Molecular Detection and Identification of Mycobacteria
Overview

• Direct detection of *Mycobacterium tuberculosis* complex (MTBC) in specimens

• Identification of MTBC and other mycobacteria from culture
Direct Detection

- Direct Detection is the testing of a primary specimen by nucleic acid amplification (NAA) to detect MTBC DNA
  - Not a replacement for culture

Sputum → DNA extraction → Amplification

MTBC detected
MTBC not detected

*Can be performed in 24-48 hours from specimen receipt*
Specimens that can be Tested for Direct Detection

- Sputum, processed or raw
- Other respiratory—bronchoalveolar lavage fluid (BAL), bronchial washing
- Gastric lavage
- Urine
- CSF
- Body fluids (e.g. pleural, pericardial)
- Tissues (fresh or formalin-fixed)

*Methods should be validated for each specimen type*
Identification

- **Identification** is testing culture growth from liquid or solid media to identify MTBC DNA or RNA and may also include differentiation between members of the complex.

- **DNA extraction or processing**

- **Amplification**

- **Hybridization**

**MTBC**
- M. tuberculosis
- M. bovis
- M. bovis BCG
- M. africanum
- M. microti
- M. canettii

**Nontuberculous Mycobacteria**

Requires culture growth and may take weeks to months from specimen collection.
Considerations for Performing Molecular Testing for MTBC

• Molecular testing can be performed within BSL-3 or BSL-2 spaces by following manufacturers instructions and proper inactivation steps when appropriate

• If samples are removed from the BSL-3 for testing in a BSL-2 space, proper inactivation protocols and inactivation studies must be in place

• Good practices for both BSL-3 and molecular testing (e.g., unidirectional workflow) must be observed
MTBC Inactivation

- All methods need to be validated to ensure that a high level (>10^6) of TB cells will be inactivated by the method.
- Individuals performing inactivation procedures should be trained for each specific procedure.
- TB inactivation protocols and validations are critical if molecular testing is performed outside of BSL-3.
- Various methods can be performed in BSL-3 for inactivation including heat and chemical treatment.
A Typical Validation Protocol

Inactivation method

- Heat or Chemical

Viability Study:
- Hold solid and liquid cultures for 8 weeks at 37°C, inoculated in triplicate (record growth or no growth)

No inactivation method

- Performed to quantify number of TB cells before inactivation

10^6 TB cells

Mycobacterium tuberculosis

Performed to quantify number of TB cells before inactivation
QC of Molecular Methods

Laboratory Developed Tests

- Extraction controls
  - Positive and negative

- PCR Controls
  - Positive control for PCR
  - No Template Control (NTC) for PCR

- Detection of inhibition
  - Plasmid/target
  - Spiking an aliquot of sample into another assay

FDA Cleared Tests

- QC protocols will be indicated and included in package inserts

- Cepheid Xpert® MTB/RIF
  - sample processing control (SPC)- monitors adequate processing and inhibition
  - Probe Check Control (PPC)- monitors reagents

Protocols and practices designed to prevent contamination are critical!
Sample Preparation and DNA Extraction

- DNA may need to be extracted from materials prior to testing
- Method selection based on downstream application, quantity and quality needed, convenience, cost, availability of reagents and equipment, and turnaround time (TAT)

<table>
<thead>
<tr>
<th>Quality of DNA produced</th>
<th>Chloroform/ Methanol (chemical) DNA Extraction</th>
<th>Fastprep (physical disruption)</th>
<th>Heat Kill</th>
</tr>
</thead>
<tbody>
<tr>
<td>High quality DNA</td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Crude DNA</td>
<td></td>
<td>X</td>
<td>X</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Quantity of DNA produced</th>
<th>Chloroform/ Methanol (chemical) DNA Extraction</th>
<th>Fastprep (physical disruption)</th>
<th>Heat Kill</th>
</tr>
</thead>
<tbody>
<tr>
<td>Large quantity</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Small quantity</td>
<td></td>
<td></td>
<td>X</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Downstream applications</th>
<th>Chloroform/ Methanol (chemical) DNA Extraction</th>
<th>Fastprep (physical disruption)</th>
<th>Heat Kill</th>
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<tbody>
<tr>
<td>PCR</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Whole Genome Sequencing</td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sequencing</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Line probe assay</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
</tbody>
</table>
DIRECT DETECTION OF MYCOBACTERIA BY NAA TESTING
Earlier laboratory confirmation of TB can lead to earlier treatment initiation, improved patient outcomes, increased opportunities to interrupt transmission, and more effective public health interventions.
Updated Recommendation

• “NAA testing should be performed on at least one respiratory specimen from each patient with signs and symptoms of pulmonary TB for whom a diagnosis of TB is being considered but has not yet been established and for whom the test result would alter case management or TB control activities.”
Direct Detection of MTBC is an Important Diagnostic Test for TB

• Added value of NAA testing compared to AFB smear microscopy
  – Greater positive predictive value (>95%) with AFB smear-positive specimens in settings in which nontuberculous mycobacteria are common
  – Ability to rapidly detect the presence of MTBC in 50%-80% of AFB smear-negative, culture-positive specimens

• Appropriate use of NAA testing is critical to rapidly detecting and reducing transmission of TB in the United States

Updated Guidelines for the Use of Nucleic Acid Amplification Tests in the Diagnosis of Tuberculosis. MMWR 2009; 58 (01): 7-10
Healthy People 2020

- Increase the proportion of culture-confirmed TB patients with a positive NAA test result reported within 2 days of specimen collection
- 2008 Baseline: 32.0% of culture-confirmed TB patients with a positive NAA test had their test results reported within 2 days of specimen collection
- Target: 77.0%

Clinical Considerations for NAA Testing

• Suspected of having pulmonary TB based on clinical evaluation
• Package inserts for FDA cleared/market authorized tests provide stipulations on number of days of treatment for use
  – Less than 7 days of therapy (MTD)
  – Less than 3 days of therapy (GeneXpert MTB/RIF)
Methods for Direct Detection of MTBC

- Hologic (previously Gen-Probe) Amplified M. tb Direct Test (MTD)
- Cepheid GeneXpert (Xpert) MTB/RIF
- Line Probe Assays (LPA)
- Laboratory Developed Tests (LDT)
  - Real-time PCR
  - Sequencing
MTD

• Commercial kits available in the United States
• Approved for *in vitro* diagnostic use by the FDA in 1995, and in 1999, enhanced for smear-negative specimens
• Assay based on target-mediated amplification and uses nucleic acid probe
• Assay time 2.5-3 hours
Transcription Mediated Amplification (TMA)
Considerations for MTD Testing

- AFB smear positive or negative respiratory sediments prepared using NALC-NaOH digestion-decontamination
- Test must be performed in conjunction with mycobacterial culture
- Analysis of non-respiratory specimens is considered “off-label” testing and would require full validation
- Specimens that are grossly bloody should not be tested
Considerations for MTD Testing (2)

- **Advantages**
  - Approved for multiple types of respiratory specimens
  - Uses same equipment as Accuprobe assays

- **Limitations**
  - Labor-intensive; minor changes in technique can cause inconclusive results or failed runs
  - Not automated, limited throughput
  - No internal inhibition control
  - Presence of *M. celatum* and *M. terrae*-like organisms can cause false-positive results
MTD Performance

<table>
<thead>
<tr>
<th></th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Smear Positive</td>
<td>97 (95-98)</td>
<td>96 (93-97)</td>
</tr>
<tr>
<td>Smear Negative</td>
<td>76 (70-80)</td>
<td>97 (96.6-97.4)</td>
</tr>
</tbody>
</table>

Greco, S. et al. Thorax 2006; 61: 783-790
Xpert MTB/RIF

- Automated commercial system for identification of MTBC and detection of mutations associated with rifampin resistance
- FDA market-authorized in July 2013 for sputum specimens
- DNA extraction, amplification, and detection in one cartridge
- Integrated positive control assures that a negative result is not due to NAA inhibitors in the specimen
Specimen Types for Xpert Testing

• At least 1 ml of raw sputum
  – Transport and store specimens at 2 to 8°C before processing whenever possible. If necessary, sputum specimens can be stored at a maximum of 35°C for up to 3 days and then at 2 to 8°C for an additional 7 days.

• At least 0.5 ml of sputum sediment
  – Use a standard NaOH-NALC method for decontamination and resuspend the sediment in a 67 mM Phosphate/ H₂O buffer
  – Store resuspended sediments at 2 to 8°C for up to 7 days

• Analysis of other specimen types is considered “off-label” testing and would require full validation
Workflow: Self contained cartridge – just add sample

1. Pour Sample Reagent into sample tube.
   Incubate for 15 minutes at room temperature.
   (Acceptable sample types: unprocessed sputum or sediment from concentrated specimen.)

2. Pipette diluted sample into cartridge.

3. Insert cartridge and start assay.

TOTAL HANDS-ON TIME = 2 MINUTES

www.cepheid.com, brochure 300-7810
Xpert MTB/RIF Assay Design

- Assay uses real-time PCR with molecular beacons
- Detects MTBC and rifampin resistance by amplifying a specific 81 base pair region of the RNA polymerase subunit B (\textit{rpoB}) gene known as the rifampin resistance determining region (RRDR)
- The amplicon is probed with five molecular beacons to detect mutations within the RRDR

\[
5\:' \text{GCACCAGCCAGCTGAGCCAATTATCTGGACCAGACAACACCGCTGCGGGTTGACCCACACAAAGCGCCGACTGTCGCGCTG - 3'}
\]
\[
3\:' \text{CGTGGTCGCTCAGCTCGTTAAGTACCTCGGCTTTGTTGCGACAGCCCAAACCTGGGTGGTCGCGCGCTGACAGCCCGAC - 5'}
\]
Xpert MTB/RIF Results for Detection of MTBC

- “MTB DETECTED” is reported when at least two probes hybridize to the amplicon

- “MTB NOT DETECTED” is reported when fewer than two probes hybridize to the amplicon
## Xpert MTB/RIF Performance

<table>
<thead>
<tr>
<th></th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Smear Positive</td>
<td>99.6%</td>
<td>97.6%</td>
</tr>
<tr>
<td>Smear Negative</td>
<td>79.0%</td>
<td>97.6%</td>
</tr>
</tbody>
</table>

Data for Xpert MTB/RIF Assay vs MTB Culture for Expectorated Sputum from Cepheid Xpert MTB/RIF Package Insert 301-1404 Rev A
Considerations for Xpert MTB/RIF

• Advantages
  – Cepheid instrument is already available in many clinical laboratories
  – Closed-system within single cartridge
  – Minimal hands on manipulation
  – Results in ~2 hours
  – Limited biosafety concerns

• Limitations
  – FDA approved for sputum specimens only
Line Probe Assay

- Uses PCR and hybridization of amplicon to nitrocellulose strip
  - Identification of MTBC and commonly-isolated NTM by reverse hybridization

- Commercially developed LPA many of which can be used for both direct detection and identification
  - Innogenetics INNO-LiPA Mycobacteria v2 (MTBC + NTM)
  - HAIN GenoType Mycobacterium CM (MTBC + 24 NTM)
  - HAIN GenoType Mycobacterium AS (19 NTM)
Line Probe Assay
Specimen Types

• Pulmonary smear-positive NaOH-NALC pre-treated patient material
  – For some assays, if the smear results are scanty or negative, the specimen must first be cultured and resulting growth used for the LPA

• Cultured isolates from various media sources
Line Probe Assay (2)

Considerations for Line Probe Assay

• Advantages
  – Some assays detect mutations associated with MTBC drug resistance
  – Relatively low implementation costs

• Limitations
  – Not FDA approved, some assays may not be available in United States
  – Open manipulation of PCR product during the protocol could result in contamination
  – Can be difficult to differentiate bands with visual inspection
Laboratory Developed Tests for Direct Detection of MTBC

- Methods include real-time PCR
- Gene targets for detection of MTBC
  - IS6110 insertion element
  - 16S rRNA
  - senX3-regX3
  - hsp65
  - rpoB
  - IS986 insertion element
  - 23S RNA
  - dnaJ
  - Region exterior to RD9
Real-time PCR Assay

• Can be validated for multiple specimen types
  – Smear positive and negative specimens
  – Respiratory and non-respiratory specimens
  – AFB-positive cultures
• Extraction method for pulmonary specimens that involves heat and silica bead lysis
• Can be validated for any real-time PCR instrument
• Some LDT include unique plasmid controls to assess inhibition
Considerations for LDT

• Advantages
  – Can multiplex to detect additional organisms (such as *M. avium* complex) and drug resistance
    • Tran et al. 2014. Diagn Microbiol Infect Dis 79 (1):43-8
  – Adaptable to multiple instruments
  – Adaptable to different specimen types

• Limitations
  – Requires expertise to develop and validate assays
  – Extensive quality management of reagents and primers needed
False Positive/False Negative Concerns for NAA Testing

• False Positive
  – Amplicon contamination
  – Cross-reactivity of some assays with other mycobacterial strains

• False Negative
  – Limited sensitivity
  – Inhibition
  – Assay not working properly (reagents, equipment)
Recommended TATs for Reporting NAA Test Results

• TAT must be rapid to maximize benefits of NAA testing
  – Measured from specimen collection to report of laboratory results

• NAA test results should be available within 48 hours of specimen collection

• Initial positive NAA test result is a critical test value
  – Immediately report the result to the clinician and public health authorities, and be available for consultation regarding test interpretation and the possible need for additional testing


Updated Guidelines for the Use of Nucleic Acid Amplification Tests in the Diagnosis of Tuberculosis MMWR 2009; 58 (01); 7-10

Optional Algorithms for NAA Testing

**Universal Testing**
- Test all incoming TB specimens
- Cost and yield must be considered, especially in low incidence areas

**Reflex Testing**
- Test specimens based on other test results
- Specimen is AFB-smear positive

**Risk-Based Testing**
- Test specimens from patients that meet criteria jointly established by your facility and TB Program
- Patient has signs, symptoms, or risk factors for active TB
- Specimen is from a high-incidence population (correctional facility, homeless shelter, etc.)

**Combination**
Respiratory Specimen

Smear Positive

- **NAA Test**
  - **Positive:** Presumed TB, Pending culture results
  
  Use clinical judgment to begin therapy while awaiting culture results.

  Consider testing another specimen

  If a second specimen is smear positive and NAA testing negative, the patient is presumed to have an infection with an NTM, pending culture results.

- **Negative**
  
  Inhibitors Detected:
  Test result is not of diagnostic help. Consider testing second specimen

- **NAA Test**
  
  **Positive:** A patient can be presumed to have TB pending culture results, if two specimens are NAA test positive.
  
  Use clinical judgment to begin therapy while awaiting results of culture and other diagnostic tests.

  Consider testing another specimen

- **Negative**

Smear Negative

- **NAA Test**
  
  **Positive:** Use clinical judgment to begin therapy while awaiting culture results.

  Consider testing another specimen

- **Negative**
  
  Use clinical judgment to begin therapy while awaiting results of culture and other diagnostic tests.

  Currently available NAA tests are not sufficiently sensitive to exclude the diagnosis of TB in AFB smear negative patients suspected of having TB.

Courtesy of David Warshauer, PhD
Direct Detection Summary

• Advantages
  – Rapid detection of MTBC directly from clinical specimens
    • More rapid diagnosis
    • Initiation of earlier treatment
    • Cost savings with reduced patient isolation
    • Faster reporting to TB Programs
    • Reduced transmission
  – Good sensitivity for smear-positive specimens
  – Specificity is greatly improved over smear
Direct Detection Summary (2)

• Limitations
  – Does not distinguish between live and dead organisms
  – Not routinely used to differentiate between members of the TB complex
  – Limited sensitivity for smear-negative specimens
  – Not indicated for use to determine bacteriologic cure or to monitor response to therapy
NAA Testing Does Not Replace AFB Smear and Culture

• Smear needed for interpretation of test results
  – Indicates relative infectiousness of patient

• Culture is still the “gold standard” for TB diagnosis

• Culture is necessary for phenotypic drug susceptibility testing and genotyping
MOLECULAR DETECTION AND IDENTIFICATION OF MYCOBACTERIA
Identification from Culture Growth

• Accurate and prompt identification of culture growth is important for patient management and public health response
Identification Methods

- Line Probe assays*
- Real-time PCR*
- Hologic (previously Gen-Probe) Accuprobe
- MALDI-TOF MS
- DNA Sequencing
  - Sanger sequencing
  - NGS

*Line Probe assays and Real-time PCR follow same methodologies for identification as mentioned in direct detection section
Real-time PCR Assays Can Be Multiplexed

- **MTBC/MAC**
  - Multiplex assay allowing identification of MTBC and MAC within same PCR run

- **MTBC species**
  - “Five-plex” multiplex assay
  - Multiplex assay for differentiation of *M. tuberculosis*, *M. bovis*, *M. bovis* BCG, *M. africanum*, *M. microti* and *M. canettii*
  - Presence or absence of Regions of Difference (RD) allows for differentiation between members of the complex
    - RD1, RD4, RD9, RD12 and a region exterior to RD9

*Halse et al. 2011 JCM 49 (7): 2562-2567*
Identification within MTBC

- Laboratories should consult with healthcare providers and TB program to decide the necessity of differentiating among members of *Mycobacterium tuberculosis* complex
  - Due to unique epidemiology and inherent PZA drug resistance, laboratories should consider differentiating *M. bovis* and *M. bovis BCG* from other members of MTBC
    - *M. bovis* infection is often associated with unpasteurized dairy products
    - *M. bovis* BCG is used in bladder cancer treatment and, internationally, for vaccination
  - Genotyping results may help to distinguish *M. bovis* and *M. bovis BCG*
Accuprobe

- In-solution hybridization assay for identification of culture growth
  - Nucleic acids are extracted after organisms are lysed and made non-viable
  - Single-stranded labeled DNA probes are allowed to anneal to target ribosomal RNA
  - If present, RNA:DNA hybrids are detected by chemiluminescence

- Commercially available kits for identification of MTBC, *M. avium* complex, *M. gordonae*, *M. kansasii*
Considerations for Accuprobe

• Advantages
  – Available for four frequently-isolated mycobacteria; three clinically significant
  – Relatively easy to use
  – FDA-cleared

• Limitations
  – No nucleic acid amplification occurs during this assay; sufficient culture growth is necessary for identification
  – Beware of relative light units (RLU) values that are near the cutoff; “high negative” values could indicate that the target organism is present in low numbers
MALDI-TOF

- Matrix-Assisted Laser Desorption Ionization Time-of-Flight

- Identification of mycobacterial species through mass spectrometry
  - Analysis of proteins and peptides depicted as mass spectra with patterns of characteristic peaks

http://www.bruker.com/
MALDI-TOF Mycobacterial Sample Preparation

• Single mycobacterial colonies from solid media or 1.2 ml from liquid media undergo an extraction protocol and are applied to a sample target plate and overlaid with matrix

• Formic acid/acetonitrile extraction
  – Necessary to extract proteins from mycobacterial cells
  – Includes physical (bead beat), chemical (formic acid), and heat lysis steps

[Diagram showing steps: Culture suspension & wash steps → Heat lysis → Ethanol wash → Bead Beat → 70% formic acid/acetonitrile addition]
MALDI-TOF MS Results

- Dedicated software analyzes mass spectra against library of stored profiles

**Bruker Daltonics Biotyper**
Scoring interpretation
- 2.00-3.00 = Secure genus and species identification
- 1.70-1.99 = Probable genus identification
- 0-1.699 = Unreliable identification

<table>
<thead>
<tr>
<th>Analyte Name</th>
<th>Analyte ID</th>
<th>Organism (best match)</th>
<th>Score Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1 (++)(C)</td>
<td>BTS</td>
<td><em>Escherichia coli</em></td>
<td>2.40</td>
</tr>
<tr>
<td>A2 (++)(C)</td>
<td>BTS</td>
<td><em>Escherichia coli</em></td>
<td>2.60</td>
</tr>
<tr>
<td>A3 (+)(A)</td>
<td>M. avium</td>
<td><em>Mycobacterium avium</em></td>
<td>2.90</td>
</tr>
<tr>
<td>A4 (+)(A)</td>
<td>M. avium</td>
<td><em>Mycobacterium avium</em></td>
<td>2.90</td>
</tr>
<tr>
<td>A5 (-)(C)</td>
<td>14mm</td>
<td>no peaks found</td>
<td>&lt;0</td>
</tr>
</tbody>
</table>

**bioMerieux Vitek MS**
Confidence values (%)
- Results have a strong match and are ready to report
- Results have low discrimination and require further review
- Low-quality results with no identification made
MALDI-TOF Safety Precautions

• Concern that mycobacteria may still be viable

• If MALDI-TOF instrument is not within BSL-3, ensure inactivation before transferring bacteria to lower BSL
  • Inactivation study should be performed prior to implementation of this method
Considerations for MALDI-TOF

• Advantages
  – Rapid identification
  – Can also be used for identification of many bacteria and fungi in the laboratory

• Limitations
  – Difficult extraction protocol for mycobacteria
  – Current database limitations
    • Library used must be built on the same extraction protocol used by the laboratory
  – Initial cost investment high
  – Cannot identify to species within the MTBC
Sanger Sequencing

- Template DNA, primers, DNA polymerase, and fluorescently labeled nucleotides are used to create DNA fragments.

- The length of the DNA fragment in combination with the specific fluorescence detected reveals the sequence of the DNA fragment.

http://www.uic.edu/classes/bios/bios100/lectures/sanger03.jpg
Sanger Sequencing (2)

- Compare nucleotide order to library of known sequences for bacterial identification
- Receive a list of potential identifications with associated percent

http://seqcore.brcf.med.umich.edu/doc/dnaseq/interpret.html

Sanger Sequencing: Common Target Genes

<table>
<thead>
<tr>
<th>Gene</th>
<th>Gene Length (bp)</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>16S rRNA</td>
<td>~500 fragment (of 1,500 gene)</td>
<td>Highly conserved gene</td>
<td>Difficulty differentiating some species with identical hypervariable regions or complete 16S sequence</td>
</tr>
<tr>
<td>23S rRNA</td>
<td>~3,100</td>
<td>Contains conserved and variable sequence regions</td>
<td>Length of gene</td>
</tr>
<tr>
<td>rpoB</td>
<td>~3,600 (fragments analyzed)</td>
<td>Gene contains mutations known to confer resistance to rifampin for MTBC</td>
<td>No consensus on which fragment is most suitable for slow-growing mycobacteria</td>
</tr>
<tr>
<td>hsp65</td>
<td>~440</td>
<td>Useful in the ID of closely related species compared to 16S</td>
<td>Small size differences between the fragments or the incidence of identical restriction patterns for closely related species and new species</td>
</tr>
<tr>
<td>gyrB</td>
<td>~1,000</td>
<td>Useful for differentiation of some species within MTB complex</td>
<td>Length of gene</td>
</tr>
</tbody>
</table>
CLSI Recommendations for Molecular Identification Results Reporting

- 16S sequencing identification based on the percent identity
  - If 100%, both genus and species may be reported
  - If 99.0-99.9%, report as “Mycobacterium most closely related to the [species listed]”
  - If 95.0-98.9%, report as “Unable to definitively identify by 16SrRNA gene sequencing, most closely related to Mycobacterium sp.”
Considerations for Sanger Sequencing

• Advantages
  – Specific primers have been well described and validated
  – Libraries are available but require careful selection

Peer-reviewed databases:
• May not be publically available
• May require subscription fee
• Entries are reviewed
• Data obtained from characterized isolates using approved nomenclature

Public databases:
• Free
• Huge number of entries
• May contain data from unusual isolates that are not fully characterized
• Know the limitations of these data

• Limitations
  – Differentiation of some Mycobacterium sp. can be difficult when highly conserved genes are used
Next Generation Sequencing

• Massively parallel sequencing of short DNA fragments
  – Genomic DNA is fragmented into smaller segments that are sequenced in millions of parallel reactions

• Nucleotide base incorporation and detection occurs in real-time

• Multiple manufacturers each with their own technology
NGS Data

• The strings of data are reassembled (aligned)
  – Reference guided – used as scaffolding
  – de novo – align sequencing to each other
    • Used in the absence of reference genome

• Data compared to library for identification

• Software packages critical in assembly and interpretation of data
## Comparison of NGS Platforms

<table>
<thead>
<tr>
<th>Platform</th>
<th>Sequencing chemistry</th>
<th>Read Length (bp)</th>
<th>Run Time</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>454 Titanium FLX (Roche)</td>
<td>Pyrosequencing</td>
<td>400</td>
<td>10 hrs</td>
<td>Long read length</td>
<td>High error rate in homopolymer; Appreciative hands on time</td>
</tr>
<tr>
<td>HiSeq 3000 (Illumina)</td>
<td>Reversible Terminator</td>
<td>2 x 150</td>
<td>1-3 days</td>
<td>High throughput; Minimal hands on time</td>
<td>Short reads; Long run time (normal mode)</td>
</tr>
<tr>
<td>SOLiD 5550XL (ABI/Life Technology)</td>
<td>Sequencing by Ligation</td>
<td>2 x 60</td>
<td>8 days</td>
<td>Low error rate; High throughput</td>
<td>Short reads; Long run time</td>
</tr>
<tr>
<td>Ion Torrent (ABI/Life Technology)</td>
<td>H+ Ion Sensititive Transistor</td>
<td>200</td>
<td>2 hrs</td>
<td>Short run times</td>
<td>Short reads</td>
</tr>
<tr>
<td>RS II (PacBio)</td>
<td>Real-time Sequencing</td>
<td>10,000</td>
<td>4 hrs</td>
<td>No PCR; Longest read length; Simple sample prep</td>
<td>High error rate</td>
</tr>
</tbody>
</table>

Data shown here represent the highest figures currently available on the company website and is likely to change.
Applications of NGS

• Whole genome
  – Wealth of information (e.g., identification, resistance profile, genotype)
  – Useful for molecular epidemiology of TB through comparison of genome sequences

• Amplicon-based
  – Also known as targeted sequencing
  – Focus on genomic area of interest that can be larger than that of Sanger sequencing
  – Keeps data amount low compared to whole genome sequencing
Considerations for NGS

• **Advantages**
  – High throughput
  – Large amount of data compiled
  – Ability to recognize new strains

• **Limitations**
  – Database management
  – Data storage
  – Bioinformatics expertise required
  – Costs
    • Initial setup costs
    • Batching to keep costs down
False Positive/False Negative Concerns for Molecular Identification Methods

• False Positive
  – Amplicon contamination

• False Negative
  – IS6110 negative strains of MTBC have been reported
  – Inhibition
Reporting of Molecular Identification Results

• CDC recommended TAT
  – Use rapid methods to identify and report isolates as MTBC in ≤ 21 days from specimen receipt

• Report MTBC identification as soon as possible to provider and TB Control Program
  – Use manufacturer’s guidance, where available, for terminology and disclaimers
Combining Molecular and Culture-based Approaches

- Process Specimen
- AFB Smear Microscopy
- Inoculate Media
- Culture Positive
- Organism Identification
- Nucleic Acid Amplification Test

Conventional Methods: 2-6 Weeks

Molecular Methods: 1-3 days
NAA Testing Resources

- CDC—Updated Guidelines for the Use of Nucleic Acid Amplification Tests in the Diagnosis of Tuberculosis
  [http://www.cdc.gov/mmwr/preview/mmwrhtml/mm5801a3.htm](http://www.cdc.gov/mmwr/preview/mmwrhtml/mm5801a3.htm)

  [http://www.cdc.gov/mmwr/preview/mmwrhtml/mm6241a1.htm](http://www.cdc.gov/mmwr/preview/mmwrhtml/mm6241a1.htm)

- APHL—Laboratory Considerations for Use of Cepheid GeneXpert MTB/RIF Assay
NTM Database Resources

- GenBank

- Ribosomal Database Project (RDP)
  [http://rdp.cme.msu.edu/](http://rdp.cme.msu.edu/)

- BioInformatics Prokaryotes Quick Phylogeny (leBIBI)
  [https://umr5558-bibiserv.univ-lyon1.fr/lebibi/lebibi.cgi](https://umr5558-bibiserv.univ-lyon1.fr/lebibi/lebibi.cgi)
NGS Resources

• Ion Torrent next generation sequencing technology
  http://www.youtube.com/watch?v=MxkYa9XCvBQ

• Illumina next generation sequencing technology
  https://www.youtube.com/watch?v=HMyCqWhwB8E