



## Microbial ID

# Microbial identification using the MicroSEQ system on the SeqStudio Genetic Analyzer

### Keywords

SeqStudio Genetic Analyzer, microbial identification, MicroSEQ ID Software, sequencing, 16S gene, bacterial and fungal identification, validated bacterial and fungal libraries, .ab1 files, 21 CFR Part 11

### In this application note we present:

- The use of the innovative Applied Biosystems™ SeqStudio™ Genetic Analyzer for capillary electrophoresis
- The simplified workflow of the Applied Biosystems™ MicroSEQ™ Rapid Microbial Identification System for highly accurate identification of bacteria and fungi
- Data analysis using Applied Biosystems™ MicroSEQ™ ID Software for SeqStudio™ Genetic Analyzer

### Introduction

Rapid and accurate identification of infectious or fastidious microbial isolates is a major challenge for clinical practice and public health surveillance. Based on the groundbreaking phylogenetic research work of Woese et al. [1] and others, sequencing of the 16S ribosomal RNA (rRNA) gene has emerged as the preferred method for taxonomic classification and identification of bacteria. Today, many products and literature reviews demonstrate the use of sequencing of this region for identification at the species level (for reviews, see references 2 and 3). There are numerous protocols and a published guideline [2] for PCR and sequencing of the 16S gene for research and applied use. In addition, several databases from public institutions and commercial companies are available for comparing the 16S sequences with typed or annotated entries.

The MicroSEQ Rapid Microbial Identification System powered by the validated and curated MicroSEQ database has long been trusted for microbial identification in validated and pharmaceutical production settings on high-throughput instruments.

The SeqStudio Genetic Analyzer provides a rapid, low-cost alternative to high-throughput instruments. The SeqStudio Genetic Analyzer is ideal for microbiology laboratories that need to sequence individual samples or small batches in a fast, easy, and economical manner. The new security, audit, and electronic signature (SAE) module is an optional component of Applied Biosystems™ SeqStudio™ Data Collection Software that offers an electronic data chain of custody for the integrity of your data and to manage system security and quality control.\*

Here we present the validated MicroSEQ ID microbial identification workflow that is optimized for use on the SeqStudio Genetic Analyzer, with downstream data analysis using MicroSEQ proprietary libraries representing more than 12,000 bacterial and fungal species reflecting up-to-date taxonomy.

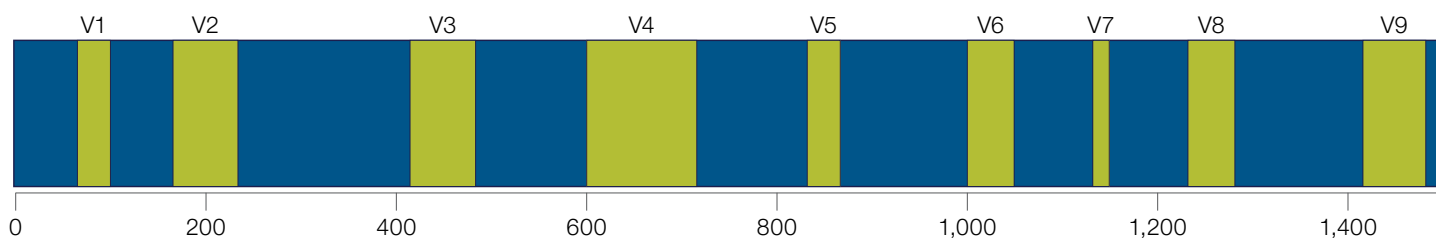
### SeqStudio Genetic Analyzer and the MicroSEQ ID microbial identification workflow

The SeqStudio Genetic Analyzer is an affordable capillary electrophoresis (CE) system that can perform both automated Sanger DNA sequencing and high-precision sizing and analysis of multicolor fluorescent DNA fragments. The instrument can accommodate a 96-well plate loaded with sequencing reactions

that are sequentially electrophoresed in batches of 4 samples per run. The sequencing samples can be processed in a 40 min medium run cycle, resulting in DNA sequences of about 500 bases each. This is sufficient to perform routine identification of bacterial species using the first 3 hypervariable regions of the 16S rRNA gene (Figure 1). Specimens are sequenced in both forward and reverse sample directions, allowing for consensus sequence for identification.

The procedure described here can be adapted to the individual lab; as few as two specimens can be run for complete bidirectional sequencing or as many as 46 can be combined to fill a single 96-well plate with forward and reverse positive and negative control reactions.

The optimized and validated workflow on the SeqStudio instrument helps reduce the time-to-results by taking advantage of the shorter run cycle and off-instrument data analysis. This maximizes the throughput of samples within a given timeframe, while leveraging the discriminatory power of the MicroSEQ libraries.



**Figure 1. Hypervariable regions within the 16S rRNA gene.** There are 9 hypervariable regions (V1–V9) within the bacterial 16S gene, indicated in green. The conserved regions are indicated in blue.

\* SAE enablement requires installation by an on-site field service engineer and a PC (desktop or laptop) purchased from Thermo Fisher Scientific. For customers with 21 CFR Part 11 compliance needs, please consult with your local sales representative for additional product offerings and service options.

## The MicroSEQ ID workflow

An overview and example timeline for the MicroSEQ ID workflow are shown in Figure 2. Starting in the morning, individual bacterial colonies are picked from agar culture plates, and PCR-ready DNA is extracted using the Applied Biosystems™ PrepMan™ Ultra Sample Preparation Reagent. PCR reactions for bacterial identification are then set up using the Applied Biosystems™ Fast MicroSEQ™ 500 16S rDNA PCR Kit followed by a 25 min program. A 5 min enzymatic cleanup using the Applied Biosystems™ ExoSAP-IT™ Express PCR Product Cleanup Reagent prepares the reactions for sequencing. Sequencing reactions are set up using the MicroSEQ 500 16S rDNA Sequencing Kit. The reactions are then purified with the Applied Biosystems™ MicroSEQ™ ID Ultra Sequencing Clean-up 8-Strips or Plates Kit. Single cleanup cartridges are available when sample numbers are low. The samples are electrophoresed on the SeqStudio Genetic Analyzer immediately following cleanup. The instrument continuously generates a dataset of 4 sequences every 40 min when operating in medium-run mode. The sequence files are then available for secondary data analysis using the MicroSEQ ID Software for SeqStudio Genetic Analyzer v1.0 to basecall the .ab1 files, create a consensus sequence for the specimen, and interrogate the libraries to identify the organism.

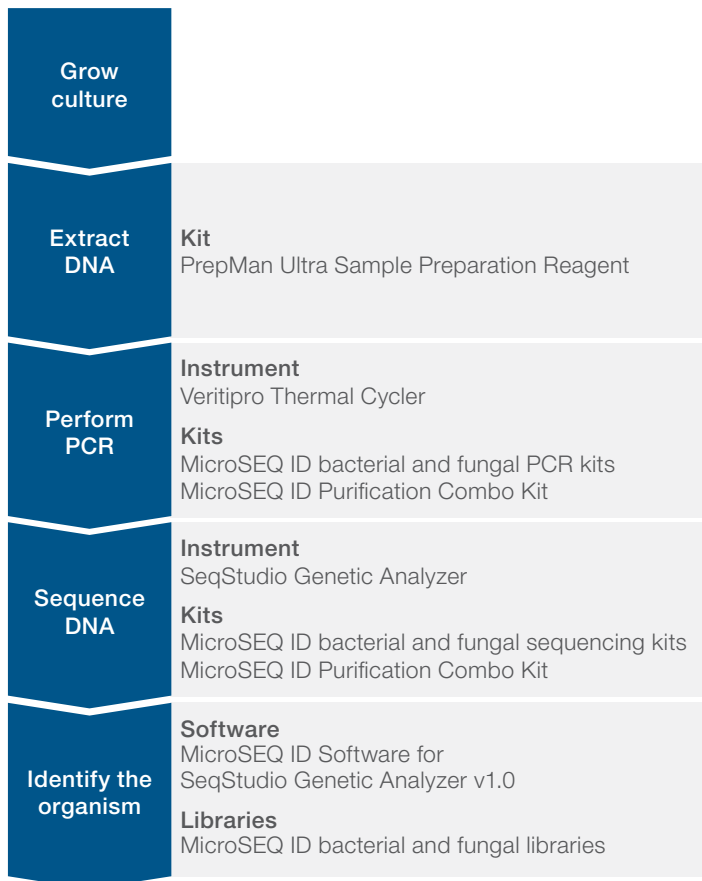


Figure 2. MicroSEQ system workflow with SeqStudio Genetic Analyzer.

## Materials and methods

### DNA

The genomic DNA (gDNA) used for this research was from bacterial strains of known species obtained from ATCC and diluted to 10 ng/μL in Invitrogen™ TE buffer (Tris 10 mM, EDTA 0.1 mM). To prepare PCR-ready DNA from bacteria growing on an agar plate or other biological or environmental matrix, the PrepMan Ultra Sample Preparation Reagent is a suitable product that is fast, economical, and easy to use. Other options are described at [thermofisher.com/gdna](http://thermofisher.com/gdna).

### PCR

For PCR of the V1–V3 region of the 16S rRNA gene, we used the Fast MicroSEQ 500 16S rDNA PCR Kit. The kit includes a proprietary master mix containing primers for easy reaction setup (Table 1). The use of the fast master mix allows rapid cycling, completing the first step in <30 min with the parameters shown in Table 2.

Table 1. Preparation of PCR reactions.

Reaction type	Volume for one reaction
Negative controls	• 15 μL of fast PCR master mix
	• 15 μL of negative control (provided in kit)
Samples	• 15 μL of fast PCR master mix
	• 15 μL of 1:100 dilution of supernatant prepared using PrepMan Ultra reagent
Positive controls	• 15 μL of fast PCR master mix
	• 15 μL of positive control DNA (provided in kit)

The advantage of this kit is that in 4 sequencing reactions, complete forward and reverse sequencing results are obtained for two full specimens. This allows the customer to stage important samples for first runs, prioritizing their analysis. In addition, the SeqStudio instrument empowers the user to customize sample injections during the plate run. This allows for higher-priority samples to be moved forward so that the data can be analyzed sooner, and the results reported as quickly as possible.

Table 2. Thermal cycling protocol using the fast master mix.

Initial step	Each of 30 cycles		Final extension	Final step
Hold	Melt	Anneal	Hold	Hold
95°C	95°C	64°C	72°C	4°C
10 sec	0 sec	15 sec	1 min	∞

As an optional step after PCR, 15 μL of low-EDTA TE buffer was added to the reaction and 2.5 μL (~10%) was used for electrophoresis on a 2% Invitrogen™ E-Gel™ agarose gel to verify the presence of amplicon “A” (~830 bp) or amplicon “B” (~750 bp) as a clearly visible, single band in an estimated amount of 20–50 ng.

High-resolution Sanger sequencing with the MicroSEQ Rapid Microbial Identification System has been demonstrated to produce clear base resolution close to the primer at both the 5' and 3' ends of the sequences. This allows for full coverage of the expected region of interest, enabling the customer to achieve the recommended 80% consensus length of the sample matched to the library entry. Sequencing was achieved using the cycling parameters in Table 3.

**Table 3. Sanger sequencing parameters.**

Initial step	Each of 25 cycles			Final step
Hold	Melt	Anneal	Extend	Hold
96°C	96°C	50°C	60°C	4°C
1 min	10 sec	5 sec	1 min 15 sec	∞

After cycle sequencing, the reactions were prepared for placement on the SeqStudio Genetic Analyzer by centrifugation using the MicroSEQ ID Ultra Sequencing Clean-up 8-Strips Kit. This product is ideally suited to match the number and placement of the samples going onto the sequencer while reducing the cost of reagents. The strips were centrifuged with an equally set balance plate in a benchtop centrifuge for 3 minutes at 850 x g. Then, the waste plate was discarded, the injection plate placed on the bottom of the cleanup strip plate, and the samples added to the wells containing the cleanup gel. Following a second 3 min centrifuge at 850 x g, the samples were placed directly onto the SeqStudio Genetic Analyzer for CE.

Alternative cleanup options for fewer samples include the Applied Biosystems™ MicroSEQ™ ID Sequencing Clean-up Cartridges for individual sample cleanup. A plate format is also available to handle high-throughput sequencing.

### Sequencing by CE on the SeqStudio Genetic Analyzer

The sequencing reactions were run under the MediumSeq (approximately 40 min) run module. Complete and accurate assembly of the first 500 bases of the 16S rRNA gene sequence was reliably achieved with high-quality (quality value (QV) >35), medium-length run data. After completion of a CE run cycle, the 4 sequencing files for the forward and reverse specimen samples were immediately available for data analysis and also remotely retrievable through internet cloud connectivity. This helps enable urgent identification analysis away from the lab or after lab hours.

### Data analysis using MicroSEQ ID software

MicroSEQ ID Software for SeqStudio Genetic Analyzer v1.0 is part of a streamlined workflow for microbial identification of bacteria and fungi. MicroSEQ chemistry kits are used to generate PCR products that are sequenced on a SeqStudio Genetic Analyzer. The software offers the MicroSEQ ID Run wizard to set up specimens and export the plate for the instrument run. All specimen data from the genetic analyzer are analyzed automatically in the software after the user confirms the run's completion. MicroSEQ ID Software for SeqStudio Genetic Analyzer v1.0 compares the sequencing data to validated microbial libraries and generates an identification report with percent similarity. The report includes a final identification list of organisms that closely match the unknown sample and a phylogenetic tree. Results are presented in an easy-to-read "Standard Report" format or may be customized in a report format of the user's choice (Figure 3). Security, audit, and e-signature capabilities are included to help enable 21 CFR Part 11 compliance.

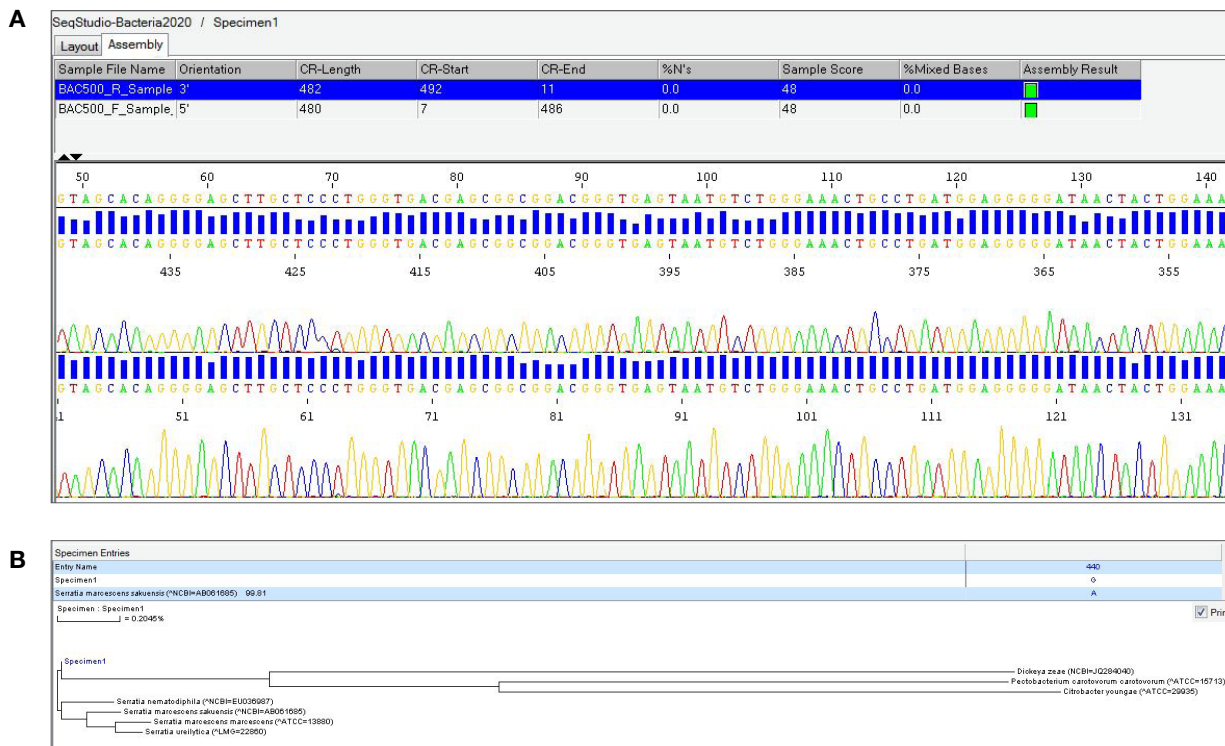


Figure 3. Example of (A) high-quality data and (B) data in report format.

## Conclusions

The SeqStudio Genetic Analyzer is the low-throughput option in the family of Applied Biosystems™ CE instruments featuring Sanger sequencing and fragment analysis capabilities.

Here we have demonstrated that the platform is compatible with the MicroSEQ Rapid Microbial Identification System and MicroSEQ database, which have long been trusted as the gold standard for microbial identification. The optimized workflow on the SeqStudio instrument helps reduce the time-to-results

by taking advantage of the shorter run cycle and off-instrument data analysis. This maximizes the throughput of samples within a given time frame, while leveraging the discriminatory power of the MicroSEQ libraries.

The SeqStudio Genetic Analyzer is an affordable, easy-to-use, low-maintenance instrument that can be an asset for any microbiology laboratory that needs to sequence individual samples or small batches in a fast, easy, and economical manner.

## References

1. Woese CR et al. (1990) Towards a natural system of organisms: proposal for the domains Archaea, Bacteria, and Eucarya. *Proc Natl Acad Sci USA* 87:4576–4579.
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