DCLS

Division of

Consolidated Laboratory Services

DGS

DEPARTMENT OF GENERAL SERVICES

Real-time PCR detection of *Mycobacterium tuberculosis* complex at the DCLS TB Laboratory

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October 26, 2023

DCLS
Virginia State Public Health Laboratory

- Located in Richmond, VA
- Clinical Testing
- Emergency Testing
- Environmental Testing
- Food Testing
- Newborn Screening Program
- https://dgs.virginia.gov/division-of-consolidated-laboratory-services/

- TB Laboratory
  - ~3,000 specimens per year
  - 200-250 TB patients per year
    - Identification and susceptibilities performed
  - Six full-time scientists

Outline

- Nucleic acid amplification testing (NAAT) for *Mycobacterium tuberculosis* complex (MTBC)
- Workflow and testing scheme
- Verification/validation requirements
- Real-time PCR for MTBC/MAC validation at DCLS
- Post implementation of assay

Rapid Detection of MTBC

- Ability to quickly detect MTBC impacts patient diagnosis, isolation precautions and treatment
- NAAT allows detection of MTBC within hours to days compared to weeks
- CDC TB elimination grant objectives (healthy people 2030)
  - Test every patient by a rapid detection method
  - Report MTBC detection within 48 hours of specimen receipt
- Laboratory developed test method – Wadsworth Center NY State Department of Health

MTBC/MAC Real-time Polymerase Chain Reaction (PCR) Assay

Tests for:

- *Mycobacterium tuberculosis* complex DNA
- *Mycobacterium avium* complex DNA

Tests what:

- Clinical sputum specimens
- Isolates from culture

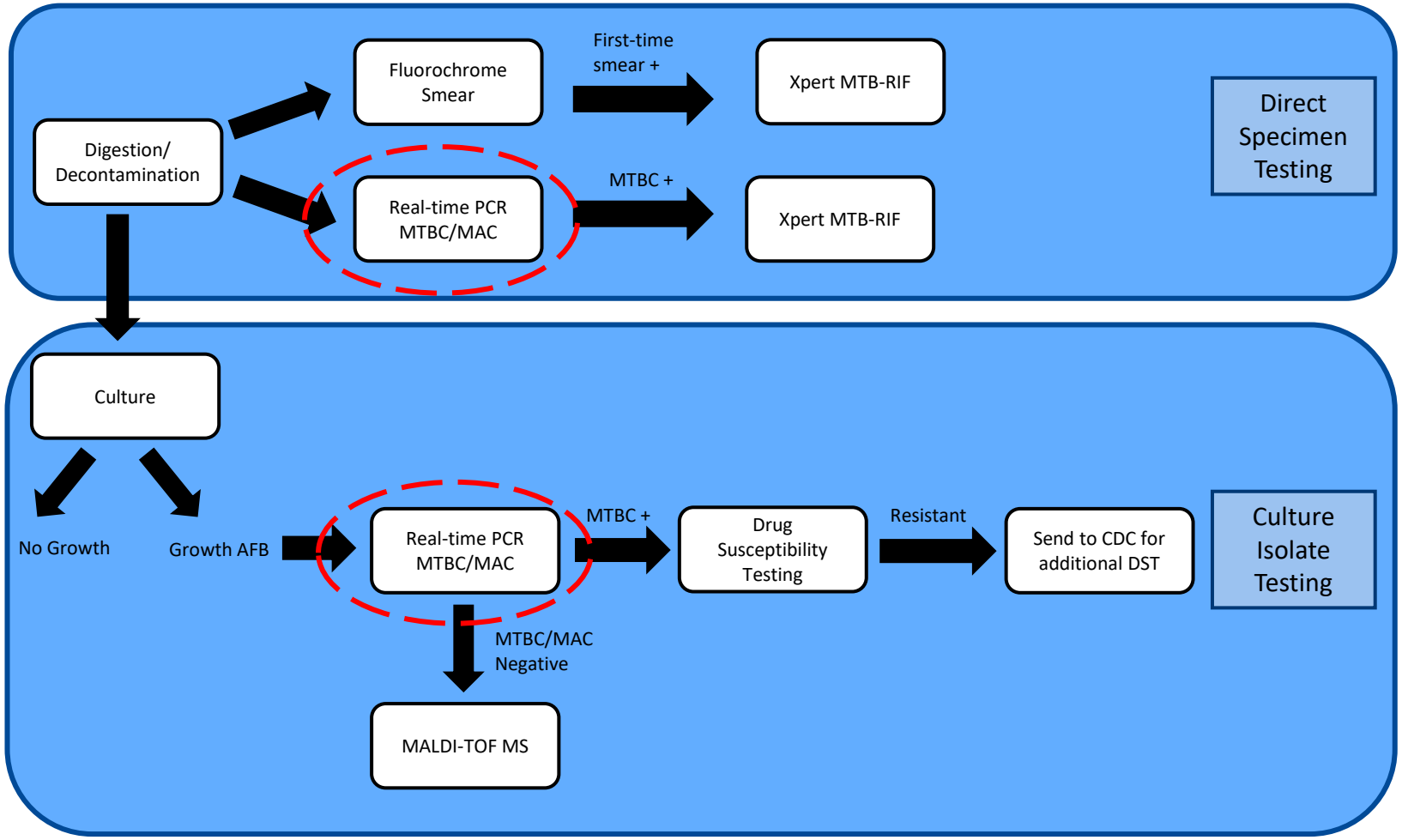
Tests Who:

- Every patient
- Once + PCR result, will test again after 12 months

DCLS TB Laboratory Workflow

Pre-validation

Post-validation



Verification/Validation of Laboratory Test

- Verification – the process to ensure that the laboratory can meet/reproduce the specifications as stated by the manufacturer (FDA-approved)
- Validation – the process to ensure that the test specifications are determined when not using an FDA-approved method (laboratory developed method)

Clinical Laboratory Improvement Amendments (CLIA)

- 493.1253 Standard: Establishment and verification of performance specifications
  - (b)(2) Each laboratory that modifies an FDA-cleared or approved test system, or introduces a test system not subject to FDA clearance or approval (including methods developed in-house and standardized methods such as textbook procedures), or uses a test system in which performance specifications are not provided by the manufacturer must, before reporting patient test results, establish for each test system the performance specifications for the following performance characteristics, as applicable:

- Accuracy
- Precision
- Analytical sensitivity (limit of detection)
- Analytical specificity (interfering substances)
- Reportable range (quantitative only)
- Reference range

Direct Specimen Validation for Real-time PCR Assay

Clinical Samples	Number Tested	+MTBC PCR Result	+MAC PCR Result	Negative PCR Result	Inconclusive PCR Result	Inhibition PCR Result
All specimens	120	53	5	38	4	20

Accuracy of PCR to Detect MTBC DNA

Compared to Culture Results	Number Tested	+ MTBC PCR Result	% Accuracy
MTBC Final	55	45	81.81
Fluorochrome Positive	37	37	100
Fluorochrome Negative	18	8	44.44

High Accuracy

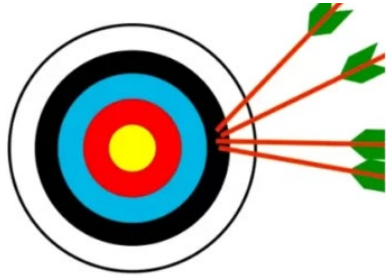
https://proleantech.com/precision-vs-accuracy/

Method Comparison	+MTBC PCR	-MTBC PCR
+MTBC Xpert MTB-RIF	21	0
-MTBC Xpert MTB-RIF	0	5

100% agreement with Xpert MTB-RIF

Precision

- Samples tested on five different days, by two different analysts, on five different instruments
- Repeatability
- Reproducibility



High Precision

https://proleantech.com/precision-vs-accuracy/

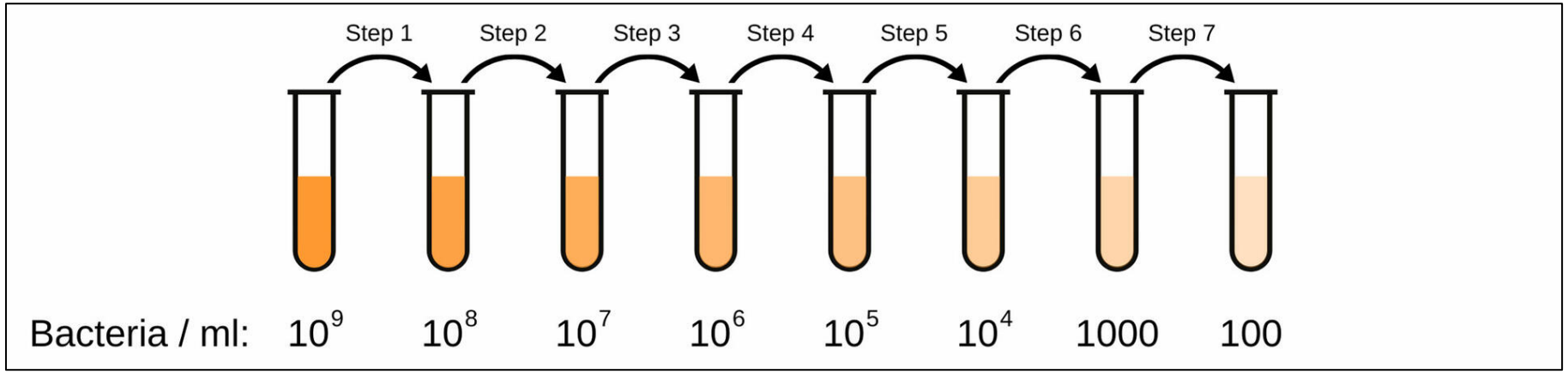
ID	ABI 9			
	Analyst I - 7/30/21			
	Mac	Mtb	Ext-RD9	Bicoid IC
8		16.65/16.70	18.49/18.44	
28				32.77/32.45
40	29.15/29.40			
47		16.27/16.21	18.40/18.29	
58				32.41/33.29
L7		14.10/14.11	15.33/15.39	
L10		14.17/14.23	16.02/15.97	
L21	21.8161/22.24			
L24	21.74/21.93			
L29				30.95/31.80
M8		14.86/14.72	16.51/16.39	
M13		15.85/15.62	17.33/17.19	
M20	21.73/21.67			
M23	23.56/23.82			
M26				32.10/31.70
S5		30.19/30.22	29.83/29.89	
S16		32.94/33.23	34.22/34.91	
S55				33.62/33.97
S78				33.57/33.20
S102	31.67/31.75			
S107	31.40/31.19			
	Analyst II - 8/3/21			
	Mac	Mtb	Ext-RD9	Bicoid IC
8		16.35/16.40	19.49/19.50	
40	29.33/29.02			
L7		14.43/14.10	17.02/17.02	
M23	23.57/23.29			
S5		3049/30.26	3154/31.42	
S55				32.72/31.72
S78				32.24/33.35
S107	31.94/31.32			

ID	ABI 10			
	Analyst I - 8/2/21			
	Mac	Mtb	Ext-RD9	Bicoid IC
8		16.5/16.33	18.28/18.17	
28				32.59
40	29.33/29.89			
47		16.11/16.42	18.17/18.63	
58				33.38/33.75
L7		14.17/14.35	15.37/15.63	
L10		14.8/14.35	15.37/15.63	
L21	21.70/22.08			
L24	21.49/21.91			
L29				31.16/31.25
M8		15.56/15.59	17.37/17.36	
M13		16.32/16.29	17.82/17.85	
M20	21.90/21.79			
M23	23.72/23.82			
M26				31.37/30.79
S5		30.44/30.52	30.27/30.11	
S16		33.28/33.51	35.80/35.2	
S55				33.76/33.29
S78				34.29/33.90
S102	32.13/33.02			
S107	31.26/31.18			
	Analyst II - 7/30/21			
	Mac	Mtb	Ext-RD9	Bicoid IC
8		17.05/16.37	18.72/18.42	
40	29.35/29.36			
L7		14.15/14.10	15.28/15.27	
M23	24.47/23.99			
S5		30.37/29.97	29.42/29.44	
S55				32.56/32.46
S78				33.14/32.21
S107	31.37/30.80			

ID	ABI 11			
	Analyst I - 8/3/21			
	Mac	Mtb	Ext-RD9	Bicoid IC
8		15.42/15.43	16.64/16.59	
28				31.95/33.14
40	26.82/26.72			
47		15.64/15.89	17.15/17.42	
58				31.81/33.05
L7		13.76/13.69	14.51/14.47	
L10		13.95/14.00	15.00/15.02	
L21	22.28/22.14			
L24	21.59/22.01			
L29				31.40/36.03
M8		14.76/14.74	15.97/15.94	
M13		15.47/15.404	16.35/16.32	
M20	21.42/21.90			
M23	23.37/24.84			
M26				32.58/34.47
S5		29.67/29.55	28.80/28.63	
S16		32.81/32.29	35.02/34.51	
S55				32.58/32.40
S78				32.45/33.23
S102	32.69/32.84			
S107	31.85/31.91			
	Analyst II - 8/2/21			
	Mac	Mtb	Ext-RD9	Bicoid IC
8		16.88/16.76	19.3/19.25	
40	28.87/29.07			
L7		15.02/15.06	16.73/17.04	
M23	23.93/23.61			
S5		30.77/3089	31.20/31.27	
S55				33.41/33.01
S78				32.30/33.06
S107	31.28/31.03			

Limit of Detection

LOD study	Sample	Last Dilution Detected	LOD (CFU/mL)
Sputum	*M. tuberculosis* (4.22 x 10^7) *M. avium* (6.39 x 10^8)	10^{-7} 10^{-5}	4.22 6,390



https://www.integra-biosciences.com/china/en/blog/article/how-do-serial-dilutions-including-calculations

Interfering Substances

	1:1 Ratio			10:1 Ratio					
Sample	MTB: MAC	MTB: NTM	MAC: NTM	MAC: MTB	MAC: NTM	MTB: MAC	MTB: NTM	NTM: MAC	NTM: MTB
PCR Result	MTB+/ MAC+	MTB+	MAC+	MTB+/ MAC+	MAC+	MTB+/ MAC+	MTB+	MAC+	MTB+

Interfering Substances

	Sputum				No matrix (DNA Extract)	
Sample	High TB/ Low MAC	**PCR Result**	High MAC/ Low TB	**PCR Result**	High TB/ Low MAC	**PCR Result**
		MTB+ MAC-		MTB+ MAC+		MTB+ MAC-

- The assay can detect MTBC at the LOD if a high concentration of MAC is present
- The assay cannot detect MAC at the LOD if a high concentration of MTBC is present

Real-time PCR MTBC Validation Performance Characteristics Summary

Characteristic	Total Sample Results	Acceptance Criteria	Observed Performance
Accuracy	Direct: 120 Xpert: 21 Isolates: 150	>90%	Direct: 82% Xpert: 100% Isolates: 100%
Precision	Direct: 6 Isolates: 15	>90%	100%

Analytical Sensitivity (LOD)	MTBC: 4.22 CFU/mL
Analytical Specificity (Interfering substances)	No interfering substances found

False negatives: Culture is more sensitive = gold standard

False positives: PCR will detect non-viable DNA that will not grow in culture

Overall Accuracy = 94%

Standard Operating Procedure

Inactivate sediments and culture aliquot

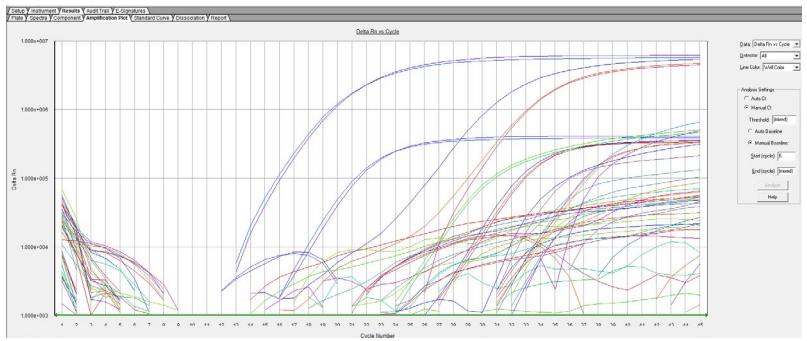
Extract DNA - Bead beat and heat lysis

Reagent Name	Amount per PCR reaction (µL)
5X perfeCTa qpCR Multiplex ToughMix	5
Primer: TBC-F (45µM)	0.25
Primer: TBC-R (45µM)	0.75
Primer: TbextRD9-F (45µM)	0.25
Primer: TbextRD9-R (45µM)	0.25
Primer: MAC-F (45µM)	0.5
Primer: MAC-R (mix of 5 primers at 100µM)	1.25
Probe: TBC-P(FAM-mgb) (25µM)	0.25
Probe: TbextRD9-P (Cy5 (LNA))(25µM)	0.125
Probe: MAC-P (mix of 2 probes at 25µM) (VIC-mgb) (25µM)	0.25
Probe: Bicoid-P (TexRd-bhq) (25µM)	0.25
Water	6.125

Prepare Mastermix of PCR Reagents

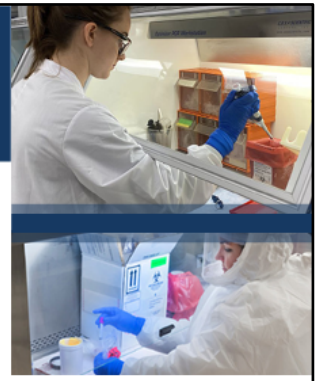
Add samples/controls and mastermix to plate

Run plate on ABI and results analysis



RESULT	INTERPRETATION
Detected	DNA is present
Undetected	DNA is not present or below the limit of detection
Inconclusive	DNA was not reliably detected; above the cut-off for a positive result. Possibly due to low amount of DNA, cross-reactivity
Inconclusive Due to Inhibition	DNA presence not able to be determined due to inhibitors in the specimen

DCLS Nucleic Acid Amplification Testing (NAAT) for *M. tuberculosis*



Guidelines for the use of *M. tuberculosis* NAATs

CDC recommends that NAAT testing be performed on at least one respiratory specimen from each patient with signs and symptoms of pulmonary tuberculosis (TB) when:

- A diagnosis of TB is being considered but has not yet been established
- The test result would alter case management or TB prevention activities, like contact investigations

NAAT utilized at DCLS on Sputum Specimens

Real-time PCR (non-FDA approved)

- Detects *M. tuberculosis* complex (MTBC) and *M. avium* complex (MAC)
- Direct specimen testing
  - o 48-72 hour turn-around-time (TAT) from specimen receipt
  - o If MTBC detected, sample reflexes to Xpert MTB/RIF
- Isolate testing
  - o Used for identification of MTBC and MAC in culture

Xpert MTB/RIF (FDA-approved)

- Detects *M. tuberculosis* complex (MTBC)
- Detects mutations associated with rifampin resistance
- If rifampin resistance detected, sample reflexes to CDC molecular detection of drug resistance testing

NAAT should NOT be used on the following patients:

- Patients recently diagnosed with TB disease (patients with a previous positive MTBC result (NAAT and/or culture) in the past 12 months will not be tested again by direct real-time PCR at DCLS)
- Patients currently receiving anti-TB treatment (which can cause false-negative results)
- Patients recently treated for TB disease (which can cause false-positive result)

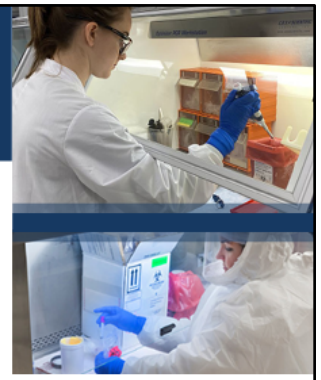
Benefits of NAAT

- Greater positive predictive value (>95%) with AFB smear positive specimens as compared to smear alone
- Ability to rapidly detect the presence of *M. tuberculosis* in 50-80% of AFB smear-negative, culture-positive specimens
- Detects the presence of *M. tuberculosis* in days, compared to weeks in culture

Importance of smear and mycobacterial culture

- Even if NAAT are performed, AFB smears and cultures should be performed on three respiratory specimens
- Culture remains the gold standard for diagnosis, and is still necessary for drug susceptibility testing and strain genotyping
- A negative NAAT result does not exclude the possibility of MTBC in culture
- A negative NAAT result for MTBC and a positive AFB smear are good indicators of the presence of nontuberculous mycobacteria

DCLS Nucleic Acid Amplification Test (NAAT) Results



Direct Specimen NAAT

M. tuberculosis complex DNA and *M. avium* complex DNA

- Detected
- Not detected
- Inconclusive -DNA was not reliably detected; above the cut-off for a positive result. Possibly due to low amount of DNA, cross-reactivity
- Inconclusive due to inhibition - DNA presence not able to be determined due to inhibitors in the specimen

Xpert MTB/RIF

M. tuberculosis complex DNA

- Detected
- Not detected

Rifampin resistance (only reported if *M. tuberculosis* complex DNA detected)

- No *rpoB* gene mutations detected; probably rifampin susceptible
- A mutation in *rpoB* gene has been detected; indicating possible rifampin resistance
- Presence of *rpoB* gene mutations cannot be accurately determined

Culture Isolate NAAT

M. tuberculosis complex DNA and/or *M. avium* complex DNA

- Detected

Notification

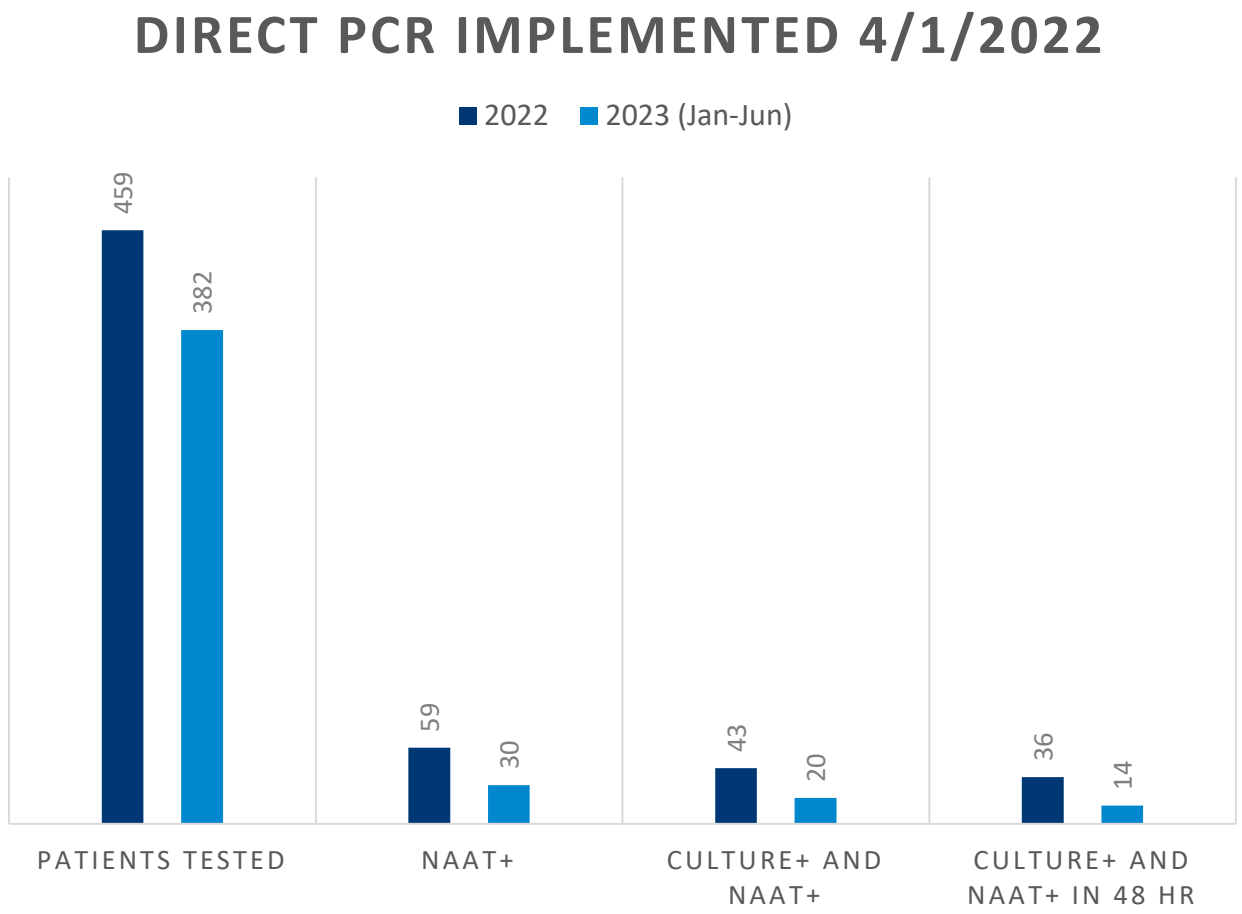
- Same day verbal notification of the initial MTBC detected result (either direct specimen or culture isolate) and *rpoB* mutation detected result
- Preliminary laboratory report issued for all NAAT results

NOTE: Clinicians should interpret all laboratory results on the basis of the clinical situation. A single negative NAAT test result should not be used as a definitive result to exclude TB, especially when the clinical suspicion of TB is moderate to high. Rather, the negative NAAT test result should be used as additional information in making clinical decisions, to expedite testing for an alternative diagnosis, or to prevent unnecessary TB treatment. Consultation with a TB expert should be considered if the clinician is not experienced in the interpretation of NAAT tests or the diagnosis and treatment of TB.

References

- Centers for Disease Control and Prevention (CDC). Updated guidelines for the use of nucleic acid amplification tests in the diagnosis of tuberculosis. MMWR Morb Mortal Wkly Rep.2009 Jan 16;58(1):7-10. Available at: http://www.cdc.gov/mmwr/preview/mmwrhtml/mm5801a3.htm
- Los Angeles County TB Control Program. Guidelines for the use of Mycobacterium tuberculosis nucleic acid amplification tests, including Xpert MTB/RIF. September 15, 2015. Available at: http://ph.lacounty.gov/tb/docs/NAATs/NAATGuidelines09-15-2015f.pdf

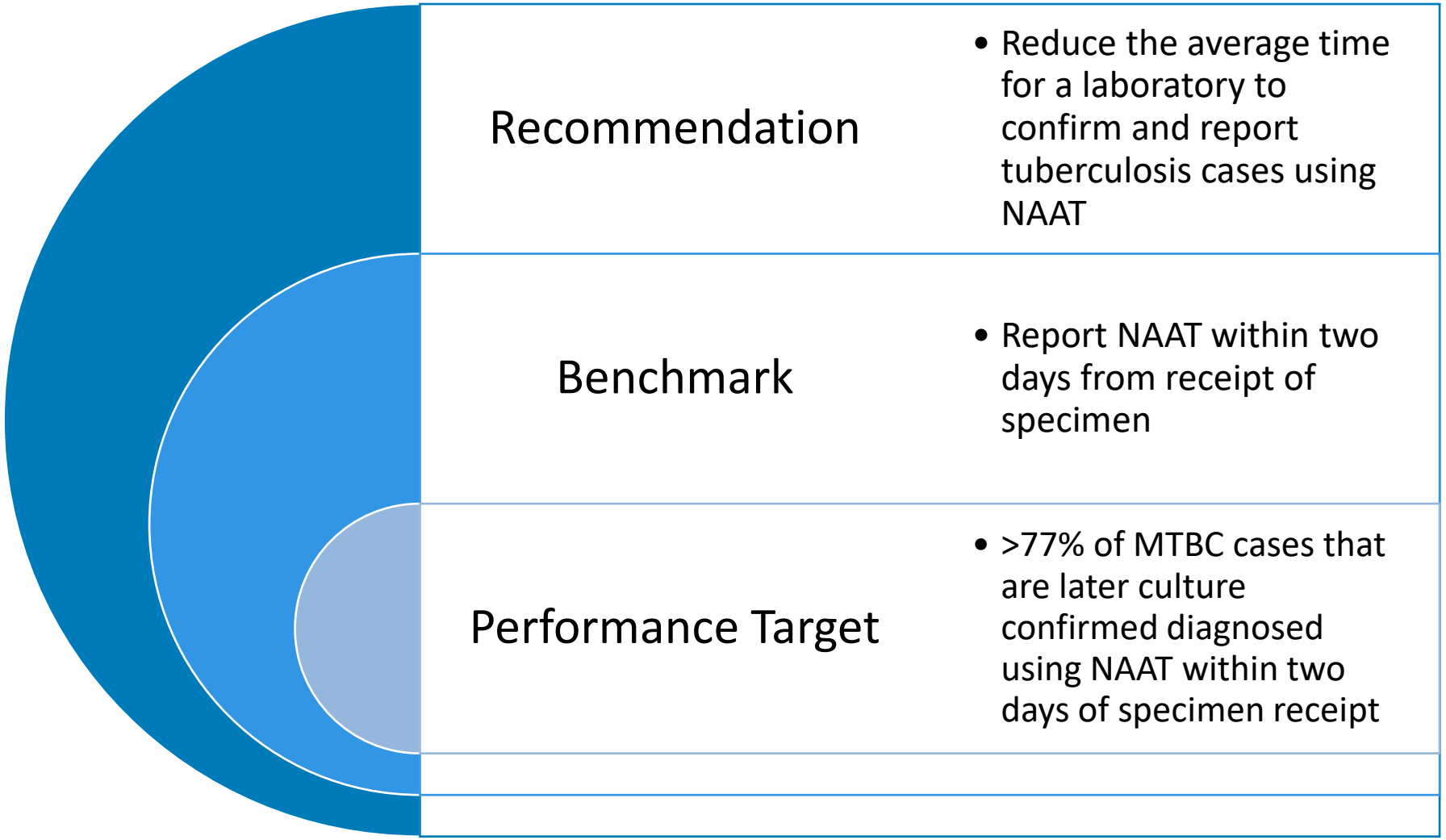
Post-Implementation of Real-time PCR Assay



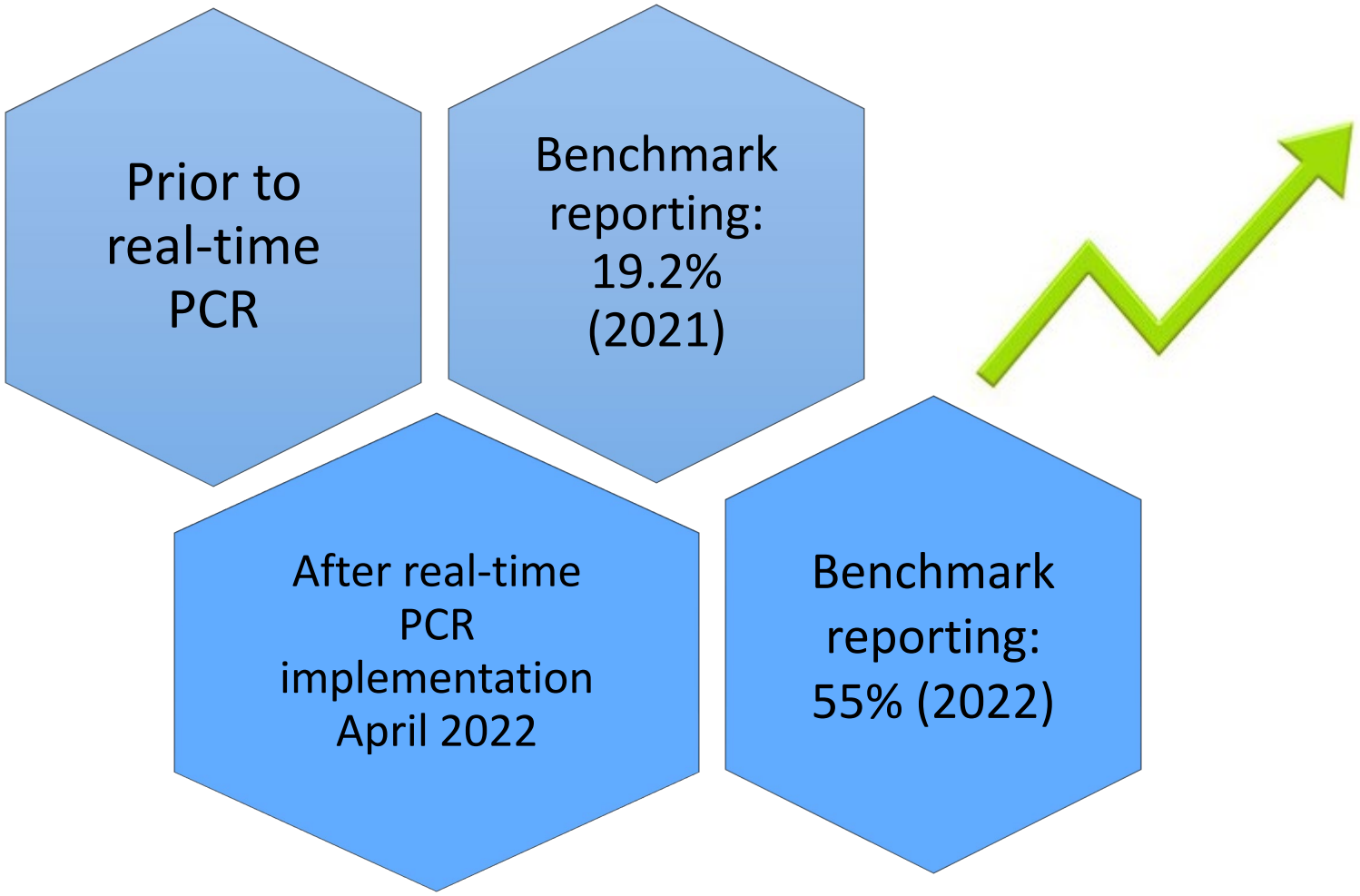
Year	Average days to diagnose TB from receipt of specimen
2021	15
2022	9

Year	TAT
2022	On average, for a culture positive patient, PCR was able to detect MTBC 10.2 days faster
	Range: 3-20 days faster
	Average to report PCR: 1.5 days

Healthy People 2030 Background



DCLS Healthy People Benchmark



Questions

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