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Fungal DNA Barcoding Kit (KIT-1200-50)

Fungal DNA Barcoding Kit

Product No: KIT-1200-50

This kit allows crude nucleic acid extraction from pure isolate of fungal sample from agar culture or cell pellet from liquid culture, followed by PCR amplification of the ITS gene for Sanger sequencing. It is a solution-based extraction method that utilizes high salt to extract nucleic acid for PCR applications. The kit includes PCR primers, PCR reagents and optimized PCR protocol to generate end products for Sequencing+ PLUS Services from 1st BASE sequencing services. Customer can align and BLAST the obtained sequencing results to their choice of database for barcoding purposes. If customer choose to send their PCR products for other sequencing provider, the PCR products need to be purified before sequencing. It is an ideal kit to perform DNA barcoding of various fungal samples readily.

For Research Use Only. Not for use in Diagnostic Procedures.

Kit Contents

No	Product	KIT-1200-50	Storage
1	Fungal Lysis Buffer	50 mL	Room temperature
2	TE Buffer	11 mL	
3	Proteinase K Solution	180 µL	-20 °C
4	ITS Primer Mix	1 mL	
5	ITS Enzyme	15 µL	
6	ITS PCR Buffer	300 µL	
7	Fungal Plasmid Positive Control	25 µL	
8	Sequencing Forward Primer, SPF (10 µM)	150 µL	
9	Sequencing Reverse Primer, SPR (10 µM)	150 µL	

Product Specification

	KIT-1200-50
Sample	0.5 cm x 0.5 cm cut agar **or** Cell pellet from ≤ 2 mL fresh liquid culture
Elution	50 – 100 µL
Duration	a) Nucleic acid extraction: Overnight b) PCR amplification: ~ 45 minutes
Storage	- 20 °C and Room Temperature (21 °C – 25 °C)

Materials Supplied by Users

- ✓ Thermo block to set at 56 °C
- ✓ Micro-centrifuge (non-refrigerated) with minimum speed of 14,000 × *g*
- ✓ Thermocycler
- ✓ Electrophoresis reagents and system
- ✓ Spectrophotometer
- ✓ Sterile nuclease-free 1.5 mL micro-centrifuge tubes (2x units per sample)
- ✓ Sterile nuclease-free 0.2 mL PCR tubes or 96-well plate
- ✓ Sterile nuclease-free pipette and pipette tips
- ✓ Isopropanol
- ✓ 70% ethanol

Precautions for Users

- ✓ Always wear a lab coat, disposable gloves, anti-fog protective goggles and surgical mask.
- ✓ For fungal sample that is potentially contagious, suitable protective PPE lab coats and face shield must be equipped. The extraction works should be conducted in BSL2 or higher grade, which is according to interim guidelines of laboratories.

Protocol

Lysis	1. Add 500 µL of **Fungal Lysis Buffer** into 1.5mL micro-centrifuge tube that contains the recommended sample size of fungus. *Tips:* ✓ *1 mL filter pipette tip is recommended to be used to add Lysis Buffer.* ✓ *Ensure the entire sample size is 100% submerged into Lysis Buffer.* ✓ *Handle each sample size one by one. Do not open ≥ two tubes together at the same time to avoid cross contamination.* 2. Add 3 µL of **Proteinase K solution**. Vortex to mix and spin down briefly. 3. Incubate at 56 °C for overnight. 4. Centrifuge the lysate at 14,000 – 16,000 × *g* for 10 minutes.
Washing	5. Transfer ~500 µL of supernatant to a new 1.5 mL micro-centrifuge tube, which contains 500 µL of **isopropanol**. Invert the tube several times to mix gently. 6. Centrifuge at 14,000 – 16,000×g for 10 minutes and discard the supernatant. 7. Add 1 mL of **70% ethanol**. Centrifuge again at 14,000 – 16,000 × *g* for 5 minutes and discard the supernatant. *Tip:* 70% ethanol should be prepared freshly or less than a week. 8. Air dry the pellet for 3 minutes.
Elution	9. Re-suspend the dried DNA pellet with 50 µL **TE Buffer** and incubate at 56°C for < 1 hr. *Tip: If necessary, increase the elution volume using* not more than 100 µL TE Buffer *to dissolve the DNA pellet completely.* 10. Measure the Optical Density (OD) reading using spectrophotometer. Dilute the nucleic acid to 15 – 25ng/ µL. Use 2 µL of this diluted nucleic acid as DNA Template for PCR.

PCR	11. Prepare the **PCR Mix** according to Table 1. 12. Add 2 µL of DNA Template from Step 10 with each 23 µL of **PCR Mix** into 0.2mL tube or 96-well plate. 13. Run the PCR Cycle Protocol on Thermocycler according to Table 2. 14. After the PCR cycle is completed, check the present of ~700 bp PCR products on 1% agarose gel according to Figure 1.
Sequencing	15. For the unpurified PCR products that shows single band at ~700 bp on agarose gel electrophoresis, they are ready to send for 1st BASE Sequencing+ PLUS Services using the provided SPF and SPR sequencing primers. *Tips:* ✓ *If there is no amplification or no PCR products generated from your fungal sample, please refer Troubleshooting Guidelines.* ✓ *1st BASE Sequencing+ PLUS Services has included PCR clean-up before sequencing. The turnaround time is ~3 – 4 working days from the day of the unpurified PCR products received by 1st BASE.*
Barcoding	16. After the sequencing results are ready, trim off the reads with Quality Value (QV) < 20, align the Forward and Reverse sequencing results. 17. BLAST the aligned sequence against your preferred database, e.g. NCBI, Greengenes or others. *18.* The identification of the fungus is reliable up to genus level and it typically appears within the top-10 of nucleotide BLAST results.

Table 1: Preparation of PCR Mix

Number of Reactions	ITS Primer Mix (µL)	ITS Enzyme (µL)	ITS PCR Buffer (µL)	Total PCR Mix (µL)
4	71.0	1.0	20	92
6	106.5	1.5	30	138
8	142.0	2.0	40	184
10	177.5	2.5	50	230
12	213.0	3.0	60	276
14	248.5	3.5	70	322
16	284.0	4.0	80	368
18	319.5	4.5	90	414
20	355.0	5.0	100	460

Note 1:

- ✓ The PCR Mix must be freshly prepared.
- ✓ The recommended DNA template amount in each PCR is 30 – 50 ng.
- ✓ Each PCR consists of 23 µL of PCR Mix and 2 uL of diluted DNA Template.
- ✓ Both NTC (No Template Control) and positive control reactions are recommended to be included into each round of PCR preparation.
- ✓ For each positive control reaction, use 1 µL of the provided plasmid positive control (5 ng/µL) as DNA Template.
- ✓ For each NTC reaction, use 1µL of the provided TE Buffer as DNA Template.

Table 2: PCR Cycle Protocol

Step	PCR Process	Time	Temp. (°C)	Number of Cycle
1	Initial Denaturation	2 min	98	1
2	Denaturation	15 sec	98	25 cycles
3	Annealing	30 sec	60	
4	Extension	30 sec	72	
5	Final Extension	10 min	72	1

Note 2:

- ✓ Always check the present of PCR end products, which is ~700 bp of size on agarose gel electrophoresis before send for 1st BASE Sequencing+ PLUS Services.
- ✓ If you have alternative sequencing service provider, please purify the PCR products (PCR purification reagents not provided with this kit) before sequencing.

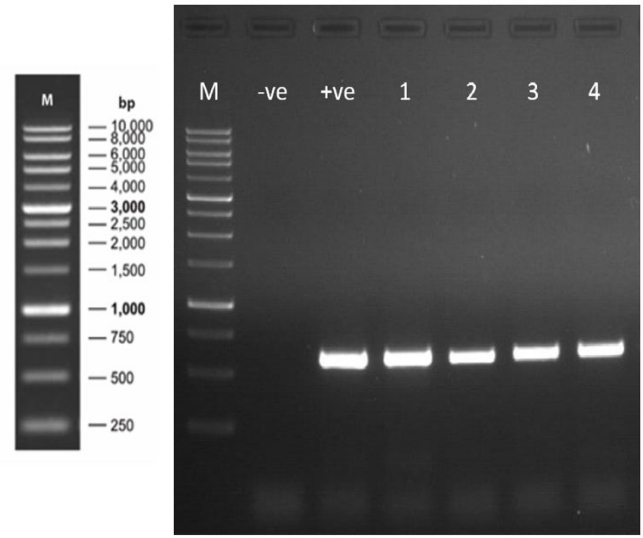

Figure 1. 1uL of un-purified PCR products on 1% TAE Agarose Gel. PCR was performed in 25uL according to Table 2.

-ve: NTC (No Template Control)
+ve: Fungal Plasmid Positive Control, 1uL per reaction
1 to 4: Fungal Nucleic Acid, 30 to 50ng per reaction

Troubleshooting Guidelines

Problems	Reasons
Low yield of nucleic acid	a) The culture agar exceeds the recommendation cut size of 0.5cm x 0.5cm. The sample size not submerged entirely in Fungal Lysis Buffer. b) For lysis of certain fungal strains, additional enzymatic treatment, which is not included in this kit, may be required. For fungal strains that not able to be lysed using the provided Fungal Lysis Buffer, it is recommended to use alternative commercial yeast/ fungal nucleic acid extraction to repeat the DNA extraction.
No PCR amplification	▪ Presence of PCR inhibitors: a) Check whether dilution was performed correctly in Step 10; or b) Perform 5x – 20x dilution in Step 10 and repeat PCR using the diluted DNA Template.
NTC shown amplification	▪ PCR mix in Table 1 must be prepared freshly. Do not use the premix that was prepared overnight.
Sanger sequencing results shown mixture of signal	▪ More than 1 different copy of ITS gene were detected due to presence of more than 1 type of fungus. a) Re-isolate single colony from the fungal culture. Repeat the process of extraction and sequencing using the new PCR products amplified from pure culture; or b) If the mixture of fungus is less than 5, by using the remaining PCR products, you may order cloning prior sequencing services using product code: **MBS-3006.** If the mixture of fungus is a lot more complex, you may consider our next-generation sequencing services using product code: **NGS-7008**. New gDNA is required.

The kit has been tested with wide range of fungal fresh culture in ***Table 3.***

Table 3:

Aspergillus	*Fusarium*	*Penicillium*	*Polyporales*	*Toluraspora*
Candida	*Ganoderma*	*Perenniporia*	*Rigidoporus*	*Trametes*
Cunninghamella	*Lachancea*	*Pleurotus*	*Schizophyllum*	*Trichoderma*

For fungus strain listed in Table 4 below, it is recommended to use Fungal DNA Barcoding PCR Kit (Product No: **KIT-1210-50**) with your choice of alternative commercial yeast/ fungal nucleic acid extraction kit. Contact us at mbs@apicalscientific.com for more information.

Table 4:

Alternaria	*Cladosporium*	*Exophiala*	*Lodderomyces*	*Rhizopus*
Bipolaris	*Curvularia*	*Lasiodiplodia*	*Pleosporales*	*Talaromyces*

Product Ordering Information

Product Name	*Packaging Size*	*Product No.*
Fungal DNA Barcoding Kit	50 preps	KIT-1200-50
Fungal DNA Barcoding PCR Kit, without DNA Extraction	50 preps	KIT-1210-50
1st BASE Sequencing+ PLUS Services	2 sequencing reactions for each sample	SS1201
PCR Product Cloning Service PLUS (up to 1.5kb) + Cloning of unpurified PCR product into pJET1.2/ Blunt vector + Colony PCR screening and pick 5 positive colony PCR products for bi-directional sequencing.	1 sample	MBS-3006
Amplicon Sequencing Lite, partial gene (ITS v2) with Basic Bioinformatics Analysis.	1 sample	NGS-7008
Fungal DNA Barcoding Services from pure isolates, ITS gene + Extraction of gDNA + PCR amplification and Purification + Bidirectional PCR product sequencing + Data analysis (BLAST to show the top 10 matches from database)	1 sample	MBS-5004
Fungal DNA Barcoding Services from mixed isolates, ITS gene + Extraction of gDNA, PCR Amplification and Purification + Cloning and 8 positive clones of plasmid DNA to send for Forward Single Pass Sequencing + Data analysis (Alignment & BLAST to show the top 10 matches from database)	1 sample	MBS-5104
<Optional> Custom DNA Barcoding Services	Minimum 5 samples	MBS-5007

Customization of DNA Barcoding kit for your choice of organism is available. Please contact us at http://www.base-asia.com/find-us for more information.

Note

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