ATCC® NGS Standards and Their Applications
How Standards Help Establish Reliable Workflows for Microbiome Analysis

Leka Papazisi, DVM, PhD
Principal Scientist, ATCC

Credible Leads to InCredible™
About ATCC®

- Founded in 1925, ATCC® is a non-profit organization with HQ in Manassas, VA, and an R&D and Services center in Gaithersburg, MD

- World’s premier biological materials resource and standards development organization
  - 5,000 cell lines
  - 80,000 microorganisms
  - Genomic & synthetic nucleic acids
  - Media/reagents

- ATCC® collaborates with and supports the scientific community with industry-standard biological products and innovative solutions

- Growing portfolio of products and services

- Sales and distribution in 150 countries, 19 international distributors

- Talented team of 450+ employees, over one-third with advanced degrees
The microbiome field is rapidly moving toward translational research pertinent to human health and disease, therapeutics, and personalized medicine.
Challenges in Microbiome Research and Applications

**Sample Collection**
- Storage
- Handling
- Processing

**DNA Extraction**
- Cell lysis
- DNA/RNA recovery
- Quality & quantity

**Library Preparation**
- Amplicon vs non-amplicon
- Choice of primers
- Library preparation

**Sequencing**
- Platform
- Chemistry
- Depth

**Data Analysis**
- Read quality
- Algorithm
- Database

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Development of ATCC NGS Standards

Strain selection criteria:
- Ability to lyse
- Taxonomy
- GC content
- Genome size
- Spore formation
- Aerobic/anaerobic
- Diagnostic relevance
- Assembled genomes
- ITS variability
- Genome complexity
- 16S rRNA copy number
- Microbiome site

Whole Cell Standards:
- Authenticated ATCC cultures
- Growth and image cytometry cell counting
- Mixed in even proportion-based cell numbers cells
- Storage at 4°C

Genomic DNA Standards:
- Authenticated ATCC nucleic acids
- Fluorescent dye-based quantification
- Mixed in even proportions-based genome copy number
- Storage at -20°C

Assay development, optimization, verification, and quality control
# ATCC® NGS Standards Portfolio

<table>
<thead>
<tr>
<th>Preparation</th>
<th>ATCC® Catalog No.</th>
<th>Number of Organisms</th>
<th>Composition</th>
<th>Complexity</th>
<th>Utility</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genomic DNA</td>
<td>MSA-1000™</td>
<td>10</td>
<td>Even</td>
<td>Medium</td>
<td>Standards for assay development and optimization</td>
</tr>
<tr>
<td></td>
<td>MSA-1001™</td>
<td>10</td>
<td>Staggered</td>
<td>Medium</td>
<td></td>
</tr>
<tr>
<td></td>
<td>MSA-1002™</td>
<td>20</td>
<td>Even</td>
<td>High</td>
<td></td>
</tr>
<tr>
<td></td>
<td>MSA-1003™</td>
<td>20</td>
<td>Staggered</td>
<td>High</td>
<td></td>
</tr>
<tr>
<td>Whole cell</td>
<td>MSA-2003™</td>
<td>10</td>
<td>Even</td>
<td>Medium</td>
<td></td>
</tr>
<tr>
<td></td>
<td>MSA-2002™</td>
<td>20</td>
<td>Even</td>
<td>High</td>
<td></td>
</tr>
<tr>
<td>Genomic DNA</td>
<td>MSA-4000™</td>
<td>11</td>
<td>Staggered</td>
<td>Medium</td>
<td>NGS-based pathogen detection</td>
</tr>
<tr>
<td>Genomic DNA</td>
<td>MSA-3000™</td>
<td>6</td>
<td>Even</td>
<td>Low</td>
<td>Environmental studies</td>
</tr>
<tr>
<td></td>
<td>MSA-3001™</td>
<td>10</td>
<td>Even</td>
<td>Medium</td>
<td></td>
</tr>
<tr>
<td></td>
<td>MSA-3002™</td>
<td>10</td>
<td>Staggered</td>
<td>Medium</td>
<td></td>
</tr>
</tbody>
</table>
## ATCC® Site-specific NGS Standards

<table>
<thead>
<tr>
<th>Standard</th>
<th>Preparation</th>
<th>ATCC® Catalog No.</th>
<th>Number of Organisms</th>
<th>Importance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oral</td>
<td>Whole cell</td>
<td>MSA-2004™</td>
<td>6</td>
<td>Mock microbial communities representing the oral, skin, gut, and vaginal microbiomes</td>
</tr>
<tr>
<td>Oral</td>
<td>Genomic DNA</td>
<td>MSA-1004™</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Skin</td>
<td>Whole cell</td>
<td>MSA-2005™</td>
<td>6</td>
<td>Comprises normal and atypical flora</td>
</tr>
<tr>
<td>Skin</td>
<td>Genomic DNA</td>
<td>MSA-1005™</td>
<td></td>
<td>Anaerobic and aerobic microbial strains</td>
</tr>
<tr>
<td>Gut</td>
<td>Whole cell</td>
<td>MSA-2006™</td>
<td>12</td>
<td>A combination of Gram-positive and Gram-negative bacterial cultures</td>
</tr>
<tr>
<td>Gut</td>
<td>Genomic DNA</td>
<td>MSA-1006™</td>
<td></td>
<td>Even composition</td>
</tr>
<tr>
<td>Vaginal</td>
<td>Whole cell</td>
<td>MSA-2007™</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>Vaginal</td>
<td>Genomic DNA</td>
<td>MSA-1007™</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
**ATCC® Virome Standards**

<table>
<thead>
<tr>
<th>Composition of Virome Standards</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human herpesvirus 5 strain AD169 (ATCC® VR-538™)</td>
</tr>
<tr>
<td>Human mastadenovirus strain F (ATCC® VR-931™)</td>
</tr>
<tr>
<td>Influenza B virus strain B/Florida/4/2006 (ATCC® VR-1804™)</td>
</tr>
<tr>
<td>Zika virus strain MR 766 (ATCC® VR-1838™)</td>
</tr>
<tr>
<td>Reovirus 3 strain Dearing (ATCC® VR-824™)</td>
</tr>
<tr>
<td>Human respiratory syncytial virus strain A2 (ATCC® VR-1540™)</td>
</tr>
</tbody>
</table>

**Table: Standard Preparation**

<table>
<thead>
<tr>
<th>Standard</th>
<th>Preparation</th>
<th>ATCC® Catalog No.</th>
<th>Number of Organisms</th>
<th>Specification (ddPCR™)</th>
<th>Applications</th>
</tr>
</thead>
<tbody>
<tr>
<td>Virome</td>
<td>Virus Mix</td>
<td>MSA-2008™</td>
<td>6</td>
<td>2 x 10^3 genome copies/µL per virus</td>
<td>Standards for virome assay development, optimization, verification, and validation; evaluating reproducibility; and use as a daily run quality control</td>
</tr>
<tr>
<td></td>
<td>Nucleic Acid Mix</td>
<td>MSA-1008™</td>
<td>6</td>
<td>2 x 10^4 genome copies/µL per virus</td>
<td></td>
</tr>
</tbody>
</table>

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## ATCC® Spike-in and Mycobioime Standards

<table>
<thead>
<tr>
<th>Standard</th>
<th>Preparation</th>
<th>ATCC® Catalog No.</th>
<th>Number of Organisms</th>
<th>Application</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spike-in</td>
<td>Whole cell</td>
<td>MSA-2014™</td>
<td>3</td>
<td>▪ Microbiome measurements and data normalization</td>
</tr>
<tr>
<td></td>
<td>Genomic</td>
<td>MSA-1014™</td>
<td></td>
<td>▪ 16S rRNA and shotgun assay verification, validation, and quality control</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Standard</th>
<th>Preparation</th>
<th>ATCC® Catalog No.</th>
<th>Number of Organisms</th>
<th>Application</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mycobioime</td>
<td>Whole cell</td>
<td>MSA-2010™</td>
<td>10</td>
<td>▪ Fungal mock community standards for assay development, optimization, verification, validation; and use as a daily run quality control</td>
</tr>
<tr>
<td></td>
<td>Genomic</td>
<td>MSA-1010™</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
ATCC® Genome Portal

A cloud-based platform that enables users to easily browse authenticated and traceable reference genomes and metadata.

- Download whole-genome sequences and annotations of ATCC materials
- Search for nucleotide sequences or genes within genomes
- View genome assembly metadata and quality metrics

genomes.atcc.org

3,238 Authenticated Microbial Reference Genomes

- 2,778 bacteria
- 250 viruses
- 206 fungi
- 4 protists

New genomes released every month!

REST-API for bioinformatics applications available

Free for non-commercial research use (RUO) purposes. Commercial use licenses available. Registration required.


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Utility and Application of NGS Standards

- Evaluating DNA extraction methods and kits
- Evaluating 16S rRNA and WGS library kits
- Evaluating NGS platforms
Microbiome Workflow, Biases, and Standardization

Sample Acquisition
- Storage
- Handling
- Processing

DNA Extraction
- Cell lysis
- DNA/RNA recovery
- Quality & quantity

Library Preparation
- Primers
- Amplification
- Library prep

Sequencing
- Platform
- Chemistry
- Depth

Data Analysis
- Algorithm
- Read quality
- Database

Whole cell standards – Full process controls, including DNA Extraction

Genomic DNA standards – Library preparation, sequencing

One Codex analysis

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Evaluating DNA Extraction Methods and Kits
Genomic Versus Whole Cell Standards

DNA extraction methods are not perfect

Shotgun metagenomic analysis of the Oral Microbiome Genomic Mix

DNA extraction from the Oral Microbiome Whole Cell Mix with two different kits followed by shotgun metagenomic analysis
Evaluating 16S rRNA and WGS Library Kits
16S Amplicon-based Analysis: Primer Selection

Compare different primer sets, optimize amplification steps, and validate 16S analysis methods

16S rRNA analysis of the Oral Genomic DNA Standard via two primer sets

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Comparing Library Preparation Kits

Nextera Flex enables uniform coverage of genomes of low GC content

Oral Microbiome Genomic DNA (ATCC® MSA-1004™)

**Nextera™ XT Workflow**
- Free enzyme Tagmentation
- PCR Amplification
- Normalization
- Sequencing
- Analysis

**Nextera™ Flex Workflow**
- Bead-Linked Tagmentation
- PCR Amplification
- Sequencing
- Analysis

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**Sample Composition**

**Nextera™ XT Workflow**
- Actinomyces odontolyticus: 46.07%
- Prevotella melaninogenica: 16.09%
- Streptococcus mitis: 16.04%
- Veillonella parvula: 12.65%
- Haemophilus parainfluenzae: 7.89%
- Fusobacterium nucleatum: 1.34%

**Nextera™ Flex Workflow**
- Streptococcus mitis: 18.83%
- Veillonella parvula: 18.42%
- Fusobacterium nucleatum: 17.13%
- Haemophilus parainfluenzae: 15.68%
- Actinomyces odontolyticus: 15.54%
- Prevotella melaninogenica: 14.40%

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The Gut Whole Cell Microbiome Standard (ATCC® MSA-2006™) can be used as a full process control for shotgun and 16S rRNA assays.
Evaluating NGS Platforms
Short-read Sequencing Platform: Illumina®

Assay reproducibility through different Illumina sequencing platforms

Shotgun Metagenomic Data (ATCC® MSA-3001™)

Expected

MiniSeq™

MiSeq®

NextSeq®

HiSeq®

Relative Abundance (%)

0%

10%

20%

30%

40%

50%

60%

70%

80%

90%

100%

Sequencing Platform

Enterococcus faecalis

Staphylococcus epidermidis

Micrococcus luteus

Halofex volcanii

Halobacillus halophilus

Bacillus subtilis

Pseudoalteromonas haloplanktis

Escherichia coli

Chromobacterium violaceum

Pseudomonas fluorescens

Data courtesy of Dr. Stefan Green, UIC (ABRF-MGRG)
Long-read Sequencing Platform: PACBIO®

16S rRNA (full-length) and shotgun data on the PacBio Sequel Platform using ATCC® MSA-1003™

Data courtesy of Dr. Joan Wong, PACBIO®
Shotgun Metagenomic Analysis: Short vs Long Reads

ATCC® NGS Standards are technology agnostic

ATCC® NGS Standards are technology agnostic

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Fungal Mock Community Standards for Mycobiome Studies
# Mycobiome Composition: Fungal Mock Community

## Fungi strains selection attributes and clinical relevance

<table>
<thead>
<tr>
<th>ATCC® No.</th>
<th>Species Name</th>
<th>Genome Size (Mb)</th>
<th>Relevancy</th>
</tr>
</thead>
<tbody>
<tr>
<td>MYA-4609™</td>
<td><em>Aspergillus fumigatus</em></td>
<td>28.8</td>
<td>Opportunistic, airborne pathogen that is responsible for fungal infections in immunocompromised patients.</td>
</tr>
<tr>
<td>10231™</td>
<td><em>Candida albicans</em></td>
<td>17.1</td>
<td>Commensal fungus of the oral cavity that can form biofilms on denture surfaces, leading to mucosal infections.</td>
</tr>
<tr>
<td>2001™</td>
<td><em>Candida glabrata</em></td>
<td>12.3</td>
<td>Commensal fungus of the oral cavity and human gut that can acquire resistance toazole antifungals, leading to infection.</td>
</tr>
<tr>
<td>208821™</td>
<td><em>Cryptococcus neoformans var. grubii</em></td>
<td>18.9</td>
<td>Responsible for cryptococcal meningitis in immunosuppressed patients.</td>
</tr>
<tr>
<td>MYA-4612™</td>
<td><em>Malassezia globosa</em></td>
<td>9.0</td>
<td>Part of the normal skin flora but can be responsible for skin diseases such as dandruff, dermatitis, and folliculitis.</td>
</tr>
<tr>
<td>201390™</td>
<td><em>Saccharomyces cerevisiae</em></td>
<td>12.2</td>
<td>Bakers’ and brewers’ yeast originating in food. Emerging pathogen in immunocompromised patients.</td>
</tr>
<tr>
<td>9533™</td>
<td><em>Trichophyton interdigitale</em></td>
<td>21.9</td>
<td>Can infect skin and nails to cause chronic infections such as athlete’s foot and ringworm.</td>
</tr>
<tr>
<td>204094™</td>
<td><em>Cutaneotrichosporon dermatis (T. dermatis)</em></td>
<td>23.3</td>
<td>Emerging opportunistic agent of invasive fungal infections, particularly in severely immunocompromised patients.</td>
</tr>
<tr>
<td>10106™</td>
<td><em>Penicillium chrysogenum</em></td>
<td>32.5</td>
<td>Spore-former, less prevalent, but can be responsible for intestinal infection in immunosuppressed patients.</td>
</tr>
<tr>
<td>36031™</td>
<td><em>Fusarium keratoplasticum</em> (<em>F. solani</em> complex)</td>
<td>48.6</td>
<td>Filamentous, opportunistic pathogen that causes fungal keratitis.</td>
</tr>
</tbody>
</table>
## ATCC® Mycobiome NGS Standards

**Product description, research use, and applications**

<table>
<thead>
<tr>
<th>Standard</th>
<th>ATCC® Catalog No.</th>
<th>Preparation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mycobiome</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MSA-2010™</td>
<td></td>
<td>Whole cell</td>
<td>Even mixture of whole cells comprising 10 fungal strains (2 x 10^6 cells of each organism)</td>
</tr>
<tr>
<td>MSA-1010™</td>
<td></td>
<td>Genomic DNA</td>
<td>Even mixture of genomic DNA comprising 10 fungal strains (2 x 10^6 genome copies of each organism)</td>
</tr>
</tbody>
</table>

**DNA Extraction**
- Storage
- Cell lysis
- DNA/RNA recovery
- Quality & quantity

**Sample Collection**
- Sequencing
  - ITS and Shotgun
    - Choice method
    - Choice of primers
    - Library preparation
    - Platform
- Data Analysis
  - Bioinformatics
    - NGS quality
    - Depth and coverage
    - Pipelines & Algorithm
    - Database

**MYCOBIOME WORKFLOW**

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Mycobiome Standards: Evaluation of DNA Extraction kits

Challenges with the development of whole cell standards and fungal DNA extraction methods

 Shotgun Metagenomic Analysis of Mycobiome Standards (ATCC® MSA-2010™ and MSA-1010™)

- Fusarium keratoplasticum
- Penicillium chrysogenum
- Trichosporon dermatis
- Trichophyton interdigitale
- Saccharomyces cerevisiae
- Malassezia globosa
- Cryptococcus neoformans
- Candida glabrata
- Candida albicans
- Aspergillus fumigatus

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Conclusions about the ATCC Mycobiome Standards

- Whole cell standards can help identify biases introduced during DNA extraction and can be used as full-process controls.

- Genomic DNA standards can be used for comparing various library preparation methods and sequencing platforms.

- The data analysis for mycobiome profiling is challenging due to the lack of complete fungal reference genomes and the limited availability of analyses pipelines.
Spike-in Internal Controls: Synthetic 16S Tagged Strains
The Output of a Metagenomic Data is a Relative Abundance

Relative abundance does not reflect the quantity of the microbial community and the inter-sample differences among taxa.
Engineering Synthetic 16S Tag Into Bacterial Genome

<table>
<thead>
<tr>
<th>Bacterial Strains</th>
<th>Gram Stain</th>
<th>Genome G/C (%)</th>
<th>Insertion loci</th>
<th>BSL</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Escherichia coli</em> Tag1</td>
<td>Negative</td>
<td>50.8</td>
<td>Beta galactosidase</td>
<td>1</td>
</tr>
<tr>
<td><em>Clostridium perfringens</em> Tag2</td>
<td>Positive</td>
<td>29.0</td>
<td>Theta-toxin</td>
<td>2</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em> Tag3</td>
<td>Positive</td>
<td>32.8</td>
<td>O-antigen polymerase</td>
<td>2</td>
</tr>
</tbody>
</table>

**16S copy**

- *E. coli* Tag1 (7+1)
- *C. perfringens* Tag2 (10+1)
- *S. aureus* Tag3 (6+1)
### Spike-in Standards (3 Strain Tagged Mix)

<table>
<thead>
<tr>
<th>ATCC® Catalog No.</th>
<th>Preparation</th>
<th>Specification</th>
<th>Importance</th>
</tr>
</thead>
<tbody>
<tr>
<td>MSA-1014™</td>
<td>Genomic DNA</td>
<td>6 x 10^7 genomes copies/vial ± 1 log</td>
<td>- Microbiome measurements and data normalization</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>- 16S rRNA and shotgun assay verification, validation, and quality control</td>
</tr>
<tr>
<td>MSA-2014™</td>
<td>Whole cells</td>
<td>6 x 10^7 cells/vial ± 1 log</td>
<td></td>
</tr>
</tbody>
</table>

#### Spike-in Composition

<table>
<thead>
<tr>
<th>Species</th>
<th>Gram Stain</th>
<th>Genome size (Mb)</th>
<th>Tag size (bp)</th>
<th>G/C Content (%)</th>
<th>16S Copies</th>
<th>Tag copies</th>
<th>Cells per vial</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Escherichia coli Tag1</em></td>
<td>Negative</td>
<td>4.59</td>
<td>829</td>
<td>50.8</td>
<td>7</td>
<td>1</td>
<td>2 x 10^7</td>
</tr>
<tr>
<td><em>Clostridium perfringens Tag2</em></td>
<td>Positive</td>
<td>3.25</td>
<td>799</td>
<td>29.0</td>
<td>10</td>
<td>1</td>
<td>2 x 10^7</td>
</tr>
<tr>
<td><em>Staphylococcus aureus Tag3</em></td>
<td>Positive</td>
<td>2.70</td>
<td>833</td>
<td>32.8</td>
<td>6</td>
<td>1</td>
<td>2 x 10^7</td>
</tr>
</tbody>
</table>
Development of Spike-in Standards and Quality Control

Relative abundance of the genomic DNA and whole cell spike-in standards
Relative Abundance of a Mock Community with Spike-in

The spike-in doesn’t have obvious impact on 16S relative abundance

ATCC® MSA-1014™ was mixed with MSA-1000™ at ~1:10 and ~1:100
Relative Abundance of a Mock Community with Spike-in

The spike-in doesn’t have an obvious impact on whole genome shotgun analysis

ATCC® MSA-1014™ was mixed with MSA-1000™ at ~ 1:10 and ~1:100
Comparison of Absolute Quantitation by ddPCR and Normalized WGS

Three tagged genomic DNA mixed with 10 even genomic DNA (ATCC® MSA-1000™)
Mock Microbial Communities
- Genomic DNA and whole cell standards
- Even and staggered mixtures comprising 10 or 20 strains
- Environmental and pathogen mixtures

Site-specific Standards
- Genomic DNA and whole cell standards
- Even mixtures of 6-12 strains
- Bacterial strains prevalent in the oral, skin, gut, and vaginal microbiome

Spike-In Standards
- Recombinant strains with a unique DNA tag stably integrated into the chromosome
- Recombinant standards include the Gram-negative and Gram-positive bacteria

Virome and Mycobiome Standards
- Genomic DNA and whole cell standards comprising diverse and clinically relevant strains
- Even mixtures of 6-10 strains

Bundled with data analysis on the One Codex platform
Microbiome Research

Optimize your research with the right controls

The complexities involved in 16S rRNA community profiling and shotgun metagenomics methods pose significant challenges for microbiome research. Significant biases can be introduced at each stage of the microbiome workflow, affecting data interpretation and reproducibility.

**NGS Standards** provide a solution to this problem. From sample collection to data analysis, NGS Standards enable you to optimize your diverse research applications with confidence and improve the consistency and reproducibility of your data, run after run.

The robust applicability of these controls, combined with the ATCC commitment to authentication and characterization, make NGS Standards ideal tools for standardizing data from a wide range of sources and generating consensus among microbiome applications and analyses.
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- Scott Tighe, UVM
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- Rohan Patil, Microbiome Analysis Center, GMU
- Joan Wong, PACBIO®
- Tash Rodrigues, Diversigen
- Emily Hollister, Diversigen

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Questions?

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