Instruction Manual

respiraDNA

real time PCR Kit

For research use only.
For the *in vitro* detection of the DNA of *Adenovirus, Mycoplasma pneumoniae, Chlamydophila pneumoniae* in clinical specimens.

REF	G01085-32	G01085-96
Σ	32	96

- 18°C

gerbion gmbH & Co. KG
Remsstr. 1
70806 Kornwestheim
Germany
phone: +49 7154 806 20 0
fax: + 49 7154 806 20 29
e-mail: info@gerbion.com
www.gerbion.com

Index

1 Components

The reagents supplied are sufficient for 32 or 96 reactions respectively.

Table 1: Components of the respiraDNA real time PCR Kit.

	Label	Lid Colour	Content	
			32	96
K1	Reaction Mix	yellow	1 x 512 µl	2 x 768 µl
K2	Positive Control 1 *Chlamydophila*	red	1 x 50 µl	1 x 100 µl
K3	Positive Control 2 *Mycoplasma*	red	1 x 50 µl	1 x 100 µl
K4	Positive Control 3 *Adeno*	red	1 x 50 µl	1 x 100 µl
K5	Negative Control	green	1 x 50 µl	1 x 100 µl
K6	Control DNA	red	1 x 160 µl	2 x 240 µl

2 Abbreviations

PCR Polymerase Chain Reaction
DNA Deoxyribonucleic acid

3 Transport and Storage

The **respiraDNA** real time PCR Kit is shipped on dry ice. All components must be stored at -18°C in the dark immediately after receipt.
Do not use reagents after the date of expiry printed on the package. After initial usage, reagents are stable for up to six months. To avoid a loss of sensitivity, the reagents should not be thawed and frozen more than two times. If necessary aliquot kit components K1, K2, K3, K4 and K6.

4 Intended Use

The **respiraDNA** real time PCR Kit is a screening assay for the detection of the DNA of *Adenovirus*, *Mycoplasma pneumoniae*, and *Chlamydophila pneumoniae* in clinical specimens (e.g. throat swabs, nasal swabs, bronchial lavage) using real time PCR microplate systems.

5 Sample Material

Starting material for the assay is DNA isolated or released from clinical specimens (e.g. throat swabs, nasal swabs, bronchial lavage).

6 Quality Control

In accordance with gerbion's ISO-certified Quality Management System, each lot of the **respiraDNA** real time PCR Kit is tested against predetermined specifications to ensure consistent product quality.

7 Product Warranty

gerbion guarantees the performance of all products when used according to the instructions given in the Instruction Manual. The purchaser must determine the suitability of the product for its particular use. Should any product fail to perform satisfactorily due to any reason other than misuse, gerbion will replace it free of charge or refund the price. We reserve the right to change, alter, or modify any product to enhance its performance and design.

8 Introduction

respiraDNA is a multiplex real time PCR for the detection of causative agents of respiratory diseases. The **respiraDNA** real time PCR is designated for pathogens with a DNA genome: *Chlamydophila pneumoniae*, *Mycoplasma pneumoniae*, and *Adenovirus*.

In combination with **NukEx PLUS 2.0** (gerbion Cat. No. G05016) for the preparation of throat swabs and nasal swabs, **respiraDNA** real time PCR allows for fast, efficient and cost effective diagnostics.

Chlamydophila pneumoniae

Chlamydophila pneumoniae (formerly *Chlamydia pneumoniae*) are obligate intracellular bacteria which are mainly transmitted by droplet infection. *C. pneumoniae* is a common cause of pneumonia around the world; it is typically acquired by otherwise healthy people and is a form of community-acquired pneumonia. The infestation of the human population is very high. Antibodies against *Chlamydophila pneumoniae* can be found in around 70 to 80% of the 60 year olds, and in around 30% of ten year old school children.

Because its treatment and diagnosis are different from historically recognized causes, such as *Streptococcus pneumoniae,* pneumonia caused by *C. pneumoniae* is categorized as an „atypical pneumonia". Most infections remain without or only mild symptoms. Bronchitis, sinusitis, chronical obstructive

respiratory diseases can occur. Typical early symptoms of a *Chlamydophila pneumoniae* infection are dry mucous membranes of mouth, nose, and eyes. 4 to 6 weeks after the primary infection, post infectious arthritis and tenosynovitis might occur.
In immuno-suppressed persons, such as cancer, HIV, or organ transplant patients, and in elderly people severe complications can lead to fatal outcome.

Mycoplasma pneumoniae

Mycoplasma pneumoniae is a very small bacterium which causes the disease mycoplasma pneumonia, a form of atypical bacterial pneumonia. Tracheobronchitis, laryngitis, meningitis, otitis media, and other symptoms can be caused by *Mycoplasma pneumoniae* infection. It is also associated with disorders of the haematopoietic system, the central nervous system, the liver and the pancreas, and with cardiovascular syndromes. Furthermore, a connection with asthma is suspected.
Mycoplasma pneumoniae does usually not occur in healthy persons, however, it is highly contagious and is transmitted by droplet infection. Children are mainly at risk.

Adenovirus

Adenoviruses mainly cause infections of the respiratory system. Dependent on the Serotype, numerous other diseases can be caused, such as gastroenteritis, keratoconjunctivitis epidemica, cystitis, rhinitis, pharyngitis, and diarrhoea. Respiratory symptoms range from mild flu to acute bronchitis and pneumonia. Immuno-suppressed patients are prone to severe complications, such as acute respiratory distress syndrome. Although the epidemiological characteristics of Adenoviruses vary from type to type, all types are transmitted by direct contact, feacal-orally, and rarely by water. Some types cause persistent, asymptomatic infections of the palatine and pharyngeal tonsils, and the gastro-intestinal tract. Spreading of the virus can occur over months or years.

9 Principle of the Test

The **respiraDNA** real time PCR Kit contains specific primers and probes labelled with a fluorescent dye for the analysis of the DNA of *Chlamydophila pneumonia, Mycoplasma pneumonia* and *Adenovirus* isolated or released from clinical specimens (e. g. throat swabs, nasal swabs, bronchial lavage).
The detection of the amplification is carried out in real time via hybridization and subsequent hydrolysis of the pathogen-specific fluorescent probes. The

fluorescences are measured in the FAM (Adenovirus), ROX (Mycoplasma pneumoniae) and Cy 5 channels (Chlamydophila pneumoniae).

Furthermore, the **respiraDNA** real time PCR Kit contains a Control DNA (K6), which is detected in a heterologous amplification system.
Added during DNA extraction, the Control DNA (K6) allows not only for the detection of PCR inhibition but also detects possible mistakes during DNA extraction. This greatly reduces the risk of false-negative results. The amplification of the Control DNA (K6) is measured in the VIC®/HEX/JOE™/TET channel.

10 Equipment and Reagents to be Supplied by User

- DNA isolation kit (e.g. **NukEx Pure** RNA/DNA, gerbion Cat. No. G05004) or **NukEx PLUS 2.0** Nucleic Acid Release Reagent, gerbion Cat. No. G05016
- Sterile microtubes
- Pipets (adjustable volume)
- Sterile pipet tips with filter
- Table centrifuge
- Vortexer
- real time PCR instrument
- Optical PCR reaction tubes with lid
- Optional: Liquid handling system for automation

11 Important Notes

- The **respiraDNA** real time PCR must be performed by qualified personnel only.
- Good Laboratory Practice (GLP) has to be applied.
- All samples must be regarded as potentially infectious material and all equipment used has to be treated as potentially contaminated.

12 General Precautions

- Stick to the protocol described in the Instruction Manual.
- Set up different laboratory areas for the preparation of samples and for the set up of the PCR in order to avoid contaminations.
- Pipettes, tubes and other materials must not circulate between those different laboratory areas.
- Always use filter tips.

- Regulary decontaminate equipment and benches with ethanol-free decontaminant.
- Do not combine **respiraDNA** real time PCR Kit components of different lot numbers.

13 Isolation of DNA

The **respiraDNA** real time PCR is suitable for the detection of *Chlamydophila pneumoniae, Mycoplasma pneumonia* and *Adenovirus* DNA isolated or released from clinical specimens (e. g. throat swabs, nasal swabs, bronchial lavage) with appropriate isolation methods.
Commercial kits for DNA isolation are recommended, e.g.:

- **NukEx Pure** RNA/DNA (gerbion Cat. No. G05004)

Alternatively DNA can be released from throat or nasal swabs with **NukEx PLUS 2.0** Nucleic Acid Release Reagent (gerbion Cat. No. G05016). This is the fastest and most convenient method for the release of nucleic acid from swabs, because column based purification of the DNA can be omitted. More information can be found on www.gerbion.com.

Important: In addition to the samples always run a „water control" in your extraction, possible contaminations during DNA extraction will be detectable. Treat this water control analogous to a sample.

Please note the chapter ‚Control DNA' on page 7.
If the real time PCR is not performed immediately, store extracted DNA according to the instructions given by the DNA extraction kit's manufacturer.
Further information about DNA isolation is to be found in the extraction kit manual or from the extraction kit manufacturer's technical service.

14 Control DNA (K6)

The **respiraDNA** real time PCR Kit contains a Control DNA (K6) which allows the user to control the DNA isolation procedure and to check for possible real time PCR inhibition.

Control DNA (K6) used as Extraction Control:
respiraDNA Control DNA (K6) is added prior to the DNA extraction.
To this end, multiply the buffer volume needed per extraction with the number of samples (including at least one water control) (N) plus 1 to compensate for

inaccuracies in pipetting (N+1). Add 5 µl Control DNA (K6) per extraction (5 µl x (N+1)). Mix well. Perform the DNA isolation according to the manufacturer's instructions.

If the extraction protocol includes an incubation step of the sample in the first buffer, the Control DNA (K6) is to be added to each sample individually after incubation.

The Control DNA (K6) must not be added to the sample material directly.

Control DNA (K6) used as Internal Control of the real time PCR:

If crude **NukEx PLUS 2.0** lysates are being used or control of the DNA extraction is not desired, the Control DNA (K6) can be used as Internal Control of the real time PCR only. To that end, the Control DNA (K6) is to be added directly to the real time PCR Master Mix.

15 Real time PCR

15.1 Important Points Before Starting:

- Please pay attention to the ‚Important Notes' on page 6.
- Before setting up the real time PCR familiarise yourself with the real time PCR instrument and read the user manual supplied with the instrument.
- The programming of the thermal profile should take place before the PCR set up.
- In every PCR run at least one of each Positive Control (K2, K3, K4) and one Negative Control (K5) should be included.
- Before each use, all reagents should be thawed completely at room temperature, thouroughly mixed (do NOT vortex the Reaction Mix (K1) but mix by pipetting up and down repeatedly), and centrifuged very briefly. Then place all reagents on ice or on a cooling block (+2 to +8°C).

15.2 Procedure

If the Control DNA (K6) is used to control both the real time PCR and the DNA isolation procedure, please follow protocol A. If the Control DNA (K6) is solely used to detect possible inhibition/failure of the real time PCR, please follow protocol B

Protocol A

The Control DNA (K6) was added during DNA extraction (see ‚Control DNA', page 7). In this case, prepare the Master Mix on ice or in a cooling block (+2 to +8°C) according to Table 2.

The Master Mix contains all of the components needed for PCR except the sample. Prepare a volume of Master Mix for at least one sample more than required, in order to compensate for pipetting inaccuracy.

Table 2: Preparation of the Master Mix (Control DNA (K6) was added during DNA extraction)

Reaction Volume	Master Mix Volume
16.0 µl Reaction Mix (K1)	16.0 µl x (N+1)
0.0 µl Control-DNA (K6)	0.0 µl x (N+1)

Protocol B

The Control DNA (K6) is used for the control of the real time PCR only (see ‚Control DNA', page 7). In this case, prepare the Master Mix on ice or in a cooling block (+2 to +8°C) according to Table 3.

The Master Mix contains all of the components needed for PCR except the sample. Prepare a volume of Master Mix for at least one sample more than required, in order to compensate for pipetting inaccuracy.

Table 3: Preparation of the Master Mix (Control DNA (K6) is added directly to the Master Mix)

Reaction Volume	Master Mix Volume
16.0 µl Reaction Mix (K1)	16.0 µl x (N+1)
0.5 µl Control DNA (K6)*	0.5 µl x (N+1)*

*The increase in volume caused by adding the Control DNA (K6) is not taken into account when preparing the PCR assay. The sensitivity of the detection system is not impaired.

Protocol A and B: real time PCR set up

- Put the number of optical PCR reaction tubes needed into the cooling block.
- Pipet **16 µl** of the Master Mix into each optical PCR reaction tube.
- Add **4 µl** of the eluates from the DNA isolation (including the eluate of the water control) or the crude NukEx PLUS 2.0 lysates, the Positive Controls (K2, K3, K4), and the Negative Control (K5) to the corresponding optical PCR reaction tube (Table 4).
- Close the optical PCR reaction tubes immediately after filling in order to reduce the risk of contamination.

Table 4: Preparation of the real time PCR

Component	Volume
Master Mix	16.0 µl
Sample	4.0 µl
Total Volume	20.0 µl

15.3 Instrument Settings

For the real time PCR use the thermal profile shown in Table **5.**

Table 5: real time PCR thermal profile

Discription	Time	Temperature	Number of Cycles
Initial Denaturation	5 – 15 min*	95°C	1
Amplification of DNA			
Denaturation	10 sec	95°C	45
Annealing and Extension	40 sec Aquisition at the end of this step	60°C	

*When using crude **NukEx PLUS 2.0** lysates that have not been heat inactivated or purified, the initial denaturation has to be increased to 15 min. For purified DNA samples 5 min denaturation is sufficient.

Samples can be tested for pathogens with a RNA genome in the same PCR run– e.g. with the respiraRNA real time RT-PCR Kit – when a reverse transcription step is run prior to the amplification cycles. The thermal profile has to be programed according to Table 6.

Important: Crude NukEx PLUS 2.0 lysates must be heat inactivated prior to adding them to the real time (RT-) PCR mix when performing a reverse transcriptase step prior to the initial denaturation!

Table 6: real time RT-PCR thermal profile

Discription	Time	Temperature	Number of Cycles
Reverse Transcription	10 min	45°C	1
Initial Denaturation	5 min	95°C	1
Amplification of DNA			
Denaturation	10 sec	95°C	45
Annealing and Extension	40 sec Aquisition at the end of this step	60°C	

Dependent on the real time instrument used, further instrument settings have to be adjusted according to Table 7.

Table 7: Overview of the instrument settings required for the respiraDNA real time PCR.

Real time PCR Instrument	Parameter	Detection Channel	Notes
LightCycler 480II	Adenovirus	483-533	Color Compensation Kit Multiplex 1 (G07OMP1-cc) required
	M. pneumoniae	558-610	
	Control DNA	615-670	
	C. pneumoniae	523-568	
Stratagene Mx3000P / Mx3005P	Adenovirus	FAM	Gain 8
	M. pneumoniae	ROX	Gain 1 Reference Dye: None
	Control DNA	HEX	Gain 1
	C. pneumoniae	Cy5	Gain 4
ABI 7500	Adenovirus	FAM	Option Reference Dye ROX: NO
	M. pneumoniae	ROX	
	Control DNA	JOE	
	C. pneumoniae	Cy5	
Rotor-Gene Q, Rotor-Gene 3000 Rotor-Gene 6000	Adenovirus	Green	
	M. pneumoniae	Orange	
	Control DNA	Yellow	
	C. pneumoniae	Red	

16 Data Analysis

The *Chlamydophila pneumoniae* specific amplification is measured in the Cy 5 channel, the *Mycoplasma pneumoniae* specific amplification in the ROX channel and the *Adenovirus* specific amplification in the FAM channel. The amplification of the Control DNA (K6) is measured in the VIC®/HEX/JOE™/TET channel.

Following results can occur:

- A signal in the FAM channel is detected:
  The result is positive, the sample contains *Adenovirus* DNA.

  In this case, detection of a signal of the Control DNA (K6) in the VIC®/HEX/JOE™/TET channel is inessential, as high concentrations of virus DNA may reduce or completely inhibit amplification of the Control DNA (K6).

- A signal in the ROX channel is detected:
  The result is positive, the sample contains *Mycoplasma pneumoniae* DNA.

  In this case, detection of a signal of the Control DNA (K6) in the VIC®/HEX/JOE™/TET channel is inessential, as high concentrations of bacterial DNA may reduce or completely inhibit amplification of the Control DNA (K6).

- A signal in the CY 5 channel is detected:
  The result is positive, the sample contains *Chlamydophila pneumoniae* DNA.

  In this case, detection of a signal of the Control DNA (K6) in the VIC®/HEX/JOE™/TET channel is inessential, as high concentrations of bacterial DNA may reduce or completely inhibit amplification of the Control DNA (K6).

- No signal in the FAM, ROX and Cy 5 channel, but a signal in the VIC®/HEX/JOE™/TET channel is detected:
  The result is negative, the sample does neither contain *Chlamydophila pneumoniae* DNA nor *Mycoplasma pneumonia* DNA, nor *Adenovirus* DNA.

The signal of the Control DNA (K6) excludes the possibilities of DNA isolation failure (in case the Control DNA (K6) is being used as an Extraction Control) and/or real time PCR inhibition. If the C_T value of a sample differs significantly from the C_T value of the water control, a partial inhibition occured, which can lead to negative results in weak positive samples (see „Troubleshooting“, page 16).

- **Neither in the FAM, ROX, Cy 5 nor in the VIC®/HEX/JOE™/TET channel a signal is detected:**
  A diagnostic statement cannot be made.

  The DNA isolation was not successful or an inhibition of the PCR has occurred. In case the Control DNA (K6) was added during DNA isolation and not directly to the PCR Master Mix, the Negative Control (K5) is negative in both channels.

Figure 1 and Figure 2 show examples for positive and negative real time PCR results.

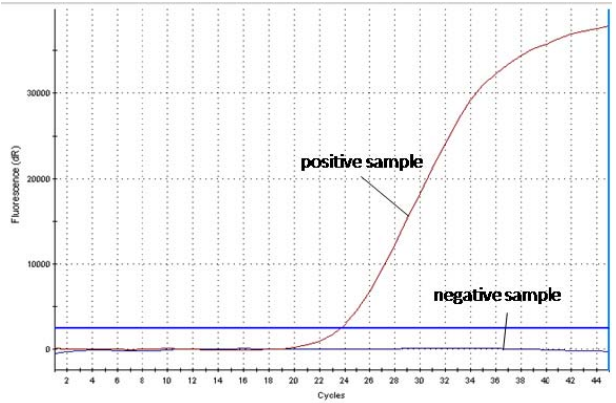

Figure 1: The positive sample shows bacteria specific amplification in the FAM channel whereas no fluorescence signal is detected in the negative sample.

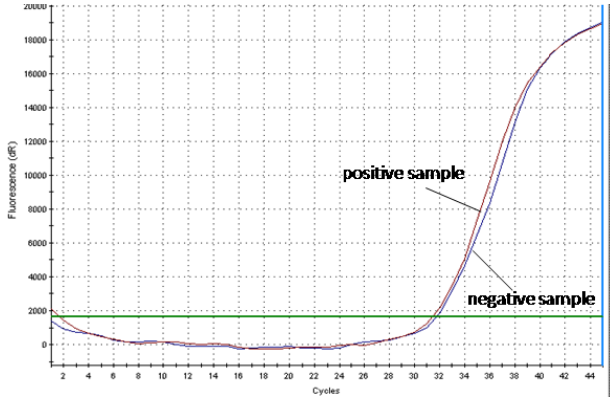

Figure 2: The positive sample as well as the negative sample show a signal in the Control DNA-specific VIC®/HEX/JOE™/TET channel. The amplification signal of the Control DNA (K6) in the negative sample shows, that the missing signal in the bacteria-specific FAM channel is not due to PCR inhibition or failure of DNA isolation, but that the sample is a true negative.

17 Troubleshooting

The following troubleshooting guide is included to help you with possible problems that may arise when performing a real time PCR. If you have further questions, please do not hesitate to contact our scientists on info@gerbion.com.

No fluorescence signal in the FAM, ROX, Cy 5 channel of the Positive Controls (K2, K3, K4)	
The selected channel for analysis does not comply with the protocol	Select the FAM channel for analysis of the *Adenovirus* specific amplification, the ROX channel for the *Mycoplasma pneumoniae* specific amplification, the Cy 5 channel for the *Chlamydophila pneumoniae* specific amplification and the VIC®/HEX/JOE™/TET channel for the amplification of the Control DNA (K6).
Incorrect configuration of the real time PCR	Check your work steps and compare with ‚Procedure' on page 8).
The programming of the thermal profile is incorrect	Compare the thermal profile with the protocol (Table 5, page 10).
Incorrect storage conditions for one or more kit components or kit expired	Check the storage conditions and the date of expiry printed on the kit label. If necessary, use a new kit and make sure kit components are stored as described in ‚Transport and Storage', page 3
Weak or no signal of the Control DNA (K6) and simultaneous absence of a signal in the FAM, ROX or Cy 5 channel	
real time PCR conditions do not comply with the protocol	Check the real time PCR conditions (page 8).
real time PCR inhibited	Make sure that you use an appropriate isolation method (see ‚Isolation of DNA', page 7) and follow the manufacturer's instructions. Make sure that the ethanol-containing wash buffer of the isolation kit has been completely removed. An additional centrifugation step at high speed is recommended before elution of the DNA. Dilute NukEx PLUS 2.0

	lysates 1:3 in $_dH_2O$ or NukEx Universal Dilution Buffer (gerbion, Cat. No. G01014). Alternatively, purify the lysates with e.g. NukEx Pure RNA/DNA Kit (gerbion, Cat. No. G05004).
Initial denaturation too short	When using crude NukEx PLUS 2.0 lysates the initial denaturation step must be performed for 15 minutes in order to heat inactivate the enzymatic component of NukEx PLUS 2.0.
DNA loss during isolation process	In case the Control DNA (K6) was added during extraction, the lack of an amplification signal can indicate that the DNA isolation was not successful. Make sure that you use an appropriate isolation method (commercial kits are recommended) and stick to the manufacturer's protocol.
Incorrect storage conditions for one or more components or kit expired	Check the storage conditions and the date of expiry printed on the kit label. If necessary, use a new kit and make sure kit components are stored as described in ‚Transport and Storage', page 3.
Detection of a fluorescence signal in the FAM, ROX or Cy 5 channel of the Negative Control (K5)	
Contamination during preparation of the PCR	Repeat the real time PCR in replicates. If the result is negative in the repetition, the contamination occurred when the samples were pipetted into the optical PCR reaction tubes. Make sure to pipet the Positive Controls (K2, K3, K4) last and close the optical PCR reaction tube immediately after adding the sample. If the same result occurs, one or more of the kit components might be contaminated. Make sure that work space and instruments are decontaminated regularly. Use a new kit and repeat the real time PCR.

18 Other Products

A number of products related to real time PCR and nucleic acid isolation is available from gerbion GmbH & Co. KG. More information as well as the complete Product Catalogue is available on www.gerbion.com.

Product	Description	Cat. No..
NukEx Pure RNA/DNA	Spin column-based kit for the isolation of RNA and DNA from a variety of sample matrices. For 50 or 200 extractions.	G05004-50 G05004-200
NukEx PLUS 2.0	Reagent for the enzymatic release of nucleic acids from swabs and cell culture suspensions. Very fast and convinient protocol! Including NukEx Stop for chemical inactivation.	G05016
NukEx Collection Tubes	500 NukEx Collection Tubes for use with NukEx Spin Columns.	G06008
NukEx Universal Dilution Buffer	Diluent for samples for real time (RT-) PCR.	G01014
NukEx Pestle 1.5 ml	100 disposable PBTP pestles for use in 1.5 ml reaction tubes. Individually packed. DNase-free, RNase-free, non-pyrogenic.	G06006
NukEx TS	Shredding material aliquoted in 1.5 or 2.0 ml safe lock tubes or 2.0 ml screw cap tubes for the manual or automated preparation of samples such as tissue or insects.	G06007-1.5 G06005-2.0 G06005-2.0 sc
Proteinase K	Proteinase K from *Tritirachium album*. 100 mg.	G07001