# Validation of the NTM-DR Line Probe Assay Employing Nontuberculous Mycobacterial Whole Genomic Sequence Data as Reference Standard

L. Elaine Epperson[1], Rebecca M. Davidson[1], Sara Kammlade[1], Nabeeh A. Hasan[1], Sophie E. Nick[1], Iara M. P. Machado[2], Valerie H. Rodriguez[2], Aaron Appleman[2], Niels K. Helstrom[2], Michael Strong[1], Max Salfinger[2]

[1]Center for Genes, Environment and Health, and [2]Mycobacteriology, Advanced Diagnostics Laboratories
National Jewish Health, Denver, CO

## Introduction

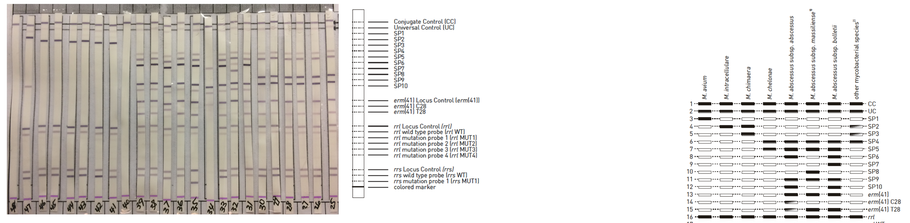
### Objective

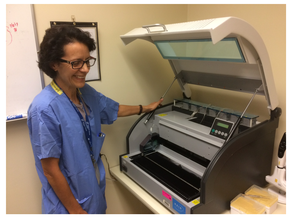
Nontuberculous mycobacteria (NTM) are increasingly isolated from sputum of cystic fibrosis (CF) patients. Effective treatment depends on accurate species identification and determination of macrolide (MA) and aminoglycoside (AG) resistance. Current approaches use time-consuming Sanger sequencing and culture-based antimicrobial susceptibility testing. An accurate test to identify NTM species and resistance markers in one day is a significant unmet need. The Hain NTM-DR line probe assay (LPA) achieves this with a rapid hybridization method. We tested and validated the LPA against a panel of whole genome sequences from 284 NTM isolates.

### Methods

Genomic DNAs from the Colorado CF Research and Development Program (RDP) collection and non-CF control DNAs were PCR amplified, hybridized, developed, and interpreted according to the manufacturer protocol (Hain Lifescience, Nehren, Germany.) Results were compared to whole genome phylogenomic trees to identify species. The LPA antimicrobial resistance results were compared to corresponding sequences of three extracted NTM genomic loci: *erm*(41), 16S rRNA (rrs), and 23S rRNA (rrl).

## Line Probe Assay NTM-DR version 1.0



Dr. Iara Machado with the line probe assay hybridization instrument, capable of processing 48 samples at once

**Table 1:** Mutations in the *rrl* gene and the corresponding wild type and mutation bands [2]

| Failing wild type band | Analyzed nucleic acid position | Developing mutation band | Mutation | Phenotypic resistance |
|---|---|---|---|---|
| *rrl* WT | 2058-2059 | *rrl* MUT1 | A2058C | Macrolides |
| | | *rrl* MUT2 | A2058G | |
| | | - | A2058T | |
| | | *rrl* MUT3 | A2059C | |
| | | *rrl* MUT4 | A2059G | |
| | | - | A2059T | |

*rrs*
The *rrs* gene is examined for detection of resistance to aminoglycosides [kanamycin, amikacin, gentamicin].
The wild type probe comprises the most important resistance region of the *rrs* gene [see table 2]. When the wild type probe stains positive, there is no detectable mutation within the examined region. In case of a mutation, the respective amplicon cannot bind to the wild type probe resulting in the absence of the wild type probe signal.
The mutation probe detects the most common resistance-mediating mutation [see table 2].
Each pattern deviating from the wild type pattern [see evaluation example 1] indicates, as far as is known, an aminoglycoside resistance of the tested strain.

**Table 2:** Mutations in the *rrs* gene and the corresponding wild type and mutation bands [3-5]

| Failing wild type band | Analyzed nucleic acid position | Developing mutation band | Mutation | Phenotypic resistance |
|---|---|---|---|---|
| *rrs* WT | 1406-1409 | *rrs* MUT1 | A1408G | Aminoglycosides |
| | | - | T1408A | |
| | | | C1409T | |

## NTM Species Identification Results

| NTM species ID | LPA v WGS n (284) | sens † | spec |
|---|---|---|---|
| *M. abscessus* subsp. *abscessus* | 122 | 100 | 100 |
| *M. abscessus* subsp. *massiliense* | 36 | 100 | 100 |
| *M. abscessus* subsp. *bolletii* | 5 | 100 | 100 |
| *M. intracellulare* | 27 | 100 | 98.8 |
| *M. avium* | 75 | 100 | 100 |
| *M. chimaera* | 10 | 60 | 100 |
| *M. chelonae* | 1 | 100 | 100 |
| Other NTM | 8 | 100 | 99.6 |

Table 1: Hain Genotype NTM-DR Ver. 1.0 results compared to whole genome sequencing (WGS) phylogenomic results. sens, % sensitivity; spec, % specificity; n, number of samples in each category. Number of samples for each species (n), Line Probe Assay (LPA). † Sensitivity and specificity are reported in percentages.

## Antimicrobial Resistance Results

| locus | LPA v WGS n | sens | spec | n, resistant |
|---|---|---|---|---|
| 16S rDNA (*rrs*) AG | 254 | 100 | 100 | 22 (9%) |
| 23S rDNA (*rrl*) MA | 256 | 100 | 100 | 7 (3%) |
| *erm*(41) MA | 127* | 100 | 100 | 109† (86%‡) |

Table 2: Hain Genotype NTM-DR Ver. 1.0 results compared to site-specific extractions of whole genome sequence results for three loci interrogated by the line probe assay, 16S rRNA, 23S rRNA, and *erm*(41) **M. abscessus* subspp. *abscessus* and *bolletii* only, †T at position 28 of full length *erm*(41) indicates inducible macrolide resistance, ‡In spite of their full length *erm*(41) gene, 14% of samples possessed C at position 28, indicating macrolide susceptibility for these strains

## Whole Genome Sequencing of Mycobacteria The Colorado Cystic Fibrosis Research and Development Program

**Clinical Core**
Has enrolled >70 patients in the PREDICT Trial for NTM diagnosis (PRospective Evaluation of NTM Disease In CysTic Fibrosis, NCT02073409), and the PATIENCE Trial for standardized treatment of MAC and *M. abscessus* (Prospective Algorithm for Treatment of NTM in Cystic Fibrosis, NCT02419989) to provide longitudinal samples of very well-phenotyped NTM infection and treatment.

**Culture and Biorepository Core**
Has received, organized, cultured, and stored aliquots of >1800 mycobacterial isolates

**Molecular and Genomics Core**
Has sequenced >900 CF isolates and >300 non-CF isolates to >40X average core genome coverage. Data and species IDs are made available to submitting labs and collaborators

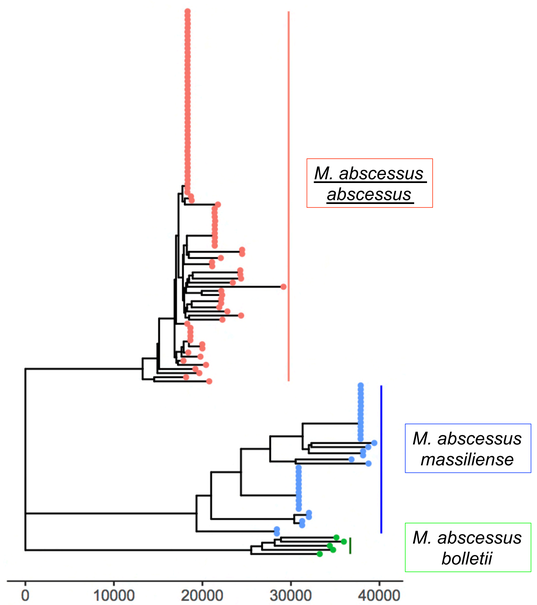
Surveillance: Data mapped to reference genomes to identify species, subtypes, and clade if known. Phylogenomic trees used to determine the proximity of each isolate to other closely related genomes in the database; transmitted strains or those from a common source of exposure are expected to have very few SNP differences.

**Research Collaborations**
https://www.nationaljewish.org/colorado-cf-research-and-development-program/home

## Phylogenomic Identification of NTM Species Using Whole Bacterial Genome Sequences

Illumina reads were trimmed to a phred score minimum cutoff of 20 using Skewer [1]. Trimmed reads were assembled in SPAdes [2]. Assemblies were annotated using Prokka [3]. Full-length 16S rRNA and 23S rRNA genes were extracted from Prokka annotations. The *erm*(41) gene was not identified by Prokka so was extracted from genome assemblies using a custom script.

A phylogenomic tree was created using data from over three million core genome positions to illustrate the *M. abscessus* subspecies identifications by the CF-RDP Molecular and Genomics Core. Shared genomic positions across all isolates were aligned, and a phylogenetic tree was constructed using Neighbor-joining with 100 bootstrap replicates in Molecular Evolutionary Genetics Analysis (MEGA; version 6) [3]. Phylogenetic trees were annotated and visualized with ggtree [4].




## Identification of single nucleotide polymorphisms that are queried by the NTM-DR LPA

For the 16S and 23S rRNAs, full-length sequences from all isolates were aligned using Clustal Omega. For the *erm*(41) gene, only full-length sequences from *M. abscessus* subspp. *abscessus* and *bolletii* were included. Nucleotide calls at positions corresponding to the NTM-DR LPA probes (positions 1406, 1408 and 1409 of the 16S rRNA [5], 2058 and 2059 of the 23S rRNA [6], and position 28 from *erm*(41) [6]), were analyzed and scored for each isolate. Base calls from genome-derived sequences were then compared to those from the NTM-DR LPA to compute sensitivity (sens) and specificity (spec) for each locus.

## Conclusions

The NTM-DR line probe assay offers an accurate and faster alternative to currently available approaches for identifying the most frequently encountered NTM species and subspecies of *M. abscessus* and the *M. avium* complex. This assay promises to be an asset for health care providers who require an understanding of antimicrobial susceptibility to implement the most effective treatments for CF patients.

## Citations

[1] Jiang, H, et al. 2014 (Skewer) PMCID: PMC4074385 [2] Nurk, Sergey, et al. 2013 PMCID: PMC3791033 [3] Kumar S, et al. 2016 PMID: 27004904 [4] Yu G, et al. GGTREE Methods in Ecology and Evolution 2017, 8, 28–36 [5] Nessar, R. et al. 2011 PMCID:PMC3133489 [6] Bastian, S. et al. 2011 PMCID:PMC3028756