MicroSeq® 500

16S rDNA Bacterial Identification Kits

Protocol

Applied Biosystems

For Research Use Only. Not for use in diagnostic procedures.

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Contents

Preface

This preface contains:

Safety

Safety Alert Words

Four safety alert words appear in Applied Biosystems user documentation at points in the document where you need to be aware of relevant hazards. Each alert word–**IMPORTANT, CAUTION, WARNING, DANGER**–implies a particular level of observation or action, as defined below:

IMPORTANT! – Indicates information that is necessary for proper instrument operation, accurate chemistry kit use, or safe use of a chemical.

CAUTION – Indicates a potentially hazardous situation that, if not avoided, may result in minor or moderate injury. It may also be used to alert against unsafe practices.

WARNING – Indicates a potentially hazardous situation that, if not avoided, could result in death or serious injury.

DANGER – Indicates an imminently hazardous situation that, if not avoided, will result in death or serious injury. This signal word is to be limited to the most extreme situations.

Chemical Hazard Warning

WARNING **CHEMICAL HAZARD**. Some of the chemicals provided in your reagent kit may be hazardous. Before handling the reagents, read the material safety data sheets (MSDSs) that accompany your first shipment. Always follow the safety precautions (wearing appropriate protective eyewear, clothing, and gloves, etc.) presented in each MSDS. To receive additional copies of MSDSs at no extra cost, use the contact information below.

WARNING **CHEMICAL HAZARD**. Some of the chemicals used in this protocol are hazardous. Follow the safety precautions stated in the specific chemical warnings that appear throughout this protocol.

WARNING **CHEMICAL HAZARD**. Some of the chemicals referred to in this protocol may not have been provided with your kit. If the chemicals are not provided, they are not manufactured or sold by Applied Biosystems. Please obtain the material safety data sheets from their manufacturers.

Chemical Safety Guidelines

To minimize the hazards of chemicals:

- Read and understand the Material Safety Data Sheets (MSDS) provided by the chemical manufacturer before you store, handle, or work with any chemicals or hazardous materials. (See “About MSDSs.”)
- Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (for example, safety glasses, gloves, or protective clothing). For additional safety guidelines, consult the MSDS.
- Minimize the inhalation of chemicals. Do not leave chemical containers open. Use only with adequate ventilation (for example, fume hood). For additional safety guidelines, consult the MSDS.
- Check regularly for chemical leaks or spills. If a leak or spill occurs, follow the manufacturer’s cleanup procedures as recommended on the MSDS.
- Comply with all local, state/provincial, or national laws and regulations related to chemical storage, handling, and disposal.

About MSDSs

Chemical manufacturers supply current Material Safety Data Sheets (MSDSs) with shipments of hazardous chemicals to *new* customers. They also provide MSDSs with the first shipment of a hazardous chemical to a customer after an MSDS has been updated. MSDSs provide the safety information you need to store, handle, transport, and dispose of the chemicals safely.

Each time you receive a new MSDS packaged with a hazardous chemical, be sure to replace the appropriate MSDS in your files.

Obtaining MSDSs

You can obtain from Applied Biosystems the MSDS for any chemical supplied by Applied Biosystems. This service is free and available 24 hours a day.

To obtain MSDSs:

1. Go to **https://docs.appliedbiosystems.com/msdssearch.html**.
2. In the Search field, type in the chemical name, part number, or other information that appears in the MSDS of interest. Select the language of your choice, then click **Search**.
3. Find the document of interest, right-click the document title, then select any of the following:
   - **Open** – To view the document
   - **Print Target** – To print the document
   - **Save Target As** – To download a PDF version of the document to a destination that you choose
4. To have a copy of a document sent by fax or e-mail, select **Fax** or **Email** to the left of the document title in the Search Results page, then click **RETRIEVE DOCUMENTS** at the end of the document list.
5. After you enter the required information, click **View/Deliver Selected Documents Now**.

Chemical Waste Hazard

CHEMICAL WASTE HAZARD. Some wastes produced by the operation of the instrument or system are potentially hazardous and can cause injury, illness, or death.

Chemical Waste Safety Guidelines

To minimize the hazards of chemical waste:

- Read and understand the Material Safety Data Sheets (MSDSs) provided by the manufacturers of the chemicals in the waste container before you store, handle, or dispose of chemical waste.
- Provide 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.)
- Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (for example, safety glasses, gloves, or protective clothing). For additional safety guidelines, consult the MSDS.
- Minimize the inhalation of chemicals. Do not leave chemical containers open. Use only with adequate ventilation (for example, fume hood).For additional safety guidelines, consult the MSDS.
- Handle chemical wastes in a fume hood.
- After emptying the waste container, seal it with the cap provided.
- Dispose of the contents of the waste tray and waste bottle in accordance with good laboratory practices and local, state/provincial, or national environmental and health regulations.

Waste Disposal

If potentially hazardous waste is generated when you operate the instrument, you must:

- Characterize (by analysis if necessary) the waste generated by the particular applications, reagents, and substrates used in your laboratory.
- Ensure the health and safety of all personnel in your laboratory.
- Ensure that the instrument 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

BIOHAZARD. Biological samples such as tissues, body fluids, and blood of humans and other animals have the potential to transmit infectious diseases. Follow all applicable local, state/provincial, and/or national regulations. Wear appropriate protective eyewear, clothing, and gloves. Read and follow the guidelines in these publications:

- U.S. Department of Health and Human Services guidelines published in *Biosafety in Microbiological and Biomedical Laboratories* (stock no. 017-040-00547-4; **http://bmbl.od.nih.gov**)
- Occupational Safety and Health Standards, Bloodborne Pathogens (29 CFR§1910.1030; **http://www.access.gpo.gov/nara/cfr/waisidx_01/29cfr1910a_01.html**).

Additional information about biohazard guidelines is available at: **http://www.cdc.gov**

How to Obtain Services and Support

For the latest services and support information for all locations, go to **http://www.appliedbiosystems.com**, then click the link for **Services and Support**.

At the Services and Support page, you can:

- Search through frequently asked questions (FAQs)
- Submit a question directly to Technical Support
- Order Applied Biosystems user documents, MSDSs, certificates of analysis, and other related documents
- Download PDF documents
- Obtain information about customer training
- Download software updates and patches

In addition, the Services and Support page provides access to worldwide telephone and fax numbers to contact Applied Biosystems Technical Support and Sales facilities.

Introduction

Product Description

The MicroSeq® 500 16S rDNA Bacterial Identification PCR Kit (PN 4381502) and the MicroSeq® 500 16S rDNA Bacterial Identification Sequencing Kit (PN 4381503) provide all the reagents necessary to sequence the first 500 base pairs of the 16S ribosomal RNA bacterial gene (16S rDNA).

The resulting DNA sequence is analyzed and compared to a library of 16S rDNA bacterial gene sequences using MicroSeq ID Analysis Software. Variation within this region is sufficient to identify most organisms.

Identifying bacteria through comparative sequence analysis yields accurate and reproducible results, especially for biochemically inert species or “fall through” samples.

Unlike other bacterial identification systems, the MicroSeq 500 kits do not require gram stains, biochemical information, or special growth conditions to identify bacteria. DNA extraction is greatly simplified by using the Applied Biosystems PrepMan® Ultra Sample Preparation Reagent (PN 4322547), which can be used for all types of bacteria.

Available Products

The MicroSeq 500 kits are part of the MicroSeq Microbial Identification System, which is based on phylogenetic analysis of rRNA gene sequences. The following are the products that are part of the MicroSeq system.

Table 1 Available products

Description	Part Number
MicroSeq® 500 16S rDNA Bacterial Identification kits	
MicroSeq® 500 16S rDNA Bacterial Identification PCR Kit Includes PCR reagents sufficient for 60 amplifications (positive and negative controls), Quick Reference Card, and this Protocol **Note:** For reagents only (without documentation), order Part Number 4348228.	4381502

Table 1 Available products *(continued)*

Description	Part Number
MicroSeq® 500 16S rDNA Bacterial Identification Sequencing Kit Includes Sequencing reagents sufficient for 55 identifications, Quick Reference Card, and this Protocol **Note:** For reagents only (without documentation), order Part Number 4379284.	4381503
MicroSeq® 500 16S rDNA Bacterial Identification Kit Protocol	4379853
MicroSeq® 500 16S rDNA Bacterial Identification Kit Quick Reference Card	4379748
MicroSeq® Full Gene 16S rDNA Bacterial Identification kits	
MicroSeq® Full Gene 16S rDNA Bacterial Identification PCR Kit Includes PCR reagents sufficient for 20 amplifications (positive and negative controls), Quick Reference Card, and Protocol **Note:** For reagents only (without documentation), order Part Number 4349155.	4381540
MicroSeq® Full Gene 16S rDNA Bacterial Identification Sequencing Kit Includes Sequencing reagents sufficient for 15 identifications, Quick Reference Card, and Protocol **Note:** For reagents only (without documentation), order Part Number 4379286.	4381541
MicroSeq® Full Gene 16S rDNA Bacterial Identification Kits Protocol	4379737
MicroSeq® Full Gene 16S rDNA Bacterial Identification Kits Quick Reference Card	4379750

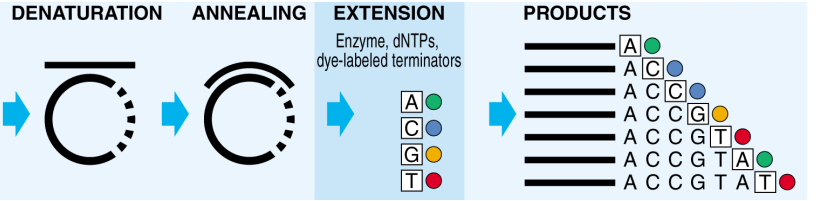
Table 1 Available products *(continued)*

Description	Part Number
MicroSeq® D2 LSU rDNA Fungal Identification kits	
MicroSeq® D2 LSU rDNA Fungal Identification PCR Kit Includes PCR reagents sufficient for 60 amplifications (positive and negative controls), Quick Reference Card, and Protocol **Note:** For reagents only (without documentation), order Part Number 4349153.	4381542
MicroSeq® D2 LSU rDNA Fungal Identification Sequencing Kit Includes Sequencing sufficient for 55 identifications, Quick Reference Card, and Protocol **Note:** For reagents only (without documentation), order Part Number 4379288.	4381543
MicroSeq® D2 LSU rDNA Fungal Identification Kits Protocol	4379854
MicroSeq® D2 LSU rDNA Fungal Identification Kits Quick Reference Card	4379749
MicroSeq® ID Analysis Software, Application and Libraries	
MicroSeq® ID Analysis Software v1.0, Application and Libraries Includes MicroSeq® ID Analysis Software v1.0, MicroSeq® ID 16S rDNA 500 Library v1.0, MicroSeq® ID 16S rDNA Full Gene Library v1.0, and MicroSeq® ID Fungal Gene Library v1.0	4379749
MicroSeq® ID Analysis Software v2.0, Application and Libraries Includes MicroSeq® ID Analysis Software v2.0, MicroSeq® ID 16S rDNA 500 Library v2.0, MicroSeq® ID 16S rDNA Full Gene Library v2.0, and MicroSeq® ID Fungal Gene Library v2.0	4371298

About Dye Terminator Chemistry

The MicroSeq 500 Sequencing kit uses Applied Biosystems BigDye® Terminator v1.1 chemistry. The Forward and Reverse Sequencing Mixes, which are part of the Sequencing kit, contain dye-labeled 3′- dideoxynucleotide triphosphates (dye terminators).

With dye-terminator labeling, each of the four dideoxy terminators (ddNTPs) is tagged with a different fluorescent dye. When dye-labeled terminators are present in the reaction mix, extension products are simultaneously terminated and labeled with the dye that corresponds to that base, as shown in the following figure.



For more information about dye terminator and other sequencing chemistries, refer to the *ABI PRISM® Automated DNA Sequencing Chemistry Guide* (PN 4305080).

Instrument Platforms

The MicroSeq 500 16S rDNA Bacterial Identification Sequencing Kit can be used with the:

- ABI PRISM® 310 Genetic Analyzer
- ABI PRISM® 3100 and 3100-*Avant* Genetic Analyzers
- Applied Biosystems 3130 and 3130*xl* DNA Analyzers
- Applied Biosystems 3730 and 3730*xl* DNA Analyzers
- GeneAmp® PCR System 9700
- Applied Biosystems 9800 Fast Thermal Cycler

Protocol Overview

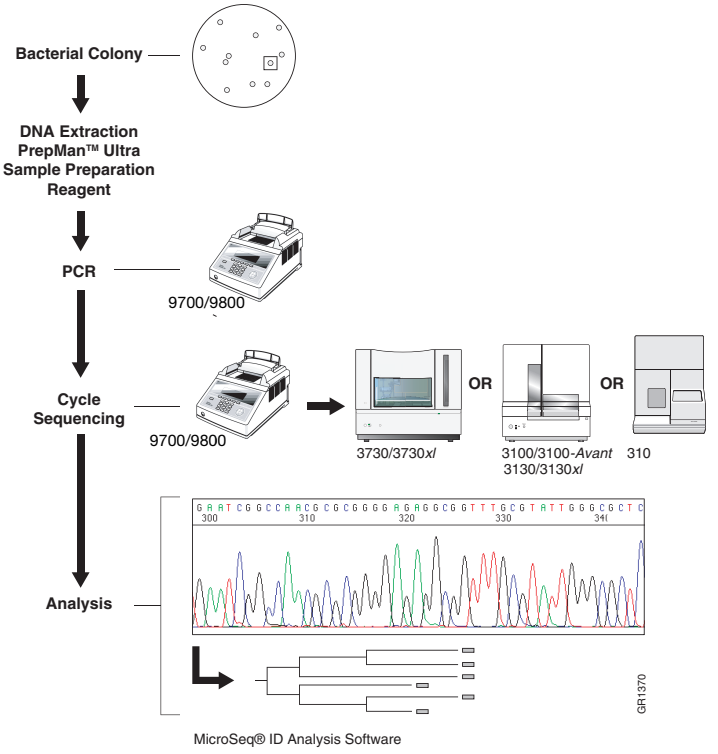
About This Protocol

This protocol provides:

- A list of materials and equipment required to determine the sequence of the first 500 base pairs of the bacterial rRNA gene
- Instructions for performing PCR and cycle sequencing, and purifying PCR and extension products
- Information about interpreting results

Procedure Workflow

The following provides a simplified overview of the procedure for using the MicroSeq 500 bacterial identification kits.



Preventing Contamination

PCR techniques require special laboratory practices to avoid false positive amplifications (Kwok and Higuchi, 1989). The high throughput and repetition of these techniques can lead to amplification of a single DNA molecule (Saiki et al., 1985; Mullis and Faloona, 1987).

Follow these recommended general PCR practices:

- Wear a clean lab coat (not previously worn while handling amplified PCR products or used during sample preparation) and clean gloves when preparing samples for PCR amplification.
- Change gloves whenever you suspect that they are contaminated.

- Maintain separate areas, dedicated equipment, and supplies for:
  - Sample preparation and PCR setup
  - PCR amplification and post-PCR analysis
- Never bring amplified PCR products into the PCR setup area.
- Open and close all sample tubes carefully. Try not to splash or spray PCR samples.
- Quick-spin PCR samples whenever residual sample is present on the inside lid (such as after dropping a tube or when there is condensation on the tube from heating or thawing)
- Keep reactions and components capped as much as possible.
- Use aerosol-resistant or positive-displacement pipette tips.
- Clean lab benches and equipment periodically with freshly diluted 10% chlorine bleach.

Materials and Equipment

Kit Contents

The table below describes the contents of the two kits:

- The MicroSeq 500 16S rDNA Bacterial Identification PCR Kit
- The MicroSeq 500 16S rDNA Bacterial Identification Sequencing Kit

PCR Kit (PN 4348228)

Component	Description
PCR Master Mix	One tube containing 2× PCR Master Mix, which is designed to amplify 16S rRNA gene sequences from bacterial genomic DNA. This tube contains enough PCR Master Mix to perform 60 PCR amplifications (positive and negative control assays).
Positive Control DNA	One tube of Positive Control DNA at 1 ng/µL, sufficient to perform ten positive control assays.
Negative Control (Water)	One tube of Negative Control

Sequencing Kit (PN 4379284)

Component	Description
Forward Sequencing Mix	Two tubes, containing enough mix to perform a total of 55 reactions.
Reverse Sequencing Mix	Two tubes, containing enough mix to perform a total of 55 reactions.

Storage Guidelines

Use the following guidelines for storing the MicroSeq 500 kits:

- Store the MicroSeq 500 kits at –15 to –25 °C.
- After the first use, store the PCR kit at 2 to 6 °C in a PCR clean room. Store the Sequencing kit at –15 to –25 °C.
- Avoid excess freeze-thaw cycles. Aliquot reagents in smaller amounts, if necessary.
- Before each use of the kits, allow the frozen stocks to thaw at room temperature.

  IMPORTANT! Do not heat the reagents.

- Mix the contents of each tube thoroughly, but do not vortex vigorously. Centrifuge the tubes briefly to collect the liquid at the bottom of the tube.
- Whenever possible, keep thawed materials on ice during use.

Equipment and Materials Not Included

The items in the following two tables are required in addition to the reagents supplied in the MicroSeq 500 kits.

Instruments from Applied Biosystems

Instrument	Source
One of the following genetic analyzers: • ABI PRISM® 310 Genetic Analyzer • ABI PRISM® 3100 Genetic Analyzer • ABI PRISM® 3100-*Avant* Genetic Analyzer • Applied Biosystems 3130/3130*xl* DNA Analyzer • Applied Biosystems 3730 DNA Analyzer • Applied Biosystems 3730*xl* DNA Analyzer	Contact your local Applied Biosystems sales office.
• GeneAmp® PCR System 9700 • Applied Biosystems 9800 Fast Thermal Cycler	

User-supplied materials

Materials	Source
310/377/3100/3100-*Avant* Genetic Analyzer Sequencing Standard BigDye® Terminator v1.1 (for 310 instruments)‡	Applied Biosystems (PN 4336791)
BigDye® Terminator v1.1 Sequencing Standard (for the 3100 and 3100-*Avant* instruments only)‡	Applied Biosystems (PN 4336824)
3700/3730 BigDye® Terminator v1.1 Sequencing Standard (for the 3730 and 3730xl instruments only)‡	Applied Biosystems (PN 4336799)
Hi-Di™ Formamide (optional, for greater stability of extension products)	Applied Biosystems (PN 4311320)

User-supplied materials *(continued)*

Materials	Source
Plates, covers, tubes, and caps as needed if using the GeneAmp® PCR System 9700:	
To run MicroSeq in 96-Well Plates:	
• MicroAmp® 96-Well Optical Reaction Plate with Barcode	Applied Biosystems (PN 4306737)
• MicroAmp® clear adhesive film	Applied Biosystems (PN 4306311)
• MicroAmp® Caps, 8 Caps/Strip or 12 Caps/Strip	Applied Biosystems (PN N8010535 or N8010534)
• MicroAmp® Cap Installing Tool (Handle)	Applied Biosystems (PN 4330015)
• MicroAmp® adhesive film applicator	Applied Biosystems (PN 4333183)
• MicroAmp® optical film compression pad	Applied Biosystems (PN 4312639)
• MicroAmp® Splash-Free 96-well base	Applied Biosystems (PN 4312063)
To run MicroSeq in MicroAmp Reaction tubes:	
• MicroAmp® Reaction tubes, 0.2-mL or MicroAmp® 8-tube Strip, 0.2-mL	Applied Biosystems (PN N8010533 or N8010580)
• MicroAmp® 96-Well Tray/Retainer set	Applied Biosystems (PN 403081)
• MicroAmp® Caps, 8 Caps/Strip or 12 Caps/Strip	Applied Biosystems (PN N8010535 or N8010534)
• MicroAmp® Cap Installing Tool (Handle)	Applied Biosystems (PN 4330015)
• MicroAmp® 96-Well Support Base or MicroAmp® Splash-Free 96-Well base	Applied Biosystems (PN 8010531 or 4312063)

User-supplied materials *(continued)*

Materials	Source
Plates, covers, tubes, and caps as needed if using the Applied Biosystems 9800 Fast Thermal Cycler:	
To run MicroSeq in 96-Well Plates:	
• Optical 96-Well Fast Thermal Cycling Plate with Barcode	Applied Biosystems (PN 4346906)
• MicroAmp® clear adhesive film	Applied Biosystems (PN 4306311)
• MicroAmp® Caps, 8 Caps/Strip or 12 Caps/Strip	Applied Biosystems (PN N8010535 or N8010534)
• MicroAmp® Cap Installing Tool	Applied Biosystems (PN 4330015)
• MicroAmp® adhesive film applicator	Applied Biosystems (PN 4333183)
• MicroAmp® optical film compression pad	Applied Biosystems (PN 4312639)
• MicroAmp® Splash Free 96-well base	Applied Biosystems (PN 4312063)
To run MicroSeq in MicroAmp Reaction tubes:	
• MicroAmp® Fast 8-Tube Strip, 0.1-mL	Applied Biosystems (PN 4358293)
• MicroAmp® Fast 96-Well Tray	Applied Biosystems (PN 4358305)
• MicroAmp® 96-Well Support Base or MicroAmp® Splash Free 96-Well Base	Applied Biosystems (PN 8010531 or 4312063)
• MicroAmp® Caps, 8 Caps/Strip or 12 Caps/Strip	Applied Biosystems (PN N8010535 or N8010534)
• MicroAmp® Cap Installing Tool	Applied Biosystems (PN 4330015)

User-supplied materials *(continued)*

Materials	Source
PrepMan® Ultra Sample Preparation Reagent	Applied Biosystems (PN 4322547)
Nuclease-Free Water (not DEPC treated)	Applied Biosystems (PN AM9937)
No-Stick RNase-Free 1.5-mL microfuge tubes	Applied Biosystems (PN AM12450)
Table-top centrifuge, with 96-tube tray adaptor	Eppendorf (5804) or equivalent
Fixed-angle microcentrifuge	Eppendorf (5415D) or equivalent
E-Gel® Single Comb Starter Pack 2% Agarose, General Purpose	Invitrogen (G5000-02)
ExoSAP-IT® Reagent	USB§ (78200)
Montage® PCR Filter Unit	Millipore (UFC7 PCR 50)
DyeEx™ 2.0 Spin Kit	Qiagen (PN 63204)
DyeEx™ 96 Kit	Qiagen (PN 63181)
Performa® DTR Gel Filtration Cartridges	Edge Biosystems (PN 98780)
Performa® DTR Ultra 96-Well Plate Kit	Edge Biosystems (PN 54789)
Vacuum centrifuge (optional)	Savant Speedvac (DNA100) or equivalent
Vortexer	Major Laboratory Supplier (MLS)

‡. BigDye® Terminator Sequencing Standards are required for:
- Spectral calibration of the sequencing instrument with BigDye terminator v1.1
- Verification of instrument performance

Please refer to the instrument manual for more information.

§. United States Biochemical. Check the USB Web site at **http://www.usbweb.com**

Isolating Bacterial Genomic DNA

PrepMan® Ultra Sample Preparation Reagent

Isolate bacterial genomic DNA using Applied Biosystems PrepMan® Ultra Sample Preparation Reagent (PN 4322547). Follow the instructions in the *PrepMan® Ultra Sample Preparation Reagent Protocol* (PN 4367554).

WARNING **CHEMICAL HAZARD**. **PrepMan Ultra** contains a material that may cause eye, skin, and respiratory tract irritation, and adverse effects on the kidneys and blood and central nervous system. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

Note: For additional information on isolating genomic DNA, go to www.microseq.com.

Storing the DNA Sample

The isolated DNA (supernatant) can be stored at –20 °C indefinitely or at 4 °C for up to 1 month.

IMPORTANT! The ideal colony size is 2 to 3 mm. For smaller colonies, decrease the amount of PrepMan Ultra Sample Preparation Reagent to 50 µL from the recommended 200 µL.

Preparing the Working Stock of Bacterial Genomic DNA

At the end of the PrepMan Ultra protocol, you obtain a supernatant that contains bacterial genomic DNA.

To make the working stock of bacterial genomic DNA:

1.	Pipette 495 µL of nuclease-free water (AM9937) into a 1.5-mL microcentrifuge tube (AM12450).
2.	Add 5 µL of the supernatant to get a 1:100 dilution.
3.	Vortex the tube to mix the solution.
4.	Store the remaining supernatant at –20 °C.

Amplifying the 16S rDNA Region

Performing PCR

PCR amplifies the first 500 base pairs of the 16S ribosomal RNA gene (16S rDNA) in the GeneAmp® PCR System 9700 (in 9600 emulation mode) as explained in the following procedure.

Note: You can also use a Applied Biosystems 9800 Fast Thermal Cycler in STD mode instead of a GeneAmp® PCR System 9700.

CAUTION **CHEMICAL HAZARD**. **MicroSeq 500 16S rDNA PCR Master Mix** may cause eye and skin irritation. Exposure may cause discomfort if swallowed or inhaled. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

To amplify the 16S rDNA region:

1. Prepare samples and controls in 0.2-mL MicroAmp PCR tubes or 96-well trays as follows:

If preparing...	Then combine...
Negative Controls	• 15 µL PCR Master Mix • 15 µL nuclease-free water
Positive Controls	• 15 µL PCR Master Mix • 15 µL of the *E. coli* positive-control DNA
Samples	• 15 µL PCR Master Mix • 15 µL working stock (1:100 dilution of PrepMan Ultra supernatant) **Note:** The 15 µL of working stock solution is intended to provide an amount of DNA close to the 25 ng that is optimal for the PCR Master Mix.

2. Cap the tubes or seal the 96-well tray with the 96-well clear adhesive film, then place them in the thermal cycler.

To amplify the 16S rDNA region: ***(continued)***

3. Use the following thermal-cycling conditions:

Initial Step‡	Each of 30 Cycles§			Final Extension	Final Step
	Melt	Anneal	Extend		
HOLD	CYCLE			HOLD	HOLD
95 °C 10 min	95 °C 30 sec	60 °C 30 sec	72 °C 45 sec	72 °C 10 min	4 °C ∞

‡. Required to activate the AmpliTaq Gold® DNA Polymerase
§. You can increase the number of cycles to increase the PCR yield, but doing so can cause additional background signal from the negative control

4. Set the reaction volume for thermal cycling to 30 µL.
5. Start the run.
6. Store the PCR products at – 15 to – 25 °C until you are ready to use them.

Analyzing PCR Products

Determine if a PCR product is present in your samples by running a 2% agarose gel. Loading 10 µL of the PCR product per lane is sufficient to detect amplified DNA with ethidium bromide staining.

Note: Running a 2% E-Gel (Invitrogen G5000-02) is a time-efficient way to determine if PCR products are present. Read and understand the Material Safety Data Sheets (MSDS) provided by the chemical manufacturer before you store, handle, or work with any chemicals or hazardous materials.

The positive control and samples should display a PCR product between 460 to 560 bp, depending on the bacterial species. No product should be visible for the negative control.

If your samples show no PCR product, PCR inhibition is the most likely cause. "Troubleshooting" on page 24 provides more information about potential PCR problems and solutions.

Preparing PCR Products for Cycle Sequencing

If the PCR product is present, you must remove unused dNTPs and primers from the remaining 20 µL of PCR product mixture before proceeding to cycle sequencing.

Clean-up the PCR product using one of the following methods:

- ExoSAP-IT® (USB PN 78200)

  Read and understand the Material Safety Data Sheets (MSDS) provided by the chemical manufacturer before you store, handle, or work with any chemicals or hazardous materials.
- Montage® PCR Filter Unit (Millipore PN UFC7 PCR50)

Be sure you follow the guidelines for the starting sample volume for clean-up as directed in the product literature.

Cycle Sequencing

Cycle sequencing occurs when successive rounds of denaturation, annealing, and extension in a thermal cycler result in linear amplification of extension products. The products are then loaded into a genetic analyzer to determine the sequence.

For additional information about cycle sequencing chemistries, refer to the *Automated DNA Sequencing Chemistry Guide* (PN 4305080).

Performing Cycle Sequencing

Prepare one forward and one reverse sequencing reaction for each PCR product, as explained in the following procedure.

WARNING **CHEMICAL HAZARD. MicroSeq 500 Sequencing Mixes (Forward and Reverse)** cause eye, skin, and respiratory tract irritation. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

To perform cycle sequencing:

1. In a 0.2-mL microcentrifuge tube or 96-well tray, combine
   - 7 µL of the purified PCR product (approx 5–20 ng)
   - 13 µL of the forward or reverse sequencing mix
2. Cap the tubes or seal the 96-well tray with the 96-well clear adhesive film, then place them in the thermal cycler.
3. Program the GeneAmp® PCR System 9700 (in 9600 emulation mode) using the following thermal-cycling conditions:

Each of 25 Cycles			Final Step
Melt	Anneal	Extend	
CYCLE			HOLD
96 °C 10 sec	50 °C 5 sec	60 °C 4 min	4 °C ∞

Note: You can use an Applied Biosystems 9800 Fast Thermal Cycler in STD mode instead of a GeneAmp® PCR System 9700.

To perform cycle sequencing: ***(continued)***

4.	Set the reaction volume for thermal cycling to 20 µL.
5.	Start the run.
6.	If necessary, store the extension products overnight at 4 °C before purifying them. You can store extension products at –20 °C for up to 1 week.

Purifying Extension Products

After cycle sequencing, you must remove excess dye terminators and primers from the cycle sequencing reactions using one of the following products:

If you performed PCR in ...	Then use...
MicroAmp®tubes	DyeEx® 2.0 Spin Kit (Qiagen PN 63204) or Performa® DTR Gel Filtration Cartridges (Edge Biosystems PN 98780)
96-well plates	DyeEx® 96 Kit (Qiagen PN 63181) or Performa® DTR Ultra 96-Well Plate kit (Edge Biosystems PN 54789)

Follow the guidelines and procedures that accompany the kits.

Electrophoresis and Sequencing of Extension Products

Preparing Samples for Electrophoresis

Load samples directly on the sequencing instrument. After purification, transfer 15 to 20 µL of extension product into a 96-well plate. Load the plate on the sequencing instrument and start the run.

This method may require optimization. Please refer to page 26 for more information.

Dry the samples and resuspend them in Hi-Di™ Formamide. This method ensures greater stability of extension products and is highly recommended when more than four runs are set per plate.

WARNING **CHEMICAL HAZARD. Formamide.** Exposure causes eye, skin, and respiratory tract irritation. It is a possible developmental and birth defect hazard. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

To prepare samples for electrophoresis:

1.	Spin-down the microcentrifuge tubes or plates containing the purified extension products in a speed vac. Centrifuge time and speed depend on the number of samples and the type of speed vac used. Typical times range from 30 to 60 minutes. **IMPORTANT!** Do not overdry the DNA pellet. Further, do not use heat when drying the pellet.
2.	Resuspend the DNA in 15 µL of Hi-Di™ Formamide (PN 4311320). **Note:** Formamide disrupts hydrogen bonds in double-stranded DNA. This activity inhibits secondary structure and DNA conglomeration, resulting in cleaner and more consistent electrophoresis runs.
3.	Load the resuspended DNA samples onto the sequencing platform.

Instrument Configuration for Electrophoresis

To correctly analyze data, the MicroSeq ID Analysis Software must use the appropriate DyeSet/Primer file (also called mobility file), basecaller, and run module. The following table indicates the required configuration for each of the supported platforms when using BigDye Terminator Chemistry v1.1.

Instrument	Filter Set	Run Module	Basecaller‡	DyeSet/Primer (Mobility File)
310	E	Seq POP6 (1 mL)	KB.bcp	DTPOP6{BD}.mob
3100 3100-*Avant*	E	StdSeq50_POP6	KB.bcp	KB_3100_POP6(BD)b2.mob
3130 3130*xl*	E	StdSeq50_POP6_1	KB.bcp	KB_3130_POP6_BDTv1.mob
3730 3730*xl*	E	StdSeq50_POP7_1	KB.bcp	KB_3730_POP7_BDTv1.mob

‡. Applied Biosystems recommends that you use the basecaller and mobility file names listed in the table with the Data Collection 2.0 (DC 2.0) and Data Collection 3.0 (DC 3.0) Software. Certain basecaller and mobility files issued for previous releases of the software are compatible with the DC 2.0 and DC 3.0 software. These files are listed in Appendix A.

Refer to the MicroSeq ID Analysis Software Online Help for more information about naming conventions for Basecaller and DyeSet/Primer files.

Applied Biosystems recommends that you use the standard 50-cm sequencing capillary length. Please refer to your instrument user guide for more information.

Analyzing Data

MicroSeq ID Analysis Software

MicroSeq® ID Analysis Software enables you to analyze sequences obtained with any of the MicroSeq® Microbial Identification Kits, including the MicroSeq® 500 16S rDNA Bacterial Identification Kits, the MicroSeq® Full Gene 16S rDNA Bacterial Identification Kits, and the MicroSeq® D2 rDNA Fungal Identification Kits.

The software assembles the 16S rDNA sequence for the unknown, then compares the sequence with 16S rDNA sequences in the MicroSeq® ID 16S rDNA 500 Library (v1.0 and v2.0). Based on the comparison, the software provides an ID for the unknown bacterial 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 help you meet 21 CFR Part 11 compliance requirements.

MicroSeq Proprietary Libraries

The MicroSeq® ID 16S rDNA 500 Library (v1.0) includes 1435 and the MicroSeq® ID 16S rDNA 500 Library (v2.0) includes 1716 validated 16S rDNA sequences. All sequences and strains are carefully checked and quality controlled to achieve maximum reliability. Polymorphic positions are taken into account to ensure the highest degree of accuracy.

Custom Libraries

In addition to accessing the proprietary libraries, MicroSeq® ID Analysis Software allows you to create custom libraries. You can create custom libraries using data generated by the MicroSeq® ID software, or you can download sequences from public databases. Custom libraries are easy to import and export, making information sharing convenient.

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

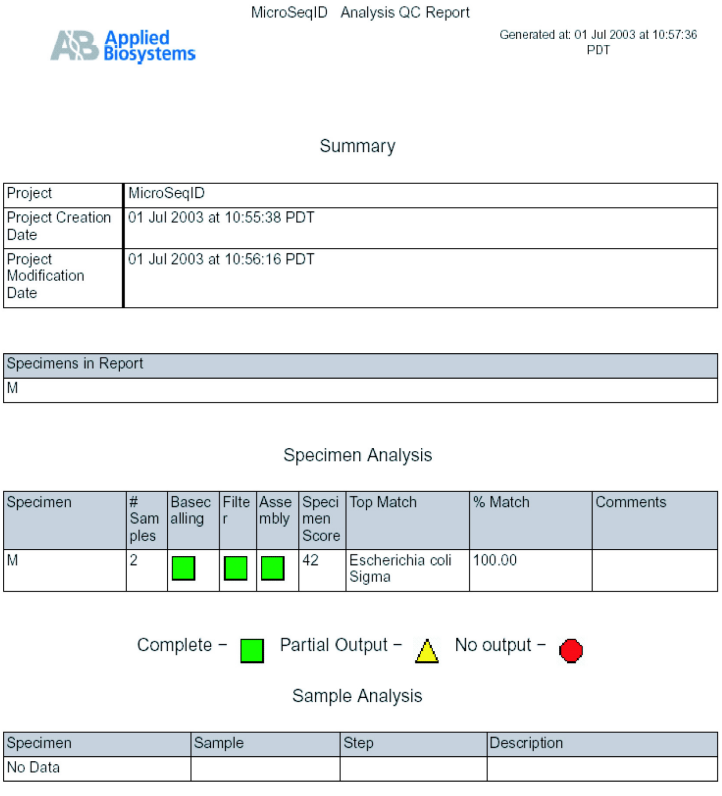
Generated Reports

MicroSeq® ID Analysis Software (v1.0 and v2.0) generates three 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. Figure 1 on page 22 shows a sample Analysis QC Report.
- **Library Search Report** – Provides more detailed information about the libraries searched, including a list of all the top matches and the total number of bases searched. Figure 2 on page 23 shows a sample Library Search Report.
- **Audit Trail Report** – Tracks changes made to projects following analysis.

All three reports can be generated on a project level and on a per specimen level.

Note: MicroSeq® ID Analysis Software (v2.0) generates an additional detailed report, the Electronic Signature History Report. For information, refer to the MicroSeq® ID Analysis Software Version 2.0 Online Help.



MicroSeqID Analysis QC Report

Applied Biosystems

Generated at: 01 Jul 2003 at 10:57:36 PDT

Summary

Project	MicroSeqID
Project Creation Date	01 Jul 2003 at 10:55:38 PDT
Project Modification Date	01 Jul 2003 at 10:56:16 PDT

Specimens in Report
M

Specimen Analysis

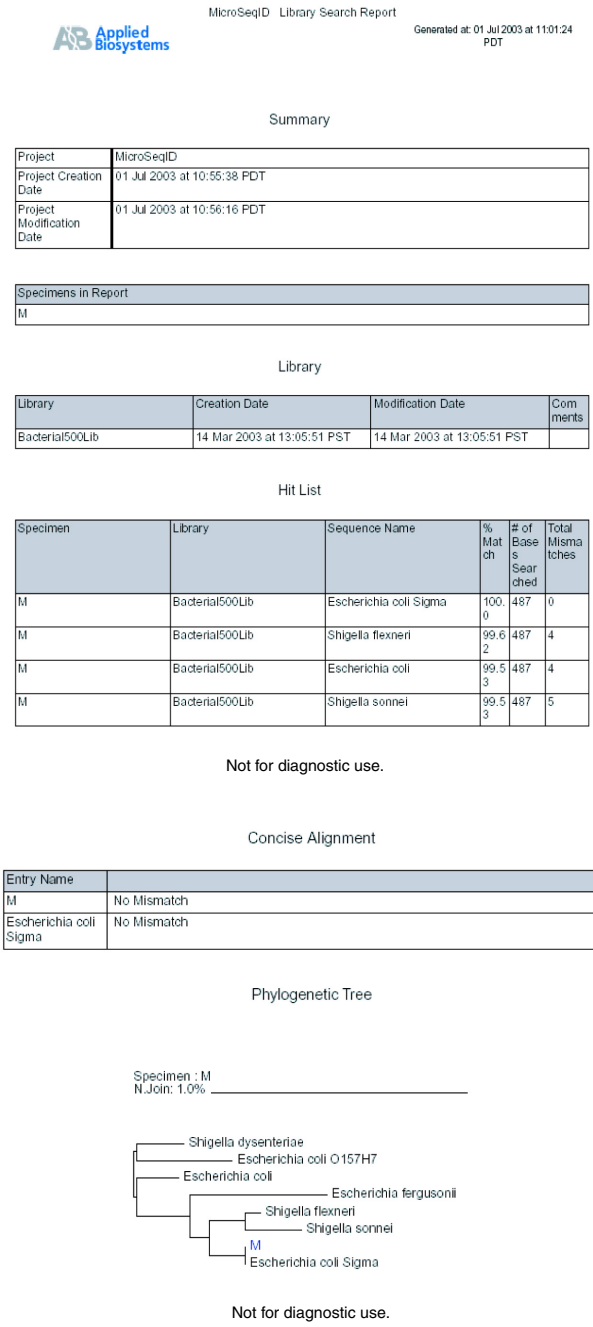
Specimen	# Samples	Basecalling	Filter	Assembly	Specimen Score	Top Match	% Match	Comments
M	2				42	Escherichia coli Sigma	100.00	

Complete – Partial Output – No output –

Sample Analysis

Specimen	Sample	Step	Description
No Data			

Figure 1 Sample Analysis QC Report



MicroSeqID Library Search Report

Applied Biosystems

Generated at: 01 Jul 2003 at 11:01:24 PDT

Summary

Project	MicroSeqID
Project Creation Date	01 Jul 2003 at 10:55:38 PDT
Project Modification Date	01 Jul 2003 at 10:56:16 PDT

Specimens in Report
M

Library

Library	Creation Date	Modification Date	Comments
Bacterial500Lib	14 Mar 2003 at 13:05:51 PST	14 Mar 2003 at 13:05:51 PST	

Hit List

Specimen	Library	Sequence Name	% Match	# of Bases Searched	Total Mismatches
M	Bacterial500Lib	Escherichia coli Sigma	100.0	487	0
M	Bacterial500Lib	Shigella flexneri	99.62	487	4
M	Bacterial500Lib	Escherichia coli	99.53	487	4
M	Bacterial500Lib	Shigella sonnei	99.53	487	5

Not for diagnostic use.

Concise Alignment

Entry Name	
M	No Mismatch
Escherichia coli Sigma	No Mismatch

Phylogenetic Tree

Specimen : M
N.Join: 1.0%

Shigella dysenteriae
Escherichia coli O157H7
Escherichia coli
Escherichia fergusonii
Shigella flexneri
Shigella sonnei
M
Escherichia coli Sigma

Not for diagnostic use.

Figure 2 Sample Library Search Report

Troubleshooting

Problem	Possible Cause	Solution
No PCR product	• No biomass • PCR inhibition • Fungal sample • Cells were not disrupted by the PrepMan® Ultra Kit method	• Use more bacterial cells • Make additional dilutions (1:10, 1:100, 1:1000) of the PrepMan Ultra kit supernatant before proceeding to PCR. • Use the MicroSeq® D2 LSU rDNA Fungal Identification Kits. • Use the bead-beating method to isolate bacterial genomic DNA.
Short sequence, the first part of which is very bright and off-scale and the remainder of which has very low intensity	High starting amount of DNA or too much DNA template in the sequencing reaction	Decrease the amount of bacterial cell material (smaller colony or pellet).
Both my results and raw data show occasional high spikes for all four dye colors	Bubbles in the capillary	Check the instrument manual.
Large regions of overlapping sequence	DNA sample is contaminated (that is, the DNA is derived from more than one species of bacteria)	Clone the PCR product (using a kit such as the Invitrogen™ Topo® PCR Cloning Kit) before performing sequencing.
Small regions of overlapping sequence	• In bacterial species with multiple copies of the rRNA gene, insertions or deletions in a subset of the genes can result in a shift by 1 to 3 bp. • Similarly, in bacteria with multiple copies of the rRNA gene, the gene can be polymorphic, resulting in overlap of up to 1% of the sequence.	N/A
Cannot call bases for large regions of sequence	DNA sample is contaminated (that is, the DNA is derived from more than one species of bacteria)	Clone the PCR product (using a kit such as the Invitrogen™ Topo® PCR Cloning Kit) before performing sequencing.

Frequently Asked Questions

Sensitivity and Quantification

What is the sensitivity of the MicroSeq® 500 kits?

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

Can I use the MicroSeq 500 Kits to quantify bacteria?

No. The PCR is an endpoint assay.

Sample Preparation and Storage

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 prepare bacterial samples. Extra dilutions of the working fungal stock are sometimes necessary.

Which kits should I use to identify yeast samples?

Use the MicroSeq® D2 LSU rDNA Fungal Identification Kits to sequence and identify yeast samples.

Are there alternative methods for preparing genomic DNA?

If the PrepMan Ultra kit method does not successfully disrupt cells, you can use the bead-beating method to isolate bacterial genomic DNA from bacterial colonies or cultures. Use 100 µM zirconia/silica beads (Biospec Products PN 11079101Z). Refer to **http://www.biospec.com** for the bead-beating protocol.

Alternatively, you can use a DNA extraction kit (available from vendors like Qiagen) 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 2 to 3 mm. For smaller colonies, you can decrease the amount of PrepMan Ultra Sample Preparation Reagent to 50 µL from the suggested 200 µL in the P*repMan® Ultra Sample Preparation Reagent Protocol* (PN 4367554).

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 kit-isolated DNA?

Applied Biosystems recommends storing DNA at –20 °C, although you may safely keep it at room temperature overnight.

Electrophoresis and Sequencing

Is there another way to prepare samples for electrophoresis and sequencing?

Direct loading of extension products after purification with Edge DTR or DyeEx products was successful in internal tests. If the resulting signal strength is too high, you might have to adjust injection time. Refer to the appropriate instrument manual for the guidelines on the injection times.

IMPORTANT! For direct loading method, you must use highly purified water (such as Nuclease-free Water, PN AM9937).

Note: For the latest support information, go to **http://www.appliedbiosystems.com**, then click **Support**.

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 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® PCR Cloning Kit from Invitrogen™.

Overlapping Sequences

My sequence has large regions of overlap. What does this mean?

The presence of large regions of overlapping sequence indicates that the DNA is derived from more than one species of bacteria. You can still derive the sequence of each of the bacterial species by cloning the PCR products (using a kit such as the Invitrogen Topo® PCR Cloning Kit).

My sequence has small regions (up to 1%) of overlap. What does this mean?

Some bacterial species can have multiple copies of the rRNA gene. Small regions of overlapping sequence can be caused by insertions or deletions in a subset of these genes, resulting in a shift of 1 to 3 bp. Similarly, the gene can be polymorphic, leading to overlap of up to 1% of the sequence.

PCR Product Size

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

PCR products can vary, depending on the species.

Expected product sizes for the:

- **MicroSeq 500 Kit** – 1 band at 460 to 560 bp
- **MicroSeq Full Gene Kit** – 1 band at 460 to 560 bp and 2 bands at 700 to 780 bp
- **Fungal Kit** – 1 band at 300 to 500 bp

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

You can increase the number of cycles to increase the PCR yield, but doing so can cause additional background signal from the negative control.

BigDye® Terminator Chemistry

What is the difference between the BigDye Terminator chemistry currently used in the kit and the dRhodamine chemistry used in previous versions of the kit?

BigDye Terminator chemistry allows you to read clear sequences close to the primer regions. Additionally, overall signal strength is increased. Refer to the *ABI PRISM® Automated DNA Sequencing Chemistry Guide* (PN 4305080) for more information about BigDye Terminator chemistry.

Species Libraries

How are species in the Applied Biosystems libraries validated?

Refer to the MicroSeq® Validation Statement link at **www.microseq.com**.

Where are the species in the Applied Biosystems libraries derived from?

The species are derived from the American Type Culture Collection (ATCC) and the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (German Collection of Microorganisms and Cell Cultures).

Are there pathological species included in the libraries?

Yes.

What is the difference between the libraries for the MicroSeq Full Gene Kits 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 these kits. The sequences in the library for the MicroSeq Full Gene kits, on the other hand, are ≈1440 bp—the maximum sequence length that the kits allow you to determine.

How often is the library updated?

Yearly.

How many bacterial species are included in the libraries for the MicroSeq 500 and Full Gene kits? How many fungal species are included in the library for the MicroSeq Fungal kit?

Kit	Library	Number of Species
MicroSeq 500	• MicroSeq® ID 16S rDNA 500 Library v1.0	1435
	• MicroSeq® ID 16S rDNA 500 Library v2.0	1716
MicroSeq Full Gene	• MicroSeq® ID 16S rDNA Full Gene Library v1.0	1225
	• MicroSeq® ID 16S rDNA Full Gene Library v2.0	1261
MicroSeq Fungal	• MicroSeq® ID Fungal Gene Library v1.0	900
	• MicroSeq® ID Fungal Gene Library v2.0	1113

Note: The three libraries are available as part of the MicroSeq® ID Analysis Software v1.0, Application and Libraries (PN 4345387), and the MicroSeq® ID Analysis Software v2.0, Application and Libraries (PN 4371298). For more information, see http://www.appliedbiosystems.com.

Instrument Configuration

I have older versions of the basecaller and mobility files. Will these continue to work with DC 2.0 software?

Applied Biosystems recommends that you use the basecaller and mobility files specified in "Instrument Configuration for Electrophoresis" on page 19. However, some older versions of the files are compatible with DC 2.0 software; these files are listed in Appendix A.

Additional Documentation

Where can I find additional information about MicroSeq ID Analysis Software?

Additional information about MicroSeq ID Analysis Software is available in the:

- *MicroSeq® ID Analysis Software Version 1.0 Quick Reference Card* (PN 4345868)
- *MicroSeq® ID Analysis Software Version 1.0 Getting Started Guide* (PN 4345867)
- *MicroSeq® ID Analysis Software Version 2.0 Quick Reference Card* (PN 4364624)
- *MicroSeq® ID Analysis Software Version 2.0 Getting Started Guide* (PN 4364623)
- Online Help for the software

Appendix A Basecaller and Mobility Files

The following table lists the recommended basecaller and mobility files for Data Collection 2.0 and Data Collection 3.0 software and the corresponding files for earlier releases of the software.

Instrument	Basecaller		DyeSet/Primer (Mobility File)	
	DC 2.0/3.0	Previous Releases	DC 2.0/3.0	Previous Releases
310	KB.bcp	310POP6.bcp	DTPOP6{BD}.mob	DTPOP6{BD}.mob
3100 3100-*Avant*	KB.bcp	3100POP6SR.bcp	KB_3100_POP6(BD)b2.mob	DT3100POP6(BD)v2.mob
3130 3130*xl*	KB.bcp	N/A	KB_3130_POP6_BDTv1.mob	N/A
3730 3730*xl*	KB.bcp	Basecaller_3730POP7LR.bcp	KB_3730_POP7_BDTv1.mob	DT3730POP7(BD).mob

"Instrument Configuration for Electrophoresis" on page 19 contains additional information required for instrument configuration.

References

Kwok, S. and Higuchi, R., 1989. Avoiding false positives with PCR. *Nature* 339:237–238.

Mullis, K.B. and Faloona, F. A., 1987. Specific synthesis of DNA in vitro via a polymerase-catalyzed chain reaction. *Methods Enzymol.* 155:335–350.

Saiki, R.K., Scharf, S., Faloona, F., *et al.*, 1985 Enzymatic amplification of β-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. *Science* 230:1350–1354.

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