -70°C**

Date: 09.04.20

Date: 09.04.20

(b) (6)

## Appendix 2: FACS gating strategy