



## **Validation of the *Mycoplasma pneumoniae* PCR Diagnostic Kit Venor<sup>®</sup>Mp**

Study Number MB012001.01VMP

18.04.2001

## Summary

The Venor<sup>®</sup>Mp PCR test system for direct clinical diagnosis of infection by the pathogen *Mycoplasma pneumoniae* within humans was developed by the firm Minerva Biolabs GmbH. The production has also been executed at Minerva Biolabs GmbH. The kit was validated and is officially registered by the In-Vitro Diagnostic Directives 98/79/EU under No. DE/CA73/084175.

This report summarizes the results of the validating investigations. It covers the specifications for specificity and sensitivity of the test under comparison to a further commercially available system for direct detection of the pathogen on the basis of ELISA technology.

500 throat swab samples of patients suspected of Influenza infection arrived for the commencement of the investigation. Seven of these patients were tested positively for *Mycoplasma pneumoniae* with Venor<sup>®</sup>Mp. Specificity was confirmed by sequence-specific samples hybridizing at the amplicons. 103 samples of the total sample number were randomly selected, as well as the 7 samples positively tested with Venor<sup>®</sup>Mp, and were examined with the ELISA test system. None of the samples could be tested positively using the ELISA test system. False negative tests could be excluded from the 110 samples with the Venor<sup>®</sup>Mp through the use of a sensitive internal amplification control. The sensitivity of Venor<sup>®</sup>Mp was determined with a  $1.6 \cdot 10^3$  particles/ml PCR sample due to the multicopy P1 operon as the template. The test enabled a reproducible diagnostic from  $1.6 \cdot 10^3$  to  $8.2 \cdot 10^{10}$  particles/ml PCR sample. Venor<sup>®</sup>Mp detects *M. pneumoniae* Subtype I and Subtype II, but not *M. oral*, *M. fermentans*, *M. hominis*, and *Candida albicans*.

## 1. Introduction

### 1.1 Clinical relevance of the pathogen *Mycoplasma pneumoniae*

Pathogens of the upper respiratory tract infections cause the majority of the human infections. *Mycoplasma pneumoniae* is common globally and causes up to 20 % of all bronchial infections. The only reservoir of the pathogen are humans. The transmission occurs particularly via droplet infection. The occurrence is endemic in closely settled areas. Small epidemic diseases in homes, barracks and with very young children are usual. Every 3-4 years a more expanded epidemic occurrence is observed.

An infection of the mouth and upper respiratory tract cannot fundamentally be excluded by clinical observation or with only small specificity on the releasing pathogen. For economic reasons, such diagnosis is frequently omitted. The clinical diagnosis from throat swabs and sputum was performed via culture methods and serological techniques, and was highly time consuming, labor intensive and provided unreliable results. Serological investigations later prove the etiology in approximately 50% of the cases. For economic reasons and to adhere to medical practice compliance, one usually will conduct the diagnosis without a micro-biological diagnostic. Instead under empirical criteria, antibiotics are given. This procedure results an inadequate therapy, which can be avoided by an early and reliable pathogen diagnostic in a substantial portion of the cases.

### 1.2 Principles of the Venor<sup>®</sup>Mp detection procedure

Venor<sup>®</sup>Mp functions on the basis of a nucleic acid test for the direct qualitative confirmation of *Mycoplasma pneumoniae* in clinical sample material. The test is based on the principle of a polymerase chain reaction. The Venor<sup>®</sup>Mp test system is a nucleic acid test for the qualitative diagnosis of *Mycoplasma pneumoniae* in clinical samples. The test system also allows for a fast diagnostic of infections by *Mycoplasma pneumoniae*. The contained primers are specific to a section of the multicopy P1 operon region of the *Mycoplasma* genome. The amplified PCR product has a size of 207 BP and can be made directly visible in the agarose gel.

The kit contains the following reaction components:

- Primer/Nucleotide mix: Primer and deoxynucleotidetrphosphate dATP, dCTP, dGTP and dTTP, aliquoted for 25 tests, lyophilised
- PCR reaction buffer: sterile, 1300 µl, 10 mM Tris-HCl (pH 8.5), 50 mM KCl, 2.5 mM MgCl<sub>2</sub>
- Internal control DNA: Plasmid DNA from *E. coli* with primer-specific sequence segment and more integrated 263 bp template-DNA, not infectious, lyophilised
- Positive Control DNA: DNA-fragment from *Mycoplasma pneumoniae*, by means of manufactured PCR, not infectious, lyophilised

## 2. Base Data of the Study

|   |                        |   |
|---|------------------------|---|
| A | Title:                 | Validation of the <i>Mycoplasma pneumoniae</i><br>PCR Diagnostic kits Venor®Mp  |
| B | Study Number:          | MB 012001.01VMP   |
| C | Product Tested:        | Venor®Mp <i>Mycoplasma pneumoniae</i><br>Diagnostic Kit, Lot No. 14S1031, 14S2031<br>Order number VMP-025, VMP-100, VMP-250   |
| D | Sample Origin:         | throat swabs of Influenza suspected patients,<br>made available by Robert-Koch-Institute, Berlin, FB Virologie<br><br><i>Mycoplasma pneumoniae</i> (NCTC 010119) Batch No. 08<br><br>DNA extract:<br>a) by means of extraction kit up-cleaned DNA <i>Mycoplasma pneumoniae</i> (NCTC 010119), Batch No. 3, subtype I<br><br>b) from Dr. Jorgen Jensen of the National Serum Institute in Copenhagen, Denmark, placed for the order subtype II |
| E | Test system:           | Biometra Thermocycler (Cat.No. 050-801)<br>Eppendorf Mastercycler (Cat.No. 5333 000.018)<br>GelStar® Nucleic Acid System (Cat.No. 850535)<br>FLU-O-BLU Imaging System (Cat.No. 970000)  |
| F | Sponsor:               | Minerva Biolabs GmbH<br>Köpenicker Str. 325, 12555 Berlin, Germany  |
| G | Test Locations:        | Minerva Biolabs GmbH<br>Köpenicker Str. 325, 12555 Berlin<br>ISO9001-certified, CERT-10137-2001-AQ-ESN-TGA<br><br>Robert-Koch-Institute<br>Fachbereich Virologie<br>Nordufer 20, 13353 Berlin   |
| H | Personnel              |   |
|   | Director of the Study: | Dr. Dirk Vollenbroich   |
|   | Technical execution:   | Dipl. Ing. Christine Schramm,<br>MTA Doreen Wachauf   |
|   | Author of the Study:   | Dipl.Ing. Christine Schramm   |
|   | Translation:           | David Kazmierczak   |

- I      Schedule:
- |                                     |            |
|-------------------------------------|------------|
| Samples received:                   | 18.09.2000 |
| Commencement of Study:              | 19.09.2000 |
| Commencement of<br>laboratory work: | 19.09.2000 |
| Conclusion of laboratory<br>work::  | 18.04.2001 |
| Conclusion of Study:                | 18.04.2001 |
- J      Raw Data:      All raw data, protocol and reports are kept by Minerva Biolabs GmbH. The keeping of the original and the resulting samples is incumbent on the discretion and responsibility of Minerva Biolabs GmbH
- K      Archive:      The study documents are property of Minerva Biolabs, Köpenicker Str. 325, 12555 Berlin

### 3. Methods

#### 3.1 Sample preparation and storage

The throat swabs of Influenza suspected patients were present as suspensions. The sample material was obtained in the winter season of 1999/2000. The samples were obtained from various medical doctors from the Berlin/Brandenburg area in the form of throat swabs rinsed in a 5 ml buffer solution. 1ml of this solution was submitted for analysis. The samples were stored for further use at -18 °C. Using 200 µl this solution, a DNA extraction was accomplished by means of the QIAamp DNA Blood mini kit from the company Qiagen (Cat.No. 51104). The DNA excerpts were stored for analysis and/or for further use at -18 °C.

#### 3.2 Execution of the Venor<sup>®</sup>Mp Diagnosis

5 µl of the sample material was used for the PCR. The reaction volume of the PCR was 25 µl, which contained 10 mM Tris-HCl, pH 8.5, 50 mM KCl, 2.5 mM MgCl<sub>2</sub>, 0.1 mM dNTPs, 0.2 µM of each primer and 1 unit of a *Taq* DNA polymerase from the company Life Technologies (Cat. No. 10966-034). The PCR reaction was executed with either the Biometra Thermocycler or the Eppendorf Mastercycler. The first cycle began with a denaturation process at 94°C for 1 minute. This was followed by a primer insertion process of a two minute duration at 65°C, and by a chain prolongation at 72°C for 1 minute. In the following 34 cycles, the denaturation process for 30 seconds at 94°C was followed by an insertion process of 30 seconds at 65°C, and by a prolongation process for 30 seconds at 72°C. Finally a chain extension was performed for 3 minutes at 72°C. After that a temperature of 4°C was maintained. An earlier amplified DNA-fragment of *Mycoplasma pneumoniae* was used as a positive control, and as a negative control the sample was substituted by distilled water from the Eppendorf company.

#### 3.3 Analysis of the Results

The amplified PCR product was separated into a 1.5% agarose gel. For each PCR reaction 5 µl was applied. The electrophoresis was stopped when the bromophenolblue-marker created an approximately 2 cm bandwidth.

A mycoplasma contamination is indicated through a band at 207 bp. This band must continue for the same length as the band of the positive control, however a low mycoplasma concentration can be indicated with a weaker intensity of the fluorescence signal. A negative sample displays no band at 207 bp. Through the use of the internal control, a successfully executed PCR is indicated through a band at approximately 263 bp.

The analysis was optically made. In this manner a fluorescence signal, which was obviously prominent, was acknowledged by the minimum of two independent people and has been interpreted as a band.

### **3.4 General Reference**

The high sensitivity of the PCR method increases the danger of false-positive results, e.g. through cross contamination or through non-sterile work. Thus, all work has been executed under the GLP conditions with a PCR pipette bench. Gloves and mouth protection have been worn. Sterile and filtered pipette tips and sterile, deionised water have been used. The production of the PCR reaction mix and the aliquote of the different test methods was executed locally, and separate from the sample preparation.

## 4. Determination of the Test Parameters

### 4.1 Determination of the specificity of Venor<sup>®</sup>Mp

Throat swabs were examined from patients with a suspected Influenza infection. The DNA from the samples was extracted and submitted for diagnosis with Venor<sup>®</sup>Mp. The amplification products of positive test beginnings were identified and tested for further comparison with randomly selected samples with a commercially available ELISA system.

#### 4.1.1 Repeating precision of the Venor<sup>®</sup>Mp diagnostic kit

The above described samples were prepared by two coworkers at different points in time with different laboratory instruments and kit loads. The 110 samples were undertaken by both coworkers.

Table 1: Result of the diagnostics of throat swabs with Venor<sup>®</sup>Mp in two independent test series

|                    | Venor <sup>®</sup> Mp-Diagnostics<br>M. LaCelle | Venor <sup>®</sup> Mp-Diagnostics<br>Ch. Schramm |
|--------------------|---|--|
| total number       | 500   | 110  |
| negative           | 493 [98.6 %]                                    | 103 [93.6 %]                                     |
| negative agreement | 103 [100 %]                                     |  |
| positive           | 7 [1.4 %]                                       | 7 [6.4 %]  |
| positive agreement | 7 [100 %]                                       |  |

For these samples, corresponding negative and positive results were received with both tests commencements. The 110 samples of the second PCR test series were conducted with the internal control, which was not yet available for the first PCR test series. An inhibition of the PCR was not observed in any of the DNA extracted attempts.

#### 4.1.2 Hybridizing analysis

The DNA extracts of the samples that were positively tested with the Venor<sup>®</sup>Mp diagnostics kits were submitted for a real time PCR. The primer system corresponded to that of the Venor<sup>®</sup>Mp diagnostic kit, however a fluorescence-marked probe was attached for the detection of the amplified product. The sample sequence is homologous to a highly conserved sequence section of the P1-operon, and lies within the presumed amplification range.

**Table 2:** Identification of the positive samples by probe hybridization

|   | <b>Venor<sup>®</sup>Mp-Diagnostik<br/>M. LaCelle</b> | <b>Venor<sup>®</sup>Mp-Diagnostik<br/>Ch. Schramm</b> |
|---|--|---|
| positive  | 7  | 7   |
| positive agreement  | 7 [100 %]  |   |
| positive agreement through hybridization sample with the TaqMan <sup>®</sup> -System      | 7<br>[100%]  | 7<br>[100%]   |
| positive agreement through hybridization sample with the LightCycler <sup>™</sup> -System | 7<br>[100%]  | 7<br>[100%]   |

The conventional PCR amplicons could be identified by the following real time PCR hybridized with the LightCycler and with the ABI PRISM 7700 SDS clearly and specifically for *M. pneumoniae*. Also, the samples inserted directly into the real time PCR were clearly positive in both PCR systems.

#### 4.1.3 Comparison with the ELISA-Direct Pathogen Detection

The output suspensions of the throat swabs were tested with a commercially available ELISA system. A direct antigen test came for *Mycoplasma pneumoniae* from the enterprise Serion (Cat.No. A127).

110 samples were tested in duplicate. Execution took place after instruction. The prepared samples as well as the carried controls were submitted in the cavities of the antibody coated test strip contained in the kit and incubated for 60 minutes at 37°C. Afterwards the cavities were incubated and washed (SLT-96 PW) after the addition of the conjugation solution for 30 minutes at 37°C and incubated with the TMB substrate for 20 minutes at ambient temperature. The reaction was then stopped and the wavelength was measured at 450 nm with an ELISA reader (SLT spectra). 620 nm was selected as a reference wavelength. The cut-off was accepted with Ext. Neg. Control + 0.15.

**Table 3:** Comparison of the test results for the Venor<sup>®</sup>Mp-PCR and the Serion-ELISA

|   | <b>Direct active proof<br/>SERION ELISA, Fa. Virion</b> | <b>Venor<sup>®</sup>Mp- Diagnostic<br/>Ch. Schramm</b> |
|---|---|--|
| total number                                      | 110   | 110  |
| negative  | 110 [100 %]   | 103 [93.6 %]   |
| negative agreement<br>ELISA/ PCR                  | 103 [100 %]   |  |
| positive  | 0 [0 %]   | 7 [6.4 %]  |
| positive agreement ELISA/PCR                      | 0 [0 %]   |  |
| positive confirmation through probe hybridization | n.a.  | 7 [100%]   |

7 of the 110 samples were clearly detected as positive with the Venor<sup>®</sup>Mp test system. None of these 110 samples resulted in a positive result with the ELISA test. Controls included with the ELISA test kit were clearly positive. The ELISA test was conducted at the Robert-Koch Institute through the support of technical coworkers, who possess many years of experience with a broad range of clinical ELISA

diagnostics. None with the PCR tested samples were thus false negative. A failure of the PCR could be excluded by the internal control.

#### 4.1.4 Evaluation of the sample processing procedure

As raw material a vital cell culture of *Mycoplasma pneumoniae* in Hayflick medium was used. A dilution row with PBS was made on the basis of a culture with a titer of  $1 \times 10^9$  mycoplasma/ml. A commercial sterile cotton swab was immersed into each dilution for 10 seconds under twisting and washed afterwards in 5 ml 10 mM Tris-HCl buffer, pH 8.5. The washing solution was kept over night at 2 to +8°C. 200 µl of this washing solution was used for a DNA extraction with the QIAmp Blood mini extraction kit from the company Qiagen (concentration factor: 3.3) and afterwards submitted for PCR with Venor<sup>®</sup>Mp. 5 µl for each excerpt was used for a PCR reaction. The amplified products were isolated by means of standard agarose gel electrophoresis in 1.5% (w/v) agarose gels and made visible using the Gelstar Nucleic Acid Gel Stain.

Table 4: Result of the spiking tests with Venor<sup>®</sup>Mp

|  | Venor <sup>®</sup> Mp |
|--|-----------------------|
| total number of tested dilution stages | 10                    |
| number of repetitions                  | 3                     |
| positive                               | 6                     |
| reproductibility                       | 100%                  |
| appropriate dilution                   | $1 \times 10^{-6}$    |
| appropriate mycoplasma dilution        | 1000 mycoplasma/ml    |

To execute all necessary sample reprocessing and following PCR with Venor<sup>®</sup>Mp, it was apparent that a titer of 1000 mycoplasma/ml was necessary in order to ensure a safe diagnostics. The test does not lose sensitivity by the extensive processing .

#### 4.2 Sensitivity regulations by investigation of dilution series of genomic *Mycoplasma pneumoniae*-DNA, Subtyp 1.

The sensitivity of the Venor<sup>®</sup>Mp was determined by dilution rows of purified DNA of a different origin. The determination of the initial concentration took place photometrically. The dilution row was established using 10 mM Tris-HCl buffer, pH 8.5, and dilution steps of 1:5. 5 µl of each dilution was used for a PCR reaction. The amplified products were isolated by means of standard agarose gel electrophoresis in 1.5% (w/v) agarose gels, and made visible using the Gelstar Nucleic Acid Gel Stain.

#### 4.2.1 Precision

For the accuracy, determination of the results attained with Venor<sup>®</sup>Mp, three concentrations (4.6 ng/ml, 0.9 ng/ml, 0.2 ng/ml) of a *Mycoplasma pneumoniae* DNA were used in 10mM of Tris-HCl buffer, pH 8.5 triple repeated for a PCR reaction. The following gel evaluation took place optically and was independently confirmed by three people. All three concentrations examined displayed equal strength in each repetition within the fluorescent gel bands. No recognizable deviations in the test results arose.

#### 4.2.2 Middle Precision

The middle precision of the Venor<sup>®</sup>Mp test system was determined on the basis of the reproducibility of the test results by different persons on different days under use of varying laboratory equipment. Three laboratory coworkers each examined three different concentrations (72.0 ng/ml, 14.4 ng/ml, 2.9 ng/ml) of *Mycoplasma pneumoniae* DNA in 10 mM of Tris-HCl buffer, pH 8.5.

Table 5: Middle precision of Venor<sup>®</sup>Mp

|                                 | <b>D. Wachauf</b> | <b>D. Vollenbroich</b> | <b>Ch. Schramm</b> |
|---------------------------------|-------------------|------------------------|--------------------|
| number of tested concentrations | 3                 | 3                      | 3                  |
| number of repetitions           | 1                 | 1                      | 1                  |
| positive                        | 3                 | 3                      | 3                  |
| reproductibility                | 100%              |                        |                    |

The examined concentrations were equally detected by all three laboratory coworkers. The natural variance between the coworkers and the laboratory equipment employed does not have any detectable influence on the results of the Venor<sup>®</sup>Mp test system.

#### 4.2.3 Linearity

The linearity of a conventional PCR system shows up during optical evaluation of the gel picture by even reduction of the fluorescence intensity of the bands on investigation of a dilution row. In this study the dilution row of the DNA from *Mycoplasma pneumoniae* described above was used. To verify the results the test was repeated by three laboratory coworkers.

All three coworkers obtained gel pictures which displayed bands of decreasing intensity in the case of declining DNA concentrations. No dilution caused a precipitous removing of the fluorescence signal.

#### 4.2.4 Detection limit

For regulatory purposes, the detection limit of the Venor<sup>®</sup>Mp test system was determined using *Mycoplasma pneumoniae* DNA with a concentration of 72.2 µg/ml, gradually diluted to 1:5 and then submitted for PCR.

**Table 6:** Detection width of Venor<sup>®</sup>Mp

|                                 | Venor <sup>®</sup> Mp |
|---------------------------------|-----------------------|
| number of tested concentrations | 15                    |
| number of repetitions           | 3                     |
| positive                        | 11                    |
| reproductibility                | 100%                  |
| upper detection limit           | 72.2 µg/ml            |
| entsp. copies per beginning     | 4*10 <sup>8</sup>     |
| lower detection limit           | 1.5 pg/ml             |
| entsp. copies per beginning     | 8                     |

The detection range was located with 8 copies to 4\*10<sup>8</sup> copies per PCR cycle. The detection limit was accepted with the triple cut-offs and thus amounted to 24 copies per PCR cycle for aqueous suspensions.

#### 4.3 Cross reactions

*Mycoplasma pneumoniae* are well-known for subspecies 1 and 2. These must be recognized reliably. For common pathogens of the respiratory tract, a cross reactivity must be excluded.

**Table 7:** Cross reactivity of tested pathogens

|                        |  |
|------------------------|--|
| Gram-negative Bacteria | <i>Bacillus subtilis</i>   |
| Yeast                  | <i>Candida albicans</i>  |
| <i>Mycoplasma</i>      | <i>M. hominis</i> , <i>M. fermentans</i> , <i>M. orale</i> , <i>M. salivarium</i> ,<br><i>Acholeplasma laidlawii</i> (weak sequence homologies in the P1 operon) |

All pathogens of the respiratory tract specified in table 7 were negative in the Venor<sup>®</sup>Mp. The purified DNA from *Mycoplasma pneumoniae* Subtype 2 came from the laboratory of Dr. Jorgen Jensen at the National Serum Institute in Copenhagen, Denmark, and reacted with Venor<sup>®</sup>Mp very positively. The detection limit for this sub species was comparable with that of the Subtype 1.

#### 4.4 Stability

For the stability determination, the Venor<sup>®</sup>*Mp* test system was exposed to temperatures of 25°C or 50°C for durations of 1, 2, 3 or 4 weeks followed by the subsequent appropriate application.

**Table 8:** Result of the stability test after incubation at 25°C

| <b>Duration of the Incubation at 25°C [d]</b> | <b>Venor<sup>®</sup><i>Mp</i>-Test Results</b> |
|---|--|
| 7   | +++  |
| 14  | +++  |
| 21  | +++  |
| 28  | -  |

**Table 9:** Result of the stability test after incubation at 50°C

| <b>Duration of the Incubation at 50°C [d]</b> | <b>Venor<sup>®</sup><i>Mp</i>-Test Results</b> |
|---|--|
| 7   | +++  |
| 14  | +++  |
| 21  | +++  |
| 28  | +++  |

The investigation demonstrated that the Venor<sup>®</sup>*Mp* test system can be maintained over longer time periods at high ambient temperatures without loss of its qualitative characteristics. The test system resists an ambient temperature of 25°C during a period of 21 days. Therefore it can be transported over far distances without further cooling. For dispatch of the test system into regions far removed, it is recommended that a transportation facility is selected, in which the test system is maintained for no longer than 21 days at temperatures over +8°C. Venor<sup>®</sup>*Mp* obviously tolerates temperatures around 50°C better than more mild temperatures. At such temperatures the kit was still applicable after 28 days with qualitatively high-quality results. During the summer time, transport vehicles achieve these higher temperatures without cooling systems. The Venor<sup>®</sup>*Mp* test system is therefore stable during dispatch without cooling, provided this does not persist longer than 21 days. However, the kit must subsequently be stored at a maximum temperature of +8°C following transport.

## 5. Discussion

The Venor<sup>®</sup>*Mp* PCR test system from the company Minerva Biolabs GmbH was validated as a valuable instrument to the clinical diagnostics of human infections by the pathogen *Mycoplasma pneumoniae* within this study.

The PCR technology employed by the Venor<sup>®</sup>*Mp* is characterised by a particularly high sensitivity. The sensitivity was determined with 8 copy/PCR beginning. The system works precisely as verified through the inter-laboratory reproductibility of these investigations. Thereby, it was clearly superior in sensitivity to the established ELISA method.

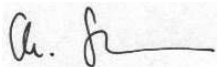
The linear detection range from 8 to  $4 \cdot 10^8$  genome copies/test beginning covers expected titers typically existing in clinical samples. With high mycoplasma concentrations, as well as in the case of a positive control, the intensity of the internal control band is reduced or is not recognizable. A repetition of the test is not required. The optimal performance of the PCR is confirmed by the band with a width of 207 bp (positive result).

The high specificity of the Venor<sup>®</sup>*Mp* diagnostic system was demonstrated through the test of the clinical sample trials. None of the tested common pathogens were detected. None of the clinical samples resulted in a false negative result by inhibitors of the sample matrices. All 7 positive samples contained *M. pneumoniae*, as the specific probe hybridizing experiments showed.

The outstanding suitability of the Venor<sup>®</sup>*Mp* *Mycoplasma pneumoniae* PCR Diagnostic Kit for the confirmation of *M. pneumoniae* in throat swab samples can be definitively confirmed. The test is simple, fast, highly sensitive and reliable. Particularly the direct proof of the pathogen is advantageous, as the diagnostic window can be closed compared with antibody pointing systems. Venor<sup>®</sup>*Mp* is particularly suitable for the early diagnosis of a *M. pneumoniae* infection through its high sensitivity.

## 6. Declaration

I accept the responsibility for the content of this study and reassure this is a description of the methods employed within the study, and that these results of the process reflect the raw data that was determined.



Berlin, 18.04.2001

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Christine Schramm, Dipl.Ing.  
Research & Development  
Minerva Biolabs GmbH

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Place, Date