THE IMPORTANCE OF STANDARDS IN MOLECULAR-BASED ASSAYS

Liz Kerrigan
Director, Standards
May 8, 2014
ATCC

- Founded in 1925, ATCC is a non-profit organization with headquarters in Manassas, VA
- ATCC serves and supports the scientific community with industry-standard products and innovative solutions
- World’s leading biological resource center and provider of biological standards
- Broad range of biological materials
  - Cell lines
  - Microorganisms
  - Native & synthetic nucleic acids
  - Reagents
Overview

Principles of assay development

Assay design
- Assay sensitivity
- Analytical specificity & microbial interference
- Controls for nucleic acid based assays

Microbial standards & tools for assay development

Challenging assay design with cancer cell lines & nucleic acids

Using Biological Standards in Proficiency Testing programs

Standards for Next-Gen sequencing
Utility for molecular testing

- Improved sensitivity
- Improved specificity
- Time to results
- Ability to multiplex
Assay development

• Assay design
  – Design goals
  – Qualitative or quantitative assay
  – Target genes
  – Relevant strains and variants
  – Updates
Analytical sensitivity, specificity & interference

- Analytical sensitivity
  - Limit of Detection (LoD)
  - Reactivity

- Analytical specificity
  - Cross reactivity

- Interference
  - Clinical specimens
Controls for nucleic acid based assays

- **Used for verification of assay performance in your analytical and clinical studies:**
  - **Negative Control:** to rule out contamination
  - **Positive Control:** to verify PCR reagents & instrument are running properly
  - **Internal Control:** to verify that a sample result is not caused by PCR inhibitors
  - **External Control:** to verify that lysis & extraction processes are functioning properly
The changing landscape of microbial genomics

**Large scale sequencing programs:**

- The Human Genome Project
- Human Microbiome Project
- JCVI Genomic Sequencing Center for Infectious Diseases (GSCID)
- MetaHIT Project
Highly characterized microbial strains for assay development

ATCC utilizes both classical and modern techniques

- Phenotypic analysis
- Genotypic analysis
- Functional analysis

No single method of identification is sufficient

**Phenotypic testing**

- Colony Morphology
- Cell Attributes
- Biochemical Analysis
- Culture Purity and Biochemical Properties

**Genotypic testing**

- Sequencing
- Toxinotyping
- Ribotyping
- Targeted Gene Sequencing
40 *Clostridium difficile* strains

<table>
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<tr>
<th>ATCC® No.</th>
<th>Strain</th>
<th>Toxinotype</th>
<th>Binary Toxin</th>
<th>EIA*</th>
<th>Ribotype</th>
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*Enzyme Immuno Assay (EIA) performed with the Wampole™ C. DIFF QUIK CHEK COMPLETE® kit or the equivalent ND= Not Detected

Native DNA is available from six different strains

Photo credit: Annie Cavanagh, David Goulding, Wellcome Trust Sanger Institute
Quantitated, frozen, *Clostridium difficile* strains

Tools for Assay Development

<table>
<thead>
<tr>
<th>ATCC® No.</th>
<th>Strain</th>
<th>Toxinotype</th>
<th>Ribotype</th>
<th>Titer (CFU/vial)</th>
<th>ddPCR™ (Bacteria/vial)</th>
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*Post preservation material is checked for total bacterial cell count (per vial) using ddPCR™*
Detecting mycoplasma

- Microbiological culture method
- DNA staining by fluorochromes (Hoechst, DAPI)
- Polymerase chain reaction
Tools for mycoplasma assay development

**Titered Mycoplasma Reference Strains Panel (ATCC® MP-7™):**

- A panel of 10 titered mycoplasma reference strains with calculated genome copy numbers
- Highly viable and dispersed
- Useful for assay development
- Useful for comparing culture- and molecular-based detection assays*

PubMed: 23910092
# Titered Mycoplasma Reference Strains Panel (ATCC® MP-7™)

<table>
<thead>
<tr>
<th>ATCC® No.</th>
<th>Species</th>
<th>Post Preservation Titer</th>
<th>GenBank (Strain Specific)</th>
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<td>15531-TTR™</td>
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<td>$1.0 \times 10^8$ cfu/mL</td>
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<td>17981-TTR™</td>
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</table>

Each strain is prepared with a low genome copy (GC) to colony forming unit (CFU) ratio, which is ideal for use in the development and validation of PCR-based methods of detection.
CRM quantitated mycoplasma genomic DNA

**Key Features:**
- Certified Reference Material
- Quantitated – Genome copy number is based on the quantification of the 16S rRNA gene from nine averaged samples using ddPCR™. (1.0x10^6-1.0x10^7 genome copies/µL)
- Extracted from the titered mycoplasma reference strains (MP-7™)

**Use as a quantitative external control for:**
- Inclusivity/exclusivity testing
- Establishing limits of detection (LoD)
- Verification or comparison of test methods
- Other molecular applications

Droplet Digital™ PCR is a trademark of Bio-Rad Laboratories, Inc.
CRM quantitated mycoplasma genomic DNA

Certified Reference Materials
- Produced under an ISO Guide 34 accredited process
- Established chain of custody

Extracted from ATCC Genuine Cultures®
- Agarose gel electrophoresis to ensure integrity
- Spectrophotometry to evaluate purity
- QX100 Droplet Digital™ PCR to calculate quantity
- PCR to confirm functional activity
- Sequence analysis of 16S rRNA to confirm species identity

Droplet Digital™ PCR is a trademark of Bio-Rad Laboratories, Inc.
# CRM quantitated mycoplasma genomic DNA

<table>
<thead>
<tr>
<th>ATCC® No.</th>
<th>Species</th>
<th>Genome Size (bp)</th>
<th>16S RNA Copy Number</th>
<th>GenBank</th>
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ATCC® Genuine Nucleics

Electrophoresis  Integrity
Spectrophotometry  Purity
PicoGreen® and RiboGreen®  Concentration
PCR  Function
Sequencing  Identity

PicoGreen® and RiboGreen® are registered trademarks of Molecular Probes, Inc.
ATCC native nucleic acids

Whole genome preparations extracted from ATCC Genuine Cultures®

- More than 750 catalog items
- Agarose gel electrophoresis to ensure integrity
- Spectrophotometry to evaluate purity
- PicoGreen® or RiboGreen® to calculate concentration
- PCR to confirm functional activity
- Sequence analysis of conserved genomic regions to confirm species identity
- Custom preparations available
- DNA from bacteria, fungi, protists, and viral strains
- RNA from viral strains

PicoGreen® and RiboGreen® are registered trademarks of Molecular Probes, Inc.
ATCC synthetic nucleic acids

- Synthetically created nucleic acids to serve as a genetic surrogate for microorganisms that are:
  - Difficult to culture
  - Can not be cultured *in vitro*
  - Select agents and/or BSL 3.
Synthetic molecular standards for Dengue virus

DENV Genome

Multiple sequence alignment

Artificial RNA synthesis

Stabilization (RNAstable®)

Sequence Verification (Next generation sequencing)

Absolute Quantification (Droplet Digital™PCR)

<table>
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<tr>
<th>ATCC Synthetic Molecular Standards</th>
<th>Advantages</th>
<th>Applications</th>
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<td>DENV-1   ATCC® VR-3228SD™</td>
<td>• ATCC fully authenticated &amp; characterized Genuine Nucleics</td>
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<td>DENV-2   ATCC® VR-3229SD™</td>
<td>• Consistent and accurate results</td>
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<td>DENV-3   ATCC® VR-3230SD™</td>
<td>• BSL-1 ready-to-use control</td>
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<tr>
<td>DENV-4   ATCC® VR-3231SD™</td>
<td>• No shipping restrictions</td>
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<td>• Quantitative format</td>
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<td>• Stabilized RNA</td>
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Bio-Rad®, CFX96™, Droplet Digital™ PCR, Real-Time PCR Detection System, and CFX Manager™ 3.0 Software are registered trademarks or trademarks of Bio-Rad Laboratories, Inc. RNAstable® is registered trademark of Biomātrica, Inc.
The changing landscape of the cancer genome

Large scale sequencing programs:

- The Cancer Genome Atlas (TCGA)
- International Cancer Genome Consortium (ICGC)
- Catalogue of Somatic Mutations in Cancer (COSMIC)
NGS leads to genomic age

Next generation sequencing

Cost per Genome

Cancer genome display

National Human Genome Research Institute
genome.gov/sequencingcosts

Moore’s Law

Circos cancer genome display
Challenges in molecular testing

- Omics data storage
- Data analysis
- Quality standards for each step from sample to answer
- Appropriate and reliable controls

Although over 1900 genetic tests are available, the majority of tests still need characterized references or QC materials.
Using authenticated cell lines as controls

- Fully authenticated
- COI and STR testing to avoid inter-species and intra-species contamination or misidentification
- Characterized tumor genetic alterations
- Stable molecular profiles
- Control FFPE process
- Control IF or IHC staining process
ATCC Molecular Signature Cell Panels

- Systematic analysis
- Gene sequencing validation
- Functional characterization
- Integrative design

Molecular Signature Cell Panels
- Driver gene focused
- Signaling pathway focused
- Integrative design

PANELS BY MOLECULAR SIGNATURE

Each panel in the molecular signature collection is composed of cell lines that have been sequenced and validated for mutations in specific genes, such as p53. These panels harness the combined forces of genomic data and highly reliable, authenticated ATCC tumor cell lines to provide solid experimental platforms for cancer research and drug discovery.

p53 hotspot mutation panels

Genetic Alteration Cell Panels

Genetic Alteration Panels
Molecular Signature Cell Panels

Growth factor receptors
- EGFR&ERBB2
- FGFR1&2
- HGFR/MET

Transcription factors
- PI3K-AKT pathway
- MAPKs pathway
- Site mutation
- Copy number change
- Gene expression
- Protein expression
- Signaling pathway

- PI3K
- AKT
- PTEN
- RAS
- BRAF
- ERK
- MYC
- p53
# EGFR cell panel characteristics

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<th>cDNA Change</th>
<th>Zygosity</th>
<th>Amino acid Change</th>
<th>EGFR copy number variation</th>
<th>ERBB2 copy number variation</th>
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<td>breast</td>
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**ATCC Certified Reference Material (CRM)**

**Reference materials are:**
- Stable with respect to one or more specified property.
- Possess a stated level of confidence for Traceability and Values of Uncertainty, where applicable.

**The intended uses of ATCC Certified Reference Materials are:**
- To challenge assay performance.
- Validate or compare test methods.
- Establish sensitivity, linearity, and specificity during assay validation or implementation.
- To benchmark critical assay performance during development/validation for regulatory submissions and production lot release.
- For use in testing and calibration in ISO 17025 accredited laboratories.

Certified Reference Materials from ATCC are accompanied by a Certificate of Analysis.
**KRAS mutation CRM cell lines and DNAs**

 KRAS mutation analysis is currently used as a predictive marker of therapeutic response

<table>
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<tr>
<th>ATCC® No.</th>
<th>Cell line name</th>
<th>AA change</th>
<th>DNA change</th>
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<td>WT</td>
<td>WT</td>
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<td>p.G12A</td>
<td>c.35G&gt;C</td>
</tr>
<tr>
<td>CRM-HTB-26™</td>
<td>MDA-MB-231</td>
<td>p.G13D</td>
<td>c.38G&gt;A</td>
</tr>
</tbody>
</table>

CRM DNAs are now available
Need for Biological Standards in Proficiency Testing

Key issues in Molecular testing

Rapidly moving field

- Test relevant, actionable targets
  - Need high quality, challenging, well characterized PT materials
  - Consistent, traceable
  - Representatives of contemporary strains
  - Genetic variations/mutations
  - New emerging strain variants

- New Technology Developments
  - NGS
  - Maldi-Tof
  - Multiplex assays
Reference material for Next-Gen Sequencing

**Characterize platforms & methods:**
- DNA sequencing
- NGS technologies and platforms
- Research applications
- Clinical diagnostic applications
  - Assay validation
- Facilitate tool development for evaluating performance
  - Somatic calls for:
    - Single nucleotide variants (SNVs)
    - Indels
    - Structural variants
    - Copy number variation
    - RNA fusion detection
## Advantages of tumor/normal pairs

<table>
<thead>
<tr>
<th>ATCC® No.</th>
<th>Name</th>
<th>Cancer Type</th>
<th>Tissue Source</th>
<th>ATCC® No.</th>
<th>Name</th>
<th>Tissue Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>CRL-1974™</td>
<td>COLO 829</td>
<td>Melanoma, malignant</td>
<td>Skin</td>
<td>CRL-1980™</td>
<td>COLO 829BL</td>
<td>B lymphoblast</td>
</tr>
</tbody>
</table>

- First comprehensive catalog of somatic mutations from an individual cancer
- Both cell lines sequenced in 2009
- Somatic calls published in 2010*

Tumor/normal cell line and DNA pair

COLO 829 / COLO 829BL

- Whole genome sequencing of COLO 829 and COLO 829BL at a depth of 90x is being generated by Illumina and TGen to build a set of consensus calls
- HiSeq 90x WGS at Illumina & TGen
- SNVs, Indels, Copy NVs, SVs
  - Confirmed by PCR & sequencing
- Complete genomic WGS
- Multiple dilution series analyzed
Conclusions
Why are Standards needed?

- To establish sensitivity, linearity, and specificity during assay validation or implementation
- To benchmark critical assay performance during development/validation for regulatory submissions and production lot release
- Move from growth based methods to molecular methods, establish method equivalence
- Evaluation of assay variability & assay comparison
- Validate or compare test methods
- For use in inter-laboratory studies and comparisons
- For use in testing and calibration in ISO 17025 accredited laboratories
- Production of laboratory reference materials
- Development of standard protocols
- Method maintenance
- External quality assessment
Thank you!

Register for more webinars in the ATCC “Excellence in Research” webinar series at www.atcc.org/webinars.

June 5, 2014
10:00 am or 3:00 pm (ET)
Dr. Doug Storts and Dr. Yvonne Reid will discuss the recent advances in STR profiling technologies and how the Standard STR protocol is transforming scientific practices.

Thank you for joining today!
Please send additional questions to tech@atcc.org