Fast MicroSEQ™ D2 rDNA Fungal Identification
USER GUIDE

using:
Fast MicroSEQ™ D2 rDNA Fungal PCR Kit and
MicroSEQ™ D2 rDNA Fungal Sequencing Kit

Catalog Numbers  4382397 (PCR kit) and 4347481 (Sequencing kit)
Publication Number  4393005
Revision  E
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IMPORTANT! Before using this product, read and understand the information in the “Safety” appendix in this document.

Revision history

<table>
<thead>
<tr>
<th>Revision</th>
<th>Date</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>D</td>
<td>August 2015</td>
<td>Updated legal and contact information. Incorporated latest thermal cycling conditions.</td>
</tr>
<tr>
<td>E</td>
<td>October 2015</td>
<td>Corrected cycle sequencing thermal cycling conditions: Initial (hold) step is 1 minute.</td>
</tr>
</tbody>
</table>
Product Information

Purpose of the product

The Applied Biosystems™ Fast MicroSEQ™ D2 rDNA Fungal PCR Kit and the Applied Biosystems™ MicroSEQ™ D2 rDNA Fungal Sequencing Kit provide all of the reagents necessary for the amplification and sequencing of the D2 region of the nuclear large-subunit (LSU) ribosomal RNA gene (rDNA). The DNA sequence of the unknown is deciphered by capillary electrophoresis on an Applied Biosystems™ Genetic Analyzer. The FAST PCR technology used in the MicroSEQ™ PCR kits allows identification in 5 hours. MicroSEQ™ ID Analysis Software compares the sequence to the validated MicroSEQ™ ID Fungal Gene Library, then generates an identification report. Variations found within the D2 region are sufficient to identify most yeast and molds to the species level.

Instrument platforms

For optimum performance of the Fast MicroSEQ™ D2 rDNA Fungal Identification, use the:

- Applied Biosystems™ Veriti™ 96-Well Thermal Cycler
- Applied Biosystems™ 3500 or 3130 Series Genetic Analyzer

For information on older instruments that can also be used, see Appendix B, “Additional Supported Instruments”.

Kit contents and storage

Table 1  Fast MicroSEQ™ D2 rDNA Fungal PCR Kit [Cat. nos. 4396787 and 4382397]

<table>
<thead>
<tr>
<th>Contents</th>
<th>Quantity</th>
<th>Storage conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>FAST MicroSEQ™ D2 LSU rDNA Fungal PCR Master Mix</td>
<td>One tube [yellow cap] sufficient for 60 PCR amplifications</td>
<td>On receipt: -15 to -25°C</td>
</tr>
<tr>
<td>FAST MicroSEQ™ D2 LSU rDNA Fungal Primer Mix</td>
<td>One tube [red cap]</td>
<td>After first use: 2 to 8°C in a PCR cleanroom</td>
</tr>
<tr>
<td>Positive Control, <em>S. cerevisiae</em>, 1 ng/µL</td>
<td>One tube sufficient for 10 positive-control assays</td>
<td></td>
</tr>
<tr>
<td>Negative Control, water</td>
<td>One tube</td>
<td></td>
</tr>
</tbody>
</table>
Table 2  MicroSEQ™ D2 rDNA Fungal Sequencing Kit (Cat. no. 4347481)

<table>
<thead>
<tr>
<th>Contents</th>
<th>Quantity</th>
<th>Storage conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>MicroSEQ™ D2 LSU rDNA Fungal Forward Sequence Mix</td>
<td>Two tubes sufficient for a total of 55 reactions</td>
<td>−15 to −25°C</td>
</tr>
<tr>
<td>MicroSEQ™ D2 LSU rDNA Fungal Reverse Sequence Mix</td>
<td>Two tubes sufficient for a total of 55 reactions</td>
<td></td>
</tr>
</tbody>
</table>

**Storage guidelines**

- Avoid excess freeze-thaw cycles. Aliquot reagents in smaller amounts, if necessary.
- Before each use of the kit, allow the frozen reagents to thaw at room temperature or on ice.

**IMPORTANT!** Do not heat the reagents.

- Whenever possible, keep thawed reagents on ice during use.
- Mix the reagents by gently vortexing the tubes. Centrifuge the tubes briefly to collect all liquid at the bottom of the tube.

**Materials and equipment required**

Contact your local MicroSEQ™ ID representative for a list of additional materials and equipment required.
Workflow

**Collect and prepare samples**
Harvest yeast or filamentous fungal colony, isolate DNA, then dilute DNA for PCR

**Amplify DNA**
Prepare reactions, perform amplification, analyze PCR products (optional), then purify PCR products

**Perform cycle sequencing**
Prepare reactions, perform cycle sequencing, then purify extension products

**Perform electrophoresis**
Configure instrument, then prepare and run samples

**Analyze data**

---

**Collect and prepare samples**

**Important procedural guidelines**

- Review “Good laboratory practices for PCR and RT-PCR” on page 23.
- When the isolated DNA (in PrepMan™ Ultra supernatant) is not in use, store it at −15 to −25°C. Before use, thaw, then vortex and centrifuge the stored supernatant. Alternatively, cover and store the supernatant at 4°C for up to 1 month.
Isolate fungal genomic DNA from yeast or filamentous fungal colonies using PrepMan™ Ultra Sample Preparation Reagent. Refer to the PrepMan™ Ultra Sample Preparation Reagent Protocol for additional information.

1. Obtain the sample and add PrepMan™ Ultra Sample Preparation Reagent:

<table>
<thead>
<tr>
<th>If starting from a ...</th>
<th>Follow this procedure ...</th>
</tr>
</thead>
</table>
| Culture broth          | 1. Pipet 1 mL of culture broth (containing less than $10^7$ cfu/mL of fungi) into a new 2-mL screw-cap microcentrifuge tube or any other microcentrifuge tube that can be tightly closed.  
2. Spin the sample for 2 minutes in a microcentrifuge at maximum speed. Aspirate and discard the supernatant.  
3. Add 100 µL of PrepMan™ Ultra Sample Preparation Reagent, then close the cap tightly. |
| Culture plate          | 1. Select a small sample amount (approximately 3 mm) from an isolated colony or from the edge of a filamentous fungus by using a 1 µL loop or the straight end of a 1 µL loop.  
2. Suspend the cells in 100 µL of PrepMan™ Ultra Sample Preparation Reagent in a 2-mL screw-cap microcentrifuge tube or any other microcentrifuge tube that can be tightly closed. |

**IMPORTANT!** The ideal colony size is 3 mm. For smaller colonies, decrease the amount of PrepMan™ Ultra Sample Preparation Reagent to 50 µL from the 100 µL suggested in the protocol.

**Note:** Fungal colonies are often cross-contaminated. When removing a sample, select a growth area that is well-separated from other colonies.

2. Vortex the sample for 10 to 30 seconds.

3. Heat the sample for 10 minute at 100°C in a heat block, then cool the sample to room temperature for 2 minutes.

4. Centrifuge the sample for 2 minutes in a microcentrifuge at maximum speed.

5. Transfer 50 µL of the supernatant into a new microcentrifuge tube.

**Dilute genomic DNA for PCR**

1. Pipet 495 µL of nuclease-free water into a 1.5-mL microcentrifuge tube.

2. Add 5 µL of the PrepMan™ Ultra supernatant to obtain a 1:100 dilution.

**Note:** For samples with low biomass, make a smaller dilution (for example, use 195 µL of nuclease-free water to make a 1:40 dilution). The minimum recommended dilution for the PrepMan™ Ultra supernatant is 1:10.

**Note:** If the PrepMan™ Ultra supernatant is colored (typically a shade of black or green), PCR inhibition may occur. See “Troubleshooting” on page 16.
Amplify the D2 LSU rDNA region

Important procedural guidelines

- Select the appropriate tubes or 96-well plates for your thermal cycler. See your instrument user guide (available at thermofisher.com).
- Using strip caps instead of 96-well adhesive plate covers may help reduce cross-contamination.
- Before preparing the PCR reactions, review “Good laboratory practices for PCR and RT-PCR” on page 23 and “Storage guidelines” on page 7 for sample and reagent handling instructions.
- If necessary after performing PCR or purifying PCR products, cover and store the PCR products at –15°C to –25°C until you are ready to use them.

Note: PCR products are stable for 6 months or longer at –15°C to –25°C.

Prepare the FAST PCR reaction mix

1. In a single tube, combine the following for each sample or control:
   - 14 μL of FAST PCR Master Mix (tube with yellow cap)
   - 2 μL of FAST Primer Mix (tube with red cap)

2. Vortex briefly, then centrifuge to collect all liquid at the bottom of the tube.

Prepare the PCR reactions

1. Vortex the diluted supernatant to mix the tube contents.

2. Using the volumes shown in the table, prepare samples and controls in MicroAmp™ reaction tubes or 96-well plates.

<table>
<thead>
<tr>
<th>Reaction type</th>
<th>Volume for one reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative controls</td>
<td>• 15 μL FAST PCR Reaction Mix&lt;br&gt;• 15 μL negative control (provided with kit)</td>
</tr>
<tr>
<td>Samples</td>
<td>• 15 μL FAST PCR Reaction Mix&lt;br&gt;• 15 μL of 1:100 dilution of PrepMan™ Ultra supernatant</td>
</tr>
<tr>
<td>Positive controls</td>
<td>• 15 μL FAST PCR Reaction Mix&lt;br&gt;• 15 μL positive-control DNA (provided with kit)</td>
</tr>
</tbody>
</table>

Note: To help avoid cross-contamination, we recommend that you pipet components in the following order: negative controls, samples, positive controls. If possible, leave empty cells between different reaction types.

3. Use strip caps and the capping tool, or adhesive film and the sealing tool, to cap the tubes or plate (see “Seal the PCR plate” on page 23). Vortex, spin briefly, then place the tubes or the plate in the thermal cycler.

IMPORTANT! Apply significant downward pressure on the sealing tool in all steps to form a complete seal.
Perform the amplification run

1. Set the appropriate ramp mode for your thermal cycler:
   - Veriti™ 96-Well Thermal Cycler — Default
     
     **Note:** To use the default mode, select Browse/New Methods ▶ New, then edit the thermal cycling conditions. See the Veriti™ Thermal Cycler User Guide for details.
   - 9800 Fast Thermal Cycler — Fast (F96)
   - GeneAmp™ PCR System 9700 — Maximum (Max)

2. Set the thermal cycling conditions:

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Melt</td>
<td>Anneal</td>
<td>HOLD</td>
</tr>
<tr>
<td>HOLD</td>
<td>CYCLE</td>
<td></td>
<td>HOLD</td>
</tr>
<tr>
<td>95°C 10 sec</td>
<td>95°C 0 sec</td>
<td>64°C 15 sec</td>
<td>72°C 1 min</td>
</tr>
</tbody>
</table>

   [1] We recommend that you use 30 cycles for most samples. If the PCR product yield is low, you may either increase the number of PCR cycles (up to 35), or prepare new PCR reactions using a 1:40 dilution of the PrepMan™ Ultra supernatant. Using 30 cycles results in lower background signal. When using 35 cycles, a background signal (resulting from non-specific PCR amplification during later cycles) is occasionally observed in the no template control (NTC).

3. Set the reaction volume to 30 μL, then start the run.

4. Before removing the caps or adhesive film, briefly centrifuge the tubes or plate.
   
   **Note:** Centrifuging helps avoid cross-contamination from liquid remaining on the caps or plate covers.
### Analyze PCR products

Analyze PCR products to confirm the presence of amplified DNA, or to estimate the PCR product yield. The cycle-sequencing protocol works best with 5 to 20 ng of amplicon input.

1. Load 5 μL of PCR product per lane on a 2% agarose gel separation (such as E-Gel™ available from thermofisher.com), or prepare your own gel.

2. Use the Mass Standard Ladder to estimate the PCR product yield. In a positive control or sample, a single fragment ranging from 300 to 500 bp in size should be detected on a gel. Actual fragment size depends on the fungal species. No product should be visible in a negative control reaction.

### Purify PCR products for cycle sequencing

Remove unused dNTPs and primers from each PCR product with ExoSAP-IT™ (Affymetrix).

**IMPORTANT!** Follow the guidelines for the starting sample volume for cleanup as directed in the product literature.
Perform cycle sequencing

Cycle sequencing occurs when successive rounds of denaturation, primer annealing, and primer extension in a thermal cycler result in the incorporation of dye terminators into extension products. The products are then loaded into a genetic analyzer to determine the D2 LSU rDNA sequence. For additional information about cycle sequencing chemistries, refer to the DNA Sequencing by Capillary Electrophoresis Chemistry Guide.

Important procedural guidelines

- Select the appropriate tubes or 96-well plates for your thermal cycler. See your instrument user guide (available at thermofisher.com).
- Using strip caps instead of 96-well adhesive plate covers may help reduce cross-contamination.
- If you are using a CentriSep™ Spin Column to purify extension products (see “Purifying Extension Products” on page 15), hydrate the column with highly purified (nuclease free) water during the cycle sequencing run.
- If necessary, cover and store the unused portions of the purified PCR products at –15°C to –25°C until you are ready to use them.

  Note: PCR products are stable for 6 months or longer at –15°C to –25°C.
- If necessary, cover and store the extension products at 4°C overnight or at –15°C to –25°C for up to 1 week before purifying them.

Prepare cycle sequencing reactions

1. Before you remove the tube or plate caps, briefly centrifuge the purified PCR products.

  Note: Centrifuging helps avoid cross-contamination from liquid remaining on the caps or plate covers.

2. In reaction tubes or a 96-well plate, prepare separate forward- and reverse-sequencing reactions for each PCR product and control:
   - **Forward-sequencing reaction** — Combine 7 μL of purified PCR product or control with 13 μL forward sequence mix.
   - **Reverse-sequencing reaction** — Combine 7 μL of purified PCR product or control with 13 μL reverse sequence mix.

  Note: To help avoid cross-contamination, pipet components in the following order: negative controls, samples, positive controls.

Perform the cycle sequencing run

1. Cap the tubes or the plate, then place the tubes or the plate in the thermal cycler.

2. Set the appropriate ramp mode for your thermal cycler:
   - **Veriti™ 96-Well Thermal Cycler** — Default
   - **9800 Fast Thermal Cycler** — Fast (F96)
   - **GeneAmp™ PCR System 9700** — Maximum (Max)
3. Set the thermal cycling conditions:

<table>
<thead>
<tr>
<th>Initial step</th>
<th>Each of 25 cycles</th>
<th>Final step</th>
</tr>
</thead>
<tbody>
<tr>
<td>HOLD</td>
<td>Melt 96°C 1 min</td>
<td>HOLD</td>
</tr>
<tr>
<td></td>
<td>CYCLE 96°C 10 sec</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Anneal 50°C 5 sec</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Extend 60°C 1 min 15 sec</td>
<td>4°C ∞</td>
</tr>
</tbody>
</table>

4. Set the reaction volume to 20 μL, then start the run.

5. Before removing the tube or plate caps, briefly centrifuge the extension products.

**Note:** Centrifuging helps avoid cross-contamination from liquid remaining on the caps or plate covers.

**Purify extension products**

After cycle sequencing, use one of the following products to remove excess dye terminators, non-incorporated nucleotides, and primers from the extension products. Select an appropriate purification product depending on whether you performed cycle sequencing in tubes or a plate. Follow the guidelines and procedures supplied with the kits.

<table>
<thead>
<tr>
<th>For cycle sequencing in ...</th>
<th>Purify using ...[1]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tubes</td>
<td>MicroSEQ™ ID Purification Combo Kit, with clean-up cartridges (Includes ExoSAP-IT™ Reagent; Cat. no. 4427807)</td>
</tr>
<tr>
<td></td>
<td>MicroSEQ™ ID Sequencing Clean-up Cartridges (36 columns; Cat. no. 4408228)</td>
</tr>
<tr>
<td>96-well plates</td>
<td>MicroSEQ™ ID Purification Combo Kit, with clean-up plates (Includes ExoSAP-IT™ Reagent; Cat. no. 4415506)</td>
</tr>
<tr>
<td></td>
<td>or MicroSEQ™ ID Sequencing Clean Up Plates (Cat. no. 4408227)</td>
</tr>
</tbody>
</table>

[1] Contact your local MicroSEQ™ ID representative for additional options.

**Perform electrophoresis of extension products**

**Important procedural guidelines**

- Use only the 50-cm capillary array length regardless of the instrument that you are using. Refer to your instrument user guide for more information.
- If you are not using a 3500 or 3130 Series Genetic Analyzer, select the appropriate parameter settings from the table in “Electrophoresis settings for additional supported instruments” on page 22. Refer to the MicroSEQ™ ID Analysis Software Online Help for more information.
- Cover and store any unused purified extension products at 4°C overnight or at –15°C to –25°C for up to 1 week.
Configure the instrument for electrophoresis

1. Configure your data collection software:
   - **Applied Biosystems™ 3500 Series Genetic Analyzers** — Use MicroSEQ™ ID Analysis Software Version 3.0 (or greater)
   - **Applied Biosystems™ 3130 and 3130xl Genetic Analyzers** — Use MicroSEQ™ ID Analysis Software Version 2.0 (or greater)
   - **For all other instruments** — Please contact your local MicroSEQ™ ID representative

   **Note:** See “Additional documentation” on page 19 for a list of MicroSEQ™ ID documentation.

2. Configure the instrument as described in the following table:

<table>
<thead>
<tr>
<th>Instrument</th>
<th>Procedure</th>
</tr>
</thead>
<tbody>
<tr>
<td>3500/3500xL</td>
<td>Create a plate using the MSID plate template in the 3500 Series Data Collection software. This plate template contains an instrument protocol/run module (POP-6™ polymer) and a basecalling protocol optimized for MicroSEQ™ ID applications.</td>
</tr>
</tbody>
</table>
   | 3130/3130xl | Specify:
   |             | • **Filter Set** – E
   |             | • **Run Module** – StdSeq50_POP6_1[1]
   |             | • **Base-caller** – KB.bcp
   |             | • **DyeSet/Primer (Mobility File)** – KB_3130_POP6_BDT v1.mob

   [1] You can use POP-7™ polymer with the StdSeq50_POP7 run module and the KB_3130_POP7_BDT v1.mob file. However, this instrument configuration reduces data quality within the first 40 bases on the 5’ end of the sequence.

Prepare samples and perform electrophoresis

**IMPORTANT!** If the electrophoresis run time will be longer than 48 hours (for example, if you are injecting more than 80 wells on a four-capillary instrument or more than 320 wells on a 16-capillary instrument), see “Prevent evaporation during electrophoresis” on page 25.

1. Before removing the tube caps or plate cover, briefly centrifuge the extension products.

2. Prepare reactions using a 1:1 ratio of purified extension product and formamide:
   a. In a 96-well plate, pipette 10 μL Hi-Di™ Formamide into each well to which you will add purified extension products or controls.
   b. Pipette 10 μL Hi-Di™ Formamide into each blank well that will be injected together with the samples.
   c. Add 10 μL of purified extension product or control to each well filled in step astep 2a, then mix by pipetting up and down.

   **Note:** Dilution in Hi-Di™ Formamide normalizes the signal strength of the sequencing reaction and stabilizes extension products. If after a 1:1 dilution you do not detect a sequencing ladder due to a low signal, run 15 μL of each sample without diluting.
3. Cover the plate, centrifuge, then load the plate into your instrument. Start the run.

**Note:** Centrifuging removes bubbles from the bottom of the wells.

4. When the run is complete, review the data using the MicroSEQ™ ID Analysis Software.

**Note:** If you are not using autoanalysis with a 3500 or 3130 Series Genetic Analyzer, refer to the *MicroSEQ™ ID Analysis Software Getting Started Guide* for data analysis instructions.

## Troubleshooting

<table>
<thead>
<tr>
<th>Observation</th>
<th>Possible cause</th>
<th>Recommended action</th>
</tr>
</thead>
<tbody>
<tr>
<td>No PCR product</td>
<td>• No biomass</td>
<td>1. If no PCR product is seen, use more fungal cells.</td>
</tr>
<tr>
<td></td>
<td>• Bacterial sample</td>
<td>2. If the problem persists, the isolate you are trying to identify may be bacteria. Amplify the sample with the Fast MicroSEQ™ 500 16S rDNA PCR Kit (Cat. no. 4370653).</td>
</tr>
<tr>
<td></td>
<td>• PCR inhibition</td>
<td>3. If the problem persists, make one or more new dilutions of the PrepMan™ Ultra supernatant, then run several PCR reactions of each dilution to increase your chance of obtaining a PCR product of the correct size. If the PrepMan™ Ultra supernatant is:</td>
</tr>
<tr>
<td></td>
<td>• Cells were not disrupted by the PrepMan™ Ultra method</td>
<td>• <strong>Clear</strong>–Make smaller dilutions (1:40 or 1:10) of the original PrepMan™ Ultra supernatant.</td>
</tr>
<tr>
<td></td>
<td>• Incorrect dilution</td>
<td>• <strong>Colored (typically a shade of black or green)</strong>– Make the following dilutions:</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- Smaller dilutions (1:40 or 1:10) of the original PrepMan™ Ultra supernatant.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- A 1:1000 dilution of the original PrepMan™ Ultra supernatant.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4. If you do not obtain a PCR product from any of the diluted samples, try one of the following solutions:</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Use a DNA extraction kit to isolate pure DNA.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>or</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Use the bead-beating method to isolate fungal genomic DNA or bacterial genomic DNA.</td>
</tr>
<tr>
<td>Observation</td>
<td>Possible cause</td>
<td>Recommended action</td>
</tr>
<tr>
<td>-------------------------------------</td>
<td>-------------------------------------------------------------------------------</td>
<td>------------------------------------------------------------------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Signal is too high</td>
<td>Too much amplicon in the sequencing reaction</td>
<td>Dilute the purified extension product with Hi-Di™ Formamide, then perform a new run. If you ran purified extension product that was:</td>
</tr>
</tbody>
</table>
|                                     |                                                                               | • Not diluted — Dilute the purified extension product at a ratio of 1:1.  
• Diluted at a 1:1 ratio — Dilute the purified extension product at a 1:10 ratio.  
• Diluted at a 1:10 ratio — Dilute the purified extension product at a 1:40 ratio.     |
| Absence of signal/blank electropherogram | Sample evaporation                                                            | See “Prevent evaporation during electrophoresis” on page 25.                                                                                           |
| The sequence is short and/or the first part of the sequence is very bright and off-scale and the remainder has very low intensity | • High starting amount of DNA  
or  
• Too much DNA template in the sequencing reaction | 1. Decrease the amount of fungal cell material using one of the following methods:  
• Use a smaller colony or pellet.  
• Further dilute the PrepMan™ Ultra supernatant.  
2. If the problem persists, estimate the PCR product yield on agarose gel and use 5–20 ng of amplicon for sequencing as described in “[Optional] Analyze PCR products” on page 12. |
| Both results and raw data show occasional high spikes for all four dye colors | Bubbles in the capillary                                                      | Check the instrument maintenance and troubleshooting guides.                                                                                           |
| Large regions of overlapping sequence or cannot call bases for large regions of sequence | • Pipetting more than one template per well  
or  
• DNA sample is contaminated (that is, the DNA is derived from more than one species of fungi)  
or  
• The organism has multiple copies of the rDNA gene, and some copies have insertions or deletions | 1. Prepare new reactions, then repeat electrophoresis.  
2. If the problem persists, sub-culture the organism to pure culture, then repeat identification.  
3. If the problem persists, clone the PCR product (using a kit such as the TOPO™ PCR Cloning Kit) before performing sequencing. |
| Small regions of overlapping sequence | In fungal species with multiple copies of the rRNA gene, the gene can be polymorphic, resulting in overlap of up to 1% of the sequence | No action needed.                                                                                                                                 |

*Fast MicroSEQ™ D2 rDNA Fungal Identification User Guide*
## Frequently asked questions

### Sensitivity and quantitation

**What is the sensitivity of the Fast MicroSEQ™ D2 rDNA Fungal Identification?**

As long as you start from a visible colony or cell pellet, MicroSEQ™ kits will work.

**Can I use the Fast MicroSEQ™ D2 rDNA Fungal Identification to quantify bacteria?**

No. The PCR is an endpoint assay.

### Sample preparation and storage

**Which kits should I use to identify yeast samples?**

Use the Fast MicroSEQ™ D2 rDNA Fungal Identification or the MicroSEQ™ D2 rDNA Fungal Identification to sequence and identify yeast samples.

**What is the best way to prepare yeast samples?**

Prepare yeast samples using the PrepMan™ Ultra Sample Preparation Reagent or bead-beating method, just as you would prepare bacterial samples. Extra dilutions of the fungal DNA supernatant are sometimes necessary.

**Are there alternative methods for preparing genomic DNA?**

If the PrepMan™ Ultra Sample Preparation Reagent method does not successfully disrupt cells, you can use the bead-beating method to isolate genomic DNA. Alternatively, you can use a DNA extraction kit (available from various vendors) to isolate pure DNA.

**Can I use less PrepMan™ Ultra Sample Preparation Reagent if I start with a smaller colony?**

Yes. The ideal colony size is 3 mm. For smaller colonies, you can decrease the amount of PrepMan™ Ultra Sample Preparation Reagent to 50 μL from the suggested 100 μL in the PrepMan™ Ultra Sample Preparation Reagent Protocol.

**Can I enrich my genomic DNA by using less PrepMan™ Ultra Sample Preparation Reagent?**

Yes. However, be careful not to overload the PCR mix. Enriched samples tend to have more cellular and other debris, which can interfere with PCR.

**At what temperature should I store my PrepMan™ Ultra-isolated DNA?**

Store isolated DNA at –15 to –25°C. (Alternatively, you can safely keep it overnight at room temperature or at 4°C.)

### Contamination

**How can I tell if my sequence is representative of a single species?**

The DNA sequence from a single species should be distinct (easy to call base pairs), without significant regions of overlapping sequence.

**If my initial DNA sample is contaminated (that is, it comes from multiple species), how can I sequence my PCR product?**

Clone the PCR product using a kit such as the TOPO™ TA Cloning™ Kit (Cat. no. K4575-J10).
Overlapping sequences

My sequence has large regions of overlap (>5% mixed bases). What does this mean?

See Troubleshooting, “Large regions of overlapping sequence or cannot call bases for large regions of sequence” on page 17.

My sequence has small regions (less than or equal to 1% of overlap). What does this mean?

See Troubleshooting, “Small regions of overlapping sequence” on page 17.

PCR product size

Can I always expect the same size PCR product for all species?

PCR products can vary from the expected product size, depending on the species.

Expected product sizes for the:
- MicroSEQ™ Fungal Kits – 1 band at 300–500 bp
- MicroSEQ™ 500 Kits – 1 band at 460–560 bp
- MicroSEQ™ Full Gene Kit – 1 band at 460–560 bp and 2 bands at 700–800 bp

Can I increase the number of cycles to increase the PCR yield?

Yes, but doing so can cause additional background signal from the negative control.

Species libraries

How are species in the MicroSEQ™ libraries validated?

Please contact your local MicroSEQ™ representative to obtain a copy of the MicroSEQ™ ID Library Validation Statement for additional information.

Where does Thermo Fisher Scientific obtain the strains used to determine the reference sequencing in the MicroSEQ™ libraries?

The strains are derived from major culture collections such as the American Type Culture Collection (ATCC) and the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ) (German Collection of Microorganisms and Cell Cultures).

What is the difference between the libraries for the MicroSEQ™ Full Gene kit and the MicroSEQ™ 500 kits?

The sequences in the library for the MicroSEQ™ 500 kits are ~500 bp, which is the expected size of the PCR products for this kit. The sequences in the library for the MicroSEQ™ Full Gene kit are ~1440 bp, the maximum sequence length that the kit allows you to determine.

Additional documentation

Where can I find additional information about MicroSEQ™ ID Analysis Software?

Refer to the following documentation for MicroSEQ™ ID Analysis Software Version 2.0 or greater:
- MicroSEQ™ ID Analysis Software Quick Reference Card
- MicroSEQ™ ID Analysis Software Getting Started Guide
- MicroSEQ™ ID Analysis Software Online Help

Note: For additional documentation, see “Customer and technical support“ on page 34.
The following products are available at [thermofisher.com](http://thermofisher.com).

<table>
<thead>
<tr>
<th>Product</th>
<th>Description</th>
<th>Cat. no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fast MicroSEQ™ D2 rDNA Fungal PCR Kit</td>
<td>This kit is the PCR component of the FAST MicroSEQ™ Fungal Identification System containing enough reagents for 50 PCR amplifications of fungal DNA unknowns. The sequencing component of the kit is required for species identification.</td>
<td>4382397</td>
</tr>
<tr>
<td>MicroSEQ™ D2 rDNA Fungal PCR Kit</td>
<td>This kit is the PCR component of the MicroSEQ™ Fungal Identification System containing enough reagents for 50 PCR amplifications of fungal DNA unknowns. The sequencing component of the kit is required for species identification.</td>
<td>4349153</td>
</tr>
<tr>
<td>MicroSEQ™ D2 rDNA Fungal Sequencing Kit</td>
<td>This kit is the sequencing component of the MicroSEQ™ Fungal Identification System, which provides an easy-to-use DNA sequence-based method to identify most fungi. It includes the primers needed to sequence the PCR products generated using the PCR component.</td>
<td>4347481</td>
</tr>
<tr>
<td>Fast MicroSEQ™ 500 16S rDNA PCR Kit</td>
<td>This kit is the PCR component of the MicroSEQ™ Bacterial Identification System containing enough reagents for 50 PCR amplifications of bacterial DNA unknowns. The sequencing component of the kit is required for species identification.</td>
<td>4370489</td>
</tr>
<tr>
<td>MicroSEQ™ 500 16S rDNA PCR Kit</td>
<td>This kit is the PCR component of the MicroSEQ™ Bacterial Identification System containing enough reagents for 50 PCR amplifications of bacterial DNA unknowns. The sequencing component of the kit is required for species identification.</td>
<td>4348228</td>
</tr>
<tr>
<td>MicroSEQ™ 500 16S rDNA Sequencing Kit</td>
<td>This kit is the sequencing component of the MicroSEQ™ Bacterial Identification System, which provides an easy-to-use DNA sequence-based method to identify most bacteria. It includes the primers needed to sequence the PCR products generated using the PCR component. There are enough reagents for 55 sequencing reactions.</td>
<td>4346480</td>
</tr>
<tr>
<td>MicroSEQ™ Full Gene 16S rDNA PCR Kit</td>
<td>This kit is the PCR component of the MicroSEQ™ Bacterial Identification System containing enough reagents for 50 PCR amplifications of bacterial DNA unknowns. The sequencing component of the kit is required for species identification.</td>
<td>4349155</td>
</tr>
<tr>
<td>MicroSEQ™ Full Gene 16S rDNA Sequencing Kit</td>
<td>This kit is the sequencing component of the MicroSEQ™ Bacterial Identification System, which provides an easy-to-use DNA sequence-based method to identify most bacteria. It includes the primers needed to sequence the PCR products generated using the PCR component. There are enough reagents for 55 sequencing reactions.</td>
<td>4347484</td>
</tr>
<tr>
<td>Product</td>
<td>Description</td>
<td>Cat. no.</td>
</tr>
<tr>
<td>----------------------------------------------</td>
<td>-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------</td>
<td>-------------------</td>
</tr>
<tr>
<td>PrepMan™ Ultra Sample Preparation Reagent</td>
<td>PrepMan™ Ultra Sample Preparation Reagent was developed for the rapid preparation of DNA template from Gram-negative food-borne pathogens for use in PCR amplification reactions. These samples often have high lipid content that can inhibit PCR amplification of the template. Using a simple boil and spin protocol, PrepMan™ Ultra Sample Preparation Reagent efficiently inactivates PCR inhibitors, significantly reducing the need to repeat the template preparation step.</td>
<td>4318930</td>
</tr>
<tr>
<td>MicroSEQ™ ID Analysis Software Version 3.0</td>
<td>This easy-to-use software enables you to identify and classify unidentified bacterial or fungal sequences by comparing them to a validated microbial library.</td>
<td>Contact your local MicroSEQ™ ID representative</td>
</tr>
</tbody>
</table>
Additional Supported Instruments

To take advantage of the reduced amplification and sequencing times allowed by the FAST PCR chemistry, we recommend that you use the Applied Biosystems™ Veriti™ 96-Well Fast Thermal Cycler and the Applied Biosystems™ 3500 or 3130 Series Genetic Analyzer with the MicroSEQ™ kits.

However, the MicroSEQ™ kits can also be used with:

- Applied Biosystems™ GeneAmp™ PCR System 9700 thermal cycler
  
  **Note:** The FAST MicroSEQ™ PCR chemistry reduces amplification time when used on the GeneAmp™ PCR System 9700 in Maximum ramp mode, but the time is further reduced when the FAST chemistry is used with the recommended thermal cycler.

  **Note:** An amplification run using a GeneAmp™ PCR System 9700 can take up to 20 minutes longer than a run using the Veriti™ or 9800 Fast Thermal Cycler.

- Applied Biosystems™ 9800 Fast Thermal Cycler
- Applied Biosystems™ 3730 and 3730x/ DNA Analyzers
- Applied Biosystems™ 3100 and 3100-Avant™ Genetic Analyzers
- Applied Biosystems™ 310 Genetic Analyzer

**Electrophoresis settings for additional supported instruments**

<table>
<thead>
<tr>
<th>Instrument</th>
<th>Filter Set</th>
<th>Run Module</th>
<th>Base-caller</th>
<th>DyeSet/Primer (Mobility File)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Applied Biosystems™ 3730 and 3730x/ DNA Analyzers</td>
<td>E</td>
<td>StdSeq50_POP7</td>
<td>KB.bcp</td>
<td>KB_3730_POP7_BDT v1.mob</td>
</tr>
<tr>
<td>Applied Biosystems™ 3100 and 3100-Avant™ Genetic Analyzers</td>
<td>E</td>
<td>StdSeq50_POP6_1</td>
<td>KB.bcp</td>
<td>KB_3100_POP6_BDT v1.mob</td>
</tr>
<tr>
<td>Applied Biosystems™ 310 Genetic Analyzer</td>
<td>E</td>
<td>Seq POP6 (1 mL) E.md4</td>
<td>KB.bcp</td>
<td>KB_310_POP6_BDT v1_50Std.mob</td>
</tr>
</tbody>
</table>
Supplemental Procedures and Guidelines

Good laboratory practices for PCR and RT-PCR

When preparing samples for PCR or RT-PCR amplification:

• Wear clean gloves and a clean lab coat (not previously worn while handling amplified products or during sample preparation).
• Change gloves whenever you suspect that they are contaminated.
• Maintain separate areas and dedicated equipment and supplies for:
  – Sample preparation and reaction setup.
  – Amplification and analysis of products.
• Do not bring amplified products into the reaction setup area.
• Open and close all sample tubes carefully. Avoid splashing or spraying samples.
• Keep reactions and components capped as much as possible.
• Use a positive-displacement pipettor or aerosol-resistant barrier pipette tips.
• Clean lab benches and equipment periodically with 10% bleach solution or DNAZap™ Solutions (Cat. no. AM9890).

Seal the PCR plate

Seal the plate with strip caps

IMPORTANT! Apply significant downward pressure on the sealing tool in all steps to form a complete seal.

Note: Use of strip caps instead of 96-well adhesive plate covers may help reduce cross-contamination.

To use the rolling capping tool:

• Roll the capping tool across all strips of caps on the short edge, then the long edge of the tray.
• Roll the capping tool around all outer rows of strips of caps.
To use the rocking capping tool:

- Slip your fingers through the handle with the holes in the tool facing down.
- Place the holes in the tool over the first eight caps in a row.
- Rock the tool back and forth a few times to seal the caps.
- Repeat for the remaining caps in the row, then for all remaining rows.

**Seal the plate with adhesive film**

**IMPORTANT!** Apply significant downward pressure on the applicator to completely seal the wells. Pressure is required to activate the adhesive on the optical cover.

1. Place an optical adhesive cover on the plate, then rub the flat edge of the applicator back and forth along the long edge of the plate.
2. Rub the flat edge of the applicator back and forth along the short edge of the plate.
3. Rub the edge of the applicator horizontally and vertically between all wells.
4. Rub the edge of the applicator around all outside edges of the plate using small back and forth motions to completely seal around the outside wells.
5. Vortex the plate on the low setting for 5 seconds. If you see liquid on the well sidewalls, spin down the plate at 2000 × g for 20 seconds using a centrifuge with a plate adapter.

**IMPORTANT!** Make sure reagents are in the bottom of the wells.
Prevent evaporation during electrophoresis

We recommend that you use Hi-Di™ Formamide to prevent sample evaporation during long electrophoresis runs. If your run time is:

- 24 hours or less, addition of formamide is not necessary
- Between 24 and 48 hours, see “Prepare a diluted sample” on page 25
- Longer than 48 hours, see “Dry-down and resuspend the sample” on page 25

Prepare a diluted sample

1. Prepare reactions using a 1:1 ratio of purified extension product and formamide:
   a. In a 96-well plate, pipette 10 μL of Hi-Di™ Formamide into each well to which you will add purified extension products or controls.
   b. Pipette 10 μL of Hi-Di™ Formamide into each blank well that will be injected together with the samples.
   c. Add 10 μL of purified extension product or control to each well filled in step 1a, then mix by pipetting up and down.

   Note: If after a 1:1 dilution you do not detect a sequencing ladder due to a low signal, rerun the sample without diluting.

2. Centrifuge the plate, load the plate into your instrument, then start the run.

   Note: Centrifuging removes bubbles from the bottom of the wells.

   Note: See “Configure the instrument for electrophoresis” on page 15 for details.

3. Cover and store the unused portion of the purified extension products overnight at 4°C or for up to 1 week at –15°C to –25°C.

When the run is complete, review the data using the MicroSEQ™ ID Analysis Software.

Note: If you are not using a 3500 or 3130 Series Genetic Analyzer, refer to the MicroSEQ™ ID Analysis Software Getting Started Guide for data analysis instructions.

Dry-down and resuspend the sample

1. Centrifuge the tubes or plate containing the purified extension products in a speed vac.

   Note: Centrifuge time and speed depend on the number of samples and the type of speed vac used. Typical times range from 30–60 minutes.

   IMPORTANT! Do not over-dry the DNA pellet, and do not use heat to dry the pellet.

2. Resuspend the DNA in 15 μL of Hi-Di™ Formamide.

   Note: Formamide disrupts hydrogen bonds in double-stranded DNA, inhibiting secondary structure and DNA conglomerate, and resulting in cleaner and more consistent electrophoresis runs.
3. Centrifuge the plate, load the plate into your instrument, then start the run.

   **Note:** Centrifuging removes bubbles from the bottom of the wells.

   **Note:** See “Configure the instrument for electrophoresis” on page 15 for details.

When the run is complete, review the data using the MicroSEQ™ ID Analysis Software.

**Note:** If you are not using a 3500 or 3130 Series Genetic Analyzer, refer to the MicroSEQ™ ID Analysis Software Getting Started Guide for data analysis instructions.
MicroSEQ™ system overview

The MicroSEQ™ Microbial Identification System combines all of the instruments, reagents, sequence libraries, and software required for automated microbial identification using DNA sequencing.

The MicroSEQ™ system is easy to use and suitable for the routine identification of all bacterial and fungal isolates, including organisms that are difficult to grow, non-viable, or unidentifiable using phenotypic methods. The MicroSEQ™ system identifies bacterial and fungal isolates from a small sample of pure culture without preliminary testing or growth on selective media.

About MicroSEQ™ ID Analysis Software

The MicroSEQ™ ID Analysis Software analyzes sequences obtained with any of the MicroSEQ™ Microbial Identification Kits.

The software assembles the D2 region rDNA sequence for the unknown, then compares the sequence with known reference D2 region rDNA sequences. For the Fast MicroSEQ™ D2 rDNA Fungal Identification, data is compared to the MicroSEQ™ ID Fungal Gene Library. Based on the comparison, the software provides a potential ID for the unknown fungal species.

With the software, you can perform:

- Basecalling with assignment of quality values
- Clear-range determination, which lets you exclude data near sequence ends (typically poor-quality data) from analysis
- Assembly and alignment of sequences to generate a high-quality consensus sequence
- Comparison of the consensus sequence to the MicroSEQ™ ID proprietary libraries to generate a list of the closest matches, including percentage match scores
- Exports of projects and consensus sequences to facilitate data-sharing between collaborators

The software also has features that assist with 21 CFR Part 11 compliance requirements.

For more information, refer to the MicroSEQ™ ID Analysis Software Online Help, MicroSEQ™ ID Analysis Software Quick Reference Card, and the MicroSEQ™ ID Analysis Software Getting Started Guide for software version 3.0 or later.
MicroSEQ™ ID library sequences are carefully validated. Polymorphic positions are taken into account and included in library species.

Custom libraries

MicroSEQ™ ID Analysis Software allows you to create custom libraries using data generated by the MicroSEQ™ ID software, or using sequences from public databases. Custom libraries are easy to import and export, making information sharing convenient.

During the analysis process, you can search proprietary and custom libraries simultaneously to determine 3–20 closest matches to the sequence of your unknown fungal species.

MicroSEQ™ ID reports

MicroSEQ™ ID Analysis Software generates four detailed reports:

- **Analysis QC Report** – Allows you to quickly scan the unknowns in a project to gather information about the samples, including the top percent identity match and specimen score to measure data quality. See Figure 1.
- **Library Search Report** – Provides more detailed information about the libraries that were searched, including a list of all the top matches and the total number of bases searched. See Figure 2.
- **Audit Trail Report** – Tracks changes made to projects after analysis.
- **Electronic Signature History Report** – Provides a summary of the electronic signatures used in a project.

All reports can be generated on project and specimen levels. In addition, the software allows you to create custom reports. For information, refer to the *MicroSEQ™ ID Analysis Software Online Help* for software version 2.0 or later.

![Image of Analysis QC Report](image)

**Figure 1** Example Analysis QC Report
About dye-labeled terminator chemistry

The MicroSEQ™ D2 rDNA Fungal Sequencing Kit uses BigDye™ Terminator v1.1 chemistry. Forward and Reverse Sequence Mixes contain sequence-terminating 3'-dideoxynucleotide triphosphates (ddNTPs). Each of the four ddNTPs is tagged with a different fluorescent dye. When the ddNTPs are incorporated into extension products during cycle sequencing, the extension products are simultaneously terminated and labeled with the dye that corresponds to the incorporated base, as shown in the following figure.
For more information about dye-labeled terminators and other sequencing chemistries, refer to the DNA Sequencing by Capillary Electrophoresis Chemistry Guide. See “Related documentation” on page 34.
WARNING! GENERAL SAFETY. Using this product in a manner not specified in the user documentation may result in personal injury or damage to the instrument or device. Ensure that anyone using this product has received instructions in general safety practices for laboratories and the safety information provided in this document.

- Before using an instrument or device, read and understand the safety information provided in the user documentation provided by the manufacturer of the instrument or device.
- Before handling chemicals, read and understand all applicable Safety Data Sheets (SDSs) and use appropriate personal protective equipment (gloves, gowns, eye protection, etc). To obtain SDSs, see the “Documentation and Support” section in this document.
WARNING! GENERAL CHEMICAL HANDLING. To minimize hazards, ensure laboratory personnel read and practice the general safety guidelines for chemical usage, storage, and waste provided below, and consult the relevant SDS for specific precautions and instructions:

- Read and understand the Safety Data Sheets (SDSs) provided by the chemical manufacturer before you store, handle, or work with any chemicals or hazardous materials. To obtain SDSs, see the “Documentation and Support” section in this document.
- Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (for example, safety glasses, gloves, or protective clothing).
- Minimize the inhalation of chemicals. Do not leave chemical containers open. Use only with adequate ventilation (for example, fume hood).
- Check regularly for chemical leaks or spills. If a leak or spill occurs, follow the manufacturer’s cleanup procedures as recommended in the SDS.
- Handle chemical wastes in a fume hood.
- Ensure use of primary and secondary waste containers. (A primary waste container holds the immediate waste. A secondary container contains spills or leaks from the primary container. Both containers must be compatible with the waste material and meet federal, state, and local requirements for container storage.)
- After emptying a waste container, seal it with the cap provided.
- Characterize (by analysis if necessary) the waste generated by the particular applications, reagents, and substrates used in your laboratory.
- Ensure that the waste is stored, transferred, transported, and disposed of according to all local, state/provincial, and/or national regulations.

IMPORTANT! Radioactive or biohazardous materials may require special handling, and disposal limitations may apply.
Biological hazard safety

**WARNING! BIOHAZARD.** Biological samples such as tissues, body fluids, infectious agents, and blood of humans and other animals have the potential to transmit infectious diseases. All work should be conducted in properly equipped facilities using the appropriate safety equipment (for example, physical containment devices). Safety equipment also may include items for personal protection, such as gloves, coats, gowns, shoe covers, boots, respirators, face shields, safety glasses, or goggles. Individuals should be trained according to applicable regulatory and company/institution requirements before working with potentially biohazardous materials. Follow all applicable local, state/provincial, and/or national regulations. The following references provide general guidelines when handling biological samples in laboratory environment.

Documentation and support

Related documentation

The following related documents are available at thermofisher.com/support:

<table>
<thead>
<tr>
<th>Document</th>
<th>Publication number</th>
</tr>
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<tbody>
<tr>
<td>Fast MicroSEQ™ D2 rDNA Fungal Identification Quick Reference</td>
<td>4393010</td>
</tr>
<tr>
<td>PrepMan™ Ultra Sample Preparation Reagent Protocol</td>
<td>4367554</td>
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<tr>
<td>Veriti™ Thermal Cycler User Guide</td>
<td>4375799</td>
</tr>
<tr>
<td>DNA Sequencing by Capillary Electrophoresis Chemistry Guide</td>
<td>4305080</td>
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<tr>
<td>MicroSEQ™ ID Analysis Software Getting Started Guide</td>
<td>v3.0: 4448336</td>
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<td>v2.2: 4445126</td>
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<td>v2.0: 4364623</td>
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<tr>
<td>MicroSEQ™ ID Analysis Software Quick Reference Card</td>
<td>v2.2: 4445420</td>
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<td>v2.0: 4364624</td>
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</table>

Note: For additional documentation, see “Customer and technical support” on page 34.

Customer and technical support

Visit thermofisher.com/support for the latest in services and support, including:

- Worldwide contact telephone numbers
- Product support, including:
  - Product FAQs
  - Software, patches, and updates
- Order and web support
- Product documentation, including:
  - User guides, manuals, and protocols
  - Certificates of Analysis
  - Safety Data Sheets (SDSs; also known as MSDSs)

Note: For SDSs for reagents and chemicals from other manufacturers, contact the manufacturer.
Limited product warranty

Life Technologies Corporation and/or its affiliate(s) warrant their products as set forth in the Life Technologies' General Terms and Conditions of Sale found on Life Technologies' website at www.thermofisher.com/us/en/home/global/terms-and-conditions.html. If you have any questions, please contact Life Technologies at www.thermofisher.com/support.