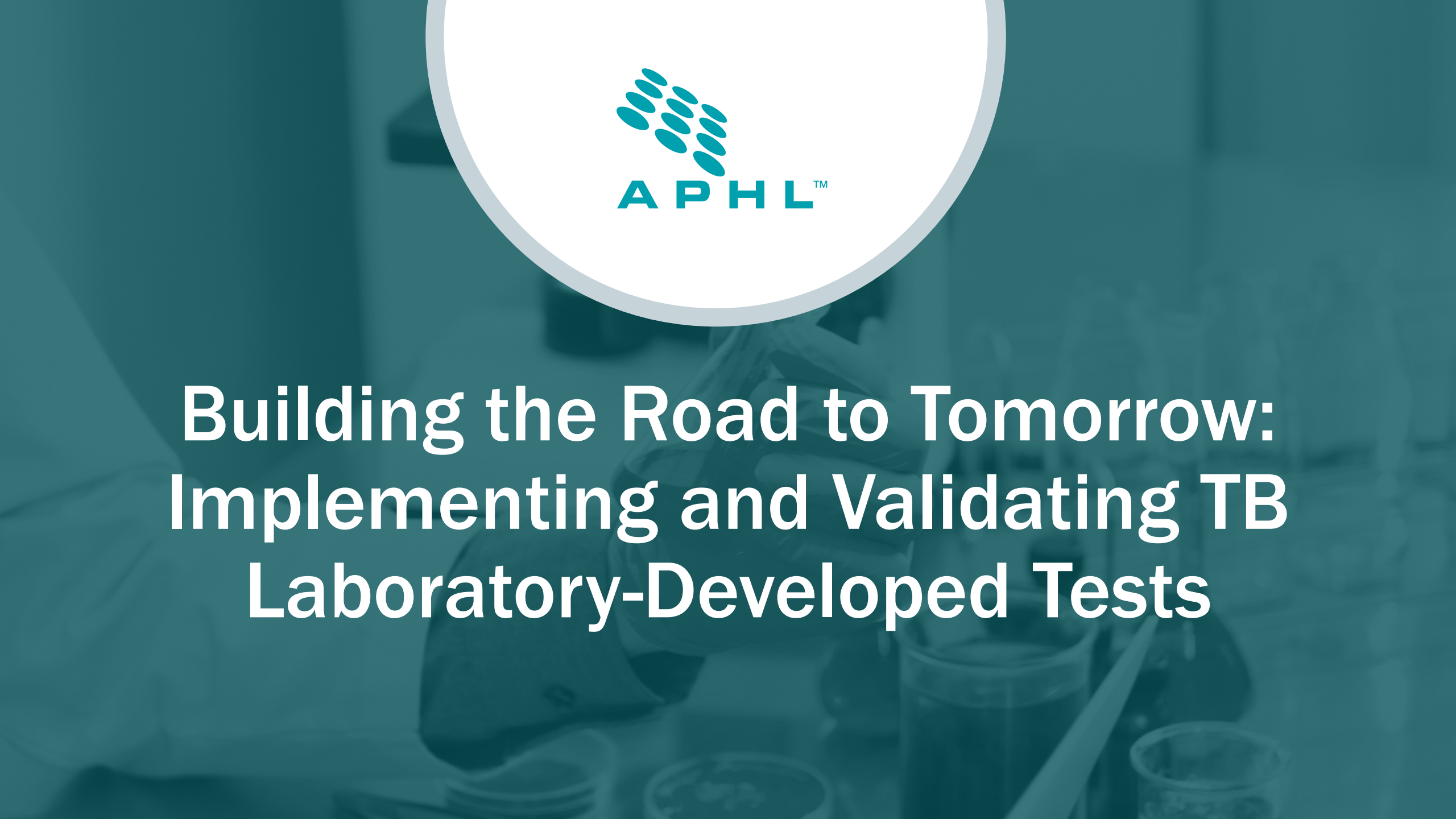

# Building the Road to Tomorrow: Implementing and Validating TB Laboratory-Developed Tests

NEW YORK STATE

Department of Health
Wadsworth Center

# Building the Road to Tomorrow: Implementing and Validating TB Laboratory-Developed Tests

Kimberlee Musser, PhD

APHL 14th National Conference on Laboratory Aspects of Tuberculosis
2026| March 10-12, 2026

# Some basics…

**Laboratory Developed Test-** Diagnostic test that is designed, manufactured, and performed entirely within a single, CLIA/CAP-certified laboratory.

**Validation-** Evidence-based process of ensuring a test, measurement, or system accurately fulfills its intended purpose, requirements, and user needs.

**Implementation of an LDT-** Differences between CLIA, CAP, NYS CLEP

- Clinical Director
- CAP Checklist
- CLEP Microbiology Molecular Checklist and Submission

# Why do we need LDTs for TB Testing?

Very few FDA approved options for identification:

- MALDI Biotyper CA System
- Cepheid Xpert MTB/RIF Assay

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# Implementing and Validating TB Laboratory-Developed Tests




- FDA Modification
- Specimen type
- Specimen volume

- Validating all aspects of an NGS
- Designing a validation study and controls for a highly multiplexed PCR assay

# Clinical Validation and LDT Test Approval

## Standard Operating Procedure

- Intended use
- Limitations
- Step-by-step procedures
- Reports
- Interpretation
- Quality control (QC)
- Assay controls
- Proficiency Testing

## Assay Validation Study

- Specificity
- Sensitivity- Limit of Detection
- Reproducibility: Intra- & Inter-assay
- Accuracy
  - Retrospective studies
  - Prospective studies
  - Comparison to gold standard

## Challenges/Questions

- How many samples do you test?
- Where can you get samples from?
- How do you handle discordance?
- What if there is no gold standard to compare to?
- What if validation is cost prohibitive?

## Building the Road to Tomorrow: Implementing and Validating TB Laboratory-developed Tests

1:30 pm – 2:30 pm • 588-706-25 • contact hours: 1.0

**Moderator:** Kimberlee Musser, PhD, Chief of Bacterial Disease, New York State Department of Health-Wadsworth Center

**Speakers:**

- Yvette Vergnetti, MS, MLS-L, MT/MLS(ASCP), Mycobacteriology Technical Supervisor, Alaska Department of Health
- John Dulany, Senior Public Health Scientist, TB and Mycology, Arizona State Public Health Laboratory
- Matthew Sylvester, PhD, PMH-CA(AAB) Research Scientist Supervisor, Microbial Diseases Laboratory, California Department of Public Health

Beyond Sputum: Validating Additional Specimen Types for GeneXpert MTB/RIF

LDT Validation of Fluoroquinolones for MGIT & Agar Proportion DSTs

Laboratory Developed Test Validation: Targeted Next Generation Sequencing (tNGS)

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# Beyond Sputum: Validating Additional Specimen Types for GeneXpert MTB/RIF

Yvette Vergnetti, MS MLSL, MT/MLS (ASCP)
TB Technical Supervisor

Alaska State Public Health Laboratories (ASPHL) - Anchorage

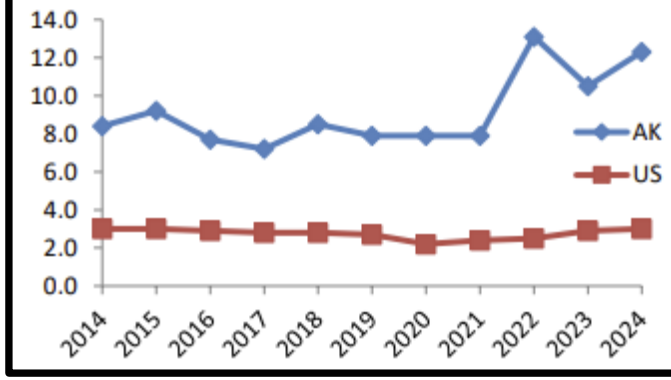
# Alaska TB

## Highest TB Rates in the nation

### *Reported Tuberculosis (TB) Cases*

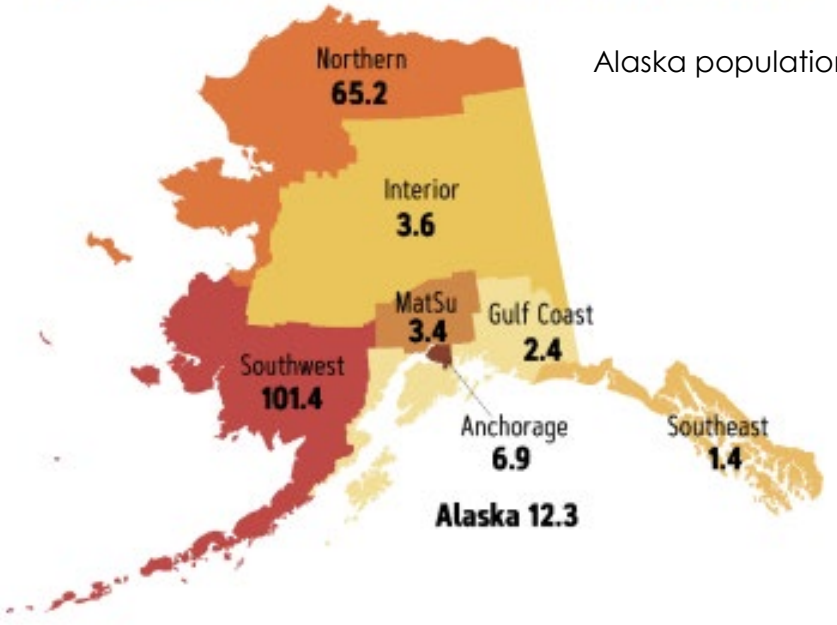
In 2024, 91 cases of TB disease were reported in Alaska, reflecting an incidence rate of 12.3 cases per 100,000 population. This represents an 18% increase in cases from the previous year. The U.S. TB incidence rate for 2024 was 3.0 cases per 100,000, with an 8% increase in cases from 2023.

**Tuberculosis Incidence Rates, Alaska & U.S., 2014-2024**




https://health.alaska.gov/media/mksh4drw/2024-ak-tb-summary-brief-final_111020255.pdf

### Regional Incidence per 100,000 population




Alaska population 739,000

77% of Alaska's 2024 TB cases were among Alaska Native communities
16% Foreign-born

# Alaska State Public Health TB Laboratory

- Only Mycobacteriology laboratory in Alaska
  - Statewide reference laboratory serving hospitals (urban, rural, military), public health clinics and the Health Department
  - CLIA-certified high-complexity laboratory with BSL3 containment
  - 3 FTEs supporting all TB diagnostics
  - Annual testing volume
    - >5,800 specimens processed annually (>7,000 in 2025)
    - >1,000 TB NAAT performed annually (both pulmonary and extrapulmonary)
      - For respiratory AFB smear negative – 2 TB NAATs are performed
  - Full-spectrum TB diagnostics: smear microscopy, solid & liquid culture, GeneXpert NAAT (TB ID), MALDI-TOF ID (MTB/NTM), and first-line drug susceptibility testing including FQs
    - Sequencing for drug resistance (including *pncA*) validation complete – launching next quarter

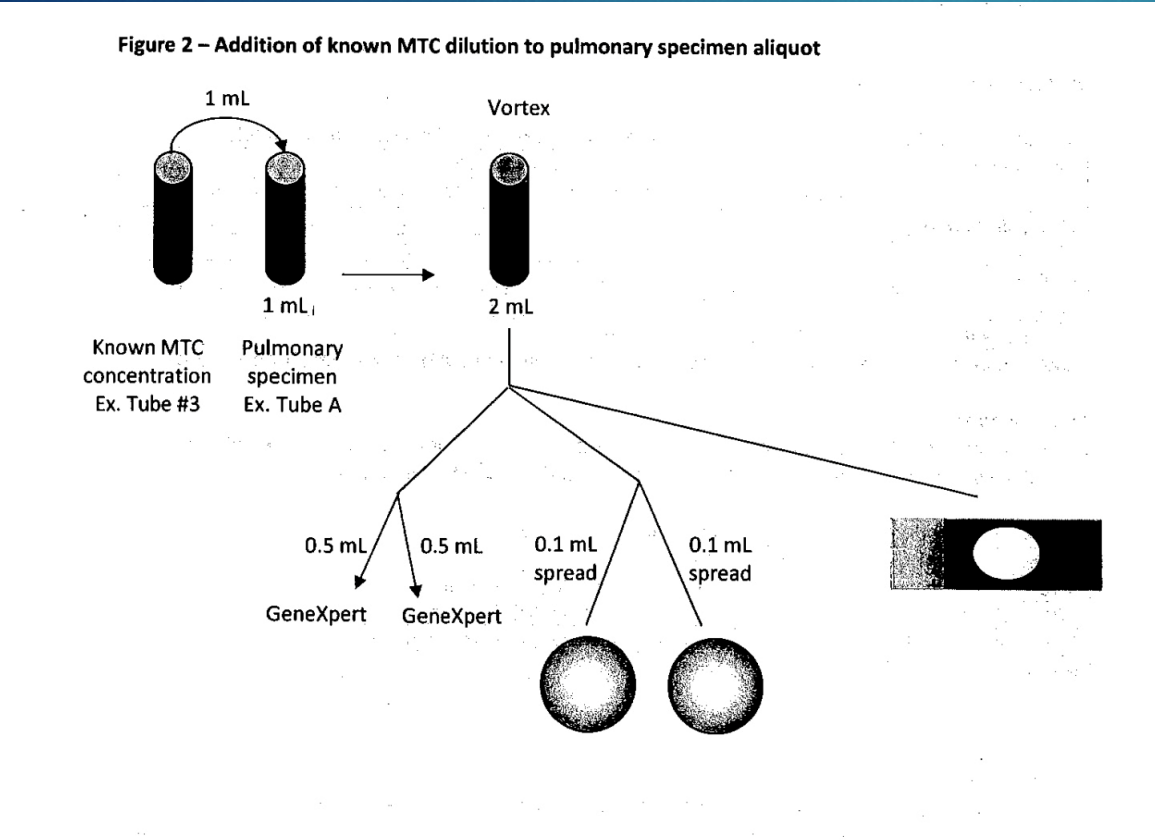
# TB NAAT in Alaska

- LDT TB NAAT implemented in 2011
  - Validated for all sources except for blood, CSF, and stool
  - Labor intensive and time consuming (~4 hours hands-on per testing day)
    - 2-3 FTEs
  - Batched testing 3 times a week
- GeneXpert MTB/RIF assay 2016
  - FDA approved for sputum on patients ≥ 18 years of age only
  - More expensive than LDT
- Maintained both testing platforms to accommodate extrapulmonary and pediatric sputa
  - Not sustainable
  - Cartridge cost justified by improved workflow and freed staff time

# GeneXpert MTB/RIF Validation - Pulmonary

- 2017 a pre-validation feasibility study performed on bronchial lavage/wash and tracheal aspirates
  - Demonstrated acceptable performance prior to formal LDT validation
- Validation Design
  - **Acceptance criteria:** Pulmonary specimens must meet package insert sensitivity for AFB smear and sputum collection method (<3,000 CFU)
  - 20 non-sputum (clinical & spiked)
    - Banked specimens stored at -80°C
    - Negative pooled processed sediments confirmed culture-negative
  - Spiking strategy/target
    - 0.5 McFarland suspensions of $1.5 \times 10^6$ CFU/mL of MTB RIF susceptible and resistant strains
      - Serial dilutions $10^{-1}$, $10^{-2}$, $10^{-3}$, and $10^{-4}$
      - Dilutions $10^{-2}$ through $10^{-4}$ tested

# Limit of Detection (LoD)




For each dilution:

- 1 mL dilution combined with 1 mL pooled culture-negative sediment
- 1 AFB smear prepared, stained, and examined
- 2 GeneXpert MTB/RIF cartridges tested
- 2 Middlebrook 7H11 plates inoculated (100 µL each) for colony enumeration
  - Observed CFUs significantly < 3,000

# Results

- Analytical Sensitivity:
  - Quantitative culture demonstrated starting suspension aligned closer to ~$3.75 \times 10^6$ CFU/mL (vs. intended $1.5 \times 10^6$)
  - Consistent with McFarland variability of >0.5 (clumping, settling, manual turbidity reading)
  - Assay detection met package insert sensitivity target (<3,000 CFU)
- Accuracy: 100% agreement between MTB/RIF and ASPHL reference methods
- Precision: 9 specimens tested by two techs on different days; 100% reproducibility
- Analytical Specificity: NTM panel tested; no cross-reactivity observed
- Conclusion: Validation confirmed GeneXpert MTB/RIF as a reliable platform beyond sputum, enabling sustainable expansion of rapid TB diagnostics in Alaska

# Lessons Learned from Pulmonary Validation

- Pilot first, validate second - small feasibility studies identify issues early
- Target concentrations are theoretical; plate counts are reality
  - Dilution math assumes ideal mixing - MTB clumping, suspension settling, and minor technique differences introduce variability which can shift CFU dramatically
- Standardize McFarland preparation and pipetting technique (be consistent)
  - For example – densitometer vs manual reading
- Decide and document variables in the validation plan
  - For example - If you don't you can end up changing prep methods mid-study
- Unexpected results are data - not failure
- Clear documentation supports regulatory compliance

# Expansion of GeneXpert Validations

- 2018 MTB/RIF Validations
  - Pediatric sputum
  - Gastric aspirates
  - Tissue
  - Body fluids (excluding blood, CSF, urine, and stool)
- 2022 – MTB-positive culture confirmation from solid and liquid media
- What's next - Stool
  - Critical for pediatric and non-expectorating patients
  - Less invasive; easier collection in remote communities
  - Supported by WHO guidance
  - Without validation, patients lack access to this testing

# Thank You


- Acknowledgements
  - ASPHL Mycobacteriology Team
  - ASPHL Laboratory Leadership
  - Alaska TB Program
  - Infectious Disease Specialists and Clinical Partners
  - Statewide submitting facilities and clinicians

- Contact Information


Yvette Vergnetti

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# Laboratory Developed Test Validation: Targeted Next Generation Sequencing (tNGS)

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Mycobacterial Drug Susceptibility Testing Unit
Mycobacterial, Mycotic, & Parasitic Diseases Section
Microbial Diseases Laboratory Branch (MDL)
California Department of Public Health
matthew.sylvester@cdph.ca.gov

**14th National Conference on Laboratory Aspects of Tuberculosis**

**March 11, 2026**

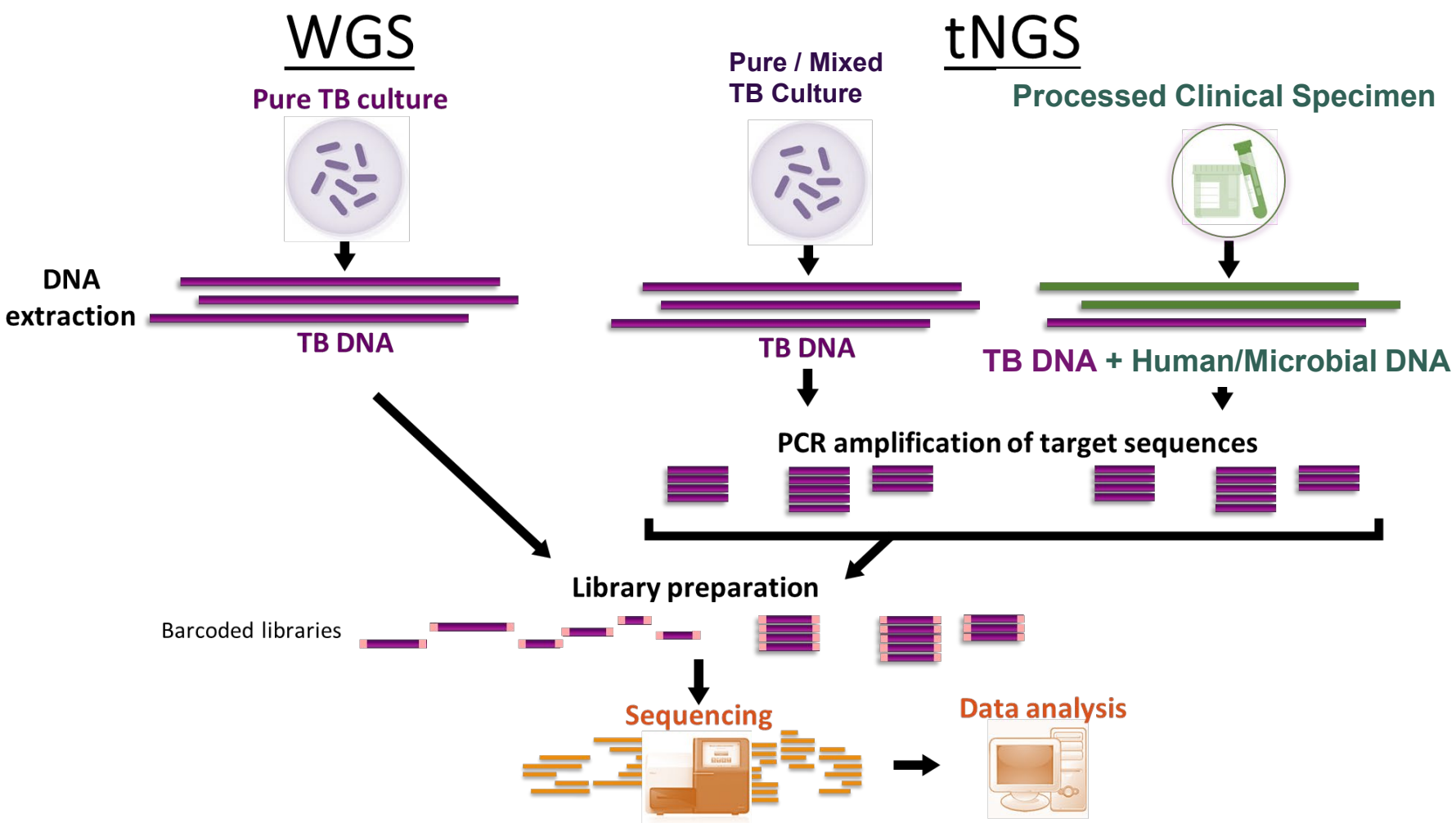
# Disclosures

- **Relevant Financial Relationships**
  - I have nothing to disclose

- **The findings and conclusions in this presentation are those of the author and do not necessarily represent the views or opinions of the California Department of Public Health (CDPH) or the California Health and Human Services Agency.**

- **The use of brand names and/or any mention or listing of specific commercial products or services herein is solely for educational purposes and does not imply endorsement by CDPH or our partners, nor discrimination against similar brands, products, or services not mentioned.**

# Targeted NGS (tNGS) LDT Assay




## tNGS at CDPH-MDL

Deeplex Myc-TB (GenoScreen, France) (May, 2024)

↓

Unavailability of Reagents: Test Discontinued (January, 2025)

↓

Modified CDC tNGS Assay (March, 2026)

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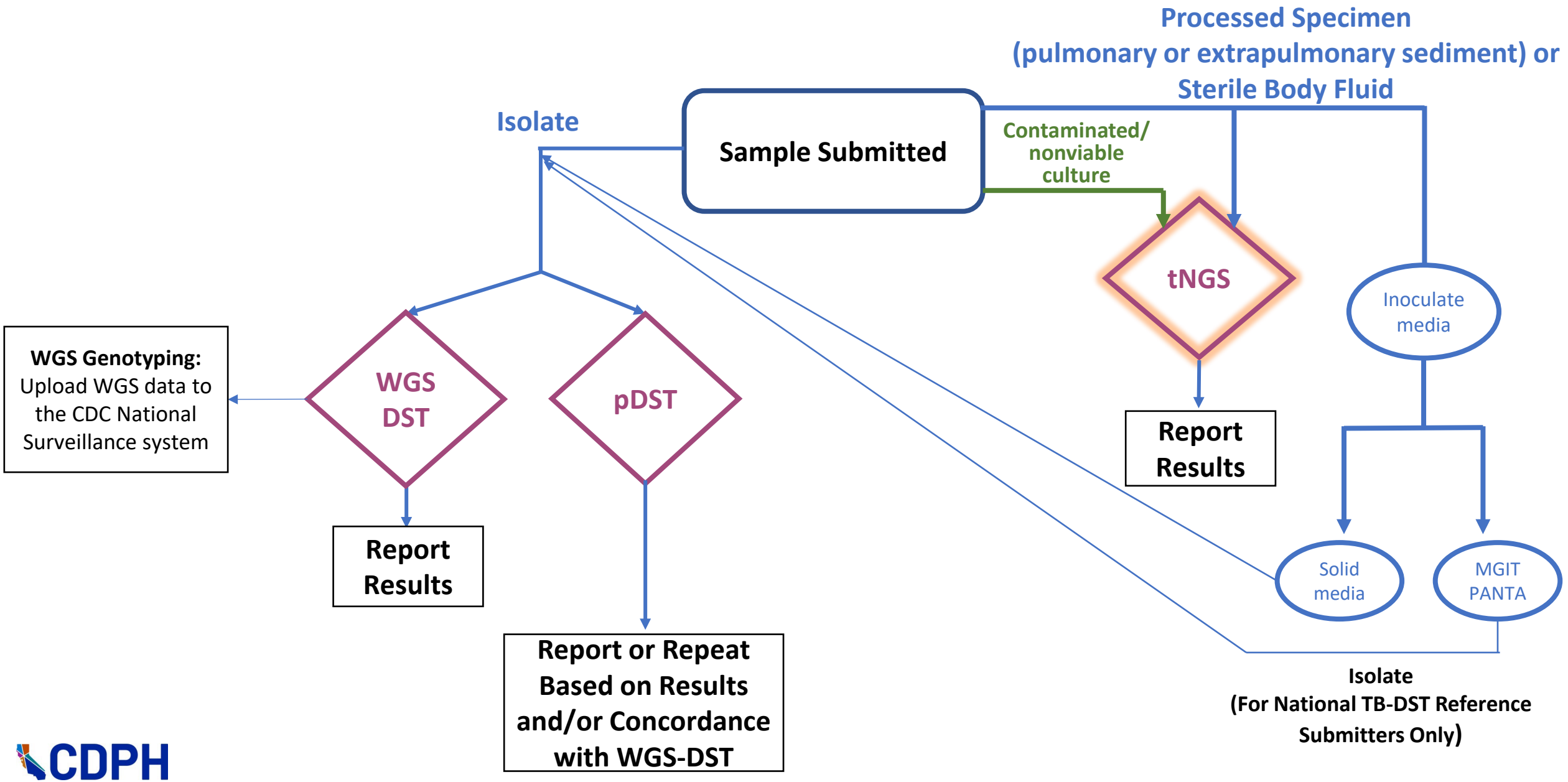
# Overall TB-DST Workflow

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# Targeted NGS LDT Assay Design: Extraction and RT-PCR

## Extraction

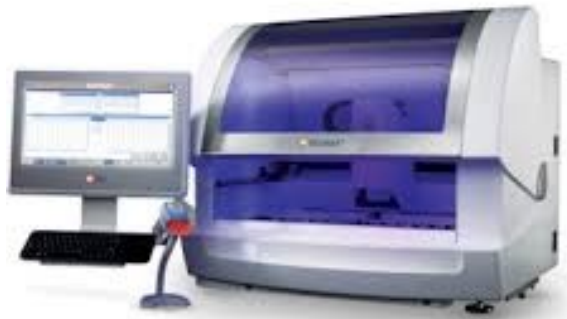
Add sample to tube with beads

Heat kill at 90°C for 30min

Bead Beat on Fastprep

Purify on Promega Maxwell

## RT-PCR



Screening RT-PCR on BDMax Platform with BioGX reagents:
- -Confirm presence of TB
- -Confirm sufficient TB load for tNGS (Ct value)

Notes:
- -Validated in parallel with tNGS
- -Ct values may not reflect those of GeneXpert MTB/RIF

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# Targeted NGS LDT Assay Design: Illumina Library Prep and Sequencing

Singleplex PCRs (20) and Illumina DNA Prep Pre-PCR

QC Checkpoint: after targeted PCR pooling and clean-up

Post-PCR Clean-up

QC Checkpoint: After library clean-up

Pool and Load

iSeq 100 v3 kit

or

MiSeq i100
5M 300-cycle or
600-cyle kits

Max: 6 samples + External Positive Control + External Negative Control

Bioinformatics Analysis

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# Bioinformatics Pipeline

Theiagen GENOMICS

Weekly Standing Meetings

CDPH California Department of Public Health

TB Profiler

FASTQ Files Uploaded to Cloud

Terra


Observed Genotype-Phenotype correlations and Expert Rules (e.g. Loss of Function)

SNPs, MNPs, small InDels, and large structural variants fed into the **Tbp-parser** script

Outputs:
1. QC reports
2. CSV file for CDPH StarLIMS upload

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# CLIA-Compliant LDT Validation Timeline

Pilot studies and write validation plan

Iterative bioinformatics analysis and development

Feb – Aug, 2025 | Sep | Oct | Nov | Dec | Jan | Feb | Mar

Wet lab sequencing runs

Finalize SOPs and write/review validation report

| | | |
|---|---|---|
| Status: FINAL | California Department of Public Health<br>Microbial Diseases Laboratory (MDL)<br><br>Mycobacterial, Mycotic & Parasitic Diseases Section (MMPDS) | Validation Report |
| | | MDL-MMPDS-DST-VR-003 |
| Version: 021126 | | Page 1 of 129 |

+28 Appendices!!!

Validation of MTBC mCDC tNGS-DST - Report

TITLE: **Validation of Targeted Next Generation Sequencing-Based Drug Susceptibility Testing of *Mycobacterium Tuberculosis* Complex from Sediments and Cultures Using Modified Centers for Disease Control and Prevention Primer Set - Report**

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# Summary Performance Parameters

| Performance Characteristic | Expected Values |
|---|---|
| Accuracy | ≥90% |
| Diagnostic Sensitivity | ≥90% |
| Diagnostic Specificity | ≥90% |
| Analytical Sensitivity<br>(Limit of detection--LOD) | N/A |
| Analytical Specificity | Cross-reactivity: None<br>Interference: None |
| Reportable Genomic Range | Within primer boundaries |
| Precision (Repeatability & Reproducibility) | 100% |

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# Reference Methods

| Validation Objective | Reference Method | Performance Parameters Calculated for: |
|---|---|---|
| Validity of tNGS Susceptible(S) / Resistance(R) predictions | Validated pDST, WGS S/R predictions, Deeplex Myc-TB tNGS S/R predictions | Each drug and overall |
| Validity of detected genomic variations (e.g. SNPs & InDels) | Previously-validated sequencing methods (WGS, Deeplex Myc-TB tNGS) | Each variation in the reportable genomic range (determined during validation) |
| Accurate identification of *M. tuberculosis* complex (not *M. bovis*) vs. *M. bovis*. | Validated sequencing methods (Pyrosequencing, WGS, Deeplex Myc-TB tNGS) | Each sample |

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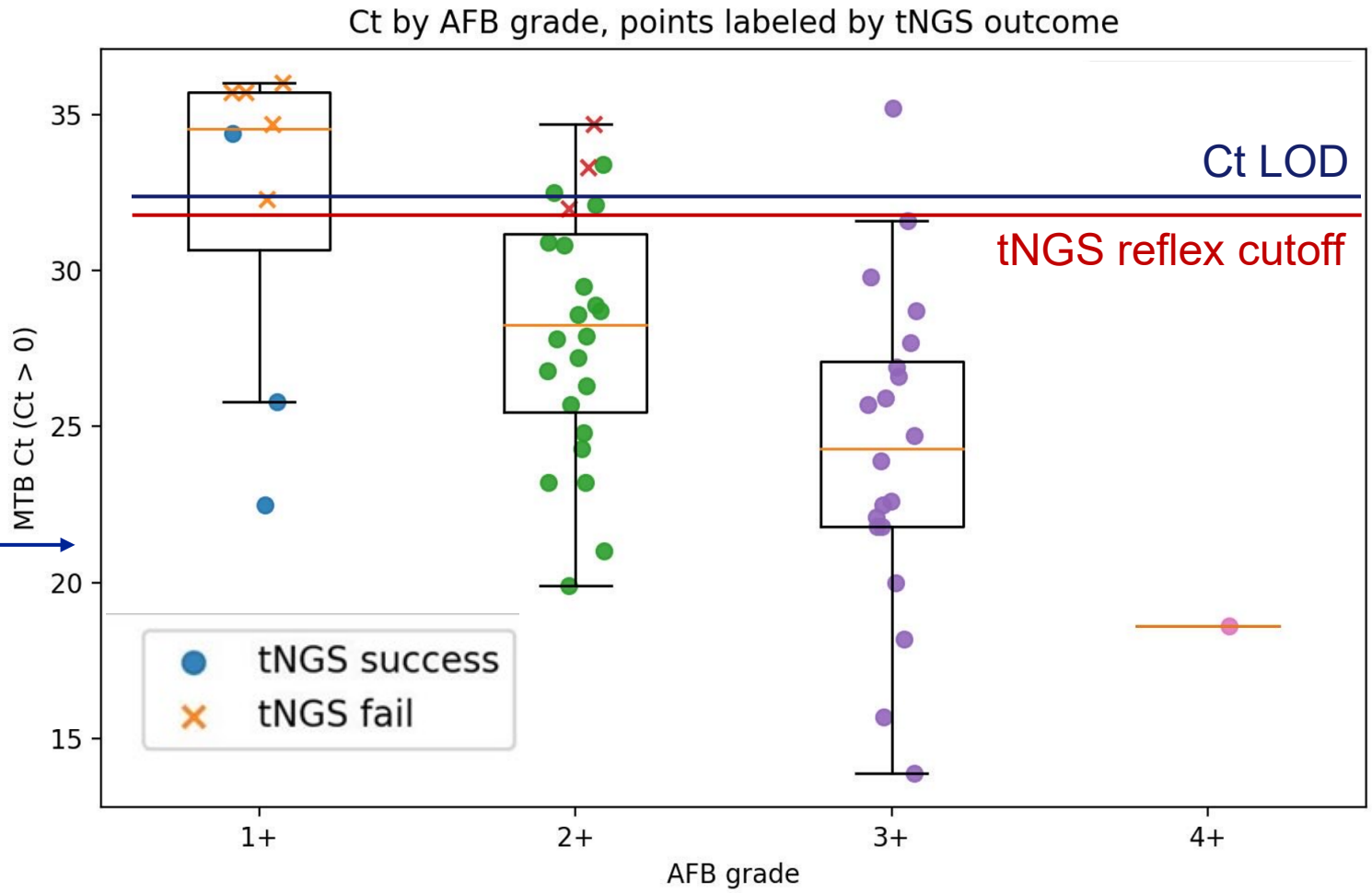
# Sample Types for Validation (Accuracy, Sensitivity, Specificity)

| Type of validation material | Patient Material Source | MTBC status | N | Additional performance parameter | Covered Variability |
|---|---|---|---|---|---|
| **Clinical Sediments:** From CDPH, Kaiser Regional Laboratory | Pulmonary | **MTBC-positive** | 34 | | 1+ to 4+ AFB grades. |
| | | MTBC-negative | 2 (NTM neg) | | |
| | Extrapulmonary samples (body fluid, processed tissue) | **MTBC-positive** | 20 | | |
| | | MTBC-negative | 10 (5 NTM neg, 5 NTM pos) | Cross-reactivity (NTM) | |
| **Contrived samples:** Cultures spiked into negative sediments at ~1 log above LOD (1:1000 of a 0.5McF) | Pulmonary | **MTBC-positive** | 18 | Repeatability /Reproducibility | Increased variety: Mostly R; *M. bovis* and *M. bovis* BCG; Naturally occurring heteroresistance; Low-level RIF-R. |
| **Cultures** | N/A (extracted from pure culture) | **MTBC strains** | 5 strains (14 tests with 3 strains x 4 media types) | MTBC ID panel: additional *M. bovis* & *M. bovis* BCG | Lowenstein-Jensen [LJ], 7H10, 7H11, early-positive MGIT |
| | N/A (simulated mixed cultures with NTM) | **MTBC-positive** | 12 | Cross-reactivity (NTM) | Mixed cultures |

Samples (N=91 unique) were chosen to cover a range of representative mutations and drug resistance

# Limit of Detection (LOD): Biological

- *In vitro* spike-in
  - H37Rv dilutions spiked into negative sediment
  - The lowest amount of MTBC yielding successful sequencing of all loci as well a accurate identification of genomic variations, correct S/R profile prediction, and MTBC ID in 95% of contrived specimens.
  - Additional contrived samples made at ~1 log above the LOD to assess other performance parameters
- *Clinical specimens*
  - The lowest amount of MTBC (AFB gradin or NAAT Ct values) yielding successful sequencing in 95% of clinical specimens.
  - LOD determined to be Ct=32.5 from the BDMax RT-PCR assay (Note: may not correlate with GeneXpert) when combinec with results from contrived samples

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# Matrix Effects

| | All Ct values | | Ct ≤Cutoff Threshold | |
|---|---|---|---|---|
| **Specimen Category** | Total | % Succeeded | Total | % Succeeded |
| **Sputum** | 37 | 91.9% | 31 | 100.0% |
| **Sterile body fluid** | 9 | 100.0% | 9 | 100.0% |
| **Tissue** | 25 | 76.0% | 16* | 87.5%* |
| **Urine** | 6 | 66.7% | 4 | 100.0% |
| **Total** | 77 | 85.7% | 60 | 96.7% |

*Matrix associated with sequencing failures but not reportable false variant calls

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# Limit of Detection (LOD): Bioinformatic

**Variant Read Support:** minimum read support permitting accurate identification of genomic variations in all tNGS DST loci with at least 95% accuracy.

Method:

- Clinical WGS sequences downsampled bioinformatically

**Heteroresistance:** Minimum allele frequency permitting accurate identification of genomic variations in all tNGS DST loci with at least 95% accuracy.

Method:

- Clinical samples with low-level heteroresistance
- Mixed WT and mutant DNA at AF=10%, 15%, 20%, 30%, undiluted

| Parameter | Method | N datapoints | Obtained range | FP | FN | LoD95 (probit) |
|---|---|---|---|---|---|---|
| **Read support** | Bioinformatic downsampling | 908 | 8x-165x | 0 | 196 | 19.65 x |
| **Allele frequency** | Different ratios of WT and mutant DNA mixed *in vitro* | 60 | 9.5% - 100% | 0 | 8 | 11.2 % SNPs / 16.7% InDels |

- 200x coverage permits detection of variants down to ~10% allele frequency
- 99.75% of reportable loci had coverage >200x

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# Cross-Reactivity with Nontuberculosis Mycobacteria

| MTBC DNA | NTM DNA | NTM | rpoB | rrs | rrl |
|---|---|---|---|---|---|
| 90% | 10% | M. avium | p.Ser431Ile; p.Ser431Tyr; p.Leu443Ser; p.Leu443Phe; p.Asp190Glu; p.Ser239Asn; p.Val243Thr; p.Val243Ile; p.Arg253fs; p.Ser254fs; p.Val262Ala; p.432; p.Gln432Gln; p.Leu443Leu; p.Arg448Arg | n.1259C>T; n.1260G>A; n.1278A>T; n.1300C>T; n.1321G>A; n.1445C>T | No mutations detected |
| 50% | 50% | M. avium | p.Ser431Pro; p.Leu443Phe; p.Asp190Glu; p.Ser239Asn; p.Val243Thr; p.Val243Ala; p.Arg253fs; p.Ser254fs; p.Val262Ala; p.Val355Ile; p.432; p.Gln432Gln; p.Leu443Leu; p.Arg448Arg | n.1259C>T; n.1260G>A; n.1278A>T; n.1300C>T; n.1321G>A; n.1445C>T | No mutations detected |
| 10% | 90% | M. avium | p.Ser431Pro; p.Ser431Cys; p.Leu443Pro; p.Leu443Phe; p.Asp190Glu; p.Ser239Asn; p.Val243Thr; p.Val243Ile; p.Val243Ala; p.Arg253fs; p.Ser254fs; p.Val262Ala; p.Val355Ile; p.432; p.Gln432Gln; p.Arg448Arg | n.1259C>T; n.1260G>A; n.1278A>T; n.1300C>T; n.1321G>A; n.1445C>T | No mutations detected |
| 90% | 10% | Mix: M. abscessus, M. kansasii, M. fortuitum | p.Thr184Ser; p.Asp190Glu; p.Ser201Asn; p.Ser201Ala; p.Ser201Gly; p.Ser201Cys; p.Ser239Asn; p.Arg253fs | n.1278A>T | n.2331A>G |
| 50% | 50% | Mix: M. abscessus, M. kansasii, M. fortuitum | p.Thr184Ser; p.Asp190Glu; p.Ser201Asp; p.Ser201Gly; p.Ser201Cys; p.Ser239Asn; p.Arg253fs; p.Val262Ala; p.Gly426Gly; p.Phe433fs; p.Gln432fs; p.Leu443Leu | n.1278A>T | n.2002G>A; n.2331A>G |
| 10% | 90% | Mix: M. abscessus, M. kansasii, M. fortuitum | p.Thr184Ser; p.Asp190Glu; p.Ser201Gly; p.Ser201Cys; p.Ser239Asn; p.Val243Asn; p.Val243Thr; p.Val243Ile; p.Arg253fs; p.Val262Ala; p.Thr264Pro; p.Met387Leu; p.Gly426Gly; p.Phe433fs; p.Gln432fs; p.Gly442Gly; p.Leu443Leu | n.1278A>T | n.2002G>A; n.2331A>G; n.2567A>G; n.2594T>C; n.2624T>C; n.2640C>T; n.2642C>T; n.2872A>G; n.2923G>A; n.2940G>A; n.2962C>T; n.2971T>A; n.3022T>C |

**False positives detected for: *rpoB, rrs, rrl, & rplC***

- Often cluster
- Single false variants may be present in other genes

**Thresholds set to minimize false variants:**

- For mutations with allele frequency (AF) = 10%-94.99% => min 500x coverage required
- For mutations with allele frequency (AF) ≥ 95% => 100x coverage required

**Rule for reporting:** Suppress reporting for all of these loci if 4 or more reportable mutations appear in one locus => 100% of FP mutations filtered

# Interfering Substances

| Substance | Results |
|---|---|
| Whole blood | No inhibition observed up to 10% (v/v) |
| Specimen Processing Reagents (i.e. AlphaTec NAC-PAC EA3) | Loci failed sequencing (low coverage) with 125µl residual processing reagents |
| Rifampin | No inhibition observed up to 25 µg/ml |
| Ethambutol | False positives observed at 25 µg/ml |

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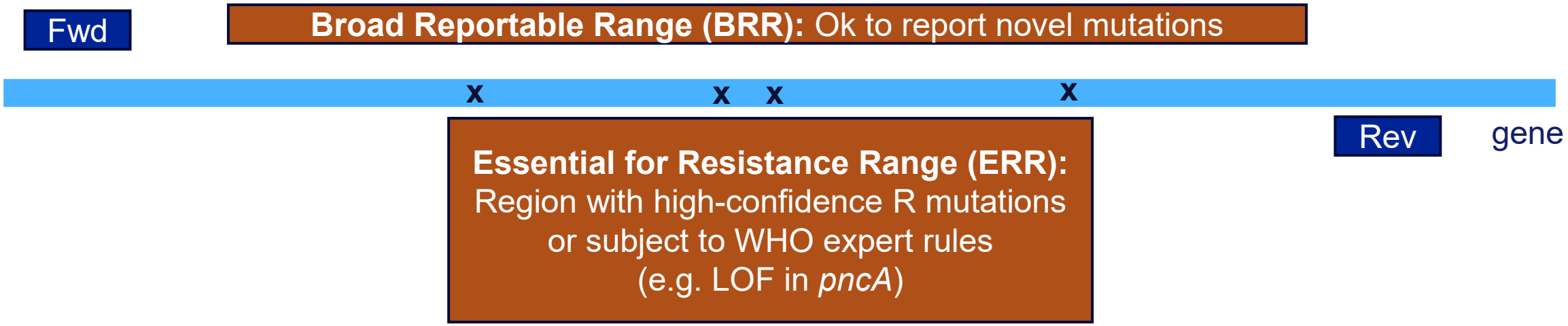
# Reportable Genomic Range

Criteria for inclusion of a position into the *broad* reportable genomic range:

- Associated with correct S/R profile in ≥ 90% of samples and correct variant detection in 95% of samples
- At least 90% of validation samples must have had the position covered by at least 50x




Of n=839 locus-level observations:

- 98.33% of observations have BRR=100%
- 99.76% have ERR=100%

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# Performance Parameter Results by Drug (Compared with Phenotypic DST)

| Drug | N | TP | TN | FN | FP | Accuracy | Sensitivity | Specificity | PPV | NPV |
|---|---|---|---|---|---|---|---|---|---|---|
| INH | 95 | 32 | 63 | 0 | 0 | **100%** | **100%** | **100%** | 100% | 100% |
| ETO | 77 | 25 | 52 | 0 | 0 | **100%** | **100%** | **100%** | 100% | 100% |
| RIF | 97 | 21 | 76 | 0 | 0 | **100%** | **100%** | **100%** | 100% | 100% |
| PZA | 97 | 31 | 65 | 1* | 0 | **99.00%** | **96.90%** | **100%** | 100% | 98.50% |
| EMB | 99 | 17 | 82 | 0 | 0 | **100%** | **100%** | **100%** | 100% | 100% |
| AMK | 83 | 9 | 74 | 0 | 0 | **100%** | **100%** | **100%** | 100% | 100% |
| KAN | 81 | 9 | 72 | 0 | 0 | **100%** | **100%** | **100%** | 100% | 100% |
| CAP | 91 | 8 | 83 | 0 | 0 | **100%** | **100%** | **100%** | 100% | 100% |
| MFX | 65 | 14 | 51 | 0 | 0 | **100%** | **100%** | **100%** | 100% | 100% |
| LFX | 80 | 14 | 66 | 0 | 0 | **100%** | **100%** | **100%** | 100% | 100% |
| BDQ | 81 | 1 | 80 | 0 | 0 | **100%** | **100%** | **100%** | 100% | 100% |
| CFZ | 84 | 1 | 83 | 0 | 0 | **100%** | **100%** | **100%** | 100% | 100% |
| LZD | 73 | 0 | 73 | 0 | 0 | **100%** | **n/a** | **100%** | n/a | 100% |
| Total | 1103 | 182 | 920 | 1 | 0 | **99.90%** | **99.50%** | **100%** | 100% | 99.90% |

*Mutation failed read support

- Also compared genomic variations

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# Summary Performance Parameters

| Performance Parameter | Expected Values | Achieved Values |
|---|---|---|
| Accuracy | ≥90% | 99.9% (S/R profile); 99.94% (Genomic Variations); 98% (MTBC ID) |
| Diagnostic Sensitivity | ≥90% | 99.5% (S/R profile); 100% (Genomic Variations); 98% (MTBC ID) |
| Diagnostic Specificity | ≥90% | 100% (S/R profile); 99.94% (Genomic Variations); 100% (MTBC ID) |
| Analytical Sensitivity (Limit of detection) | N/A | AFB 2+ (pulmonary); AFB 3+ (tissues); Ct=32.5 based on 95% of samples sequencing successfully |
| Analytical Specificity | Cross-reactivity: None<br>Interference: None | Cross-reactivity with NTM for *rpoB, rrs, rrl, rplC*<br>Interference observed with a FP mutation with EMB |
| Reportable Genomic Range | Within primer boundaries | Broad reportable range and essential for reporting range defined for each locus |
| Precision (Repeatability & Reproducibility) | 100% | 100% |

# Acknowledgements


- *CDPH Infectious Diseases Laboratory Division:*
  - *Zenda Berrada, MDL Lab Director*
  - *Varvara Kozyreva, Mycobacterial, Mycotic, and Parasitic Diseases Section Chief*
  - *Tanya Holbrook*
  - *Maftuna Gafurova*
  - *Josellin Figueroa-Sandoval*
  - *Cailin Mirabella*
  - *Alessandra Wetmore*
  - *Christian Lopez-Pena*
  - *Terry Weber*
  - *Angus Angermeyer*
- *Kaiser Permanente*
  - *Ali Aljanabi*
- *Theiagen Genomics:*
  - *Sage Wright*
  - *Theron James*
  - *Andrew Lang*
  - *Deborah Young, Krisandra Allen*
- *CDC:*
  - *Angela Starks*
  - *Jamie Posey*
  - *Lauren Cowan*
- *APHL:*
  - *Sarah Buss*
- *ARUP*
  - *Salika Shakir*
- *Wadsworth Center:*
  - *Kim Musser*
  - *Michelle Dickinson*
  - *Joseph Shea*
  - *Vincent Escuyer*
- *Jody Phelan, London School of Hygiene and Tropical Medicine*
- *Florida Bureau of Public Health Laboratories*
  - *Marie-Claire Rowlinson*
  - *Patrick Valois*
  - *Calin Chiribau*
  - *Matthew Schimenti*


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