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R&D STUDY REPORT No. R-20-0072

EXPRESSION OF LUCIFERASE-ENCODING MODRNA AFTER I.M. APPLICATION OF GMP-READY ACUITAS LIPID NANOPARTICLE FORMULATION

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Date: 27 Nov 2020

Reported by (b) (6)

Test item: modRNA encoding luciferase
Key words: COVID-19, modRNA, biodistribution, mouse, bioluminescence assay

This R&D report consists of 36 pages.

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LIST OF ABBREVIATIONS

ALC-0159	Proprietary PEG-lipid included as an excipient in the LNP formulation from Acuitas
ALC-0315	Proprietary amino-lipid included as an excipient in the LNP formulation from Acuitas
ALP	Alkaline phosphatase
ANOVA	Analysis of variance
AUC	Area under the curve
BALB/c	Mouse strain used in this study
BCIP	5-Bromo-4-chloro-3-indolyl-phosphate
BNT162	BioNTech's vaccine program against COVID-19
ConA	Concanavalin A
COVID-19	Coronavirus disease 2019
DPBS	Dulbecco's phosphate-buffered saline
ELISA	Enzyme-linked immunosorbent assay
ELISpot	Enzyme-linked immunosorbent spot
FELASA	Federation of European Laboratory Animal Science Associations
GMP	Good manufacturing practice
HRP	Horseradish peroxidase
IFN	Interferon
IgG	Immunoglobulin G
IL	Interleukin
i.m.	Intramuscularly
IP-10	Interferon-gamma induced protein 10
IVIS	*In vivo* imaging system
LNP	Lipid nanoparticle
Luc	Luciferase (from firefly *Pyractomena lucifera*)
MCP-1	Monocyte chemotactic protein 1
MHC	Major histocompatibility complex
MIP-1β	Macrophage inflammatory protein 1β
modRNA	Nucleoside-modified mRNA
NBT	Nitro blue tetrazolium
OD	Optical density
p/s	Photons per second
SD	Standard deviation
SOP	Standard operating procedure
S protein	Spike protein
saRNA	Self-amplifying mRNA
SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2
TNF	Tumor necrosis factor

RESPONSIBILITIES

Person responsible for the study:	(b) (6) (b) (6) BioNTech (b) (6) (b) (6)	26NOV2020 Date
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QA representative:	(b) (6) (b) (6) BioNTech SE	27NOV 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.

Approval of the author via e-mail according to CC-20-0081 (see attachment).
e-g (b) (6) 27NOV2020

1 SUMMARY

BioNTech is developing RNA-based vaccines designed to protect against the novel coronavirus disease that emerged in 2019 (COVID-19). The BNT162 project involves testing three RNA platforms, which are under development at BioNTech with the surface or spike protein (S protein) of the novel coronavirus (SARS-CoV-2) as the viral antigen. These RNAs will be formulated with a GMP-compatible LNP formulation provided by Acuitas.

In this study, the GMP-ready formulation containing the amino-lipid ALC-0315 and the PEG-lipid ALC-0159 (in this report referred to as LNP 8 which is the identical composition as used in BNT162) was tested in comparison with a (b) (4) (b) (4) by Acuitas, (b) (4) and an in-house formulation, (b) (4) to characterize the biodistribution of luciferase expressed by LNP-formulated nucleoside-modified mRNA (modRNA). Activation of the innate immune system, formation of antibodies against luciferase, and T-cell activation were also assessed.

Four groups of three BALB/c mice were injected intramuscularly (i.m.) with a total dose of 2 µg/animal of (b) (4) LNP8- or (b) (4) -formulated modRNA encoding luciferase or with buffer (DPBS) as control. At 6 h, 24 h, 48 h, 72 h, 6 d, and 9 d after injection, the *in vivo* luciferase expression was measured by luciferin application. Serum samples were taken 1 day before and 6 h after immunization as well as on day 9 for quantification of the activation of the innate immune system in a Luminex-based multiplex assay and antigen-binding antibody analysis in an IgG-specific ELISA. Splenocytes were isolated on 9 d to assess the T-cell response by IFN-γ ELISpot Assay.

An approximately 20-fold higher luciferase expression at the injections site was observed for modRNA-Luciferase (b) (4) and the GMP-ready modRNA-Luciferase LNP8 when compared to modRNA formulated with (b) (4) The difference between the area under the curve for (b) (4) and LNP8-formulated modRNA compared to buffer control as well as to (b) (4) formulated modRNA was statistically significant. In addition, luciferase expressed from the (b) (4) -formulated modRNA showed limited drainage to the liver compared to LNP8-formulated modRNA.

A multiplex assay showed that the innate immune system was temporally activated by (b) (4) and LNP8-formulated modRNA. The activation was more pronounced for (b) (4) formulated modRNA than for modRNA formulated with LNP8, indicating a formulation-related effect rather than a payload or expression level effect.

Treatment with modRNA with all tested LNP formulations did not induce the formation of luciferase-specific IgGs on day 9. However, a strong antigen-specific IFN-γ T-cell response was measured by ELISpot assay on day 9 for (b) (4) and LNP8-formulated modRNAs, with statistically significant differences between these test groups, the buffer control, and the (b) (4) group.

In conclusion, despite different biodistribution characteristics, both Acuitas LNPs allowed a high antigen expression level thereby inducing a strong T- but not B-cell response on day 9 post immunization.

(b) (6)		26 NOV 2020
Responsible Person: (b) (6) BioNTech (b) (6)	(b) (6)	Date

2 GENERAL INFORMATION

2.1 Sponsor and Test Facilities

Sponsor

BioNTech RNA Pharmaceuticals GmbH
An der Goldgrube 12
55131 Mainz
Germany

Test Facility

BioNTech SE
An der Goldgrube 12
55131 Mainz
Germany

2.2 Participating Personnel

Responsible person: (as defined in SOP-100-024)	(b) (6) (b) (6) BioNTech (b) (6)
Author:	(b) (6) BioNTech SE
Experimenter: Immunization, blood sampling, ELISA, ELISpot	(b) (6) BioNTech (b) (6)
Experimenter: Luminex-based multiplex assay	(b) (6) BioNTech (b) (6)

2.3 Study Dates

Start of experiments: 14 JAN 2020

Completion of experiments: 23 JAN 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.

- Animal test application approval number: G18-12-007
- SOP-030-071 Abtöten von Mäusen
- SOP-030-072 Fixiergriff und Ohrmarkierung bei Mäusen
- SOP-030-073 Betäubung bei Mäusen
- SOP-030-074 Blutentnahme bei Mäusen
- SOP-030-078 Isolierung muriner Splenozyten
- SOP-030-079 Intramuskuläre Applikation bei Mäusen
- SOP-030-110 IFN-γ ELISpot murin
- SOP-090-013 Biological safety in laboratories

2.5 Changes and Deviations

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

2.6 Documentation and Archive

Study 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\RN_R0030_AIRVAC\24_Preclinic\01_Vakzine Testing in vivo Luc\IM#88 GMP ready LNP Acuitas modRNA
- Lab books:
    - No. 1455 (complete study plan including results)
    - No. 1835 (IVIS images and quantification, Luciferase ELISA, ELISpot)
    - No. 1593 page 71-84 (Luminex-based multiplex assay)

3 INTRODUCTION

3.1 Background

In December 2019, an outbreak of pneumonia of unknown cause in Wuhan, Hubei province in China started. The disease spread rapidly and in January 2020, the agent was identified. By 1 April 2020, infection with the novel coronavirus (SARS-CoV-2) was confirmed in approximately 820,000 people with more than 40,000 casualties[1]. A vaccine is urgently needed and BioNTech decided to develop a rapid vaccine project (BNT162) with the surface or spike protein (S protein) of the virus as the viral antigen.

The development of *in vitro* transcribed RNA as an active platform for the use in infectious disease vaccines is based on the extensive knowledge of the company in RNA technology, which has been gained over the last decade. The core innovation is based on *in vivo* delivery of a pharmacologically optimized, antigen-coding RNA vaccine to induce robust neutralizing Abs and accompanying/concomitant T-cell responses to achieve protective immunization with minimal vaccine doses (Vogel et al. 2017, Moyo et al. 2018, Pardi et al. 2017).

At BioNTech, three different RNA platforms formulated with lipid nanoparticles (LNPs) are under development, namely non-modified uridine-containing mRNA (uRNA), nucleoside-modified mRNA (modRNA) and self-amplifying RNA (saRNA). In the present study, an LNP-formulated modRNA encoding luciferase was used representatively to investigate the *in vivo* biodistribution and the immune response of the vaccine candidates.

LNP formulations from a third party provider (Acuitas) were tested in comparison to the in-house formulation (b) (4) Acuitas (b) (4) (b) (4) (b) (4) Acuitas also provided an LNP formulation that is cGMP-ready, namely LNP8, which contains two proprietary lipids (ALC-0159 and ALC-0315) and has the identical composition as the LNP formulation used in the BNT162 program.

3.2 Objectives

The objective of this study was to investigate the biodistribution of luciferase expressed by the LNP-formulated modRNA using bioluminescence measurements in BALB/c mice, as well as innate immune system activation, formation of antibodies against luciferase and T-cell activation.

[1] Coronavirus disease (COVID-2019) situation report 72, World Health Organization; /www.who.int/emergencies/diseases/novel-coronavirus-2019/situation-reports

3.3 Study Design

Four groups of three BALB/c mice were injected intramuscularly (i.m.) in the right and left hind leg with each 1 µg of LNP-formulated modRNA encoding luciferase or with buffer as control on day 0. At 6 h, 24 h, 48 h, 72 h, 6 d, and 9 d after injection, the *in vivo* luciferase expression was measured by luciferin application.

In addition, serum samples were collected on day -1 and 6 h and 9 d post immunization and cytokine/chemokine level determination (multiple) and on day 1 and 9 for luciferase-specific ELISA. On day 9, spleens were resected for immunological analysis using IFN-γ ELISpot assays.

Table 1: Study design

Group	Treatment	Dose [µg/ mouse]	Formulation	Treatment schedule	End of experiment	Sample collection
1	Buffer control	N/A	N/A	Day 0	Day 9	Serum ELISA on days -1 and 9, serum for Multiplex assay on day -1, 6 h, and 9 d; splenocytes for ELISpot on day 9
2	modRNA-Luciferase (b) (4)	2 µg (1 µg/leg)	(b) (4)	Day 0	Day 9	
3	modRNA-Luciferase (b) (4)	2 µg (1 µg/leg)	Acuitas proprietary	Day 0	Day 9	
4	modRNA-Luciferase LNP8 (GMP-ready)	2 µg (1 µg/leg)	ALC-0315:ALC-0159:DSPC:Chol	Day 0	Day 9	

4 MATERIALS AND METHODS

4.1 Test Item

- LNP-formulated modRNA encoding luciferase diluted to 0.05 mg/mL to obtain a dose of 1 µg in 20 µL application volume. For CoAs see Appendix 2: Certificates of Analysis of RNA and LNPs.
- Acuitas LNPs:
  - (b) (4)
  - LNP8 modRNA Luc, RNA-EH190611-01c, batch FM-1074-D, 90% encapsulation, 1.0 mg/mL encapsulated RNA, diameter 71 nm, polydispersity 0.053, storage temperature -80°C.
- BioNTech LNP:
  - (b) (4)

Table 2: Lipid component formulations

Formulation	Lipids			
	Functional lipid 1	**Functional lipid 2**	**Structural lipid 1**	**Structural lipid 2**
(b) (4)	Acuitas proprietary	Acuitas proprietary	Acuitas proprietary	Acuitas proprietary
LNP8	ALC-0315	ALC-0159	DSPC	Cholesterol
(b) (4)	(b) (4)	(b) (4)	(b) (4)	(b) (4)

4.2 Control Item

- DPBS

4.3 Test System

- *Mus musculus*: 12 female BALB/c mice at an age of 9 weeks at study start with a body weight of approximately 25 g

4.4 Materials

Table 3: Materials

Product name	Application/specification	Article no.	Working dilution	Provider
Dulbecco's phosphate-buffered saline (DPBS)	Buffer control	14190-094	1×	Thermo Fisher Scientific
Syringes 0.3 mL 30 G	Insulin syringes for i.m. application	4144150	N/A	BD
Syringes 0.5 mL 29 G	Insulin syringes for i.p. application	324824	N/A	BD
Luciferin	Substrate for *in vivo* luciferase imaging	122799-10	150 mg/kg	Perkin-Elmer
Microvette 500 Z-gel tubes	Blood sampling	2021-01-31	N/A	Sarstedt
Xenogen IVIS® Spectrum	*In vivo* BLI imager	-	N/A	Caliper Life Sciences
MaxiSorp plate	ELISA	439454	N/A	Thermo Fisher Scientific
QuantiLum recombinant luciferase	Positive control	E1701	100 ng/µL	Promega
Casein blocking buffer	10×	B6429	1×	Sigma-Aldrich
TMB ONE ECO-TEK	Chromogenic substrate for horseradish peroxidase	4380H	N/A	BIOTREND
Sodium bicarbonate	7.5% $NaHCO_3$	S8761-100 ml	N/A	Sigma-Aldrich
HCl	Hydrochloric acid solution volumetric, 0.1 M HCl	2104-50 ml	N/A	Sigma-Aldrich
RPMI1640 medium	Cell culture medium	61870	N/A	Gibco
Biotek Epoch	ELISA plate reader	-	N/A	Biotek
Mouse IFN-γ ELISpotPLUS kit	Kit for enumeration of cells secreting mouse IFN-γ	3321-4APT-2	N/A	Mabtech
ImmunoSpot® S5 Versa Analyzer	ELISpot plate reader	-	N/A	Cellular Technology Ltd.
RPMI1640 medium	Cell culture medium	61870	N/A	Gibco
Multiplex	PROCARTAPLEX 10 PLEX	PPX-10 - MXU63C9	N/A	Life Technologies GmbH
Bio-Plex 200	Multiplex reader	-	N/A	Bio-Rad
Anti-firefly luciferase antibody (mAb21),	Assay control ELISA	ab64564	1:1,000	Abcam
Mouse IgG isotype	Assay control ELISA	0107-08	1:100 as starting dilution	Southern Biotech
Goat anti-mouse IgG HRP	Secondary antibody ELISA	115-035-071	1:15,000 (if stored in 50% glycerol 1:7,500)	Jackson

Table 4: Software

Product name	Application	Provider
Living image	*In vivo* BLI quantification	Perkin-Elmer
Prism	Analysis	GraphPad Software Inc.
Excel	Animal monitoring, raw data ELISA	Microsoft Corp.
Bio-Plex Manager (6.1)	Bio-Plex reader	Bio-Rad
Gen5	Absorbance reader	Biotek
ImmunoCapture V6.3	ELISpot analysis	Cellular Technology Ltd.

Table 5: Peptide pool for stimulation of splenocytes

Peptide pool MHC-I	
Name	**Sequence**
Firefly luciferase-1	GFQSMYTFV
Firefly luciferase-2	VPFHHGFGM
Firefly luciferase-3	VALPHRTAC

Consensus sequences from Limberis et al. 2009

4.5 Methods

4.5.1 Animal Care

4.5.1.1 General Information

BALB/c mice were delivered at the age of at least six weeks. Delivered mice were used for experiments after approximately one week of acclimatization. All experiments and protocols were approved by the local authorities (local welfare committee), conducted according to the FELASA recommendations and in compliance with the German animal welfare act and Directive 2010/63/EU. Only animals with an unobjectionable health status were selected for testing procedures.

All animals were registered upon arrival in the lab animal colony management system PyRAT (Scionics Computer Innovation GmbH, Dresden, Germany) and tracked until death. Each cage was labeled with a cage card indicating the mouse strain, gender, date of birth, and number of animals per cage. At the start of an experiment additional information was added such as the project and license number, the start of the experiment and details on interventions. Where necessary for identification, animals were arbitrarily numbered with earmarks.

4.5.1.2 Housing Condition and Husbandry

Mice were housed at BioNTech SE's animal facility under barrier and SPF conditions (An der Goldgrube 12, 55131 Mainz) in individually ventilated cages (Sealsafe GM500 IVC Green Line, TECNIPLAST, Hohenpeißenberg, Germany; 500 cm²) with a maximum of five animals per cage. The temperature and relative humidity in the cages and animal unit was kept at 20-24°C and 45-55%, respectively, and the air change (AC) rate in the cages at 75 AC/hour. The cages with dust-free bedding made of

debarked chopped aspen wood (Abedd LAB & VET Service GmbH, Vienna, Austria, product code: LTE E-001) and additional nesting material were changed weekly. Autoclaved sniff M-Z food (sniff Spezialdiäten GmbH, Soest, Germany; product code: V1124) and autoclaved water (tap water) were provided *ad libitum* and changed at least once weekly. All materials were autoclaved prior to use.

4.5.2 Animal Monitoring

Routine animal monitoring wa carried out daily and included in pection for dead animals and control of food and water supplies. Each animal's health was closely assessed at least once weekly. The general physical condition was assessed with regard to the following parameters:

- Body weight change
- Macroscopic assessment of activity level/ behavior
- Macroscopic assessment of general discomfort: drop in body temperature determined by touch and by visual inspection of ears and paws. Ears and paws appear pink in a healthy mouse, white in a mouse with discomfort indicated by reduced blood circulation
- Macroscopic assessment of fur condition and appearance of eyes, inspection of body cavities/ fluids
- Macroscopic assessment of irregularities in breathing ability
- Indication of pain
- Macroscopic assessment for signs of automutilation and or fighting

4.5.3 Endpoint of Experiment / Termination Criteria

Animals were euthanized in accordance with §4 of the German animal welfare act and the recommendation of GV-SOLAS by cervical dislocation or by exposure to carbon dioxide. Additionally, termination criteria applied according to the specification within the respective animal test approval as listed below. Body weight losses exceeding 20%, or a high severity level in any of the parameters found in Section 4.5.2 were on their own sufficient reason for immediate euthanasia.

4.5.4 Injection of Test and Control Items

Animals were anesthetized by inhalation of 2.5% isoflurane in oxygen and the injection site (hind leg) was shaved. Buffer or dissolved test item was applied i.m. into the *musculus gastrocnemius* at a volume of 20 µL. All mice received 1 µg in each leg. After injection and a short recovery phase from anesthesia, the animals were observed for any signs of discomfort due to the injection procedure.

4.5.5 Bioluminescence Measurements

The mice were monitored over a period of 9 days using *in vivo* imaging system (IVIS) measurements. Briefly, the Xenogen IVIS® Spectrum device was used for *in vivo* imaging according to the manufacturer's instruction. Approximately 6 h after LNP administration and 5 min prior to imaging, animals were injected for the first time intraperitoneally (i.p.) with luciferin (150 mg/kg, dosing volume: 300 µL, 29 G needle). Mice were anesthetized (2.5% isoflurane/O_2) and placed in the imager, first with the dorsal side exposed and then with the ventral side exposed, and luciferase activity was measured. Images were taken with exposure time and sensitivity set to 1 s, 10 s, and 1 min and bin 2, bin 4, or bin 8, respectively. The dorsal and ventral images were analyzed by visual inspection after aligning of sensitivities of each picture and used for illustration of findings. The images were analyzed using Living Image *in vivo* imaging 3.0 software, where the regions to be quantified (radiance) were drawn manually and calculated automatically (region of interest, ROI), to follow kinetics of the total fluxes (p/s) over time in a GraphPad file.

4.5.6 Blood Sampling via the *Vena Facialis*

Blood was sampled via the *vena facialis* according to SOP-030-074. In short, without prior anesthesia, mice were held tightly and using a lancet, the *v. facialis* was punctured in a precise and short movement. Blood was collected into Microvette 500 Z-gel tubes, subsequently the restraining grip was loosened. Blood samples were centrifuged at 10,000 ×g (room temperature) for 5 min and serum transferred to a pre-labeled 1.5 mL reagent tube before storage at -20°C.

4.5.7 Luminex-based Multiplex Assay (ProcartaPlex Multiplex Immunoassay)

The assay was performed according to manufacturer's protocol. Briefly, magnetic beads were added to the provided 96-well flat bottom plate and the beads were washed (wash buffer 1×) with the help of a hand-held magnetic plate washer. The antigen standard was reconstituted in universal assay buffer (1×), pooled in one tube, the volume adjusted to a final volume of 250 µL, serial diluted (4-fold serial dilution steps), and 50 µL was added to the designated wells. Serum samples were diluted 1:1 with the universal assay buffer and 50 µL added to the wells. The standard was measured in duplicates and the serum samples in triplicates. The plate was incubated on a plate shaker at 500 rpm) for 2 h covered with a black lid. After three wash steps, 25 µL of the ready-to-use detection antibody was added, incubated for 30 min on the shaker and washed three times. Streptavidin-PE (50 µL) were added and the plate incubated for 30 min on the shaker and washed three times. The beads were resuspended in 120 µL reading buffer, the plate was sealed, and data were acquired in the Bio-Plex 200 Luminex system.

4.5.8 Luciferase-specific ELISA

Luciferase-specific IgGs in serum samples obtained on study days 1 and 9 were detected using ELISA. Recombinant luciferase (100 ng/100 µL) protein was utilized to coat MaxiSorp plates at 4°C overnight. Upon washing and blocking using casein-based blocking buffer, serum samples were screened for luciferase-specific antibodies by incubation on plates for 1 h at 37°C. An anti-firefly luciferase antibody (mAb21) as well as a mouse IgG isotype were included as assay controls. Subsequently, plates were incubated with horseradish peroxidase (HRP)-labeled secondary anti-mouse IgG antibody for another 45 min at 37°C before 3, 3', 5, 5'-tetramethylbenzidine (TMB) ONE substrate was applied. Colorimetric detection was monitored and optical density read at 450 nm calculated to a wavelength reference of 620 nm (ΔOD 450–620 nm).

4.5.9 ELISpot Analysis

4.5.9.1 T-cell epitope prediction

The respective peptides for stimulation of splenocytes (Table 5) were used as published by Limberis et al. 2009, where the authors mapped the dominant and minor T-cell epitopes in BALB/c mice (GFQSMYTFV and VPFHHGFGM, VALPHRTAC, respectively) for monitoring cellular responses *in vivo*. No modifications have been added to the published peptides before peptide synthesis by JPT technologies GmbH.

4.5.9.2 Sample Collection and Processing

Spleens were removed on day 9 after euthanizing the mice, and single-cell suspensions were prepared (SOP-030-078). In brief, the removed organs were pressed through a 70 µm cell mesh using the plunger of a syringe to release the cells from the organ into a tube. After washing with PBS the cell pellet was incubated with erythrocyte lysis buffer, washed in PBS, and passed again through a 70 µm cell mesh. Resulting cells were resuspended in medium and counted.

4.5.9.3 IFN-γ ELISpot Assay

The IFN-γ ELISpot assay was used to measure IFN-γ release after *in vitro* stimulation of T cells as an indicator for the induction of antigen-specific T cells. ELISpot analysis was performed using the Mabtech Mouse IFN-γ ELISpot^{PLUS} kit. Isolated splenocytes were seeded to pre-coated ELISpot plates at 5×10^5 cells/well in 200 µL medium and stimulated with antigen-specific peptide pools composed of single peptides and a final concentration of 2 µg/mL per peptide, predicted as described in Section 4.5.9.1 overnight in a humidified incubator at 37°C. As peptide controls, splenocytes were incubated with 6 µg/mL of an irrelevant AH1 peptide derived from the endogenous retroviral gene product envelope glycoprotein 70 (gp70; AH1: amino acids 6 to 14). Splenocytes were incubated with medium alone as a negative control or with 2 µg/mL Concanavalin A (ConA) as an internal positive control, confirming the functionality of

the assay. Spots were visualized with a biotin-conjugated anti-IFN-γ antibody followed by incubation with streptavidin-alkaline phosphatase (ALP) and 5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium (BCIP/NBT) substrate. Spot numbers were counted and analyzed using the ImmunoSpot® S5 Versa ELISpot Analyzer, the ImmunoCaptureTM image acquisition software, and the ImmunoSpot® Analysis software version 5. The quality control (QC) function of ImmunoSpot analysis software was used to limit false positive spot counts. All tests were performed in triplicate and spot counts were summarized as median values for each triplicate.

4.5.10 Statistical Analysis

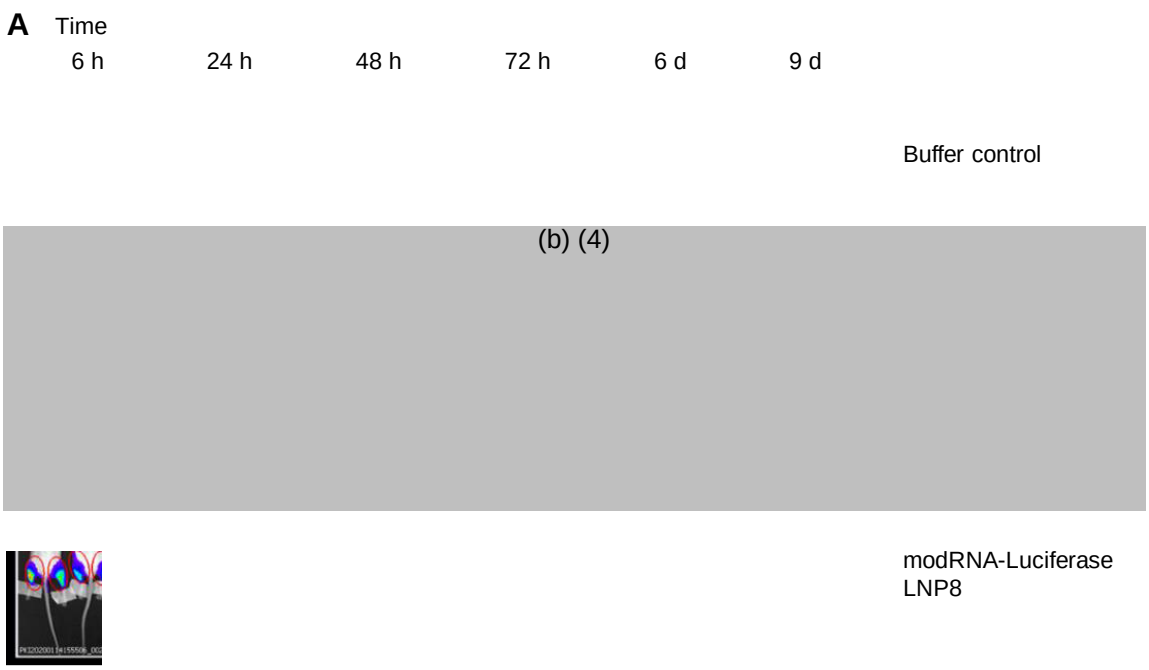
GraphPad Prism 8 software (La Jolla, USA) was used for statistical analysis and figure generation. All groups were compared by a one-way ANOVA with Tukey's multiple comparison post-test on each measurement day (area under the curve for bioluminescence assay, ELISA, and ELISpot analysis).

5 RESULTS

5.1 Bioluminescence Measurements

The biodistribution of luciferase expressed by the LNP-formulated modRNA after i.m. injection was assessed by bioluminescence measurements. Mice received a total dose of 2 µg (b) (4) LNP8, or (b) (4) -formulated modRNA, the control group received 20 µL DPBS only.

Mice were monitored over nine days and Luciferase signal was recorded and quantified (Figure 1).



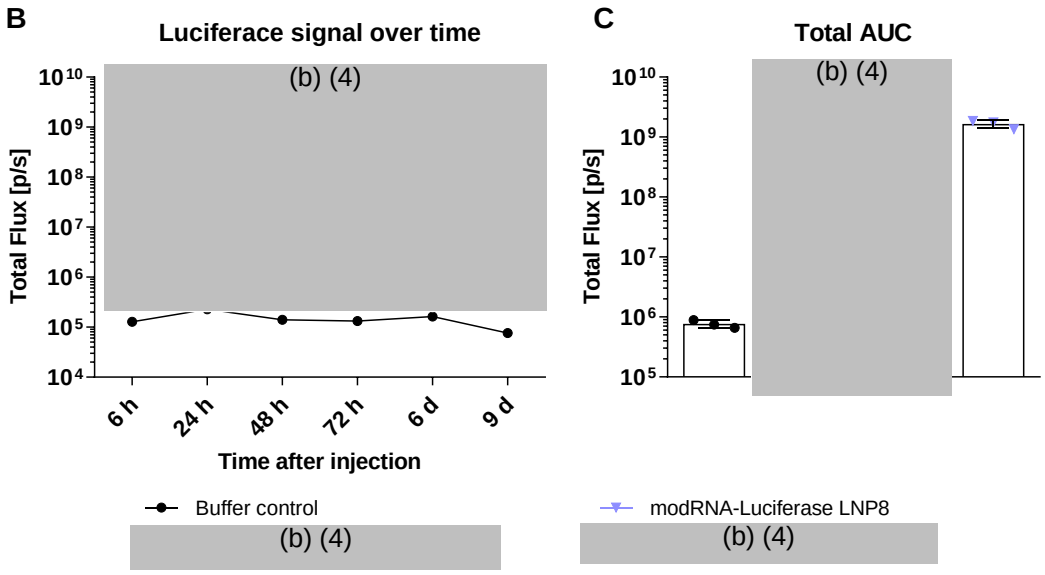


Figure 1: Bioluminescence measurement using the LNP-formulated modRNA encoding for luciferase

BALB/c mice were injected i.m. in the right and left hind leg with each 1 µg of LNP-formulated modRNA encoding luciferase or DPBS only. A) At different time points after injection, the luciferase expression *in vivo* was measured by luciferin application. After 9 d, the reporter expression dropped to background levels. B) Quantification of luciferase signal over time and C) as total area under the curve (AUC, ±SD). p/s: photons per second

All formulations resulted in a modRNA-typical expression over time (Figure 1). Highest signal was detected at the first time points after immunization at the injection site and the signal declined slowly over time until day 9 (Figure 1B). Luciferase expressed by the modRNA formulated with Acuitas LNP8 drained to the liver as visualized by luciferase expression at 6 h in the liver region (b) (4) (b) (4) Acuitas (b) (4) formulations (Figure 1A and Figure 2). Group mean luciferase expression from RNA formulated with Acuitas (b) (4) LNP8 in the muscle at 6 h was approximately 1 × 10^9 p/s, (b) (4) (b) (4) Hence, Acuitas-formulated modRNA started at about 20-fold higher signal levels, stayed more than 20-fold higher until 72 h (~7 × 10^7 p/s for (b) (4) LNP8 vs (b) (4)) and declined then to a low level (b) (4) (b) (4) on day 9 (~3–5 × 10^5 p/s).

Area under the curve calculation allowed comparing overall expression levels over the course of the experiment (Figure 1C). Statistical significance was assessed by one-way ANOVA with Tukey's multiple comparison post-test comparing all groups with each other. Total luciferase expression from modRNA formulated with (b) (4) (b) (4) while total luciferase expression from modRNA formulated with (b) (4) LNP8 was approximately (b) (4) 1.5 × 10^9 p/s, respectively. The difference between the area under the curve for (b) (4) LNP8-formulated modRNA compared to buffer control (b) (4) was

statistically significant ($p < 0.0001$). (b) (4)

(b) (4)

5.2 Liver Expression (b) (4) LNP8

As mentioned in Section 5.1, luciferase expressed by the modRNA formulated with Acuitas LNP8 drained to the liver as visualized by luciferase expression at 6 h in the liver region (b) (4) Acuita (b) (4) (Figure 2A). Here the luciferase signal of modRNA formulated with (b) (4) LNP8 was quantified for better comparison. Group mean luciferase expression from RNA formulated with Acuitas (b) (4) in the liver at 6 h was (b) (4) while luciferase expression of RNA formulated with LNP8 was at about 4.94×10^7 p/s. Hence, luciferase expression from (b) (4) Acuitas (b) (4) (b) (4) compared to LNP8 (Figure 2B). The liver luciferase expression from RNA formulated with Acuitas LNP8 dropped to 2.4×10^6 p/s at 24 h, while the luciferase signal from RNA formulated with Acuitas (b) (4) No liver signal was detected at 48 h post immunization. Statistical analysis was not performed.

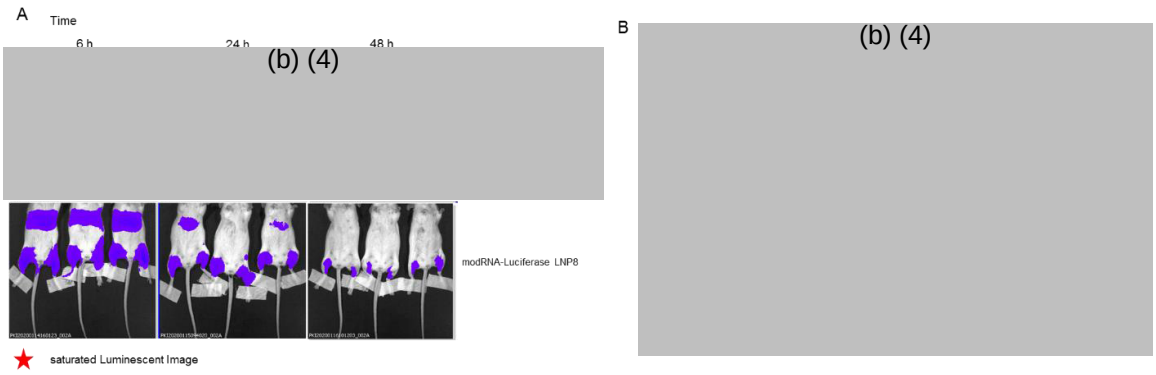


Figure 2: Bioluminescence measurement in the liver using the LNP-formulated modRNA encoding for luciferase

BALB/c mice were injected i.m. in the right and left hind leg with each 1 µg of LNP-formulated modRNA encoding luciferase. A) At 6 h, 24 h, and 48 h after injection, the luciferase expression *in vivo* was measured by luciferin application. B) Quantification of luciferase signal in the liver over time (mean ±SD). p/s: photons per second.

5.3 Luminex-based Multiplex Assay

Activation of the innate immune system was assessed in a Luminex-based multiplex assay (Procarta immunoassays). Serum samples (day -1 (pre), 6 h, and day 9) were tested for levels of the following chemokines and cytokines: MCP-1, MIP-1β, TNF-α, IFN-α, IFN-γ, IL-2, IL-6, IL-10, IL-1β, IP-10 (Figure 3). No cytokines/chemokines were detected in the pre-serum. (b) (4)

(b) (4)

(b) (4) Immunization with LNP8 induced slightly increased levels of MCP-1, IL-6, and IP-10 at 6 h post immunization (b) (4)

(b) (4) All chemokine/cytokine levels dropped to background levels at day 9. Statistical analysis was not performed for this assay.

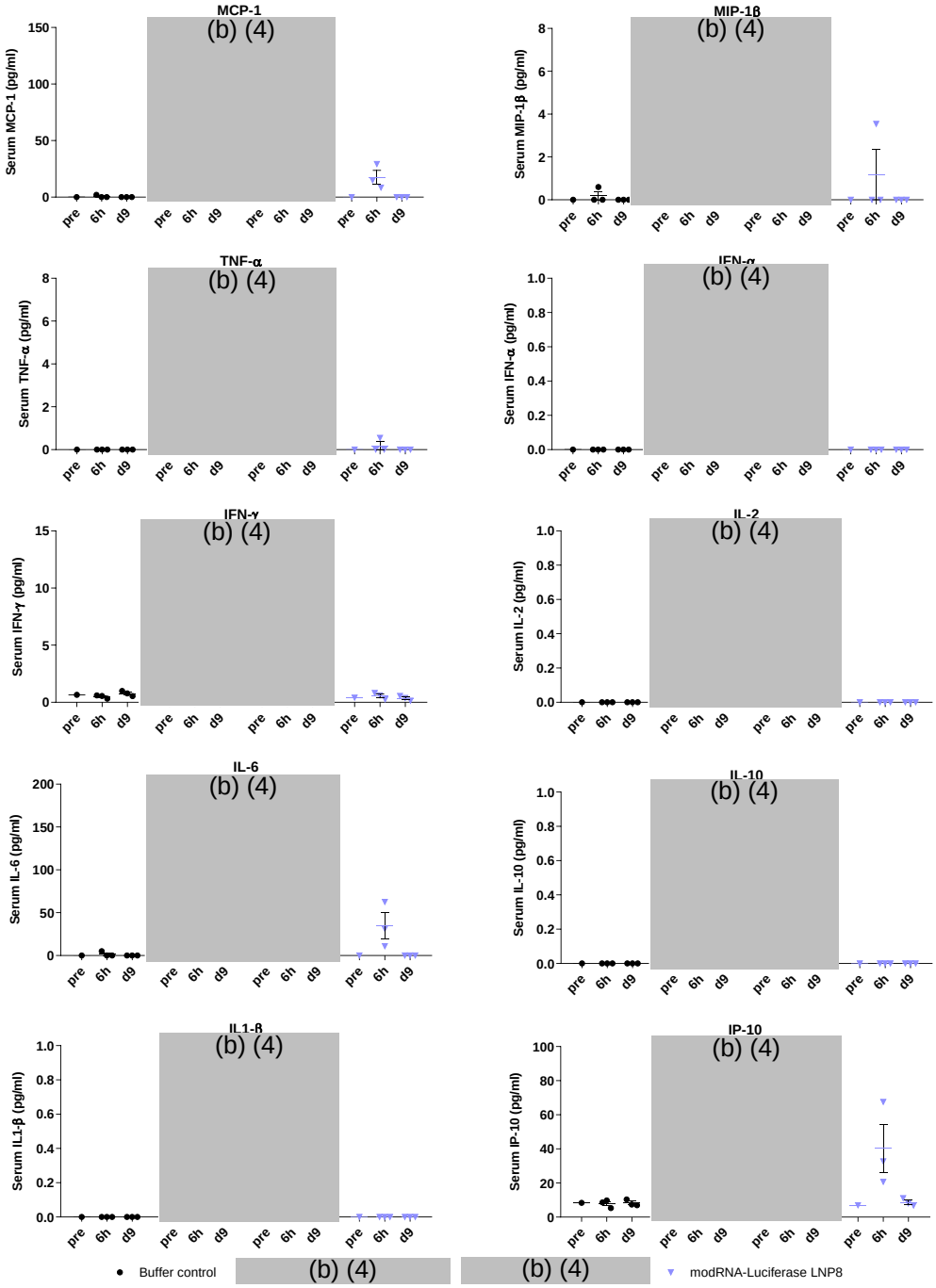


Figure 3: Activation of the innate immune system by LNP-formulated modRNA encoding for luciferase

BALB/c mice were injected i.m. in the right and left hind leg with each 1 µg of LNP-formulated modRNA encoding luciferase or DPBS only. Serum samples (day -1 (pre), 6 h, and 9 d) were assessed for presence of indicated chemokines/cytokines in a Luminex-based multiplex immunoassay.

5.4 Luciferase-specific ELISA

Luciferase-specific IgGs in serum samples obtained on study days -1 and 9 were investigated by ELISA.

Before immunization, no luciferase-specific IgGs were detected (day -1, Figure 4). Treatment with modRNA with all tested LNP formulations did not induce the formation of luciferase-specific IgGs on day 9 post immunization.

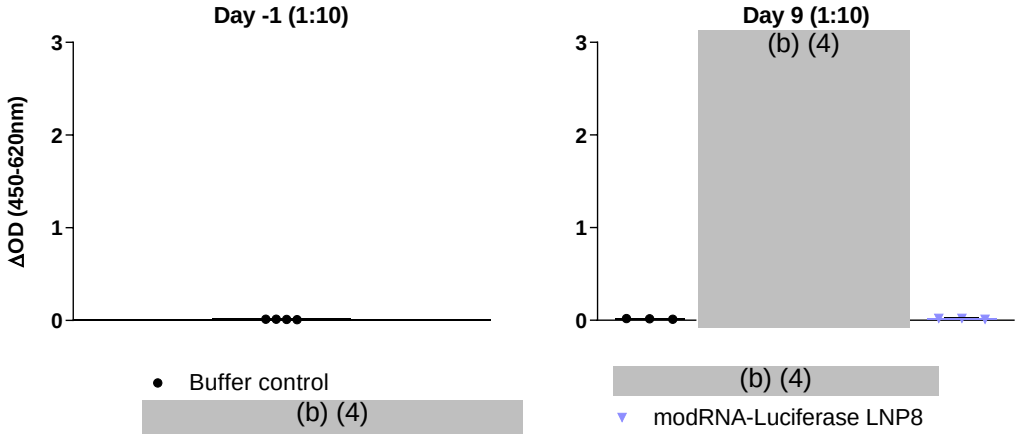


Figure 4: Luciferase-specific IgG ELISA on days -1 and 9

BALB/c mice were immunized with 1 µg/leg luciferase-encoding modRNA on day 0. Serum samples were collected on days -1 and 9 and the total amount of antigen-specific immunoglobulin G (IgG) was measured via ELISA. The serum 1:10 diluted. Individual ΔOD values are shown by dots; group mean values are indicated by horizontal bars (±SD).

5.5 IFN-γ ELISpot Assay

Mice were euthanized on day 9 and splenocytes were isolated to assess T-cell responses by ELISpot analysis. Splenocytes were stimulated with luciferase-specific peptide pools (Table 5) and IFN-γ secretion was detected. Statistical significance was assessed by one-way ANOVA with Tukey's multiple comparison post-test comparing all groups with each other. Control measurements were performed using an irrelevant peptide pool, medium only, or Concanavalin A.

Stimulation of splenocytes with MHC I-specific peptide pools induced IFN-γ responses in T cells of animals immunized with all modRNA LNP candidates (Figure 5). Group mean values of 53 spots per 5×10^5 cells were counted for animals injected with buffer control after stimulation with MHC I-specific luciferase peptide pools. The high spot count in can be attributed to reactivity of T cells of one mouse in group 1. Splenocytes of this mouse react also to the stimulation with AH1, the negative control. The group mean values are also 44 spots be 5×10^5 cells for the AH1 control, thus the response

of the control group to the luciferase-specific peptide pool can be considered unspecific. Group mean spot counts after stimulation with MHC I-specific peptide pools were (b) (4) (b) (4) 519 spots per 5 × 10^5 cells for the group treated with LNP8-formulated modRNA. The reactivity of splenocytes of the treatment groups to the negative control was very low (6-8 spots per 5 × 10^5 cells), thus activation of T-cells with luciferase peptides is highly specific in the treatment groups.

(b) (4) (b) (4) The differences between the groups treated (b) (4) LNP8-formulated modRNAs compared to buffer control were statistically significant ((b) (4) p = 0.0051 for modRNA-luciferase LNP8). The (b) (4) and LNP8 groups displayed significantly higher MHC I-specific IFN-γ secretion (b) (4) (b) (4) p = 0.0163 for modRNA-luciferase LNP8). (b) (4) (b) (4)

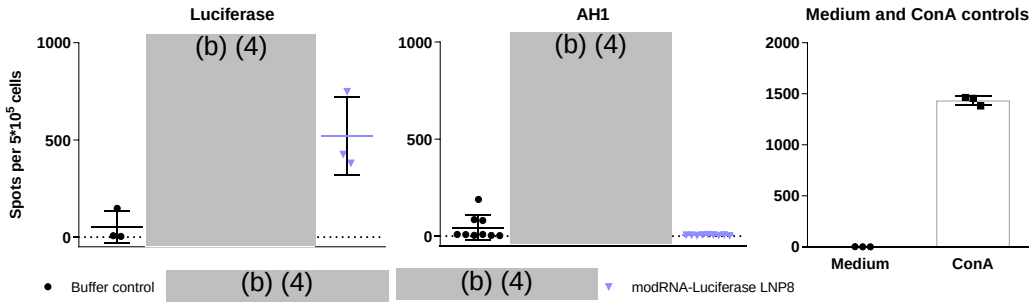


Figure 5: ELISpot analysis using splenocytes on day 9

ELISpot assay was performed using splenocytes isolated on day 9 after prime immunization. Splenocytes were stimulated with MHC I-specific luciferase peptide pools and IFN-γ secretion was measured to assess T-cell responses. Individual spot counts are shown by dots; group mean values are indicated by horizontal bars (±SD).

6 CONCLUSION

After injection of LNP-formulated modRNAs, an approximately 20-fold higher luciferase expression at the injections site was observed in a bioluminescence assay for (b) (4) the GMP-ready modRNA-Luciferase LNP8 (b) (4) (b) (4) The difference between the area under the curve for (b) (4) LNP8-formulated modRNA compared to buffer control (b) (4) was statistically significant. (b) (4) (b) (4)

A multiplex assay showed that the innate immune system was temporally activated by (b) (4) LNP8-formulated modRNA. (b) (4) (b) (4)

Treatment with modRNA with all tested LNP formulations did not induce the formation of luciferase-specific IgGs on day 9 as measured by ELISA.

However, a strong antigen-specific IFN-γ T-cell response measured by ELISpot assay on day 9 for (b) (4) LNP8-formulated modRNAs, with statistically significant differences between these test groups and the buffer control (b) (4)

7 DOCUMENT HISTORY

Reasons for changes compared to previous version:

Minor editorial changes, such as the correction of typing errors, are not listed.

Sections	Version 01	Version 02	Reason for change
1	-	More detailed information about LNP8 added	Clarification that LNP8 contains ALC-0159 and ALC-0315
3.1			
6			
4.1		Table added	Details of LNP lipid component formulations

Sections	Version 02	Version 03	Reason for change
4.1	-	Test item information updated	Diameter of LNP8 modRNA was corrected to 71 nm

8 REFERENCES

Limberis MP, Bell CL, Wilson JM. Identification of the murine firefly luciferase-specific CD8 T-cell epitopes. Gene Ther. 2009;16(3):441-7.

Moyo N, Vogel AB, Buus S, Erbar S, Wee EG, Sahin U et al. Efficient Induction of T Cells against Conserved HIV-1 Regions by Mosaic Vaccines Delivered as Self-Amplifying mRNA. Molecular therapy. Methods & clinical development. 2018; 12, 32-46.

Pardi N, Hogan MJ, Pelc RS, Muramasu H, Andersen H, DeMaso CR et al. Zika virus protection by a single low-dose nucleoside-modified mRNA vaccination. Nature. 2017; 543 (7644), 248-251.

Vogel AB, Lambert L, Kinnear E, Busse D, Erbar S, Reuter KC et al. Self-amplifying RNA vaccines give equivalent protection against influenza to mRNA vaccines but at much lower doses. Molecular therapy: the journal of the American Society of Gene Therapy. 2017; 26 (2), 446-455.

9 APPENDIX

Appendix 1: Animal Observations

		-5 days p.a.		1 days p.a.			3 days p.a.			6 days p.a.		
		09.01.2020		15.01.2020			17.01.2020			20.01.2020		
treatment	Lab ID	weight in g	health	weight in g	% change weight	health	weight in g	% change weight	health	weight in g	% change weight	health
Buffer control	1-1	21.4	No observations	21.7	101.0	No observations	21.5	100.4	No observations	21.7	101.3	No observations
	1-2	22.5	No observations	22.8	101.2	No observations	22.2	98.6	No observations	22.7	100.8	No observations
	1-3	23.0	No observations	23.3	101.4	No observations	23.5	102.3	No observations	23.4	101.5	No observations
modRNA-Luciferase (b) (4)	2-1	20.7	No observations	21.0	101.4	No observations	20.9	100.7	No observations	20.8	100.3	No observations
	2-2	21.5	No observations	22.5	104.6	No observations	22.3	103.8	No observations	23.2	107.9	No observations
	2-3	21.2	No observations	21.4	100.8	No observations	21.3	100.4	No observations	21.3	100.5	No observations
Acuitas LNPs modRNA luciferase	3-1	21.3	No observations	21.5	100.8	No observations	21.4	100.5	No observations	21.0	98.4	No observations
	3-2	21.2	No observations	21.2	99.9	No observations	21.0	99.1	No observations	21.0	99.2	No observations
	3-3	20.9	No observations	21.1	101.0	No observations	22.3	107.0	No observations	21.7	104.1	No observations
modRNA-Luciferase LNP8 (GMP-ready)	4-1	22.2	No observations	23.2	104.7	No observations	22.9	103.2	No observations	23.5	106.1	No observations
	4-2	21.6	No observations	22.4	103.8	No observations	22.5	104.3	No observations	21.9	101.6	No observations
	4-3	20.5	No observations	20.8	101.3	No observations	20.9	101.7	No observations	20.9	101.9	No observations

Appendix 2: Certificates of Analysis

Confidential

ACUITAS THERAPEUTICS

R&D Formulation Characterization Summary:

Batch ID	LNP ID	mRNA ID	Encaps (%)	Encaps mRNA (mg/mL)	Yield (mg)	mRNA/Lipid Ratio (mg/umol)	Particle Diameter (nm)	Poly-dispersity
[redacted]								
FM-1074 -D	LNP 8	mod Luc RNA-EH190611-01c	90%	1	0.36	0.025	71	0.053

Notes

Formulated 09-Dec-19

Diluent: 300 mM sucrose in PBS

Recommended storage at approximately -80 °C. Avoid repeated freeze/thaw cycles.

(b) (6)

(b) (6) Research Associate

11-Dec-19

Date

Page 1 of 1

ACD-008.01

Confidential

ACUITAS THERAPEUTICS

R&D Formulation Characterization Summary:

Batch ID	LNP Variant	mRNA ID	Encaps (%)	Encaps mRNA (mg/mL)	Yield (mg)	mRNA/Lipid Ratio (mg/umol)	Particle Diameter (nm)	Poly-dispersity
[redacted]								

(b) (4)

Notes

(b) (4)

(b) (6)

(b) (6) Research Associate

26-Nov-19
Date

Page 1 of 1 ACD-008.01

1 | Analytical Datasheet

Test items – Formulation: Analytical Data Sheet

Formulation experiment number: FSU-I#029
Responsible: MeSc, JaKs
Experimenter: MnSt, JaKs
Date of preparation: 06Jan20

Physicochemical characterisation:

Sample batch number	Formulation				pH	Osmolality (mOsmol/kg)	Size		Zeta (mV)	Encapsulation efficiency (%)	RNA conc. (mg/mL)	LNP are conform ?
	Lipid Mix	Batch	RNA	Batch			Z-Av (nm)	PDI				
[redacted]												

(b) (4)

(b) (4)

RNA Certificate of Analysis

(Version 17)

BIONTECH
RNA Pharmaceuticals

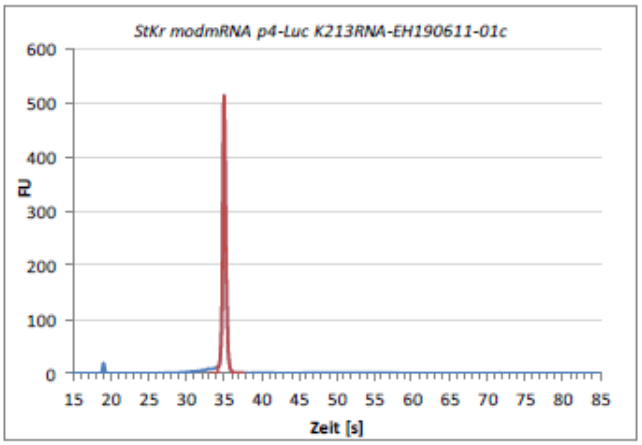
Customer	Date of Order	RNA-ID	Project Number
(b) (6)	27.05.2019	RNA-EH190611-01c	RN9095R00
Cap	**Construct**		
(b) (4)	modmRNA p4-Luc		
Purification	**Storage Buffer**		
dsRNA removal	H2O		
Modification	**Concentration [µg/µl]**	**Aliquot volume [µl]**	**Total amount [µg]**
m1Y	5,75	10600	60950

Quality Control:

Instrument:	Agilent 2100 Bioanalyzer
Run:	mRNA Nano_2019-06-14_001

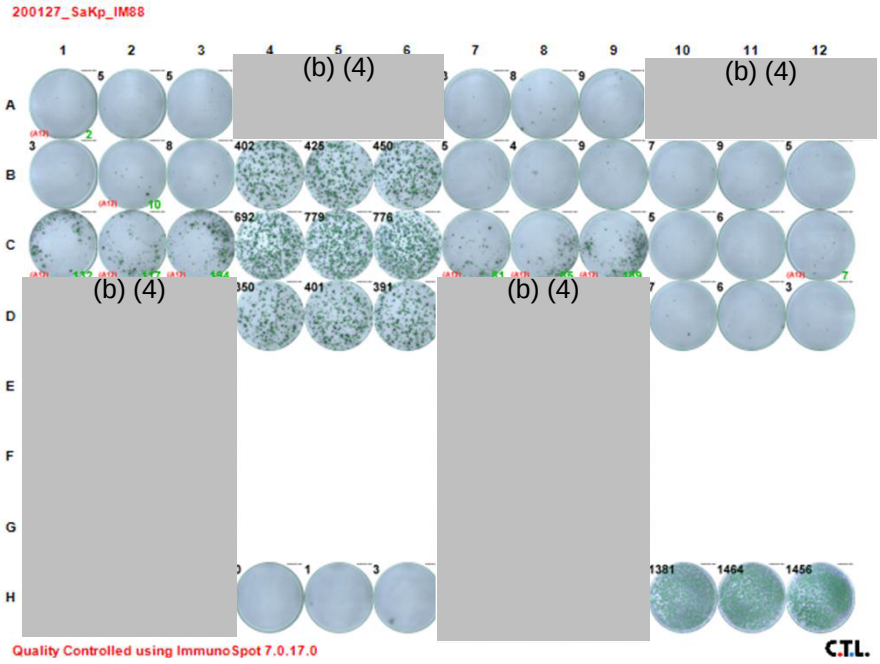
Results:

Peak height (FU):	514,963
Integral (%):	90



Appendix 3: Raw Data IFN-γ ELISpot

Plate 1: Left, luciferase peptides; right, AH1 ELISpot (irrelevant peptide)



Luc AH1

	1	2	3	4	5	6	7	8	9	10	11	12
A	Group 1, animal 1			Group 3, animal 3			Group 1, animal 1			Group 3, animal 3		
B	Group 1, animal 2			Group 4, animal 1			Group 1, animal 2			Group 4, animal 1		
C	Group 1, animal 3			Group 4, animal 2			Group 1, animal 3			Group 4, animal 2		
D	Group 2, animal 1			Group 4, animal 3			Group 2, animal 1			Group 4, animal 3		
E	Group 2, animal 2						Group 2, animal 2					
F	Group 2, animal 3						Group 2, animal 3					
G	Group 3, animal 1						Group 3, animal 1					
H	Group 3, animal 2			Medium			Group 3, animal 2			**ConA**		

Group 1: Buffer control; Group 2: (b) (4) (2x1 µg); Group 3: (b) (4) (2x1 µg); Group 4: modRNA-luciferase LNP8 (GMP-ready) (2x1 µg)

Appendix 4: Statistical Analysis

Bioluminescence assay

Group mean values, bioluminescence assay, luciferase signal over time

Time point	Buffer control	(b) (4)	(b) (4)	modRNA-Luciferase LNP8
	N = 3			N = 3
6 h	128046,667			1,2589e+009
24 h	227766,667			7,310667e+008
48 h	139995			2,1038333e+008
72 h	132585			7,8667e+007
6 d	162383,333			2920333,333
9 d	76573,333			509000

Please note that commas are used as decimal separators.

Descriptive statistics, bioluminescence assay, area under the curve

	Buffer control	(b) (4)	(b) (4)	modRNA-Luciferase LNP8
Number of values	3			3
Minimum	657790			1352000000
Maximum	889908			1848000000
Range	232118			496000000
Mean	765040			1652666667
SD	117058			264244836
SEM	67583			152561827

Please note that commas are used as decimal separators. SD: Standard deviation. SEM: Standard error of the mean.

One-way ANOVA with Tukey's multiple comparisons post-test, bioluminescence assay, area under the curve

ANOVA summary	
F	80,68
P value	<0,0001
P value summary	****
Significant diff. among means (P < 0.05)?	Yes
R square	0,9680

Tukey's multiple comparisons test	Mean diff.	95,00% CI of diff.	Significant?	Summary	Adjusted P value
		(b) (4)			
		(b) (4)			
Buffer control vs. modRNA-Luciferase LNP8	-1651901626	-2072407467 to -1231395786	Yes	****	<0,0001
		(b) (4)			
		(b) (4)			
		(b) (4)			

Please note that commas are used as decimal separators. F: F-statistic. P values ≤ 0.05 indicate statistically significant difference. R square: Coefficient of determination. CI: Confidence interval. n.s.: Not significant.

Luciferase-Specific ELISA

Descriptive statistics, luciferase-specific ELISA, day 9

	Buffer control	(b) (4)	(b) (4)	modRNA-Luciferase LNP8
Number of values	3			3
Minimum	0,0110			0,0100
Maximum	0,0190			0,0220
Range	0,00800			0,0120
Mean	0,0153			0,0177
SD	0,00404			0,00666
SEM	0,00233			0,00384

Please note that commas are used as decimal separators. SD: Standard deviation. SEM: Standard error of the mean.

One-way ANOVA with Tukey's multiple comparisons post-test, bioluminescence assay, area under the curve

ANOVA summary	
F	0,4597
P value	0,6520
P value summary	ns
Significant diff. among means (P < 0.05)?	No
R square	0,1209

No post-test for non-significant main test.

ELISpot analysis

Descriptive statistics, ELISpot analysis, day 9

	Buffer control	(b) (4)	(b) (4)	**modRNA-Luciferase LNP8**
Number of values	3			3
Minimum	4,00			381
Maximum	148			749
Range	144			368
Mean	53,0			519
SD	82,3			201
SEM	47,5			116

Please note that commas are used as decimal separators. SD: Standard deviation. SEM: Standard error of the mean.

One-way ANOVA with Tukey's multiple comparisons post-test, bioluminescence assay, area under the curve

ANOVA summary	
F	19,90
P value	0,0005
P value summary	***
Significant diff. among means (P < 0.05)?	Yes
R square	0,8819

Tukey's multiple comparisons test	Mean diff.	95,00% CI of diff.	Significant?	Summary	Adjusted P value
		(b) (4)			
		(b) (4)			
Buffer control vs. modRNA-Luciferase LNP8	-465,7	-769,0 to -162,3	Yes	**	0,0051
		(b) (4)			
		(b) (4)			
		(b) (4)			

Please note that commas are used as decimal separators. F: F-statistic. P values ≤ 0.05 indicate statistically significant difference. R square: Coefficient of determination. CI: Confidence interval. n.s.: Not significant.

(b) (6)

Von: (b) (6)
Gesendet: Freitag, 27. November 2020 07:24
An: (b) (6)
Cc: (b) (6)
Betreff: signatures: R-20-0072v3.0 (BNT162)
Anlagen: R-20-0072 Report V3.0 Bioluminescence in vivo_final_sig.pdf

Kennzeichnung: Zur Nachverfolgung
Kennzeichnungsstatus: Gekennzeichnet

Hello (b) (6)

With this email I'm giving my approval for the R&D report R-20-0072 version 3 update.

Best,
(b) (6)

(b) (6)
BioNTech SE
(b) (6)