# MycoReal Kit *Pneumocystis*

# Manual

*For Research Use Only*

**DHUF00353**

**50 reactions**

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## Explanation of symbols

| | | | |
|---|---|---|---|
| | Batch code | | Use by |
| | Catalogue number | | Manufacturer |
| | Contains sufficient for <n> tests | | Store at |
| | Corrosion, GHS05 | | Exclamation mark, GHS07 |

# 1. Intended use

MycoReal Kit *Pneumocystis* is a real-time PCR test for the detection of DNA of *Pneumocystis jiroveci*. It allows the rapid and sensitive detection of *P. jiroveci* DNA purified from bronchoalveolare lavages (BAL), tissue and paraffin embedded tissue.

# 2. Product description

MycoReal Kit *Pneumocystis* detects the mitochondrial large-subunit rRNA gene (mt LSU) of *P. jiroveci.* The test allows the rapid and sensitive detection of *P. jiroveci* DNA purified from bronchoalveolare lavages (BAL), tissue and paraffin embedded tissue. A positive PCR result indicates the presence of *P. jiroveci* in the sample. However, results should be interpreted in context with other test results and the overall clinical picture. The detection of *P. jiroveci* in high-risk patients indicates a Pneumocystis-pneumonia.

A probe-specific amplification-curve at 530 nm (FAM channel) indicates the amplification of *P. jiroveci* specific DNA. An internal DNA positive control (DNA IPC) is used as DNA extraction and real-time PCR inhibition control. The target for the DNA IPC is extracted with the sample.

This test has been validated with the ABI® 7500 instrument (Thermo Fisher Scientific) and tested with a LightCycler® 480 Instrument II (Roche) and Mx3005P® (Agilent), but is also compatible with other real-time PCR instruments which detect and differentiate fluorescence in FAM and Cy5 channel.

The test is based on real-time PCR. A specific DNA sequence of the pathogen genome is detected and amplified. The generated PCR-product is detected by an oligonucleotide-probe labelled with a fluorescent dye. This technology allows a sequence-specific detection of PCR amplificates.

Ingenetix MycoReal, BactoReal®, ParoReal and ViroReal® Kits are optimized to run under the same thermal cycling conditions. RNA and DNA can be analysed in one run.

# 3. Pathogen information

*Pneumocystis jiroveci* (formerly *Pneumocystis carinii*) is a yeast-like fungus which can be found worldwide. *Pneumocystis jiroveci* is a distinct species that only infects humans, while the related species *P. carinii* can be found in rodents and other mammals. Airborne transmission of *Pneumocystis* from host to host has been demonstrated in rodent models and several observations suggest that interindividual transmission occurs in humans. Both healthy and immunocompromised people can be colonised with *P. jiroveci*. While it does not affect healthy people, *P. jiroveci* can cause an interstitial Pneumocystis-pneumonia (PCP) in HIV-patients, persons with primary immune deficiencies, including hypogammaglobulinemia and severe combined immunodeficiency (SCID), patients receiving long-term immunosuppressive regimens for connective-tissue disorders, vasculitides, or solid-organ transplantation, patients with hematologic and nonhematologic malignancies, including solid tumors and lymphomas, and persons with severe malnutrition. Currently the diagnosis of PCP relies on microscopic methods or PCR, as *P. jiroveci* cannot be cultured in routine microbiology laboratories.

**References:**

Medrano, F.J., M. Montes-Cano, M. Conde, C. de la Horra, N. Respaldiza, A. Gasch, M.J. Perez-Lozano, J.M. Varela, E.J. Calderon. 2005. *Pneumocystis jiroveci* in general population. Emerg. Infect. Dis. 11: 245–250.

## 4. Contents of the kit, stability and storage

| Labelling | Content | Amount | Storage |
|---|---|---|---|
| Pneumocystis Assay Mix (green cap) | Primer and probe for *P. jiroveci* (FAM) detection | 1 x 50 µl | -15 °C to -25 °C |
| DNA IPC-3 Assay Mix (yellow cap) | Primer and probe (Cy5) for DNA IPC detection | 1 x 50 µl | -15 °C to -25 °C |
| DNA IPC Target (orange cap) | Target for DNA IPC (internal DNA positive control system) | 1 x 100 µl | -15 °C to -25 °C |
| Pneumocystis Positive Control (red cap) | DNA positive control for *P. jiroveci* (approx. 1000 target copies/µl) | 1 x 200 µl | -15 °C to -25 °C |
| DNA Reaction Mix (white cap) | DNA reaction mix | 2 x 500 µl | **-15 °C to -25 °C, until first use, then at +4 °C** |
| Nuclease-free water (blue cap) | Nuclease-free water | 1 x 1000 µl | -15 °C bis -25 °C |

**DNA Reaction Mix:** The Master Mix provided with the kit has been designed for reliable, high-sensitivity real-time PCR. The Master Mix contains a highly purified Taq Polymerase for rapid hot-start PCR, dNTPs with dUTP and Uracil-N glycosylase (UNG) to eliminate amplicon carryover, ROX™ dye (passive reference) and buffer components – additives optimized to handle RT-PCR inhibitors.

The components of MycoReal Kit *Pneumocystis* are stable until the expiry date stated on the label.

## 5. Additionally required materials and devices

- Reagents and devices for DNA-extraction
- Real-time PCR instrument which is able to detect and differentiate fluorescence in FAM and Cy5 channel
- Optical 96 well reaction plates or optical reaction tubes with optical closing material recommended by the manufacturer of the real-time PCR instrument

## 6. Precautions and safety information

- Clean benches and devices periodically.
- Ensure the periodical cleaning and maintenance of real-time instruments.
- Use pipette tips with filter.
- Specimens should be handled as if infectious in accordance with safe laboratory procedures. Wear protective disposable powder-free gloves when handling kit reagents and specimens.
- Use separated working areas for specimen preparation, reagent preparation and amplification. Supplies and equipment must be dedicated to each of these separate working areas and ensure workflow in the laboratory from pre- to post-PCR.
- Be careful when handling samples and positive control to avoid cross contamination. Change gloves after handling of samples or positive control. Store positive or potentially positive material separately from reagents
- Prevent contamination of work equipment and reagents with DNA/RNA, nuclease or amplification products by good laboratory practice.
- Quality of DNA has a profound impact on the test performance. Ensure that the used DNA extraction system is compatible with real-time PCR technology.
- For a valid interpretation of results, a negative control should be included during DNA-extraction (e.g. extraction of water instead of sample material), in order to exclude false-positive results due to contamination with DNA of pathogen during extraction.
- Please note the expiry date of the kit.
- Do not interchange or mix reagents from kits with different lot numbers.
- Repeated thawing and freezing of kit components should be avoided. Protect kit components from light. **Caution:** DNA IPC Target is stored in RNA/DNA stabilizer which contains Guanidinium thiocyanate/Triton X-100 (see MSDS, www.ingenetix.com).
- Use established laboratory practices according to your local safety regulations for discarding specimens, reagents and waste disposal.

## 7. Limitations

- Optimal performance of this test requires appropriate specimen collection, transport and storage, as well as an appropriate DNA extraction procedure.
- A negative test result does not exclude the possibility of a *P. jiroveci* infection, because test results may be affected by improper specimen collection, technical error, specimen mix-up or pathogen quantities below the assay sensitivity. The presence of PCR inhibitors may lead to invalid results.
- Sequence variabilities in the target-region of some subtypes (strains) may lead to false-negative or less sensitive results.
- Results should be interpreted in context with clinical and laboratory findings.

## 8. Preparation of samples and real-time PCR

- Extract samples with a DNA extraction system compatible with real-time PCR technology (see also 12. Appendix)
- An extraction negative control should be included during DNA-extraction (e.g. extraction of water).
- Include one positive control and one extraction negative control per PCR run.
- Thaw kit components at room temperature. When thawed, mix components, centrifuge briefly. Gently Mix the DNA Reaction Mix to ensure homogeneity of solution.

### 8.1. Internal DNA Positive Control (IPC)

The **DNA IPC Target** has to be added during extraction. The DNA IPC is used as a control of DNA extraction, identifies possible PCR inhibition and confirms the integrity of kit reagents.
→ Spike 1 µl of undiluted DNA IPC Target into the sample material after the lysis buffer was added, then continue the extraction procedure. **Caution:** The DNA IPC Target must not be added directly to the sample material.

### 8.2. Positive Control

Use 8 µl of Pneumocystis Positive Control. Pipette positive control at last.

### 8.3. Pipetting scheme

| | | Per sample |
|---|---|---|
| **Preparation of Master Mix 1** (mix well) | DNA Reaction Mix | 10,0 µl |
| | Pneumocystis Assay Mix | 1,0 µl |
| | DNA IPC-3 Assay Mix | 1.0 µl |
| | **Total volume of Master Mix** | **12,0 µl** |
| **Preparation of PCR** | Master Mix | 12,0 µl |
| | DNA-Probe | 8,0 µl |
| | **Total volume** | **20,0 µl** |

→ **If DNA IPC Target was not added during extraction:** Freshly dilute the DNA IPC Target 1:100 with nuclease-free water and add 1 µl per sample directly to the master mix. **Caution:** The use of more than 1 µl diluted (1:100) DNA IPC Target per reaction causes inhibition of the real-time PCR reaction.

For preparation of real-time PCR, dispense 12 µl aliquots of prepared Master Mix into the plate wells and then add 8 µl of DNA sample per well. Always pipet the Positive Control at last.

## 8.4. Programming of temperature profile

Please find further information on programming of the real-time PCR instrument in the respective operator's manual. Take into consideration that some PCR-platforms have to be calibrated with the corresponding dye before performing a multiplex-PCR.

**Sample Volume:** 20 µl

**Temperature Profile:**

| Program 1 | Program 2 | Program 3 |
|---|---|---|
| Cycles: 1<br>Analysis: None | Cycles: 1<br>Analysis: None<br>**Polymerase Activation** | Cycles: 45<br>Analysis: Quantification<br>Acquisition at 60° |
| 50°C<br>2 min[1] | 95°C<br>20 sec | 95°C<br>5 sec<br>60°C<br>1 min |

For ABI® 7500
Ramp speed: Without "fast cycling" parameter

[1]If viral RNA should be also detected in the same PCR run, program 1 has to be prolonged to 15 min at 50°C.

**Detection channels:**

**Pneumocystis Target:** FAM-Tamra (530 nm)
**IPC Target: CY5-BHQ1 (670 nm)**
**Passive reference dye, if required:** ROX (e.g. for ABI® 7500 and Mx3005P®)

- **cobas z 480 Analyzer (Roche):**
  **FAM:** Excitation at 465 nm, Emission at 510 nm
  **Cy5:** Excitation at 610 nm, Emission at 670 nm
  **Passive reference dye:** None

- **LightCycler® 480 II (Roche):**
  **FAM:** Excitation at 465, Emission at 510 nm
  **Cy5:** Excitation at 618, Emission at 670 nm
  **Detection format:** 2 Color Hydrolysis Probe
  **Passive reference dye:** None

- **Mx3005P® (Agilent)**
  **Passive reference dye**: ROX
  **Filter set gain settings:** ROX x2, Cy5 x4, FAM x8

# 9. Interpretation of PCR-data

For analysis of PCR results select fluorescence display options 530 nm (FAM channel) for the *P. jiroveci* target and 667 nm (Cy5 channel) for the DNA IPC target. Samples with positive Ct or Cp-values are considered positive. Please, also check the amplification-curves and adjust the threshold (noise band), if necessary. Samples should be inspected both in logarithmic and linear scale view and compared with the negative control.

**Table 1**: Criteria for valid controls; IPC Target was added during extraction (control of RNA extraction and RT real-time PCR)

| | **Ct FAM channel *P. jiroveci* target** | **Ct Cy5 channel IPC target** | **Interpretation** | **Action** |
|---|---|---|---|---|
| **Positive control** | 26-28 | 26-29 | Valid | - |
| **Positive control** | Negative | 26-29 | Invalid | See 10.1 |
| **Positive control** | 26-28 | Negative | Invalid | See 10.1 |
| **Negative control** | Negative | 26-29 | Valid | - |
| **Negative control** | Negative | Negative | Invalid | See 10.1 |
| **Negative control** | Positive | 26-29 | Invalid | See 10.2 |
| **Extraction negative control (optional)** | Negative | 26-29 | Valid | - |
| **Extraction negative control (optional)** | Negative | Negative | Invalid | See 10.1 |
| **Extraction negative control (optional)** | Positive | 26-29 | Invalid | See 10.3 |

Assessment of clinical specimen test results should be performed after the positive and negative controls have been examined and determined to be valid and acceptable. If the controls are not valid, the sample results cannot be interpreted.

**Table 2**: Interpretation of data with samples

| | **Ct FAM channel *P. jiroveci* target** | **Ct Cy5 channel IPC target** | **Interpretation** |
|---|---|---|---|
| **Sample** | Negative | Positive[1] | Negative for *P. jiroveci* |
| **Sample** | Positive | Positive | Positive for *P. jiroveci* |
| **Sample** | Positive | Negative[2] | Positive for *P. jiroveci* |
| **Sample** | Negative | Negative | Invalid data.<br>Information about possible error sources and their solution can be found in 10.5 Troubleshooting. |

[1)] A positive signal excludes PCR inhibition. However, IPC Ct-values should show comparable results among samples. A shift of Ct- values can indicate a partial inhibition of PCR.
[2)] High pathogen load in the sample can lead to a reduced or absent fluorescence signal of the IPC.

# 10. Troubleshooting

### 10.1. No *P. jiroveci* specific signal with positive control and with IPC

- Incorrect programming of the temperature profile of the real-time PCR instrument.
  → Compare the temperature profile with the protocol (see 8. Preparation of real-time PCR).
- Incorrect configuration of the PCR reaction.
  → Check your work steps (see 8. Preparation of real-time PCR) and repeat the PCR, if necessary.
- No Positive Control was added.
  → Repeat PCR in case all clinical samples are negative.
- For control of DNA extraction and PCR inhibition, the IPC Target must be added during extraction. If no IPC Target was added to lysis buffer during extraction:
  → Repeat PCR extraction.

### 10.2. *P. jiroveci* specific signal (FAM signal) with negative control

- A contamination occurred during preparation of the RT-PCR.
  → Repeat the real-time PCR with new reagents in replicates.
  → Strictly pipette the positive control at last.
  → Make sure that workspace and instruments are decontaminated at regular intervals.

### 10.3. *P. jiroveci* specific signal with negative control of extraction (optional)

- A contamination occurred during extraction.
  → Repeat extraction and RT-PCR using new reagents.
  → Make sure that workspace and instruments are decontaminated at regular intervals.
  → See also 11.2

### 10.4. IPC specific signal with negative control and Positive Control

- Contamination with the IPC Target
  → Make sure that workspace and instruments are decontaminated at regular intervals.

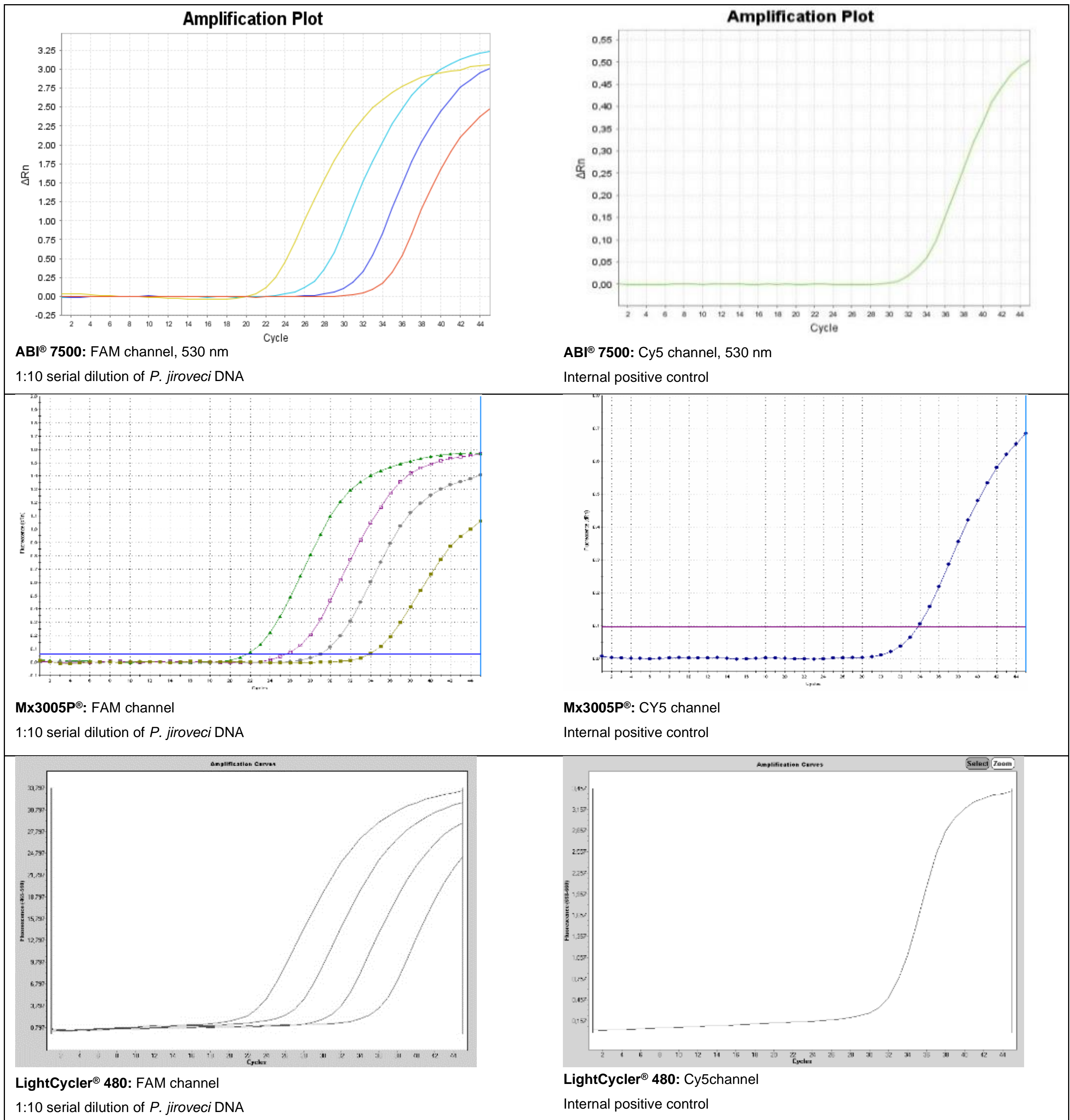
### 10.5. Valid results for controls, but no signal with IPC and *P. jiroveci* with sample

- DNA extraction has failed.
- PCR reaction was inhibited. No interpretation possible.
  → Make sure that you use a recommended method for DNA isolation and stick closely to the manufacturer's instructions.
  → If no operating mistakes during extractions can be retraced, it is recommended to repeat the PCR with lower amounts of DNA-eluate (1/5 or 1/10 of sample volume + the adequate volume of $H_2O$).
- Incorrect PCR conditions.
  → Check the real-time PCR conditions and repeat the PCR, if necessary.
- The undiluted DNA IPC Target has not been added to lysis buffer of sample.
- The extracted sample has not been added to PCR reaction.
  → No interpretation possible. Make sure you use a recommended method for DNA isolation and stick closely to the manufacturer's instructions. Check your work steps.

# 11. Specifications and performance evaluation

## 11.1. Kit performance

Figure 1 shows performance of MycoReal Kit *Pneumocystis* with an ABI® 7500 instrument (Thermo Fisher Scientific), Mx3005P® (Agilent) and LightCycler® 480 II (Roche).




**ABI® 7500:** FAM channel, 530 nm
1:10 serial dilution of *P. jiroveci* DNA

**ABI® 7500:** Cy5 channel, 530 nm
Internal positive control

**Mx3005P®:** FAM channel
1:10 serial dilution of *P. jiroveci* DNA

**Mx3005P®:** CY5 channel
Internal positive control

**LightCycler® 480:** FAM channel
1:10 serial dilution of *P. jiroveci* DNA

**LightCycler® 480:** Cy5channel
Internal positive control

**Figure 1** Performance of MycoReal Kit *Pneumocystis*

## 11.2. Analytical sensitivity – Limit of detection (LoD)

The analytical sensitivity (defined as the smallest amount of DNA that can be detected) is three target copies of *P. jiroveci*/PCR.
The LoD 95% (defined as the concentration where 95% of 20 PCR repeats/dilution were positive) is 15 target copies of *P. jiroveci*/PCR. This means, that 15 target copies of *P. jiroveci*/PCR-reaction can be detected with a probability of 95%.

## 11.3. Analytical specificity

The specificity is ensured by the selection of highly specific primers and probes. Specificity of primers and probes were validated *in silico* by carrying out the basic local alignment tool (BLAST) against the NCBI database. The primers and probes were checked for possible homologies to currently published sequences by sequence comparison analyses. This also validated the detection of so far known *P. jiroveci* strains.
Specificity was tested on different fungal isolates (Table 1). No cross reactions were observed.

## 11.4. Cut-off Value

A positive PCR result indicates the presence of *P. jiroveci* in the sample. However, for interpretation the results should be interpreted in context with other test results and the overall picture. The detection of *P. jiroveci* in high-risk patients indicates a Pneumocystis-pneumonia.

**Table 1**

| Tested isolates | Results PCR |
|---|---|
| *Candida albicans* | Negative |
| *Candida dubliniensis* | Negative |
| *Candida tropicalis* | Negative |
| *Candida krusei* | Negative |
| *Candida parapsilosis* | Negative |
| *Candida lusitaniae* | Negative |
| *Candida glabrata* | Negative |
| *Aspergillus fumigatus* | Negative |
| *Aspergillus flavus* | Negative |
| *Aspergillus terreus* | Negative |
| *Aspergillus nidulans* | Negative |
| *Aspergillus niger* | Negative |

# 12. Appendix – protocol for DNA-extraction

Kits for DNA-extraction are provided by different manufacturers. Please use the sample volume as recommended by the respective manufacturer and follow the respective manual. It has to be assured that all reagents are free of fungal DNA. It is recommended to exclude contamination of new reagent and kit lots by extraction of water instead of sample material before use with samples.

**The following DNA-extraction protocols are recommended:**

## 12.1. Extraction from BAL, tissue and paraffin-embedded tissue

DNA-extraction of *P. jiroveci* from BAL, tissue and paraffin-embedded tissue is performed with a modified protocol of the High Pure PCR Template Preparation Kit (Roche Diagnostics) including additional mechanical lysis by freeze-and-thaw cycles. See also instructions of the manufacturer.

**Additionally required materials**

- High Pure PCR Template Preparation Kit (100 extractions) (Roche Diagnostics order no. 1796828)
- Molecular Biology Grade Water (10 x 50 ml) (Eppendorf order no. 32006302)
- 1.5 ml reaction tubes (PCR-clean)
- Liquid nitrogen (or -80°C freezer)
- Isopropanol, has to be sterile filtered (e.g. Merck order no. 1009951000)
- Disposable powder-free gloves
- Pipettes (adjustable)
- Sterile pipette tips with filters
- Vortex-Mixer
- Thermomixer
- Desktop centrifuge with rotor for 2 ml reaction tubes

### 12.1.1. Extraction from BAL

- Transfer BAL (1 ml or less) in a 1.5 ml reaction tube and centrifuge for 5 min. at 13.000 x g. Discard supernatant except for 200 µl.
- Add 200 µl Binding Buffer (green cap) + 40 µl reconstituted Proteinase K, vortex.
- Incubate tube for 10 min. at 70°C.
- Freeze-and-thaw step (modification, not mentioned in protocol of extraction kit): Freeze reaction tube in liquid nitrogen or at -80°C (liquid nitrogen is more effective), then put for 1 min. at 95°-100°. Repeat these steps three times.
- Add 100 µl sterile filtered isopropanol.
- Continue as described in manual of High Pure PCR Template Preparation Kit

Recommended: Elute in 100 µl Elution Buffer* (aliquoted à 500 µl to avoid contamination with fungal DNA).
Store DNA at -20°C.

### 12.1.2. Extraction from tissue and paraffin-embedded tissue

1. Transfer tissue (ca. 0.02 mg) into a sterile petri dish and cut the sample into small pieces with a sterile scalpel. Paraffin-embedded tissue: mind to use only tissue-containing paraffin sections
2. Add tissue to 200 µl Tissue Lysis Buffer in a 1.5 ml reaction tube and mix. Add 40 µl reconstituted Proteinase K, vortex.
3. Incubate at 55°C until tissue is mainly digested.
4. Freeze-and-thaw step (modification, not mentioned in protocol of extraction kit): Freeze reaction tube in liquid nitrogen or at -80°C (liquid nitrogen is more effective), then put for 1 min. at 95°-100°C. Repeat these steps three times.
5. Add 200 µl Binding Buffer, mix well.
6. Incubate tube for 10 min. at 70°C.
7. Optional: centrifuge for 1 min. at 13.000 x g in the presence of insoluble tissue segments, use supernatant.
8. Add 100 µl sterile filtered isopropanol, mix well.
9. Continue as described in manual of High Pure PCR Template Preparation Kit. Recommended: Elute in 100 µl Elution Buffer.