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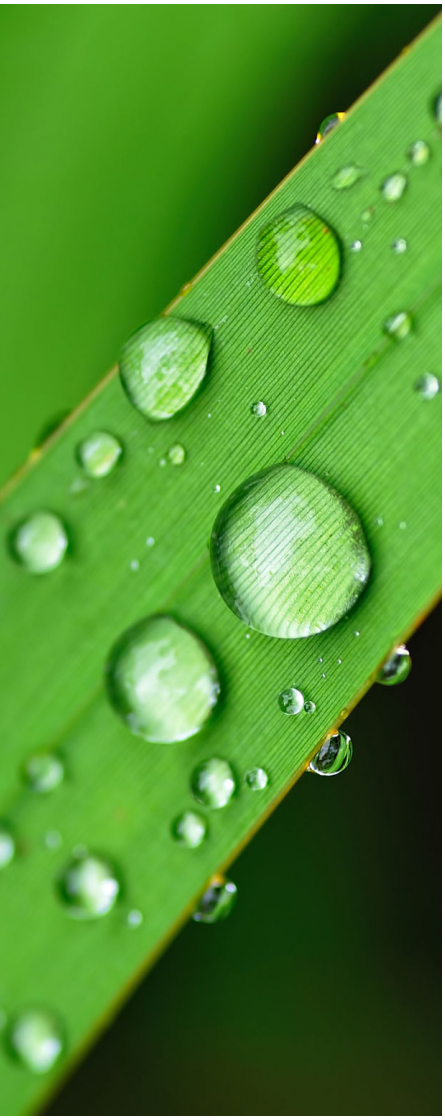
16S Sequencing Method Guide

Taxonomic profiling of bacterial communities and microbiomes through next-generation sequencing.



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Introduction

In microbiology, the 16S ribosomal RNA (16S rRNA) gene is a single genetic locus that can be used to assess the diversity of bacteria within a sample for phylogenetic and taxonomic studies. The 16S rRNA gene is approximately 1500 bp long and contains nine variable regions interspersed between conserved regions. The 16S locus acts like a 'barcode' for differentiating microbial taxa and can be used to classify bacteria taxonomically from within a heterogenous community to assess the diversity within a population and compare relative abundance across similar samples.

Using amplicon-based next-generation sequencing (NGS), researchers have been able to compile comprehensive databases for comparing sequences throughout an ecosystem, including complex environments such as the human gut microbiome.[1,2]

> Microbiome research is an emerging field that's expanding quickly. New discoveries are being made every day linking the microbiome and human disease, and how our diet and lifestyles might influence the microbiome. For these discoveries, you need advanced sequencing technology and bioinformatics tools to analyze the data. Thanks to technology developments like next-generation sequencing (NGS) systems such as the MiSeq™ System, we can now assess the diversity of microbes that live in and on our bodies faster and less expensively. That was not possible until a few years ago."
>
> **—Toni Gabaldón, PhD, Centre for Genomic Regulation (CRG) in Barcelona, Spain[3]**

Using amplicon-based NGS for 16S rRNA sequencing versus traditional methods

Amplicon sequencing is a highly targeted approach that enables researchers to analyze genetic variation in specific genomic regions. In this method, PCR is used to amplify a portion of the 16S gene from many bacteria simultaneously. The amplified regions are then interrogated through DNA sequencing. While capillary sequencing or PCR-based approaches can be used to study the 16S gene, amplicon-based NGS offers several advantages:

- Simultaneous analysis of hundreds of thousands of different 16S amplicons from a single sample
- Culture-free method to identify bacteria taxa that may not be detected using traditional methods
- Fast, high-throughput workflow enables researchers to more rapidly identify and compare bacterial populations within a microbiome or across diverse microbiome samples (Figure 1)
- Simple, one-button sequencing data analysis enables analysis of hundreds to thousands of samples in a single experiment with the 16S Metagenomics App in BaseSpace™ Sequence Hub
- Targeted approach is specific to Kingdom level and requires a minimum of sequencing data

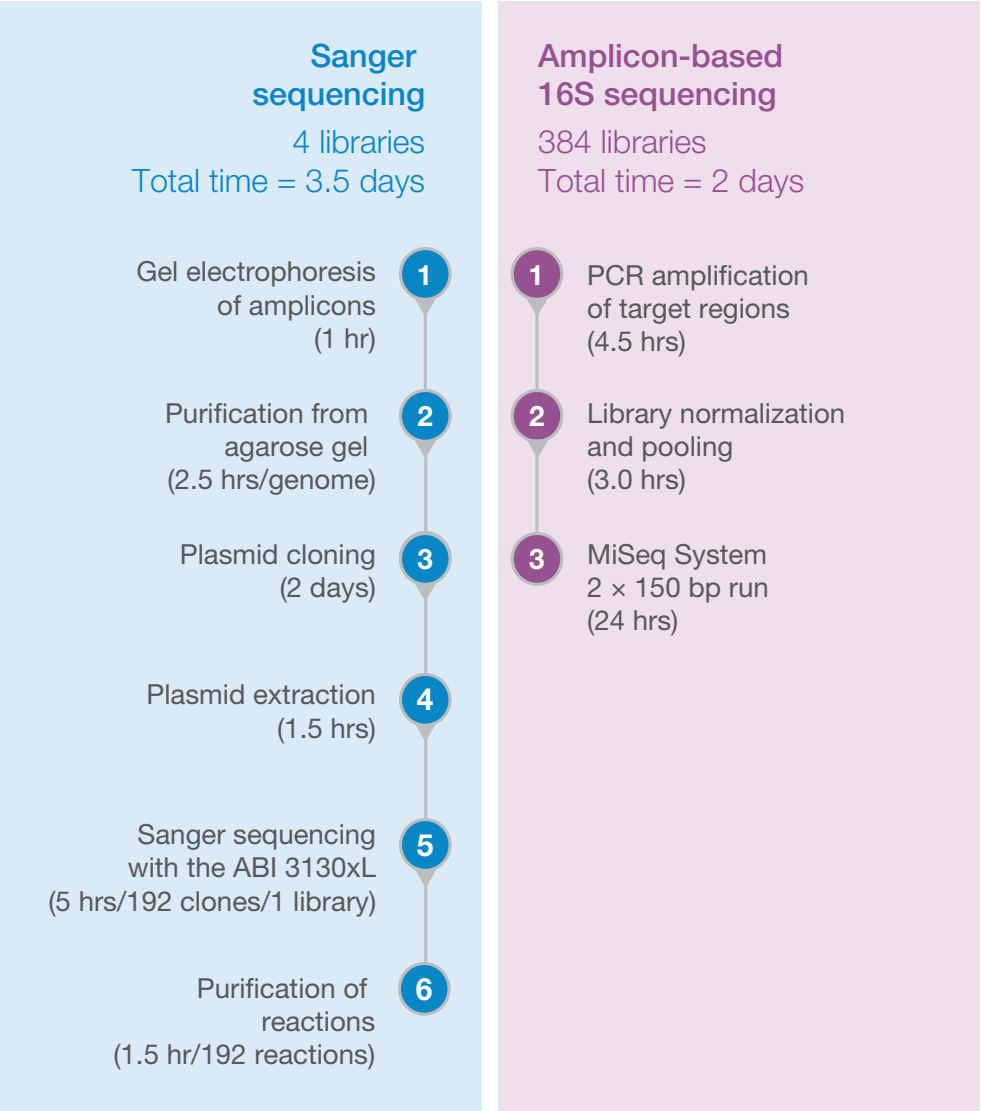


Figure 1: NGS-based 16S sequencing dramatically increases throughput and reduces turnaround time when analyzing complex microbial samples

Comparing 16S rRNA gene sequencing to shotgun metagenomics

Shotgun metagenomics is different from 16S sequencing. Shotgun metagenomics is the study of the functional genomes of microbial communities while 16S sequencing offers a taxonomic survey on the diversity of a single ribosomal gene, 16S rRNA.

Use 16S sequencing to identify bacteria and Archaea in complex samples and to estimate relative abundance within a sample. Drawing conclusions across different sample types is not recommended due to potential differences in total abundance.

Use shotgun metagenomics to analyze primary samples and compare relative abundance of all microbes, or genes, present in a sample or across sample types going beyond bacteria to include other microbes such as viruses. The limit of detection of this method is modulated by sequencing depth, read length, and data accuracy. This enables detection of microbes and genes for more comprehensive functional representations.

Accurate sequencing with streamlined analysis

Data accuracy is critical when using 16S rRNA sequencing to uncover the complexity of the bacterial population within a given sample. To deliver this high level of data accuracy, Illumina sequencing harnesses proven Illumina sequencing by synthesis (SBS) chemistry, which is used to generate more than 90% of the world's sequencing data.[4]

For high-powered data analysis in 16S rRNA studies, sequencing data can be seamlessly transferred, analyzed, and stored securely in BaseSpace Sequence Hub, the Illumina cloud-based genomic computing environment. BaseSpace Sequence Hub features a rich ecosystem of commercial and open-source apps for downstream data analysis. The 16S Metagenomics App performs taxonomic classification of 16S rRNA targeted amplicon reads using a version of the Greengenes taxonomic database curated by Illumina.

Applications for 16S rRNA sequencing

16S metagenomics powers human, animal, and environmental microbiome studies of any scale.

Human gut microbiome analysis

NIH Integrative Human Microbiome Project (HMP): The overall mission of the HMP is to generate resources to facilitate characterization of the human microbiota to further our understanding of how the microbiome impacts human health and disease. The HMP uses 16S rRNA and other sequencing methods to characterize the complexity of the human microbiome at specific body sites. Researchers can query and retrieve metagenomic, metatranscriptomic, human genetic, microbial culture, and many other data types generated by this project to use as a baseline for further research into the impacts of the microbiome on human health and disease.

American Gut Project: The human gut is estimated to contain 100 trillion microbes with over 500 unique species.[5] To examine how this microbiome impacts human health and how an individual's environment and diet impact this microbial population, researchers from the American Gut Project are using NGS to sequence 16S rRNA V4 gene fragments found in over 10K human microbiome samples collected from the United States, the United Kingdom, and Australia.[6]

Rumen microbiome analysis

The gastrointestinal tract, including the rumen and other intestinal segments of ruminants, harbors a diverse, complex, and dynamic "microbiome" that drives feed digestion and fermentation, determining feed efficiency and output of pollutants. This microbiome also plays an important role in host health. Researchers are studying rumen microbiomes to identify microbial genes that are key to better understanding methane emission and feed conversion.

Environmental metagenomics analysis

Earth Microbiome Project: The goal of the Earth Microbiome Project is to characterize the microbial populations found on Earth.[7] To accomplish this massive undertaking, the project leveraged the work of 500+ researchers from around the world. Standardized examination methods included use of 16S rRNA sequencing, allowing diversity to be analyzed and compared across multiple studies. The resulting data was used to compile a global reference database that provides a framework for future microbial diversity studies.[8]

Hospital-acquired infections (HAI) and surveying built environments

Hospital Microbiome Project: The Hospital Microbiome Project used 16S rRNA sequencing to study the microbial population found on the surfaces, staff, and patients, and in the air, of a hospital environment. The goal of this study is to understand the bacterial populations that develop and spread in the health care environment.[9]

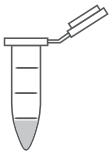
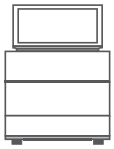
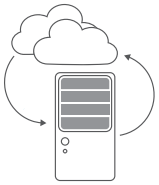
Antibiotic drug resistance

Drug resistance develops during treatment for certain viral infections, such as Tuberculosis, as the proportion of naturally occurring drug-resistant variants in the body increases. Public health experts track the development of bacterial drug resistance with targeted 16S sequencing[10].

How it works

After DNA extraction, 16S rRNA sequencing has three basic steps: library preparation, sequencing, and analysis. During library preparation, Illumina sequencing adapters and dual-index barcodes are added to the amplicon target. Prepared libraries are loaded onto a sequencing system and the resulting data are analyzed for taxonomic classification and relative abundance estimation.

Illumina solutions for 16S rRNA sequencing

Prepare library	Sequence	Analyze and report
		
16S Metagenomic Sequencing Protocol	**iSeq™ 100 System** **MiniSeq™ System** **MiSeq™ System**	**Available on Local Run Manager Software or BaseSpace Sequence Hub** 16S Metagenomics App QIIME Preprocessing and Visualization Apps EzBioCloud 16S-based MTP App CosmosID Metagenomics App
Illumina Informatics Solutions BaseSpace Sequence Hub Run management | Analysis | Reporting Clarity LIMS		

	Service contracts	Professional services
	Tiered service plans iSeq 100 System* MiniSeq System MiSeq System	**Workflow Design and Evaluation Service**

*The iSeq 100 System Advanced Exchange Service Plan includes quick, convenient replacement of instruments with issues that Technical Support cannot resolve.

Step 1 16S Sequencing Protocol (Demonstrated protocol)

Illumina does not offer library preparation kits specific for 16S rRNA sequencing. However, Illumina does provide a demonstrated protocol for preparing libraries for sequencing the variable V3 and V4 regions of the 16S rRNA gene or any other amplicon under 500 bp.[11]

Parameter	16S Sequencing Protocol[a]
Input DNA quantity	10-15 ng
Assay time	~7 hrs
Multiplexing capacity	384[b]
Genome coverage	V3 and V4 regions of the 16S rRNA gene; additional targets available depending on primer design
Sequencing method	Amplicon sequencing

a. Demonstrated protocol[10]
b. Multiplexing capacity is determined by number of indexes available and instrument capacity

Nextera™ XT DNA Index Kit

The 16S rRNA sequencing workflow begins with PCR amplification of the target regions in the 16S rRNA gene using a bacterial primer pair selected from the scientific literature.[12] Illumina sequencing adapters and dual-index barcodes are then added to the generated amplicons during PCR using the Nextera XT DNA Index Kit.

Learn more about 16S rRNA sequencing.

Step 2 Choose a sequencing system

System	iSeq 100 System	MiniSeq System	MiSeq System
Most important to me	Affordability	Onboard informatics, Capex affordability, speed	Speed, accuracy, and simplicity for far-reaching applications in microbiology
16S metagenomic samples per flow cell at 100K clusters per sample[a]	40	80 (Mid-output) 240 (High-output)	16 (Nano v2) 40 (Micro v2) 250 (Standard v2) 500 (Standard v3); limited to 384 available indexes
Recommended read length for targeted metagenomics	2 × 150 bp	2 × 150 bp	2 × 250 bp or 2 × 300 bp
Onboard informatics	●	●	●
Benchtop system	●	●	●
Max output/flow cell	1.2 Gb	2.4 Gb (Mid-output) 7.5 Gb (High-output)	0.5 Gb (Nano v2) 1.2 Gb (Micro v2) 7.5 Gb (Standard v2) 15 Gb (Standard v3)
Max clusters/flow cell[b]	4M	8M (Mid-output) 25M (High-output)	1M (Nano v2) 4M (Micro v2) 15 M (Standard v2) 25M (Standard v3)

a. Refer to demonstrated protocol for range of clusters per sample10
b. Clusters passing filter

iSeq™ 100 System

With the lowest price, smallest footprint, and fastest run time of any Illumina instrument, the iSeq 100 System offers an affordable option for researchers to expand the scope of their research. Sequence microbes, targeted genes, RNA transcripts, and more at the push of a button.

- Lowest system cost
- Suitable for small sample batches
- Fixed throughput and read length
- Suitable for amplicon sequencing and microbial whole-genome sequencing

Learn more at www.illumina.com/iSeq.

iSeq 100 System flow cell options and specifications	
Flow cell type	i1*
Output/run	144 Mb-1.2 Gb
Reads/run	4M
Max read length	2 × 150 bp
Cycles	300

*The i1 flow cell is included in the iSeq 100 Reagents; this is a ready-to-use cartridge prefilled with all the reagents needed for sequencing. Simply load the reagent cartridge directly onto the iSeq 100 System and start a run.

MiniSeq™ System

Applying industry-leading sequencing technology in a simple, small, affordable system, the MiniSeq System supports a broad range of targeted DNA and RNA applications for examining single genes or entire pathways. An intuitive user interface, load-and-go operation, and onboard data analysis make it easy to learn and easy to use.

- Affordable to acquire
- Suitable for moderate sample batches
- Range of throughput and read length
- Suitable for amplicon sequencing and microbial whole-genome sequencing

Learn more at www.illumina.com/miniseq

MiniSeq System flow cell options and specifications				
Flow cell type	Mid-output	High-output		
Output/run	2.1-2.4 Gb	1.7-1.9 Gb	3.3-3.8 Gb	6.6-7.5 Gb
Reads/run	8M	25M	25M	25M
Max read length	2 × 150 bp	1 × 75 bp	2 × 75 bp	2 × 150 bp
Cycles	300	75	150	300

MiSeq System

Combining speed, high-quality data, and the longest read lengths from Illumina, the MiSeq System is ideal for sequencing targeted panels, amplicons, and small genomes.

- Suitable for moderate sample batches
- Largest range of throughput and read lengths
- Suitable for amplicon sequencing and microbial whole-genome sequencing

Learn more at www.illumina.com/miseq

MiSeq System flow cell options and specifications								
Flow cell type	Standard v2			Micro v2	Nano v2		Standard v3	
Output/run	0.75-0.85 Gb	4.5-5.1 Gb	7.5-8.5 Gb	1.2 Gb	0.3 Gb	0.5 Gb	3.3-3.8 Gb	13.2-15 Gb
Reads/run	15M	15M	15M	4M	1M	1M	25M	25M
Max read length	2 × 25 bp	2 × 150 bp	2 × 250 bp	2 × 150 bp	2 × 150 bp	2 × 250 bp	2 × 75 bp	2 × 300 bp
Cycles	50	300	500	300	300	500	150	600

Step 3 Add biological context

16S Metagenomics App

- Enables analysis of 16S rRNA amplicon sequencing data
- Provides interactive visualization of taxonomic classification and relative abundance and raw classification output for per-sample and aggregate analyses
- Performs taxonomic classification of 16S rRNA targeted amplicon reads using a version of the Greengenes taxonomic database curated by Illumina
- Uses an algorithm that is a high-performance implementation of the Ribosomal Database Project (RDP) Classifier[13]

The 16S Metagenomics App is available on the MiSeq System as part of the MiSeq Reporter Software or through BaseSpace Sequence Hub.

16S Metagenomics Database Creator App

- Creates custom databases for the 16S Metagenomics App
- Supports FASTA and TSV input files

The 16S Metagenomics Database Creator App is a BaseSpace Labs App.*

*BaseSpace Labs Apps are developed using an accelerated development process to make them available to BaseSpace users faster than conventional Illumina Apps. Illumina may provide support for BaseSpace Labs Apps at its sole discretion. BaseSpace Labs Apps are provided AS IS without any warranty of any kind. BaseSpace Labs Apps are used at the user's sole risk. Illumina is not responsible for any loss of data, incorrect results, or any costs, liabilities, or damages that may result from the use of a BaseSpace Labs App.

For inquiries or feedback for this application, to provide a feature request, or to get support, email basespacelabs@illumina.com.

QIIME Preprocessing App

- Processes raw sequence data to generate an Operational Taxonomic Unit (OTU) table

QIIME Visualizations App

- Creates visual taxonomic summaries and alpha- and beta-diversity plots using the OTU table generated by the QIIME Preprocessing App

EzBioCloud 16S-based MTP App

- Performs comprehensive bioinformatics analysis on 16S rRNA amplicon reads using the expertly curated Chunlab reference database
- Provides bacterial taxonomic profiles at species level based on the high taxonomic accuracy and coverage of the database
- Generates taxonomic profiles with an accurate relative abundance by using optional normalization methods

The EzBioCloud 16S-based MTP App is provided by Chunlab.

CosmosID Metagenomics App

- Provides an easy-to-use, third-party application that performs microbial identification at the species, subspecies, or strain level
- Features a private, curated database

Learn more about BaseSpace Apps at www.illumina.com/basespaceapps

Ordering information

Library Prep

Product	No. indexes	No. samples	Catalog no.
Nextera XT Index Kit v2, Set A	96	384	FC-131-2001
Nextera XT Index Kit v2, Set B	96	384	FC-131-2002
Nextera XT Index Kit v2, Set C	96	384	FC-131-2003
Nextera XT Index Kit v2, Set D	96	384	FC-131-2004
PhiX Control Kit v3			FC-110-3001

a. Formerly sold as IDT for Illumina Nextera DNA UD Indexes Set A

Sequencing Systems

System	Catalog no.
iSeq 100 System	20021532
MiniSeq System	SY-420-1001
MiSeq System	SY-410-1003

Data analysis

Product	Catalog no.
BaseSpace Sequence Hub Professional Annual Subscription	20042109
BaseSpace Sequence Hub Enterprise Annual Subscription	15066411

Expand your study to include Fungi

Fungal Internal Transcribed Spacer (ITS) ribosomal RNA (rRNA) sequencing (Demonstrated Protocol)

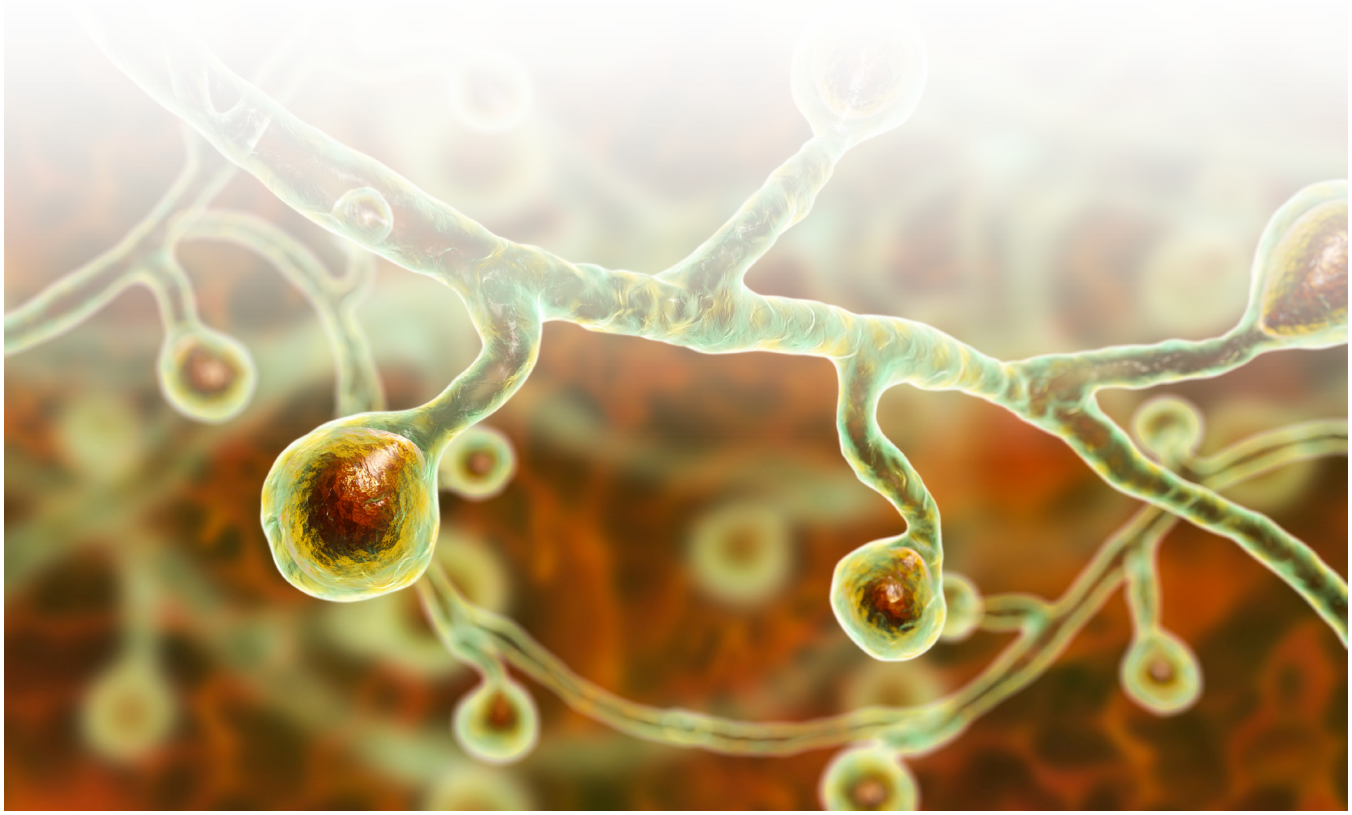
Fungal ITS amplicon sequencing is a common method used to identify and compare fungi present within a given sample. Like 16S rRNA gene sequencing, ITS is a well-established method for identifying fungi phylogeny and taxonomy from complex microbiome or environmental samples that are difficult or impossible to study using traditional methods. Illumina offers a demonstrated protocol for analyzing fungal or metagenomic samples, primer sequences, and a BaseSpace Sequence Hub analysis workflow.

View the ITS Amplicon Sequencing Demonstrated Protocol.

ITS Metagenomics App

- Performs taxonomic classification of fungal rRNA targeted amplicon reads using the UNITE taxonomic database
- Provides interactive visualizations and raw classification output for per-sample and aggregate analyses

The ITS Metagenomics App is a BaseSpace Labs App.*



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