myconostica

myconostica

MycXtra®
Fungal DNA Extraction Kit

REF 080-005

Intended Use

The MycXtra® Fungal DNA Extraction Kit is designed for the isolation and purification of fungal DNA present in human bronchoalveolar lavage (BAL), sputum and other lower respiratory tract samples.

Summary and Explanation

The kit is designed to isolate DNA of fungal origin in human lower respiratory tract samples from patients with suspected fungal infection. Fungal DNA extraction is a critical first step to analyzing a sample for fungal specific DNA. As fungal cell walls are difficult to breakdown, a combination of processes are required to optimise yield; direct mechanical forces in buffer, followed by several purification steps to remove PCR inhibiting substances, cell wall and human matrix components, followed by elution in buffer. The quality of purified DNA is suitable for Real-Time PCR analysis.

Principles of the Procedure

Fungal spores and hyphae present in the sample are concentrated by centrifugation and re-suspended in a small volume. The sample is then added to a bead beating tube containing beads, lysis solution, bead solution and Inhibitor Removal Solution. The principle is to lyse the microorganisms in the sample by a combination of detergent, and mechanical force against specialized beads. The cellular components are lysed by mechanical action on a vortex. From the lysed cells, the released DNA is bound to a silica spin filter. The filter is washed, and the DNA is recovered in a buffered solution and is now suitable for subsequent PCR analysis.

Kit Contents

- Sufficient for 10 patient samples

Description	Volume (mL)
10 x 2 mL Bead Solution tubes	0.55
Solution S1	0.65
IRS solution	2.2
Solution S2	2.75
Solution S3	14.5
Solution S4	3.3
Solution S5	1.1

- 10 x Spin filters units in 2 mL tubes
- 40 x Microcentrifuge tubes (2 mL)
- Instructions for Use

Storage

The kit should be stored at room temperature (15–30 °C) until the expiry date indicated on the kit box label, at which time it should be disposed of according to local regulations.

Warnings and Precautions

The kit is intended for use only by laboratory professionals. Biosafety Level 2 practices and procedures, containment equipment and facilities are required for non-aerosol producing manipulations of clinical specimens. All aerosol-generating activities must be conducted in a Class II or III biological safety cabinet. Pathogenic microorganisms including, but not limited to, hepatitis viruses and Human Immunodeficiency Virus, may be present in clinical specimens. Standard Precautions and institutional guidelines should be followed in handling all items contaminated with blood and other body fluids. The kit contains small quantities of: ethanol, sodium dodecyl suphate, guanidine hydrochloride and guanidine isothiocyanate. A Material Safety Data Sheet (MSDS) is available from Myconostica Ltd.

Equipment required

- Micro centrifuge (10,000 x g)
- General purpose centrifuge
- Micro-pipettes with sterile filter tips (volumes required 40 µL–1000 µL),
- Vortex Genie 2 (Scientific Industries but available from local suppliers)
- Vortex adaptor plate (available from Myconostica REF 080-015) to attach tubes to vortex.

Procedural Notes

The process takes approximately 1.5 hours. If the specimen is sputum or the BAL specimen appears viscous, then additional pretreatment is required, as described in the 'Procedure for Sputum or other viscous Respiratory Samples' section below.

Please read the entire protocol before commencing. Wear gloves at all times. All tubes must be capped when vortex mixing steps are carried out. Use micropipettes for the transfer of fluids. Take care to identify tubes appropriately when multiple patient samples are being processed.

Procedure for Clear, free-flowing, BAL Samples

Note: If the extracted DNA is subsequently to be used with a Myconostica MycAssay™ product, we recommend starting with ≥ 2 mL BAL

1. Centrifuge the BAL sample for 20 min at 3000 x *g*. Decant the supernatant and retain it.
2. Return 800 µL of the retained supernatant to the pellet, re-suspend and transfer to a microcentrifuge tube (provided).
3. Centrifuge for 2 min at 10,000 x *g* then remove any supernatant. Re-suspend the pellet in the solution remaining in the tube and transfer the entire amount to a 2 mL Bead Solution tube.
4. Gently invert to mix.
5. **Check Solution S1.** If Solution S1 has precipitated, warm the tube in the hand and vortex mix to dissolve.
6. Add 60 µL of Solution S1 to the Bead Solution tube and invert several times or vortex briefly.
7. Add 200 µL of Solution IRS (Inhibitor Removal Solution) to the Bead Solution tube.
8. Secure Bead Solution tube(s) horizontally on a vortex adapter plate (available from Myconostica REF 080-015). Vortex at maximum speed for 10 min.
9. Centrifuge the Bead Solution tube(s) at 10,000 x *g* for 30 s. **CAUTION:** Be sure not to exceed 10,000 x *g* or tubes may break. Make sure the 2 mL tubes rotate freely in the centrifuge without rubbing.
10. Transfer 450 µL of supernatant to a clean microcentrifuge tube (provided) taking care not to disturb the beads. Discard the Bead Solution tube.
11. Add 250 µL of Solution S2 to the supernatant and vortex for 5 s. Incubate at 4–8 °C for 5 min.
12. Centrifuge the tubes for 1 min at 10,000 x *g.*
13. Avoiding the pellet, transfer the entire volume of the supernatant to a clean microcentrifuge tube (provided).
14. Add 1.1 mL (2 x 550 µL) of Solution S3 to the supernatant (care is required as the tube is almost full). Mix by inverting.
15. Load approximately 650 µL onto a spin filter and centrifuge at 10,000 x *g* for 30 s. Discard the flow through, add another 650 µL supernatant to the spin filter, and centrifuge at 10,000 x *g* for 30 s. Repeat until all supernatant has passed through the spin filter. **Note**: A total of three loadings for each sample is required. On the final spin, centrifuge at 10,000 *x g* for 1 min. Discard the flow through**.**
16. Add 300 µL of Solution S4 to the spin filter and centrifuge for 30 s at 10,000 x *g*.
17. Discard the flow through.
18. Centrifuge again for 1 min to remove the last traces of S4 which will inhibit the PCR reaction.
19. Carefully place spin filter in a new clean microcentrifuge tube (provided). Avoid any carry over of Solution S4 onto the spin filter.
20. Carefully add 40 µL of Solution S5 to the centre of the white spin filter membrane. Leave at room temperature for 2 min.
21. Centrifuge for 30 s at 10,000 x g.
22. Discard the spin filter. The DNA in the tube is now ready for use in a PCR application. Store at 2–8 °C for up to 5 days, otherwise store DNA frozen at -20 °C.
23. Up to 10 BAL samples can be processed. If not all components are used on one occasion then return the kit components to the original box. Do not mix different lots.
24. When processing is complete, used kit components should be disposed of according to local health, safety and environmental regulations.

Procedure for Sputum or other viscous Respiratory Samples

It is recommended that the BD BBL™ MycoPrep™ Specimen Digestion/ Decontamination Kit for Processing of Mycobacterial Specimens (Cat. No.240862) is used with the MycXtra® protocol for the preparation of sputum specimens. The procedure below is modified to suit the MycXtra® kit sample input requirements.

Precautions

The NALC-NaOH reagent in the BD BBL™ MycoPrep™ Specimen Digestion/ Decontamination Kit for Processing of Mycobacterial Specimens contains strong alkali and can cause severe burns. Take off all contaminated clothing immediately. Gloves and eye/face protection must be worn. NaOH is irritating to the eyes and skin. In the event of eye or skin contact, rinse immediately with an eye wash system or tap water for at least 15 min and seek medical advice. If ingested, give milk, egg white or large amounts of water and seek medical advice.
Caution: Break ampoule close to its centre one time only. Do not manipulate ampoule further as the plastic bottle may puncture and injury may occur.
Storage Instructions: On receipt, store at 15–30 °C. Do not freeze. Do not open until ready to use.

Product Deterioration: Do not use reagents if ampoules are broken or there is visible evidence of deterioration. Do not use phosphate buffer if packages are torn or unsealed.

Procedure

1. Prepare the BBL™ MycoPrep™ Phosphate Buffer Solution. Pour contents of one Phosphate Buffer sachet into a glass screw-capped container and fill to the 500 mL line with molecular biology grade water. Sterilise, with cap loosened, using an autoclave at 121 °C for 15 min. Cool to room temperature and tighten cap.
2. Carefully loosen screw-cap on the BBL™ MycoPrep™ Reagent bottle. Locate ampoule in bottle, squeeze excess air from the bottle and tighten cap. With bottle in the upright position, squeeze the bottle until the ampoule breaks. Shake gently to dissolve the NALC. Avoid excessive agitation. ONCE AMPOULE IS BROKEN, USE REAGENT WITHIN 24 hours.
3. In a biological safety cabinet (minimum class 2), using a graduated 50 mL centrifuge tube with screw cap, add the specimen and twice the amount of activated NALC-NaOH solution by estimating the sputum volume. For example: add 4 mL activated NALC-NaOH solution to approximately 2 mL of specimen.
4. Cap the centrifuge tube and mix on a Vortex-type mixer until specimen is solubilised. If specimen is especially viscous, add more NALC-NaOH solution and repeat mixing.
5. Allow mixture to stand at room temperature for 15 min with occasional gentle shaking.
6. Add the prepared Phosphate Buffer to the 35 mL mark on the centrifuge tube and mix. Centrifuge for 20 min at 3000 x *g*.
7. Carefully decant all of the supernatant fluid. Using a micropipette gently remove any remaining supernatant which was not decanted.
8. Add 800 µL of prepared phosphate buffer and re-suspend the sediment. Transfer the entire amount to a microcentrifuge tube.
9. Follow the BAL procedure from step 3.

Performance Characteristics and Limitations

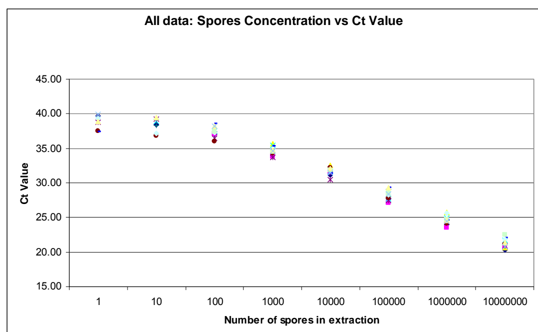
Analytical Selectivity

Using a MycXtra® kit, DNA was extracted from clinical BAL, sputum and other respiratory samples from which the following organisms had been identified by culture or microscopy: *Aspergillus fumigatus, Aspergillus terreus, Aspergillus flavus, Penicillium spp. and Pneumocystis jirovecii*. DNA was detected using the Myconostica FXG™ : RESP (Asp +) kit for Rapid Real-Time PCR detection of *Aspergillus* and *Pneumocystis* DNA in clinical respiratory samples.

Removal of Interfering Substances

Common drugs used in the treatment of respiratory disease e.g. Tobramicin, colistin sulphate, Dornase (DNAse), Salmeterol and other materials e.g. normal saline (0.9%), heparin and tannic acid can inhibit real time PCR assays. They are completely removed by the MycXtra® extraction process. Performance Evaluation trials using clinical samples indicated some PCR inhibitors do exist in respiratory samples, the nature of which is unclear, which are not removed during the extraction process.

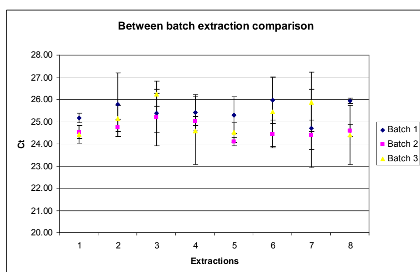
Limits of Detection and Repeatability



The MycXtra® kit extracted DNA from samples containing as few as 10 spores. Extractions of *Aspergillus fumigatus* spores in saline were carried out by 2 operators over the course of 5 days. DNA was detected with the Myconostica FXG™ : RESP (Asp +) kit which has a limit of detection of approximately 1 *A. fumigatus* genome. The results are shown in Figure 1. The overall coefficient of variation was calculated at 2.4%. The mean extraction efficiency was determined to be 8%.

Figure 1. *Aspergillus fumigatus* spore concentration versus Real-Time PCR Ct value

Reproducibility



Twenty four separate extractions were performed using each of three batches of MycXtra® kits using a standard *Aspergillus fumigatus* spore suspension. Real-Time PCR, using the Myconostica FXG™ : RESP (Asp +) kit, was then performed to determine the between and within batch variability for *Aspergillus fumigatus* DNA extraction. The results are shown in Figure 2.

Figure 2. Between and within batch extraction variation

Performance Evaluation

Aspergillus species detection

Respiratory samples (BAL) that had been collected from 2 hospitals, extracted using the MycXtra® kit, and stored were used to evaluate the performance of the MycAssay™ Aspergillus kit with clinical samples. Comparisons were made to clinical diagnosis.

The cut-off value of a Ct of 36.0 was established following review of a dataset of samples sourced from different sites and different patient populations. Different cut-offs were evaluated for the probability of differentiating between disease state and non- disease state.

PCR v Clinical Diagnosis

	Clinical positive	Clinical negative	
PCR positive	31	1	0.97 PPV
PCR negative	2	10	0.83 NPV
	0.94 Sensitivity	0.91 Specificity	

Of the samples tested, 0.8% contained PCR inhibitors as reported by the IAC, following extraction using the MycXtra® kit.

***Pneumocystis jirovecii* detection**

Clinical samples collected by bronchoaleveolar lavage (BAL) that had been obtained at 2 hospitals, extracted using the MycXtra® kit, and stored, were used to evaluate the performance of the MycAssay™ Pneumocystis kit. Comparisons were made of the PCR results to immunofluorescent microscopy.

PCR v Microscopy Diagnosis

	Microscopy positive	Microscopy negative		
PCR positive	45	8	0.85	PPV
PCR negative	2	33	0.94	NPV
	0.96 Sensitivity	0.80 Specificity		

The table represents data obtained from patients with diagnosed HIV, patients not infected with HIV and patients with undetermined HIV status. Patients with *Pneumocystis* pneumonia have highly variable amounts of organism detectable; the lower the Ct value the higher the likelihood of disease. Patients with HIV and *Pneumocystis* pneumonia tend to have higher numbers of organisms detectable than patients who are not infected with the virus, but the overlap is considerable. The scatter plot in Figure 3 below demonstrates this overlap. For completion, as the data-set in the Table included patients whose HIV status was unknown, the scatter plot for this group is included in Figure 3 (column 3):

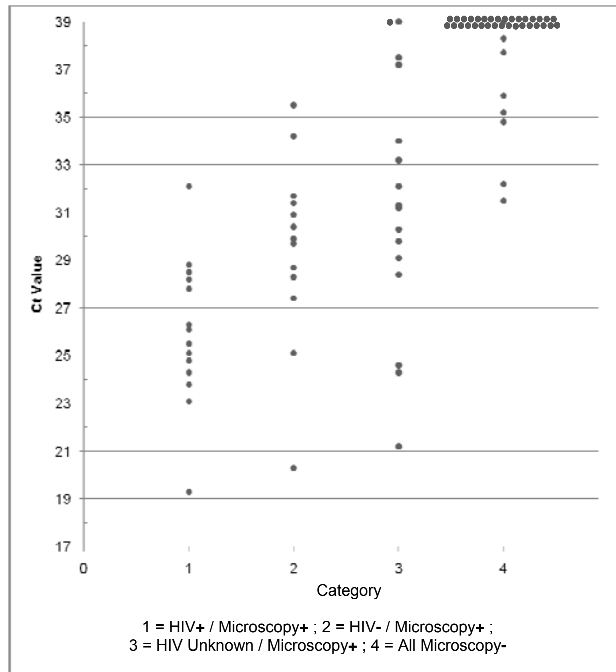


Figure 3: Scatter plot of Ct values obtained from DNA extracted from patient respiratory samples. Four groups are described.

Myconostica Limited, South Court, Sharston Road, Sharston, Manchester, M22 4SN, United Kingdom.
Telephone: +44 (0) 161 998 7239 Facsimile: +44 (0) 161 902 2496
Email: mycotech@myconostica.co.uk