Perspectives from a Public Health Laboratory

July 1, 2015

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*I have no disclosures.
Drug Resistant Tuberculosis is a Global Health Concern

- Multi-drug resistant TB (MDR): resistant to at least rifampin and isoniazid

- Extensively drug resistant TB (XDR): resistant to rifampin and isoniazid plus any fluoroquinolone and at least one of three injectable second-line drugs (i.e., amikacin, kanamycin, or capreomycin).

The Outsized Financial Toll of MDR and XDR TB
Cost increases with greater resistance:

http://www.cdc.gov/nchhstp/newsroom/2014/WorldTBDay-graphics.html
Preventing and Controlling MDR and XDR TB in the U.S. Requires:

- Better Treatment Options
- Rapid Diagnosis
- Expert Treatment of Every TB Case
- Improving Global TB Diagnosis and Treatment

http://www.cdc.gov/nchhstp/newsroom/2014/WorldTBDay-graphics.html
Why perform WGS on *Mycobacterium tuberculosis*?

- Faster turn-around time
- More comprehensive results
  - Detect mixed infections
  - Many predictors of drug resistance
  - Emerging resistance
- Cost effective
  - Replace existing assays (real-time PCR, pyrosequencing, spoligotyping)
  - Staff time savings
<table>
<thead>
<tr>
<th>Year</th>
<th>DR-TB</th>
<th>MDR-TB</th>
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<tbody>
<tr>
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<td></td>
<td></td>
</tr>
<tr>
<td>2008</td>
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<td>2009</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>2014</td>
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</table>

**TB Cases**

<table>
<thead>
<tr>
<th>Year</th>
<th>2007</th>
<th>2008</th>
<th>2009</th>
<th>2010</th>
<th>2011</th>
<th>2012</th>
<th>2013</th>
<th>2014</th>
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<tbody>
<tr>
<td>Cases</td>
<td>1175</td>
<td>1200</td>
<td>1007</td>
<td>954</td>
<td>910</td>
<td>864</td>
<td>872</td>
<td>786</td>
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</tbody>
</table>
**Primary specimens/ isolates**

- **Processed/decontaminated**
  - real-time PCR
    - MTBC/MAC
  - AFB Smear
    - Pos
    - Neg
  - MTBC member
    - real-time PCR
      - ID Member of MTBC
      - MTBC
      - NTM
    - DST 1st line drugs
      - Resistant
      - Susceptible
    - WGS
      - MALDI-TOF
        - rpoB, hsp65
      - DST 2nd line drugs
        - Identification of mutation
          - (pncA, rpoB, katG, inhA, gyrA/B)
      - Report
        - Wildtype
        - Resistance Mutation
          - MTBC member
          - Report
          - Resistant
          - Susceptible
  - Heat-killed
    - Pos MTBC
      - rpoB, katG, inhA
      - Pyrosequencing
      - No report until culture
    - Neg
      - Wait for culture
Whole Genome Sequencing
Next Generation Technologies

Extract TB DNA
Massively Parallel DNA sequencing
Bioinformatics
Validating a WGS assay for TB

Selecting validation approach, culture, optimization of DNA preparation

Library preparation and Miseq sequencing, optimizing, planning overall decisions for balancing runs (3 Illumina Miseqs)

Development, testing and continual improvements to pipeline, data storage
What to validate first?

• Isolates
  – Solid
  – MGITs
• Primary specimens
  – sputum
  – other

Need to keep in mind available testing volumes, what is needed for other tests, archiving, etc...
How Can We Mimic a Clinical Isolate?

- Grow 2 different strains in MGIT tube
  - *M. tuberculosis*: ATCC strain (ATCC)
  - *M. bovis BCG*: patient strain (BCG)
- Aliquots made and heat-killed before leaving BSL-3

10^5 to 10^6 colony-forming units per milliliter (CFU/mL)
Preparing TB DNA for WGS

- Assess methods used in lab
- Research TB WGS methods
- Assess worse case scenario
  - 1-2 ml MGIT
  - early MGIT positive (Day 0-3 flagged positive)
- Ease of use, cost
- DNA concentration
- Ultimately: WGS 40X depth and close to 100% coverage
Breaking TB Open is Critical for DNA Extraction

Important TB Characteristics

• ~24 hour doubling time
• TB clumps together
• Unique cell wall
  – Rich in lipids (>60%)
  – Mycolic acids

Initial Methods Tested

• Typical bacterial extraction
• Zymo Research Kit
  – Meant for tough to lyse fungi/bacteria
• CTAB method
  – Ideal for plant cell nucleic acid extraction/MTB

DNA yield too low, labor intensive, WGS variable results
InstaGene Matrix and Tissue Homogenizer

• **InstaGene matrix (Chelex resin)**
  – The Chelex matrix binds to PCR inhibitors rather than DNA, preventing DNA loss due to irreversible DNA binding.

• **Fastprep tissue homogenizer**
  – Good enough yield to provide reliable WGS data even with 0 day MGIT

Success!
Successful WGS

- **Depth**: Essentially the number of times the base was read; measure of confidence in correct call
  - Can be given as a genome average
  - We are aiming for 40X

- **Coverage**: A percentage that describes how much of the genome was sequenced
  - Best 100%

8X Depth

ATTGC
ATTGC
TTGCATAAAATTC
ATTGCAT
TGCTAAATTC
ATTGCAT
ATTG
TGCATAAAT

67% Coverage
Library Preparation is Another Key Factor

- Votintseva et al. suggested using 15 cycle library preparation
  - 2015 paper about WGS of early positive MGIT

<table>
<thead>
<tr>
<th>Sample</th>
<th>Method</th>
<th>stock ng/ul</th>
<th>Avg depth coverage %</th>
<th>Avg depth</th>
<th>coverage %</th>
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<tbody>
<tr>
<td><em>M. bovis BCG</em> (0day)</td>
<td>InstaGene</td>
<td>0.268</td>
<td>FAIL</td>
<td>27.66</td>
<td>97.23</td>
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<td>InstaGene</td>
<td>0.344</td>
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<td>0.346</td>
<td>FAIL</td>
<td>14.22</td>
<td>96.78</td>
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Bioinformatics Pipeline

Pascal Lapierre, PhD
Michael Palumbo, PhD
### All Mutation in screened loci (except silent mutations):

<table>
<thead>
<tr>
<th>Gene</th>
<th>Mutated nucleotide</th>
<th>Previously mutated nucleotide</th>
<th>Gene product</th>
<th>Antibiotic class</th>
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<td>7362</td>
<td>GAG -&gt; CAG</td>
<td>Glu -&gt; Gln</td>
<td>gyrA</td>
<td>Fluoroquinolones</td>
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<tr>
<td>7584</td>
<td>AGC -&gt; ACC</td>
<td>Ser -&gt; Thr</td>
<td>gyrA</td>
<td>Fluoroquinolones</td>
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<tr>
<td>9383</td>
<td>GCC -&gt; GAC</td>
<td>Gly -&gt; Asp</td>
<td>gyrA</td>
<td>Fluoroquinolones</td>
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<tr>
<td>9555</td>
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<td>Thr -&gt; Thr</td>
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<td>Fluoroquinolones</td>
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<tr>
<td>76154</td>
<td>TCG -&gt; TGG</td>
<td>Ser -&gt; Leu</td>
<td>rpoB</td>
<td>Rifampicin</td>
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<tr>
<td>764816</td>
<td>GTG -&gt; GCG</td>
<td>Val -&gt; Ala</td>
<td>rpoB</td>
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<tr>
<td>765149</td>
<td>GGG -&gt; GAG</td>
<td>Gly -&gt; Glu</td>
<td>rpoB</td>
<td>Rifampicin</td>
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<td>775639</td>
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<td>Ile -&gt; Val</td>
<td>mpI</td>
<td>Clofazimine/Bedaquiline</td>
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<td>Cys -&gt; Gly</td>
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<td>Ethambutol</td>
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<td>1416222</td>
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<td>Phe -&gt; Leu</td>
<td>embB</td>
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<td>1674046</td>
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<td>Leu -&gt; Leu</td>
<td>mabA</td>
<td>Isoniazid (Silent)</td>
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<tr>
<td>1917970</td>
<td>CTA -&gt; CTG</td>
<td>Leu -&gt; Leu</td>
<td>tlyA</td>
<td>Aminoglycosides (Silent)</td>
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<td>3336825</td>
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<td>Thr -&gt; Ala</td>
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<td>Arg -&gt; Arg</td>
<td>rcs793</td>
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<td>Val -&gt; Leu</td>
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<td>4407968</td>
<td>TTG -&gt; TCG</td>
<td>Leu -&gt; Ser</td>
<td>gidB</td>
<td>Streptomycin</td>
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</tbody>
</table>

### High confidence mutations detected:

- embB 306 ATG -> ATA Met -> Ile Ethambutol
- katG 315 AGC -> ACC Ser -> Thr Isoniazid
- mabA 203 CTG -> CTA Leu -> Leu Isoniazid
- pncA 116 CTG -> CGG Leu -> Arg Pyrazinamide
- rpoB 450 TCG -> TTG Ser -> Leu Rifampicin

### Resistance Report:

- Ethambutol: PASS Resistant
- Fluoroquinolones: PASS Sensitive
- Isoniazid: PASS Resistant
- Pyrazinamide: PASS Resistant
- Rifampicin: PASS Resistant
- Streptomycin: PASS Sensitive
Validation of TB WGS

- SOP, reports, interpretation, QC, assay controls, metrics
- Specificity, intra-assay and inter-assay reproducibility
- Retrospective testing
- Prospective testing
- Evaluate each drug
# TB WGS Reports

**Concentrated Smear (Ziehl - Neelsen/1,000 X):**

- (03/07/14): Numerous (>9 acid-fast bacilli per field)

**Direct Molecular Detection - Real-time PCR**

- **Mycobacterium tuberculosis complex DNA by real-time PCR:**
  - Detected
- **Mycobacterium avium complex DNA by real-time PCR:**
  - Not Detected

**Molecular Identification - Real-time PCR**

- **Mycobacterium tuberculosis complex species DNA identified:**
  - Mycobacterium tuberculosis

**Culture**

- (03/07/14): acid-fast bacillus was isolated

**Direct Molecular Drug Susceptibility Detection - Pyrosequencing**

- **Rifampin (rpoB):**
  - Mutation present (Ser315Leu) suggests Rifampin resistance.
  - Result must be confirmed by culture based susceptibility testing.
- **Isoniazid (inhA):**
  - Mutation absent. Culture must be performed for final susceptibility result.
- **Isoniazid (inhA):**
  - Mutation absent. Culture must be performed for final susceptibility result.

**Identification**

- (03/07/14): Mycobacterium tuberculosis was identified by culture and molecular analysis.

**Susceptibility Testing for M. tuberculosis complex (MGIT)**

- Streptomycin (1.0 ug/ml): Susceptible
- Isoniazid (0.1 ug/ml): Susceptible
- Rifampin (1.0 ug/ml): RESISTANT
- Ethambutol (5.0 ug/ml): Susceptible
- Pyrazinamide (1.0 ug/ml): Susceptible

**Whole genome sequencing**
Whole Genome Sequencing of TB: A “One Stop Shop”

**WGS**

- Single assay
- Species identification
- Genotyping *(more accurate)*
- Drug resistance mutations *(more comprehensive)*

**COST**

- Estimated around $100 per sample

**TURNAROUND TIME**

- DNA preparation (1 days)
- WGS result (4-5 days)
# WGS prediction spoligotypes and genotyping with increased resolution

<table>
<thead>
<tr>
<th>Sample #</th>
<th>Extraction Method</th>
<th>Phenotype</th>
<th>Resistance Associated Mutations (WGS)</th>
<th>Other Mutations Noted (WGS)</th>
<th>WGS Spoligotype</th>
<th>Cl. MB Sensipo NYS</th>
<th>Do results correlate?</th>
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</thead>
<tbody>
<tr>
<td>21</td>
<td>CTAB</td>
<td>SM (low level in 2nd line), INH, RIF, RBT</td>
<td><strong>aac(6)-I RIF</strong>&lt;br&gt;<strong>msl.a 88 (SM)</strong>&lt;br&gt;<strong>katA 315 (INH)</strong></td>
<td></td>
<td>S00031</td>
<td>Scheduled 12/15/14</td>
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<td>22</td>
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<td>INH (low level), RIF, RBT</td>
<td><strong>aac(6)-I RIF</strong>&lt;br&gt;<strong>integric/hyp-taqG1 115 (INH)</strong></td>
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<td>S00197</td>
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<tr>
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<td>S00204</td>
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<td>24</td>
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<td>SM, EMB, CAP, KM, AN</td>
<td><strong>rpsL (AN/SMB)</strong></td>
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<td>S00241</td>
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<td><strong>gyrA aa 94 (FLQ)</strong>&lt;br&gt;<strong>aac(6)-I RIF</strong>&lt;br&gt;<strong>integric/hyp-taqG1 115 (INH)</strong></td>
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<td>26</td>
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<td>S00031</td>
<td>S00031</td>
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## A Glimpse of the One Stop Shop in Action

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<tr>
<th>Sample</th>
<th># Day Pos</th>
<th>Qubit stock []</th>
<th>species</th>
<th>DST</th>
<th>Pyro/Sanger results</th>
<th>spoligotype</th>
<th>Depth</th>
<th>%Coverage</th>
<th>HC mutations</th>
<th>Other</th>
<th>Notable mut's</th>
<th>Spoligo / speciation match?</th>
<th>DR match?</th>
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<tbody>
<tr>
<td>15-5031</td>
<td>1</td>
<td>1.16</td>
<td><em>M. africanum?</em></td>
<td>NOT DONE</td>
<td>NOT DONE</td>
<td>S01519 (700740007 774671)</td>
<td>173.30</td>
<td>97.80%</td>
<td></td>
<td>none</td>
<td>~13Kb deletion at RD12 region</td>
<td>YES: SNP in #3 &amp; <em>africanum</em> on tree</td>
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<tr>
<td>14-26616</td>
<td>0</td>
<td>0.378</td>
<td><em>M. tuberculosis</em></td>
<td>SM, INH, ETA</td>
<td>inHA C-1ST (INH)</td>
<td>S00034</td>
<td>125.593</td>
<td>98.69%</td>
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<td>rpsL 88 (SM); mabA-15 (INH/ETA)</td>
<td>In-frame deletion iniB (INH)</td>
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<td>YES</td>
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<td>79.9</td>
<td>98.4</td>
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<td>YES</td>
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<td><em>M. tuberculosis</em></td>
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<td>168.6</td>
<td>99</td>
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<td>None</td>
<td>ethA 329, ethA 403 (ETA)</td>
<td>YES</td>
</tr>
</tbody>
</table>
Can we develop one assay capable of generating the same results...and more?
Can we do it in <1 week?
### XDR Case (November 2014)

**Spoligotype:** S00062 (777740777760771)

**Lineage:** Euro-American  
**M. tuberculosis X1 family**

**Drug Resistant phenotype:**  
FLQ (OFL, LVX, MX)  
RIF  
INH  
SM  
EMB  
PZA  
RBT  
KAN  
AMI  
CAP (11%)

<table>
<thead>
<tr>
<th>Gene</th>
<th>Genome position</th>
<th>Gene position</th>
<th>SNP</th>
<th>Res. associated</th>
<th>Codon AA change</th>
<th>Known mutation?</th>
<th>Putative mutation*</th>
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<td>rrs</td>
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<td>1400</td>
<td>A → G</td>
<td>AMI/SM</td>
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<td>gyrA</td>
<td>7362</td>
<td>61</td>
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<td>21 Glu/Gln</td>
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<td>FLQ</td>
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<td>rpoB</td>
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<td>C → T</td>
<td>RIF</td>
<td>450 Ser/Leu</td>
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<td>RIF</td>
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<td>HC mutation AGC</td>
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<td>Insertion Frameshift</td>
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<td>INH</td>
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<td>2781</td>
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<td>EMB</td>
<td>927 Arg/Arg</td>
<td>No Silent</td>
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<td>HC mutation GCC → GCC</td>
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<td>2895</td>
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<td>3165</td>
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<tr>
<td>ethA</td>
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<td>CGCGG → CGCGCGCGG</td>
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<tr>
<td>gid</td>
<td>4407934</td>
<td>269</td>
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<td>SM</td>
<td>90 Leu/Arg</td>
<td>No CTC → CGC</td>
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</table>

* Insertion Frameshift
Evolving Pipeline

- INH resistant, pyrosequencing failed
  - WGS katG gene missing
- PZA resistant, sequencing failed
  - WGS-561 nt deletion pncA, 675 nt deletion gidB (SM)
- Real-time PCR failed to differentiate MTBC
  - Regions of Deletion (RD 12, 4, 1) deletions
- Low level isoniazid, streptomycin
  - Accumulating data
- Spoligotyping shows faint bands
  - SNPs in spacer
- Missing RD4, has 3-4 alleles at gyrA90
  - FLQ resistance with no first line resistance.
- Bedaquiline mutations detected
  - Deletion in RD4
Future Directions WGS TB

• Finalize validation and implement WGS for TB MGIT testing
• Refining pipeline and data interpretation
• TB Primary specimens
• LIMS importing
• NCBI
• Data Storage
Acknowledgements

MYCOBACTERIOLOGY LAB
- Vincent Escuyer
- Donna Kohlerschmidt
- Michelle Isabelle
- Susan Wolfe
- Dennis Biggins

BACTERIOLOGY LAB
- Joe Shea
- Tanya Halse
- Tammy Quinlan
- Justine Edwards
- Linda Gebhardt

APPLIED GENOMIC TECHNOLOGIES CORE
- Matt Schudt
- Patrick VanRoey
- Pascal Lapierre
- Mike Palumbo

FUNDING

Wadsworth Center, NYSDOH
Public Health Genomics Initiative

James Posey
National Center for HIV/AIDS, Viral Hepatitis, STD, and TB Prevention

NIH
Office of Extramural Research

R03 NIH- Use of whole genome sequencing for tuberculosis diagnostics
Questions?

We are bioinformaticians, that's what we do.
TB MGIT

Centrifuge

200 ul

56°C 15 min

15 sec 2X

Proteins, inhibitors, etc...

WGS