# DNA-LNP?
# Eine Spurensuche

modarnlife.substack.com
twitter.com/a_nineties

# Gliederung

1.Herstellung

2. Messmethoden

3. Behörden

# Comirnaty-Herstellung

1. DNA-Herstellung
2. modRNA-Herstellung
3. Produkt-Herstellung
4. Fill/Finish

**Table S.2.6-9. Comparison of equipment used in process 1 and process 2**

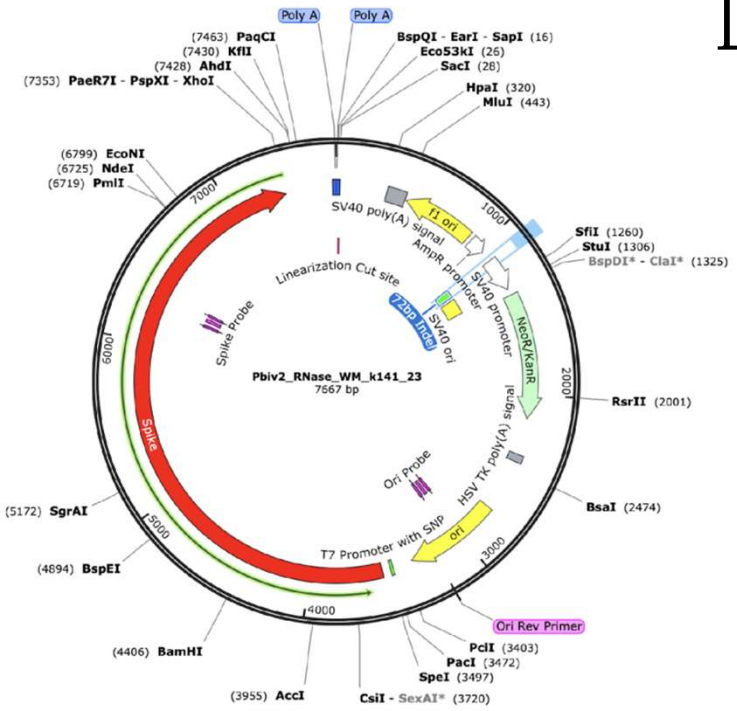
| Process step | Process 1: BNT IMFS[1] | Process 2*: Pfizer, Andover | Process 2: BNT/REN |
|---|---|---|---|
| **Step 1: In Vitro Transcription** | • Incubator (Thermo BBD 6220)<br>• Magnetic stirrer (2mag MIX 1 XL)<br>• Syringe pump (KD Scientific Legato 210 P)<br>• Centrifuge<br>Balance (Sartorius Lab Instruments) | 50L Jacketed Single Use Mixer (SUM) | Biostat STR 50L Reactor (SUM) |
| **Step 2: DNase I Digestion** | Incubator (Thermo BBD 6220)<br>Magnetic stirrer (2mag MIX 1 XL)<br>Balance (Sartorius Lab Instruments) | 50L Jacketed Single Use Mixer (SUM) | Biostat STR 50L Reactor (SUM) |
| **Step 3: Proteinase K Digestion** | - | 50L Jacketed Single Use Mixer (SUM) | Biostat STR 50L Reactor (SUM) |
| **Step 4: UFDF** | - | 200L Jacketed Single Use Mixer (SUM)<br>• SS Ultrafiltration system with 7m$^2$ 300kD membrane<br>200L SS retentate tank. | 200L Jacketed Single Use Mixer (SUM)<br>• SS Ultrafiltration system with 7m$^2$ 300kD membrane<br>200L SS retentate tank. |
| **Step 5: Final Filtration** | 0.2um filtration<br>Filter integrity tester (Pall palltronic Flowstar IV) | 200L Jacketed Single Use Mixer (SUM),<br>0.2um filtration. | 200L Jacketed Single Use Mixer (SUM),<br>0.2um filtration. |

[1] The clinical supply process includes purification of mRNA by magnetic beads hence, the process is different and Step 4 and 5 are not equal.

* Initial emergency supply is covered by process 2, in Pfizer, Andover.

| Scale (starting IVT volume) | Implemented Target/Range (rpm) | Implemented Blend time target/range (s) | Agitation rate studied range (rpm) | Blend time studied range (s) |
|---|---|---|---|---|
| Small scale 1 (8 mL)[a]<br>Small scale 2 (100 mL)[a] | 420 / 300 – 700<br>290 / 150 – 400 | 2 / 1 – 3<br>5 / 3 – 14 | 300 – 700<br>150 – 400 | 1 – 3<br>3 – 14 |
| Manufacturing scale – Pfizer (37.6 L)[b] | 80 / 60 – 110 | 13 / 5 – 17 | 30 – 210 | 5 – 33 |
| Manufacturing scale – BioNTech (37.6 L)[c] | 100 / 90 – 110 | 18 / 15 - 20 | 80 – 240 | 7 – 23 |

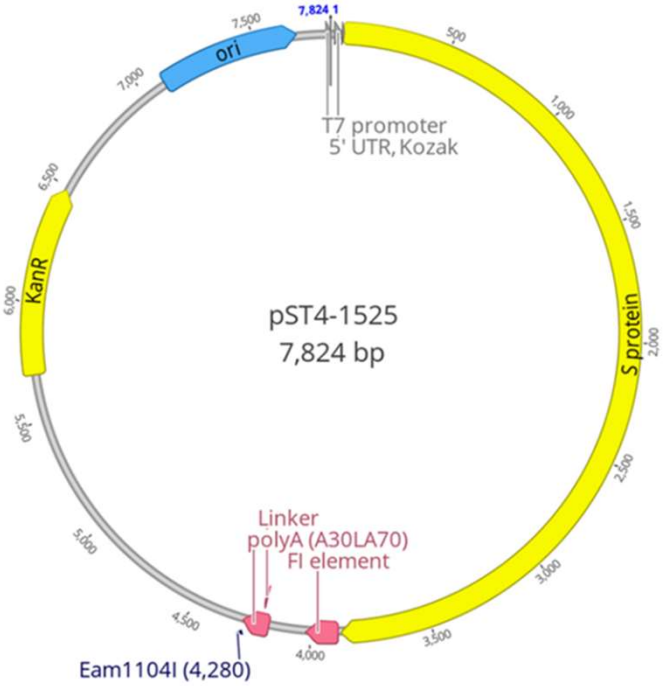
a. Studied experimentally
b. Studied in silico
c. Based on vendor supplied data




# DNA-Herstellung

## Plasmid
## Zellkultur
## Fermentierung
## Lyse
## Linearisierung

***Figure S.2.3-1. pST4-1525 Plasmid Map***



# modRNA-Herstellung

IVT (in vitro Transkription)

Adenosin, Cytosin, Guanin, N1-Methylpseudouridin

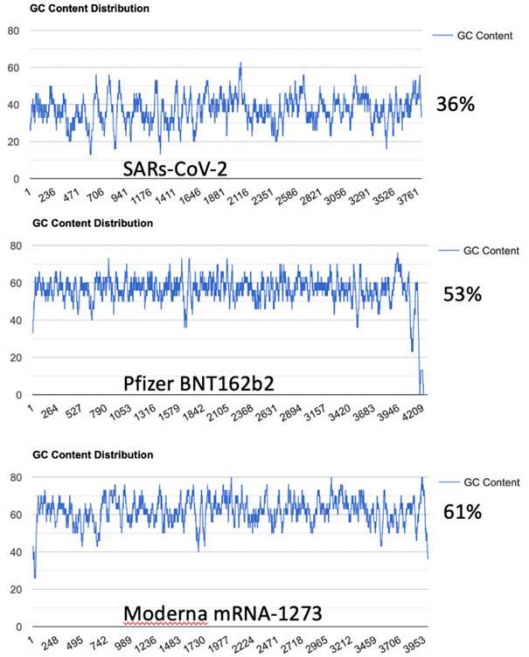
Linearisierte Plasmid DNA + RNase-Inhibitor

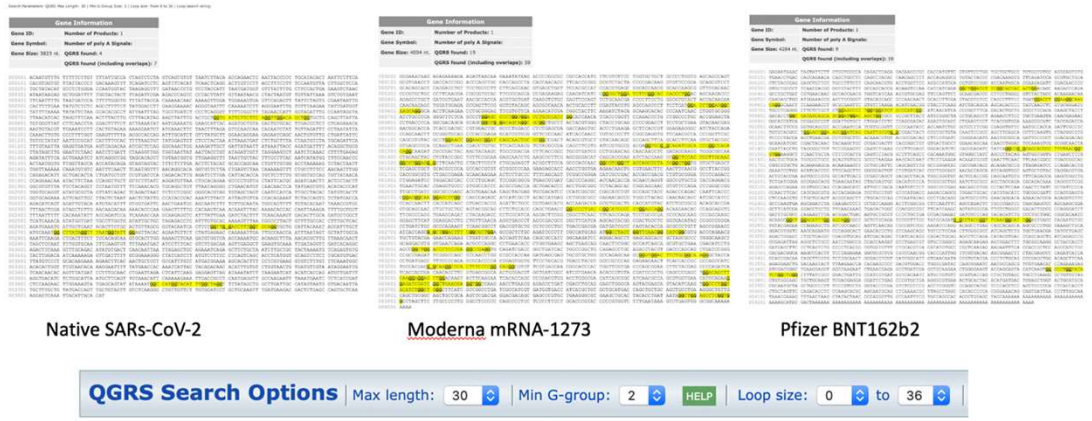
T7 Polymerase + Pyrophosphatase

EDTA-Salze

# modRNA – Zwei Problemzonen

## DNA mit hoher Guanin-Dichte ablesen

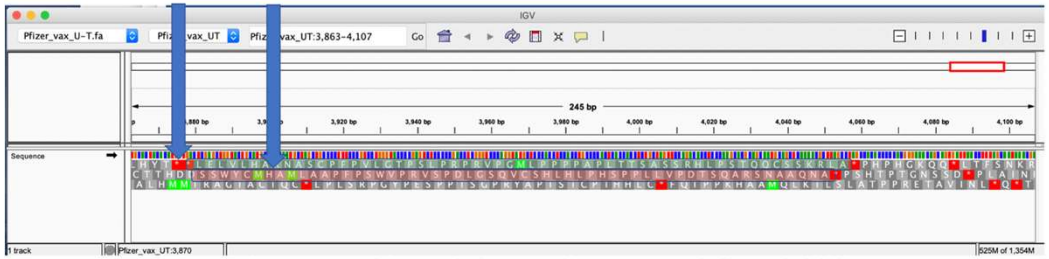



## modRNA-Stränge bilden

BNT162b2 Sequence after the Spike Stop Codon.
N1-methylpseudouridine is known to create translation errors and stop codon ablation.
Quadraplex G exacerbates these effects.

ΨGAΨGA-STOP  2 More Methionine Start Codons




**Frame 1:** LELVLHARNASCPFPVLGTPSLPRPRVPGMLPPPAPLTTSASSRHLPSTQQCSSKRLA
**Frame 2:** SSWYCMHAMLAAPFPSWVPRVSPDLGSQVCSHLHLPHSPPLLVPDTSQARSNAAQNA

**unknown [Homo sapiens]**
Sequence ID: AAG23172.1 Length: 103 Number of Matches: 1

Range 1: 37 to 83 GenPept Graphics  Next Match

| Score | Expect | Method | Identities | Positives | Gaps |
|---|---|---|---|---|---|
| 92.0 bits(227) | 2e-22 | Compositional matrix adjust. | 44/47(94%) | 44/47(93%) | 0/47(0%) |

```
Query  1   SSWYCMHAMLAAPFPSWVPRVSPDLGSQVCSHLHLPHSPPLLVPDTS  47
           S WYCMHAMLAAPFPSW PRVSPD GSQVCSHLHLPHSPPLLVPDTS
Sbjct  37  SPWYCMHAMLAAPFPSWAPRVSPDPGSQVCSHLHLPHSPPLLVPDTS  83
```

**Homo sapiens gp130 associated protein GAM mRNA, complete cds**
Sequence ID: AF072902.1 Length: 1317 Number of Matches: 1

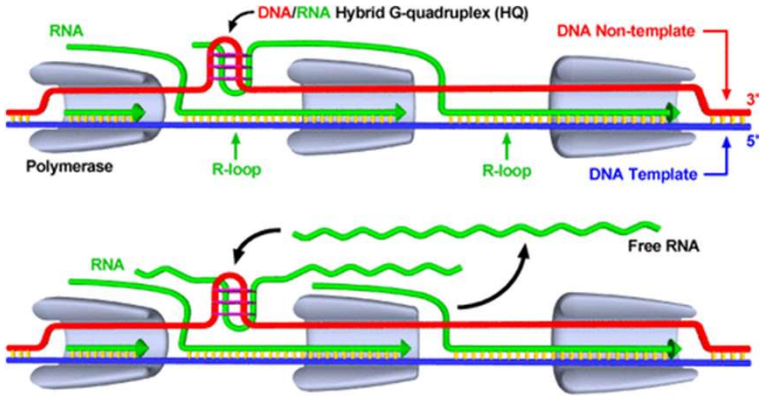
Range 1: 1052 to 1192 GenBank Graphics  Next Match

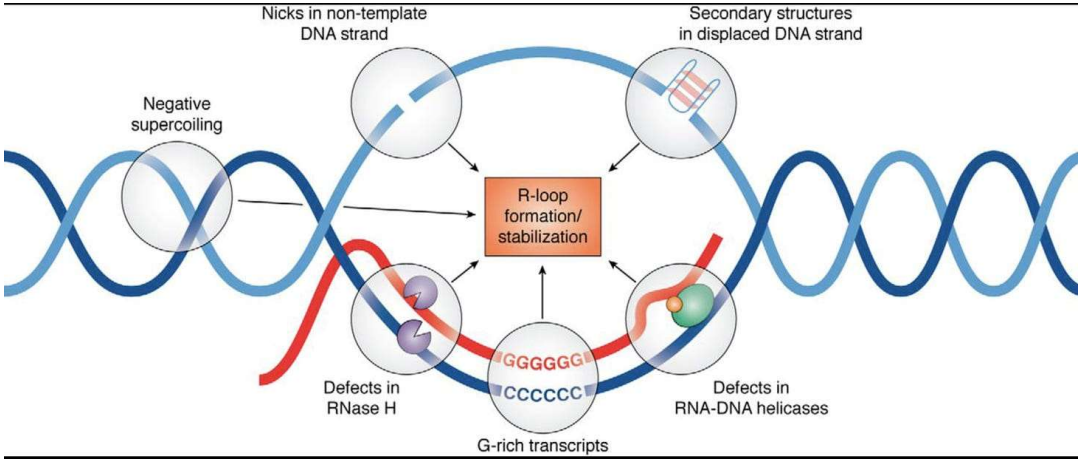
| Score | Expect | Method | Identities | Positives | Gaps | Frame |
|---|---|---|---|---|---|---|
| 65.5 bits(158) | 2e-10 | Compositional matrix adjust. | 44/47(94%) | 45/47(95%) | 0/47(0%) | +2 |

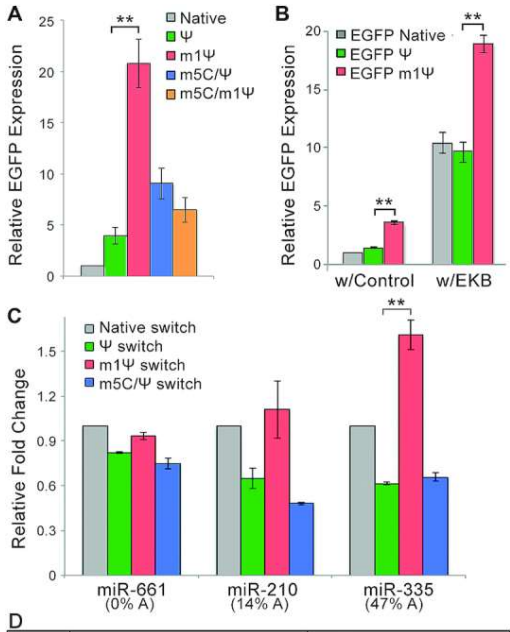
```
Query  1     SSWYCMHAMLAAPFPSWVPRVSPDLGSQVCshlhlphspplVPDTS  47
             S+WYCMHAMLAAPFPSW PRVSPD GSQVCSHLHLPHSPPLVPDTS
Sbjct  1052  STWYCMHAMLAAPFPSWXPRVSPDPGSQVCSHLHLPHSPPLLVPDTS  1192
```

# Ergebnis?

## RNA:DNA-Hybride




D

| Type | Sequence | $T_m$ (°C) |
|---|---|---|
| Native | GGCGUUUUGCG | 54.8 ± 1.2 |
| m1Ψ | GGCGm1Ψm1Ψm1Ψm1ΨGCG | 63.5 ± 0.1 |
| Ψ | GGCGΨΨΨΨGCG | [a]38.5± 4.8 63.3 ± 0.5 |

# IVT endet..

Lineare DNA

modRNA

RNA:DNA-Hybride

# Dnase-I-Schritt



-sehr geringe Wirkung auf Hybride
-Verdünnen
-länger inkubieren

accordingly. For severely contaminated RNA preparations, dilute the sample to 100 µg/ml of nucleic acid before DNase I treatment. Use 2-3 µl (4-6 units) of DNase I and incubate for one hr at 37°C.

**Table S.2.2-2. DNase I Digestion Process Parameters**

| Parameter | Acceptable Range |
|---|---|
| Temperature 1 (°C) | 34.0 – 40.0 |
| Temperature 2 (°C) | 32.0 – 38.0 |
| Agitation rate (RPM) | 90 – 110 |
| DNase I volume (mL/L starting IVT volume) | 7.20 – 8.81 |
| DNase I Incubation time (min) | 29 – 35 |

## Removing Contaminating DNA in RNA Preparations

A frequent use of DNase I is to treat RNA preparations to degrade trace to moderate amounts of genomic DNA (up to 10 µg/ml) that could otherwise result in false positive signals in subsequent RT-PCR. The amount of RNA that can be treated in a single DNase I reaction will

**Table S.2.2-1. In Vitro Transcription Process Parameters**

| Parameter | Acceptable Range |
|---|---|
| Temperature 1 (°C) | 34.0 – 40.0 |
| Initial CTP solution volume (mL/L starting IVT volume) | 85.4 – 143.8 |
| Initial ATP solution volume (mL/L starting IVT volume) | 85.4 – 135.1 |
| Initial GTP solution volume (mL/L starting IVT volume) | 4.75 – 5.25 |
| Initial N1-methylpseudo UTP solution volume (mL/L starting IVT volume) | 4.75 – 5.25 |
| Pre-enzyme agitation rate (rpm) | 40 – 80 |
| Post-enzyme agitation rate (RPM) | 90 – 110 |
| Linear DNA concentration (g/L) | 0.09 – 0.11 |
| Incubation time during GTP/N1-methylpseudo UTP bolus feeds (min) | 67 – 70 |
| Total GTP/N1-methylpseudo UTP bolus volume (mL/L starting IVT volume) | 153.2 – 187.3 |
| Final IVT incubation time (min) | 25 – 35 |

EDTA-Salz → Proteinase K Schritt

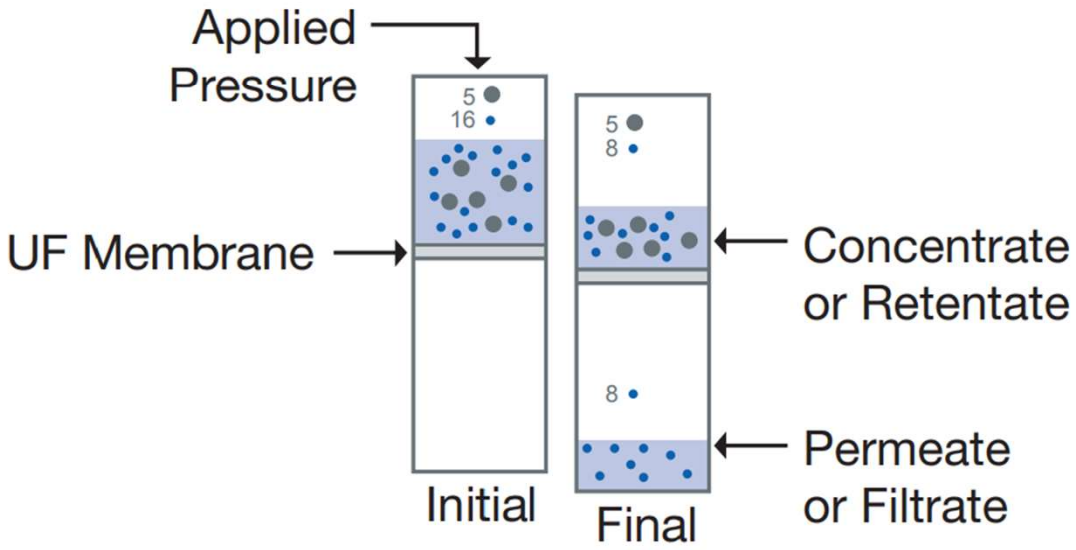
Proteinase K Pool

USA
-alles in Andover

Deutschland

Mainz → Rentschler (Laupheim)

# UFDF-Filtrierung




Ultrafiltration, a filtration method based on molecular weight differences between the target compound and impurities can be designed as a very robust method resulting in high virus yields. The scale up of the method to the industrial level is relatively straightforward and if coupled with diafiltration also provides a buffer exchange step. However, precipitation and ultrafiltration alone cannot deliver a product of sufficient purity and need to be combined with other techniques.

**DNA Removal**

Precipitation with

- Cationic detergents — e.g., cetyltrimethyl ammonium bromide (CTAB) or domiphen bromide (DB)
- Short-chain fatty acids (e.g., caprylic acid)
- Charged polymers — e.g., polyethyleneimine (PEI) and polyacrylic acid (PAA)
- Polyethylene glycol (PEG)
- Ammonium sulfate
- Tri(n-butyl)phosphate (TNBP) with Triton X-100 detergent solution

**Filtration:**

- Normal-flow filtration (NFF) with depth-charged or diatomaceous-earth–containing media
- Tangential-flow filtration (TFF)
- Ultrafiltration/diafiltration (UF/DF)

**Chromatography** and membrane adsorbers:

- Anion-exchange chromatography (AEX)
- Gel-filtration (size-exclusion) chromatography
- Hydrophobic charge-induction chromatography (HCIC)

**Degradation** with

- Enzymes
- Physical forces (shearing)
- Alkylating agents (e.g., β-propylactone)

# Messungen

Herstellungsphase

| | DNA | modRNA | Impfstoff |
|---|---|---|---|
| DNA-Gehalt | UV Spektroskopie | SYBR Green I qPCR | ------- |
| RNA-Gehalt | ------- | UV Spektroskopie | RiboGreen Fluoreszenz |

UV Spektroskopie

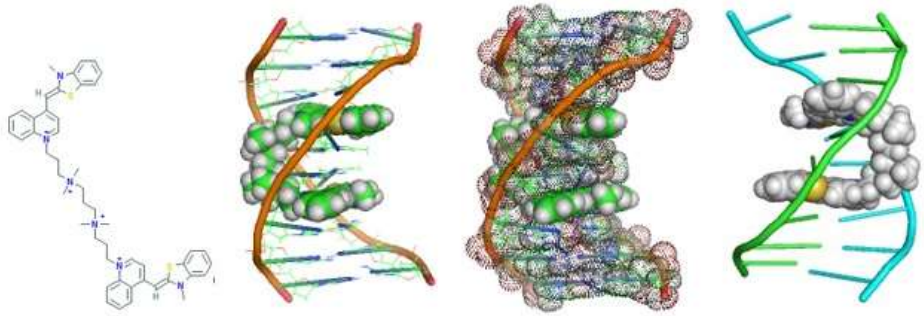
| Analytical Procedure | Acceptance Criteria |
|---|---|
| ***CIRCULAR PLASMID DNA*** | |
| **Characteristics** | |
| DNA Concentration (UV260) | 2.0 ± 0.2 mg/mL |

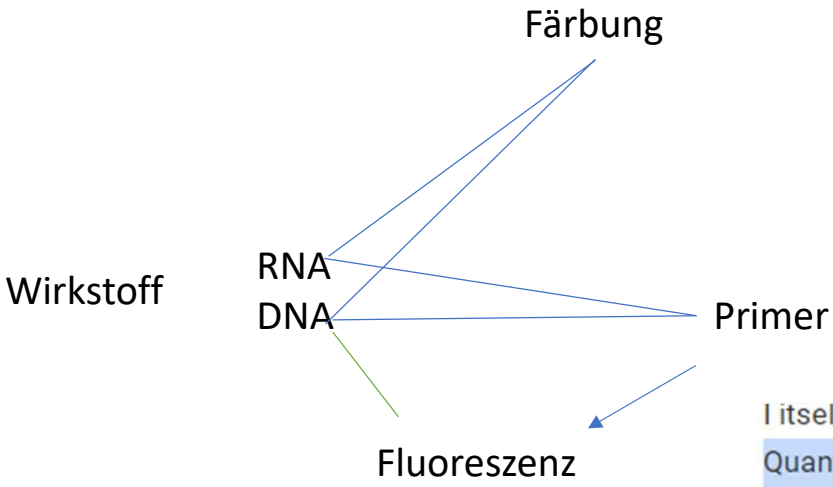
**Table 1. Drug Substance In-Process Tests (IPT-C)**

| Test | Test Method | Specification | Date of Test | Result |
|---|---|---|---|---|
| RNA Content (UFDF Pool Pre Dilution) | UV Spectroscopy | [redacted] | 20-May-2021 | 3.24 mg/mL |
| RNA Content (UFDF Pool Post Dilution) | UV Spectroscopy | | 20-May-2021 | 2.26 mg/mL |

# SYBR Green I qPCR

„96-well plate“



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United States

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**Inventor:** William Joseph ISSA, Yuxun Wang, Stephane Bancel

**Current Assignee :** ModernaTx Inc

Worldwide applications

2014 · WO EP US 2018 · US 2020 · US

I itself requires to be inactivated or removed in the subsequent process. Quantitative PCR is often applied to measure the residual DNA but it only detects the DNA molecules that contain both qPCR primers thus does not measure all other smaller DNA molecules that are partially digested. To overcome this challenge, a liquid chromatography tandem mass spectrometry (LC/MS/MS)

Messungen

**Table 1. Drug Substance In-Process Tests (IPT-C)**

| Test | Test Method | Specification | Date of Test | Result |
|---|---|---|---|---|
| RNA Content (UFDF Pool Pre Dilution) | UV Spectroscopy | [redacted] | 20-May-2021 | 3.24 mg/mL |
| RNA Content (UFDF Pool Post Dilution) | UV Spectroscopy | | 20-May-2021 | 2.26 mg/mL |

**Table 2. Drug Substance Quality Control Tests**

| Test | Test Method | Specification | Date of Test | Result |
|---|---|---|---|---|
| Clarity | Appearance (Clarity) | [redacted] | 28-May-2021 | 1 NTU |
| Coloration | Appearance (Coloration) | | 28-May-2021 | <=B9 |
| pH | Potentiometry | | 28-May-2021 | 6.9 |
| Content (RNA Concentration) | UV Spectroscopy | | 25-May-2021 | 2.27 mg/mL |
| Identity of Encoded RNA Sequence | RT-PCR | | 25-May-2021 | Confirmed |
| RNA Integrity | Capillary Gel Electrophoresis | | 25-May-2021 | 69 % |
| 5'- Cap | RP-HPLC | | 26-May-2021 | 90 % |
| Poly(A) Tail | ddPCR | | 11-Jun-2021 | 85 % |
| Residual DNA Template | qPCR | | 26-May-2021 | 220 ng DNA/mg RNA |
| Residual dsRNA | Immunoblot | | 21-Jun-2021 | NMT 40 pg dsRNA/µg RNA |
| Bacterial Endotoxin | Endotoxin (LAL) | | 24-May-2021 | NMT 1.0 EU/mL |
| Bioburden | Bioburden | | 21-May-2021 | 0 CFU/10mL |

**Abbreviations:** NTU = Nephelometric Turbidity Units; B = brown; RT-PCR = reverse transcription polymerase chain reaction; ddPCR = droplet digital PCR; qPCR = quantitative PCR; dsRNA = double stranded RNA; LAL = Limulus amebocyte lysate; EU = endotoxin unit; CFU = colony forming unit

**Table 1. Filled Vaccine Quality Control Tests**

| Test | Test Method | Specification | Date of Test | Result |
|---|---|---|---|---|
| Appearance | Appearance (Visual) | [redacted] | 01-Oct-2021 | MEETS TEST |
| Appearance (Visible Particulates) | Appearance (Particles) | | 01-Oct-2021 | MEETS TEST |
| Subvisible Particles | Subvisible Particulate Matter | | 06-Oct-2021 | 67 Particles >= 10 µm per container |
| | | | 06-Oct-2021 | 0 Particles >= 25 µm per container |
| pH | Potentiometry | | 04-Oct-2021 | 7.6 |
| Osmolality | Osmometry | | 06-Oct-2021 | 572 mOsm/kg |
| LNP Size | Dynamic Light Scattering (DLS) | | 05-Oct-2021 | [redacted] LIMS |
| LNP Polydispersity | Dynamic Light Scattering (DLS) | | 05-Oct-2021 | [redacted] LIMS |
| RNA Encapsulation | Fluorescence assay | | 05-Oct-2021 | [redacted] |
| RNA content | Fluorescence assay | | 05-Oct-2021 | 0.48 mg/m LIMS |
| ALC-0315 content | HPLC-CAD | | 06-Oct-2021 | 6.69 mg/mL LIMS |
| ALC-0159 content | HPLC-CAD | | 06-Oct-2021 | 0.83 mg/mL LIMS |
| DSPC content | HPLC-CAD | | 06-Oct-2021 | 1.42 mg/mL LIMS |
| Cholesterol content | HPLC-CAD | | 06-Oct-2021 | 2.89 mg/mL LIMS |
| Container content for injections | Vial Content (Volume) | | 01-Oct-2021 | ot less than 0.406 mL |
| Lipid identities | HPLC-CAD | | 06-Oct-2021 | MEETS TEST |

| Test | Test Method | Specification | Date of Test | Result |
|---|---|---|---|---|
| Identity of encoded RNA sequence | RT-PCR | [redacted] | 06-Oct-2021 | Identity confir LIMS |
| In Vitro Expression | Cell-based Flow Cytometry | | 08-Oct-2021 | 64 % LIMS |
| RNA Integrity | Capillary Gel Electrophoresis | | 30-Sep-2021 | 62 % LIMS |
| Bacterial Endotoxin | Endotoxin (LAL) | | 05-Oct-2021 | <5.00 EU/m LIMS |

**Abbreviations:** LNP = Lipid nanoparticles; CAD = charged aerosol detector; RT-PCR = reverse transcription polymerase chain reaction; FACS = fluorescence activated cell sorter; ddPCR = droplet digital PCR; qPCR = quantitative PCR; dsRNA = double stranded RNA; LAL = Limulus amebocyte lysate; EU = endotoxin unit

Quellen


https://modarnlife.substack.com/p/dna-lnp-eine-spurensuche

https://osf.io/bcsa6 Folie 7

https://anandamide.substack.com/p/dna-rna-hybrids-r-loops-and-nuclease Folie 8

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC1218094/ Folie 9

https://www.thermofisher.com/de/de/home/references/ambion-tech-support/nuclease-enzymes/general-articles/dnase-i-demystified.html Folie 9

https://vertassets.blob.core.windows.net/download/6ad66022/6ad66022-c1ca-4f1c-ae13-8b440bb9c866/pall_minimate_evo_diafiltrationdesalting_str_20_0408.pdf Folie 12
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