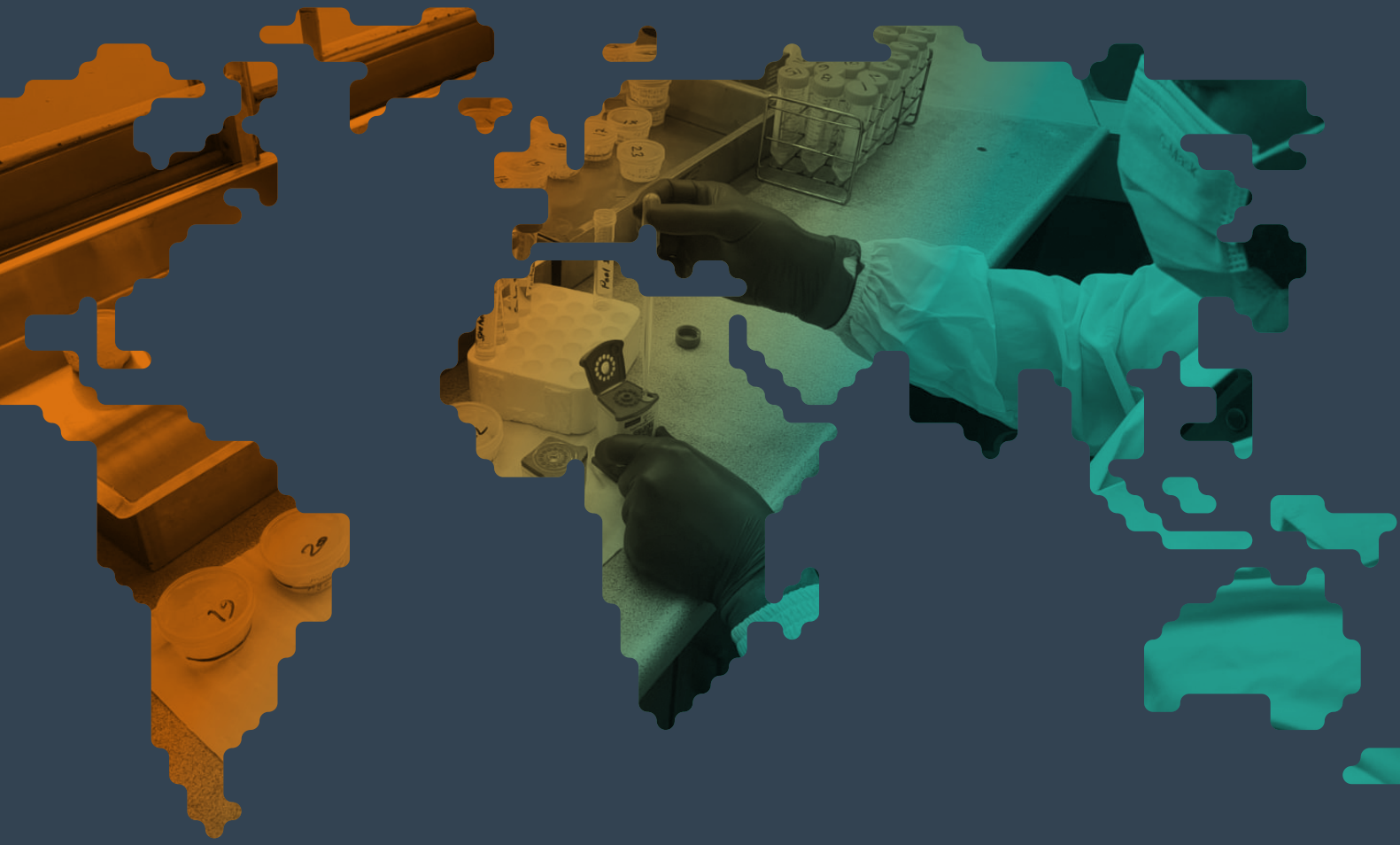
Version 1 2026

Start4All

# Pooled Sputum Testing Toolkit

A hands-on, practical guide for laboratory staff, healthcare workers, and implementers.



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Stop TB Partnership

# Toolkit Foreword

## by Unitaid Progam Manager, Smiljka De Lussigny

**At Unitaid, we invest in innovations that can shift the trajectory of global health; approaches that are ambitious, evidence driven, and designed for real world impact. The *Start4All Pooling Toolkit* exemplifies this vision.**

This toolkit reflects the strength of the partnership between Unitaid, the Liverpool School of Tropical Medicine (LSTM), and the Start4All consortium. Together, this collaboration has delivered a robust and comprehensive platform, one that we are proud to count within Unitaid's investment portfolio, designed to support countries in doing more with the resources they have, without compromising quality, equity, or impact.

What sets this work apart is its clear focus on implementation and sustainability. From the outset, Start4All was designed to respond to current programme realities while anticipating future diagnostic needs. The research underpinning this toolkit is deliberately future proofed, ensuring that the evidence and approaches generated remain relevant as technologies, policies, and country priorities evolve.

This vision is inseparable from the legacy of Professor Luis Cuevas, to whom this work is dedicated. Luis championed diagnostics that reach those most often left behind and believed deeply that innovation must translate into access and equity. His leadership and values continue to guide this work and the many people advancing it.

We are particularly encouraged that the Start4All platform enables a strong focus on vulnerable populations, including children, groups for whom diagnostic gaps remain most pronounced. By improving testing efficiency without compromising quality, pooled testing approaches such as those described here can help extend molecular diagnostics to settings and populations that have historically been underserved.

Finally, this toolkit is a testament to people: to the exceptional expertise, commitment, and motivation of the global and country teams, laboratory staff, implementers, and partners who brought Start4All to life. Their dedication to operational excellence and equity is evident throughout.

We hope this toolkit will serve not only as a practical guide, but also as an inspiration, demonstrating what is possible when rigorous science, country partnership, and a shared commitment to impact come together.

**The Unitaid team managing the Start4All investment**

(Anisa, Dessie, Irina, Jérémie, Kenny, Kristen, Mariam, Rocio, Smiljka, Tanya)

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# Executive Summary

## This Start4All Pooling Toolkit outlines the implementation of pooled sputum testing for TB.

Pooled sputum testing is a laboratory approach in which aliquots from multiple processed sputum samples are combined and tested together using WHO-recommended low-complexity automated nucleic acid amplification tests (LC-aNAATs). This strategy increases efficiency by reducing the number of cartridges and tests required, while maintaining diagnostic accuracy. It is particularly valuable in settings with constrained testing capacity and/or low to moderate TB positivity rates.

Pooled testing offers several operational advantages. It reduces the number of test cartridges required, expands testing coverage, and is especially effective in low-prevalence settings where most specimens are expected to be negative. Evidence shows that pooled testing preserves diagnostic performance while improving resource use, with minimal additional requirements for laboratories already performing LC-aNAAT testing[1].

***"In primary healthcare settings, pooled testing is a method that should be encouraged in low-income settings like ours... it's economical and helps to screen more people with the same resources."***

Cameroon KII1, Female, Programme Director

The pooled testing concept was first described in 1943 by Robert Dorfman for syphilis screening and has since been applied to HIV, hepatitis B and C, and widely adopted during the COVID-19 pandemic. In pooled sputum testing, combined samples are run as a single molecular assay. A negative pooled result allows all individuals in that pool to be considered negative, while a positive pooled result triggers reflex individual testing to identify which specimen(s) contain Mycobacterium tuberculosis.

In 2026, the World Health Organization made a conditional recommendation for the use of pooled testing to diagnose TB. This recommendation is based on pools of up to four and notes that pooled testing efficiency and cost saving decreases when test positivity exceeds 24% or when community-based sputum collection is used. The recommendation does not extend to children, people with HIV, or people at risk of drug-resistant TB.

***"Patients want quick, ready solutions. [...] The public will appreciate a system where they can get tested and treated efficiently without unnecessary delays."***

Bangladesh KII2, Male, Programme Coordinator

### Recommendation

**When resource constraints do not allow for the testing of individual samples:** In adults and adolescents with signs and symptoms for pulmonary TB or who screen positive for pulmonary TB, LC-aNAATs on (up to 4) pooled sputa may be used as the initial diagnostic strategy for diagnosing TB rather than LC-aNAATs on individual samples (conditional recommendation, high certainty of evidence).

Source: https://www.who.int/teams/global-programme-on-tuberculosis-and-lung-health/diagnosis-treatment/npoc-tongue-swabs-and-sputum-pooling-for-tb/sputum-pooling-for-low-complexity-testing

This Toolkit – or "PoolKit" as we lovingly refer to it - provides practical, step-by-step instructions on specimen handling, pool formation, laboratory workflows, result interpretation, biosafety, quality assurance, and documentation. It serves as a comprehensive guide to support safe, accurate, and streamlined implementation of pooled sputum testing within routine TB diagnostic services.

# Purpose and Scope

## Purpose

This toolkit provides guidance for implementing pooled sputum testing for the molecular detection of ***Mycobacterium tuberculosis*** (MTB) using low-complexity automated nucleic acid amplification tests (LC-aNAATs), such as Xpert MTB/RIF Ultra[2,3].

## Context

The World Health Organization (WHO) has recognised pooled sputum testing as an efficiency strategy for molecular TB testing under defined conditions[4], particularly in settings using WHO recommended LC-aNAAT platforms[5] and where testing capacity or cartridge availability is constrained. Most operational evidence for pooled sputum testing has been generated using the Xpert MTB/RIF Ultra platforms[6,7]. However, the underlying principle of pooled testing can also be applied to other WHO endorsed LC-aNAAT systems where validated protocols and appropriate operational safeguards are in place.

## Scope of Application

This toolkit describes procedures for implementing pooled sputum testing in laboratories performing molecular TB diagnosis. It applies to the processing of sputum specimens regardless of where they are collected, including both facility-based and community-based case finding activities. It is primarily intended to be used by:

- **Laboratories within the national tuberculosis programme (NTP) network**
- **Public health or reference laboratories**
- **Research or implementation programmes supporting TB diagnostic services**

While sputum collection and screening activities may take place in healthcare facilities or community settings, this toolkit focuses specifically on the laboratory procedures for pooled testing once samples are received.

Laboratories implementing pooled sputum testing should have appropriate infrastructure, trained personnel, and biosafety procedures for handling sputum specimens of people with presumptive TB and performing molecular TB testing.

## Intended Use and Target Population

Pooled sputum testing is intended as a laboratory diagnostic workflow optimisation strategy within routine TB diagnostic pathways. It should be applied to sputum specimens collected from individuals undergoing evaluation for pulmonary tuberculosis where molecular testing is indicated. Pooled testing may not be appropriate in all settings, especially contexts with high TB prevalence or where immediate individual test results are required.

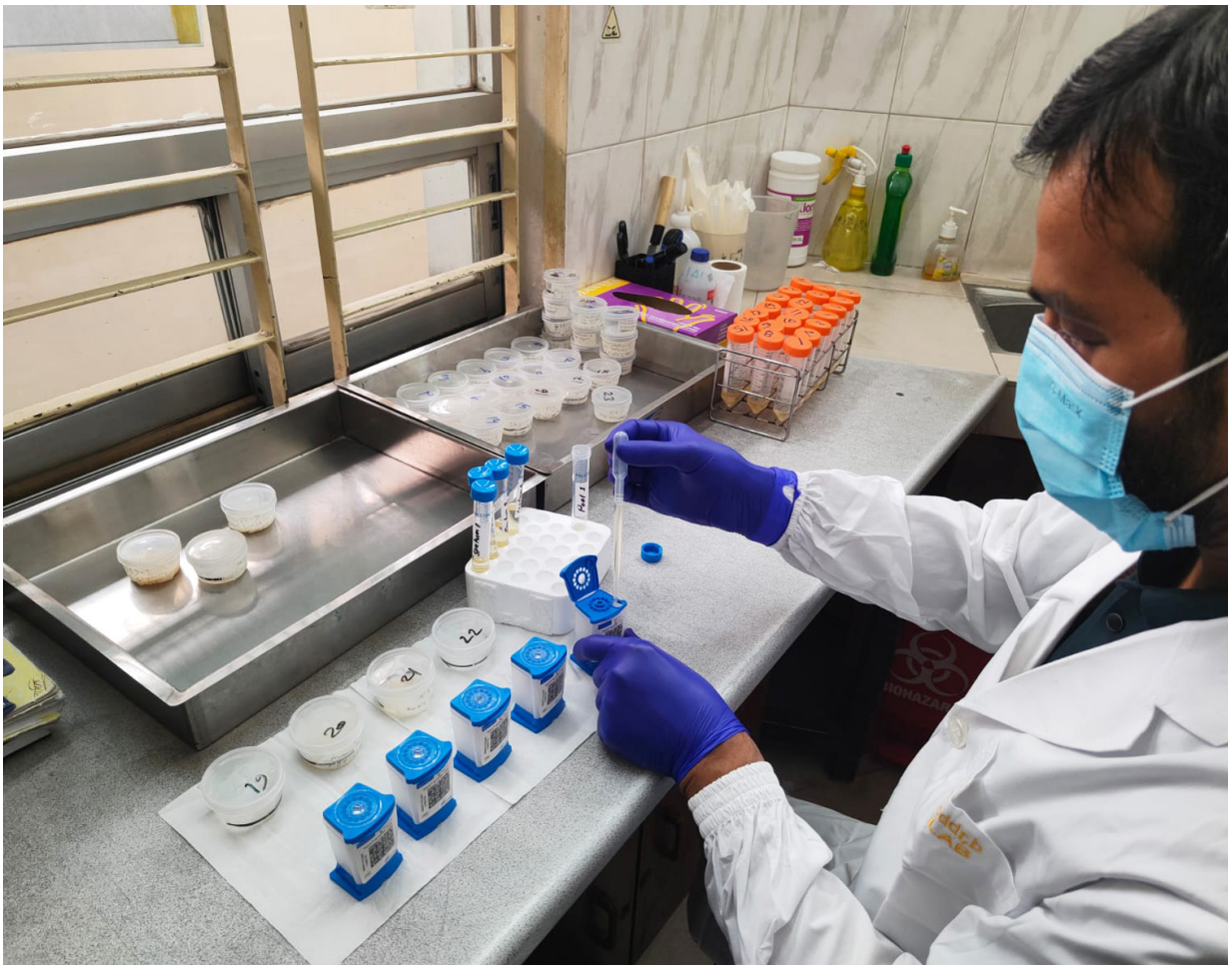
## Adaptation

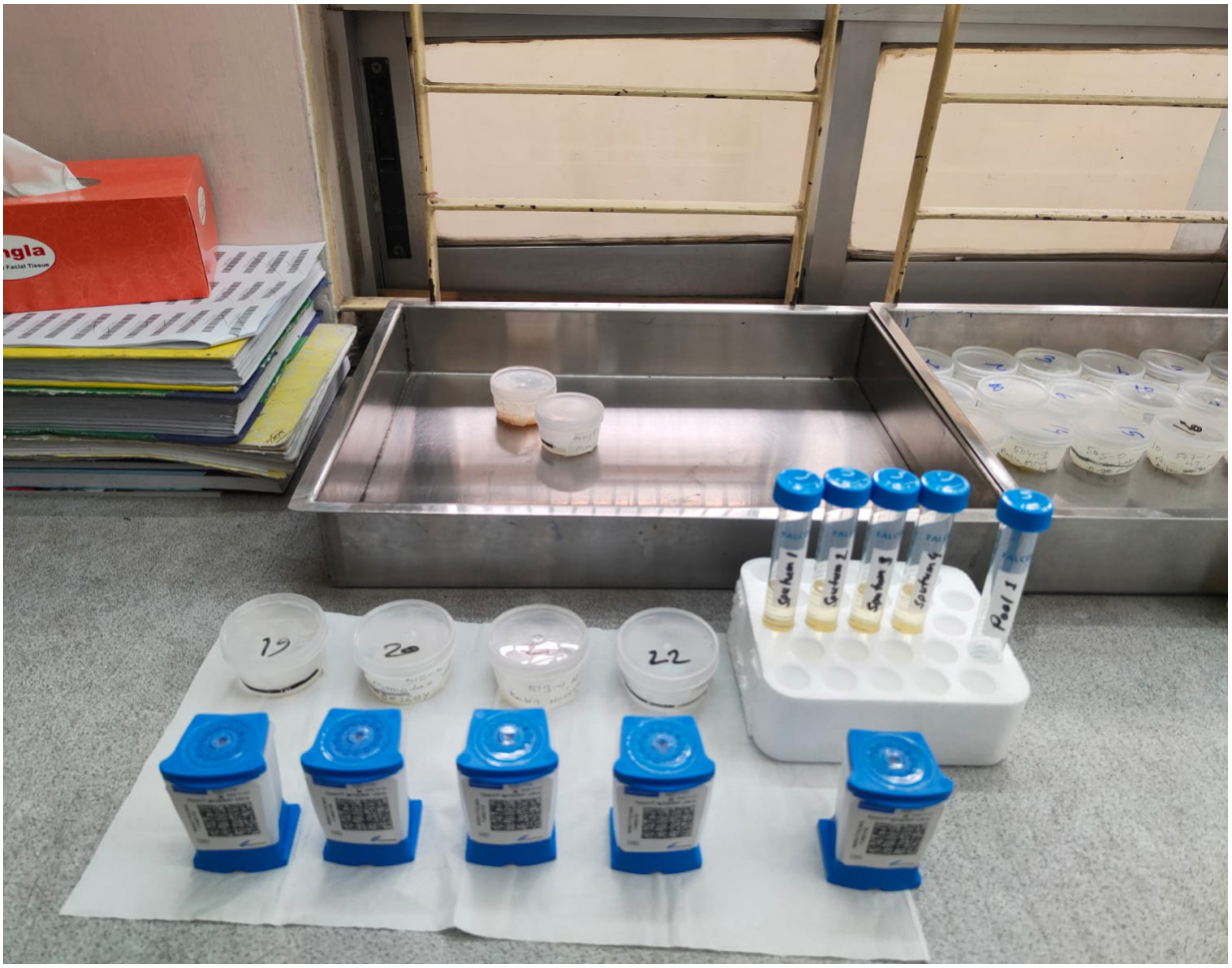
This toolkit is provided as a generic operational template and should be adapted by laboratories and programmes to align with national TB diagnostic algorithms, laboratory quality management systems, and manufacturer instructions for the molecular platform used.

The toolkit builds on operational research and implementation conducted across multiple countries, including studies undertaken within the Start4All initiative[8] and related programmes[9].

*Pictured: Pooled sputum testing by Xpert® MTB/RIF Ultra.*

*In the Start4All study, sputum samples were collected from individuals with presumptive pulmonary TB at the informal settlement areas of Dhaka metropolitan city during chest x-ray screening programmes. Healthcare worker tested the samples individually and by pooling with Xpert® MTB/RIF Ultra at the nearest TB Screening and Testing Centers (TBSTCs). Photo credit goes to Md. Sohag Mia, Research Officer of Mycobacteriology Laboratory, icddr,b.*

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Pool 1

# Toolkit Contents

# Standard Operating Procedure (SOP)

## Pooled Sputum Testing Using Low-Complexity Automated Nucleic Acid Amplification Tests (LC-aNAATs)

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| Institution: | |
| Location: | |
| Head/Responsible person: | |

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| Code: | | Version: | No. | Effective date: | |

| | Name / Designation | Signature | Date |
|---|---|---|---|
| Prepared by: | | | |
| Reviewed by: | | | |
| Verified by: | | | |
| Approved by: | | | |
| SOP Owner/Contact: | | | |
| Next Review Date: | | | |

| REVISION HISTORY | | | | | |
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# 1. Definitions and Abbreviations

| | |
|---|---|
| **Deconvolution testing** | Individual testing performed on specimens included in a pool after a positive pooled test result to identify the positive sample(s). |
| **Error, invalid or no result** | LC-aNAAT outcomes classified as non-valid results, indicating that the assay did not generate a valid diagnostic result and that repeat testing may be required. |
| **LC-aNAAT** | Low-complexity automated nucleic acid amplification test recommended by the WHO for the detection of *Mycobacterium tuberculosis*. |
| **MTB** | *Mycobacterium tuberculosis* complex. |
| **MTB detected** | LC-aNAAT result indicating that *Mycobacterium tuberculosis* DNA has been detected in the tested specimen. |
| **MTB not detected** | LC-aNAAT result indicating that *Mycobacterium tuberculosis* DNA was not detected in the tested specimen. |
| **NTP** | National Tuberculosis Programme. |
| **Pool** | Combined specimen created from several individual processed sputum samples for testing in a single molecular assay. |
| **Pooled testing** | Laboratory procedure in which aliquots from multiple individual specimens are combined and tested together in a single molecular assay. |
| **PPE** | Personal protective equipment. |
| **SOP** | Standard Operating Procedure describing standardised processes for laboratory procedures. |
| **TB** | Tuberculosis, an infectious disease caused by *Mycobacterium tuberculosis*. |
| **WHO** | World Health Organization. |
| **Xpert MTB/RIF Ultra (Xpert Ultra)** | Cartridge based molecular assay used for detection of *Mycobacterium tuberculosis* and rifampicin resistance. |

## 2. Responsibilities

Clear roles and responsibilities should be defined to ensure the correct and consistent implementation of pooled sputum testing.

### Laboratory Technician

Laboratory technicians are responsible for the operational implementation of the procedure, including:

- Receiving and verifying sputum specimens and accompanying documentation
- Preparing specimens and performing pooled testing according to this SOP and manufacturer instructions
- Ensuring accurate labelling and traceability of specimens and pools
- Recording test results in the appropriate laboratory registers and/or electronic systems
- Following appropriate laboratory biosafety and waste management procedures

### Laboratory Supervisor

Laboratory supervisors oversee the implementation of pooled testing within the laboratory. Responsibilities include:

- Ensuring laboratory staff are trained on the procedure and SOP requirements
- Supervising adherence to laboratory procedures and biosafety practices
- Monitoring implementation of quality assurance measures, including internal quality control procedures and coordination with external quality assurance (EQA) programmes where available
- Ensuring appropriate documentation and record keeping

### Laboratory Manager / Laboratory Lead

Laboratory managers or laboratory leads are responsible for the overall implementation and oversight of pooled testing within the laboratory. Responsibilities include:

- Ensuring the laboratory has the necessary infrastructure, equipment, reagents, and trained personnel
- Ensuring that procedures are aligned with NTP policies and laboratory quality management systems
- Supporting implementation, supervision, and continuous monitoring of the pooled testing strategy

### Laboratory Data and Information Management Staff

Where applicable, laboratory data personnel support documentation and data management. Responsibilities include:

- Supporting laboratory systems for recording pooled and individual test results
- Ensuring data integrity and traceability between specimens and pools
- Supporting reporting systems used by the laboratory

# 3. Principle of Pooled Sputum Testing[10]

Pooled sputum testing for TB is a laboratory strategy in which aliquots from multiple sputum specimens are combined and tested in a single molecular assay. If the pooled test result is negative, all individuals are presumed not to have the disease. If positive, each sample is retested individually to identify who has the disease. The approach aims to improve testing efficiency and optimise the use of molecular diagnostic resources while maintaining the ability to identify individuals with tuberculosis[11].

Most operational evaluations of pooled sputum testing have been conducted using molecular assays such as Xpert MTB/RIF Ultra, typically using pools of four samples[11]. This pool size reflects both empirical evidence and modelling studies, which show that for positivity rates below approximately 30%, pools of four are close to optimal in reducing the number of tests while maintaining efficiency[12]. These studies have shown that pooled testing can achieve diagnostic performance comparable to individual testing while substantially reducing the number of molecular assays required[6,13].

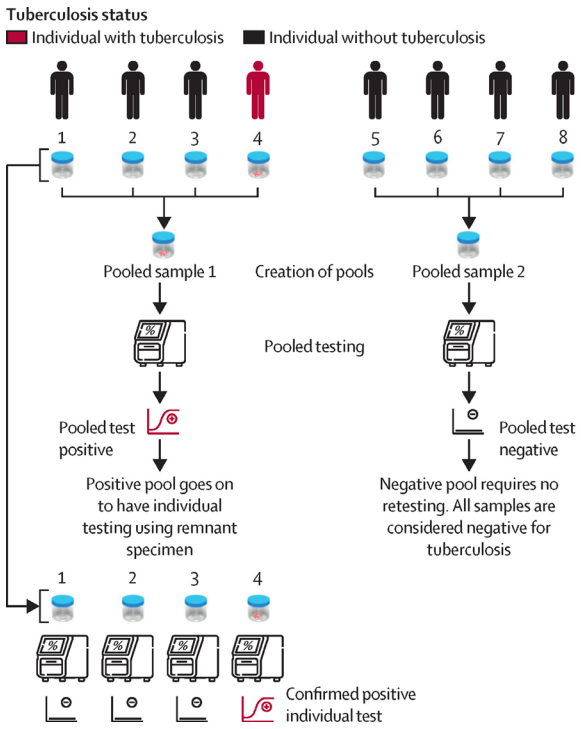


*Figure 1. Pooled testing algorithm using sputum specimens reproduced with permission from Iem et al, Lancet Respiratory Medicine 2025[10].*

Pooled testing introduces some dilution of bacterial genetic material, which may slightly reduce analytical sensitivity. Evidence suggests this effect is generally small, although detection may be lower in specimens with very low bacillary loads[11,13]. Diagnostic accuracy may also be lower when sputum is collected through CBCF compared with FCBF collection, potentially reflecting differences in bacterial load or sample quality. Programmes implementing pooled testing should therefore balance efficiency gains with potential reductions in sensitivity, particularly in populations where many specimens contain very low bacterial concentrations or where samples are collected in community settings.

The number of samples included in a pool may vary depending on operational considerations such as expected TB positivity, daily specimen throughput, and laboratory workflow. In practice, pooled testing is most efficient when the proportion of positive specimens is relatively low (<10%).

Pools of four specimens are commonly used in operational settings, although smaller pools may be selected depending on specimen availability or laboratory workflow. Larger pool sizes may offer additional gains at very low positivity levels, but increase dilution and risk of reduced sensitivity, particularly in samples with low bacterial load. As a general guide:

| | |
|---|---|
| **Individual TB test positivity <10%** | → **Use pool sizes of 3 or 4** |
| **Individual TB test positivity >10%** | → **Use pool size of 2** |
| **Individual TB test positivity >30%** | → **Use individual testing** |

Empirical data from the Start4All Viet Nam team indicate that in settings with high positivity (e.g. around 38%), pooled testing results in minimal cartridge savings and only modest cost reductions, as a large proportion of pools require individual retesting (Han Thi Nguyen et al., manuscript under review).

Laboratories that are unsure about the appropriate pool size should contact their reference laboratory for guidance.

# 4. Biosafety Considerations

Laboratories implementing pooled sputum testing should follow the same biosafety practices already required for routine molecular TB testing of sputum. These practices apply equally to testing performed in facility-based laboratories and in mobile or outreach screening programmes.

According to WHO guidance on TB laboratory biosafety[14], preparation of sputum specimens for test with LC-aNAATs (such as Xpert Ultra) is classified as a low-risk laboratory activity. These procedures have a low likelihood of generating infectious aerosols and involve a low concentration of infectious particles once sputum has been mixed with the sample reagent.

For LC-aNAAT platforms, specimen preparation may be performed on an open bench provided that adequate ventilation is ensured and standard infection prevention and control practices are followed. This allows molecular testing, including pooled sputum testing, to be safely implemented in a range of diagnostic environments, including laboratories, mobile testing units, and outreach screening programmes[7].

In pooled sputum testing, pool formation is performed after the sputum has been processed with the manufacturer's sample reagent. As a result, the biosafety risk is not increased compared with standard LC-aNAAT testing procedures routinely performed in many laboratories and near point of care environments.

Pooled testing requires transferring aliquots from several processed specimens into a single container. These additional handling steps should be performed carefully to minimise the risk of accidental spillage, particularly in high throughput or outreach settings where space may be more limited. Practical approaches to support safe and organised handling, including simple workspace layouts and sample organisation tools, are described in later sections of this toolkit.

Personnel performing pooled sputum testing should wear appropriate personal protective equipment (PPE) according to local laboratory biosafety guidelines. This typically includes laboratory coats or gowns and gloves, and other protective equipment where required.

Work surfaces and equipment used during pooled testing should be routinely cleaned and disinfected using appropriate laboratory disinfectants. Items used to organise specimens during pooled testing, such as trays, racks, or similar devices used to group samples during the process, should be disinfected after each use to prevent cross contamination.

Laboratories and programmes implementing pooled sputum testing should ensure that biosafety practices remain consistent with national TB laboratory guidance and the manufacturer's instructions for the diagnostic platform used.

# 5. Specimen Requirements and Management

## Specimen Type

Pooled sputum testing is performed on sputum specimens collected for molecular testing for tuberculosis. While molecular assays may be used on other specimen types in specific clinical situations, pooled testing has primarily been evaluated using sputum. The use of pooled testing with other specimen types is outside the scope of this SOP and is not validated under this procedure. Laboratories wishing to apply pooled testing to other specimen types must perform appropriate local validation before implementation.

## Specimen Eligibility

Specimens included in pooled sputum testing should meet the same basic criteria required for routine molecular TB testing. These typically include:

- specimens collected from individuals undergoing evaluation for pulmonary tuberculosis
- adequate sputum quality (e.g. sputum rather than saliva)
- sufficient volume to allow pooled testing and, if required, subsequent individual testing

## Specimen Rejection Criteria

The rejection criteria for pooled sputum testing are the same as those used for routine individual LC-aNAAT testing. If a specimen is considered acceptable for individual molecular testing, it can also be included in pooled testing provided that sufficient volume is available.

Laboratories should therefore follow their standard procedures for specimen acceptance and rejection according to NTP guidance and local laboratory protocols.

The following specimens should **not** be included in pooled testing and should be tested individually:

- extrapulmonary or non-sputum specimens
- samples with a volume of less than 1.0 mL
- specimens from individuals currently receiving TB treatment
- specimens requiring rifampicin resistance testing after an initial positive MTB or smear result

## Specimen Transport and Storage

Specimens should be transported and stored according to NTP guidance and the manufacturer's instructions for the LC-aNAAT platform used. Where delays occur between specimen collection and testing, appropriate storage conditions should be followed to maintain specimen integrity.

Laboratories receiving specimens from peripheral or outreach collection sites should use existing specimen transport systems and ensure that samples are correctly labelled and packaged for safe transport.

Once sputum specimens have been processed with the manufacturer's sample reagent, the specimen reagent mixture should be tested within 4 hours if kept at room temperature. If testing cannot be completed within this timeframe, the processed specimens should be stored at 2–8°C and tested within 24 hours, in accordance with LC-aNAAT platform guidance. Where delays are anticipated due to laboratory operating hours, weekends, or workload constraints, pool size and sample grouping may be adjusted to ensure timely testing within recommended storage conditions.

These conditions apply both to specimens awaiting pooled testing and to processed specimens retained for potential individual deconvolution testing following a positive pooled result.

## Specimen Volume Operational Guidance

A minimum of 1 mL of raw sputum is recommended for pooled sputum testing. After processing with the manufacturer's sample reagent at the standard 2:1 ratio (sample reagent to sputum), this typically results in approximately 3 mL of processed specimen (e.g. 1 mL sputum + 2 mL reagent).

When forming pools of four specimens, approximately 1 mL of processed specimen from each individual sample is used to create the pooled specimen. This produces about 4 mL of pooled material, allowing:

- about 2 mL for the pooled test, and
- sufficient remaining volume for repeat pooled testing if the result is error, invalid, or no result.

Because only a portion of each processed specimen is used for pooling, sufficient material remains for individual testing if the pooled result is positive.

Specimens with less than 1 mL of raw sputum may not provide enough volume for pooled testing. In such cases, pooled testing is not recommended, and laboratories should follow local procedures for managing low volume specimens, such as individual testing, specimen rejection, or requesting a new specimen, according to national or institutional guidelines.

| Pool size | Volume from each processed specimen | Total pooled volume |
|---|---|---|
| **4 specimens** | ~1 mL | ~4 mL |
| **3 specimens** | ~1 mL | ~3 mL |
| **2 specimens** | ~1.5 mL | ~3 mL |

# 6. Equipment, Materials and Reagents

Pooled sputum testing uses the same core equipment and consumables required for routine LC-aNAAT testing, with a small number of additional items to facilitate the preparation and organisation of pooled samples. The infrastructure requirements are therefore similar to those already used in laboratories performing molecular TB testing.

## LC-aNAAT Platform

Pooled sputum testing should be performed using WHO-recommended LC-aNAATs for TB detection.

The pooled testing principle may also be applicable to other LC-aNAAT systems when compatible workflows are used and appropriate validation has been conducted.

Some molecular platforms may require local validation or operational assessment before implementing pooled testing if workflows have not been formally evaluated for that platform. Laboratories should follow NTP guidance and manufacturer instructions for the diagnostic platform used.

## Core Equipment for LC-aNAAT Testing

The following equipment is typically required for routine molecular TB testing and is also used for pooled sputum testing:

- LC-aNAAT testing platform and associated instrument software
- Graduated transfer pipettes or micropipettes
- Sterile disposable pipettes or pipette tips
- Sputum containers with secure caps
- Timers
- Standard sample racks or tube holders for routine specimen handling
- Absorbent bench pads
- Biohazard waste containers
- Personal protective equipment (gloves, laboratory coat or gown, mask where required)

These items are generally already available in laboratories performing routine molecular TB testing.

## Additional Materials for Pooled Testing

A small number of additional materials can facilitate the organisation and preparation of pools:

- Pool containers or tubes for combining aliquots from multiple specimens (e.g. sputum containers)
- Additional sterile pipettes or pipette tips for aliquoting specimens into pools

Low-cost devices to group samples can help improve visual organisation and reduce the risk of mixing samples during pool preparation. Examples include:

- Repurposed muffin baking trays used to group individual specimens belonging to the same pool
- Alternatively, simple tube racks or holders where available, particularly when using conical tubes
- Locally fabricated or 3D printed racks designed to organise pooled samples

These tools are **optional and not required** for implementing pooled testing. Pool preparation can be performed directly on a clean work bench by handling one pool at a time, followed by appropriate cleaning of the working surface before proceeding to the next set of samples.

The use of trays or racks should not be a barrier to implementation. Laboratories should adopt the approach that best fits their setting, while ensuring good laboratory practice, surface decontamination, and measures to minimise cross contamination.

### Reagents

Pooled sputum testing uses the same reagents required for routine LC-aNAAT testing, including:

- Manufacturer-provided sample reagents used for sputum processing
- Test cartridges or assay-specific consumables required by the LC-aNAAT platform
- Laboratory disinfectants used for routine surface cleaning and decontamination

All reagents should be used and stored according to the manufacturer's instructions and national laboratory guidelines. Because pooled testing is performed after sputum has been processed with the sample reagent, no additional specialised reagents are required beyond those used for routine individual testing.

## 7. Procedure for Pooled Sputum Testing

The pooled sputum testing workflow follows the same principles as routine LC-aNAAT testing for tuberculosis, with additional steps for preparing and testing pooled specimens. Before starting testing, ensure the workspace is clean, organised, and free of clutter. Only the specimens that will be included in the pool should be present in the working area. This helps prevent mix-ups between samples and reduces the risk of cross contamination.


### Preparation of Specimens

Upon arrival at the laboratory, each specimen should be logged in the laboratory register or electronic system using a unique individual specimen identifier according to laboratory procedure (On page 18 in part 11. Recording and Reporting of Results see photograph of a technician in Cameroon logging the barcode of a specimen).

### Formation of Pools

Place the specimen containers in the wells of the repurposed muffin baking tray so that each group of specimens intended for one pool is arranged together in one row. Place an empty sputum container in line with the specimens to serve as the pooled sample container.

For example, when preparing pools of four specimens, place four individual specimen containers in adjacent wells of the same row. Leave the next row empty or use it for the next pool to avoid confusion. The tray helps visually organise specimens and ensures that the correct samples are combined when preparing the pooled specimen.

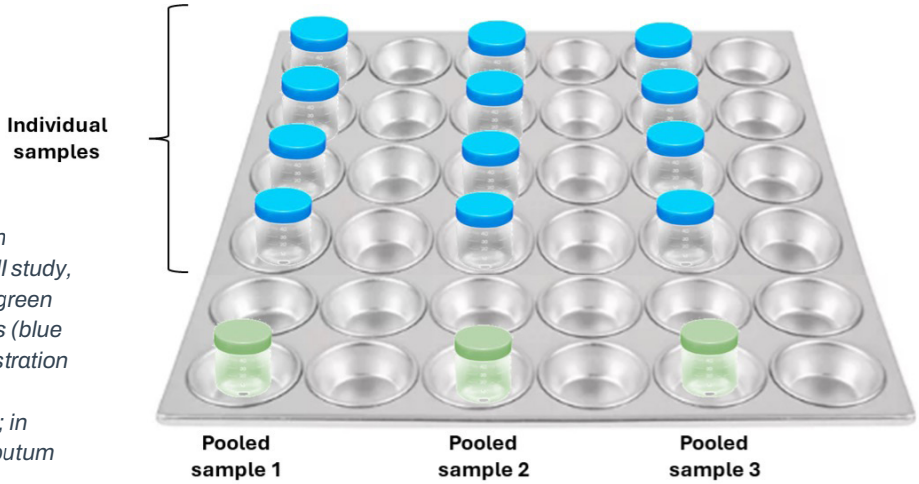


*Figure 2. An example of "muffin baking tin" used in the Start4All study, containing 3 pooled samples (green pots) from 4 individual samples (blue pots). Colours are used for illustration purposes only to distinguish pooled and individual samples; in practice, the same standard sputum containers are used.*

Each specimen should then be processed according to the standard procedure for the LC-aNAAT platform, including addition of the manufacturer's sample reagent and incubation for liquefaction[15].

Pool formation must only be performed after sputum has been mixed with the sample reagent. Raw sputum specimens should not be pooled.

Pools are created by combining equal volumes of processed sputum–reagent mixture from multiple individual specimens into a dedicated pool container. For each specimen included in the pool:

- Mix the specimen–reagent mixture briefly to re-homogenise
- Transfer an equal aliquot using a new sterile pipette or pipette tip
- Combine aliquots into the pool container

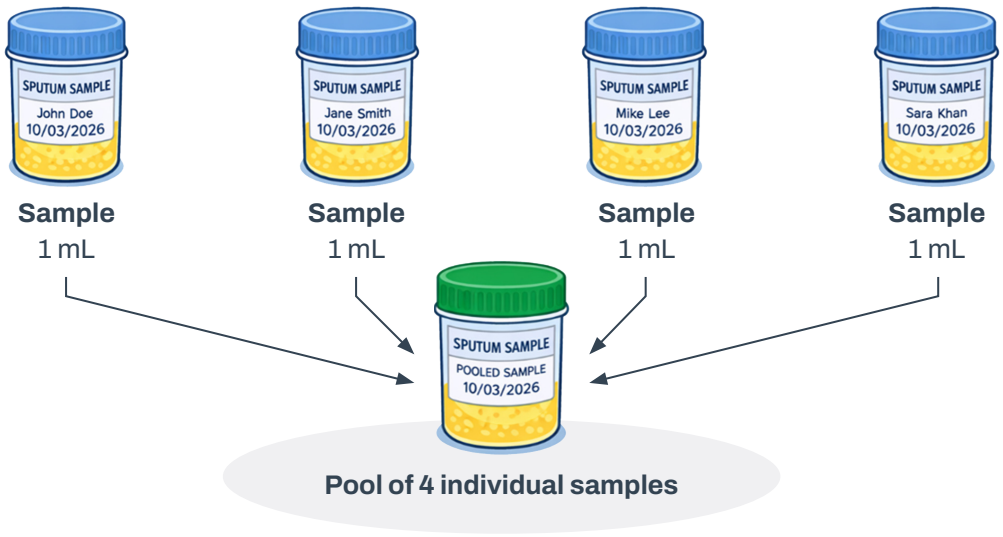


*Figure 3. Example of preparing a ~4 mL pool of four samples.*

## Testing of Pooled Samples

After aliquoting all specimens, the pooled sample should be mixed gently to ensure uniformity.

Using a sterile pipette, transfer the required volume of the pooled sample into the cartridge or reaction tube and run the test on the LC-aNAAT platform following standard procedures for individual testing.

Where possible, laboratories should maintain separate areas for pool preparation and cartridge loading to reduce the risk of cross-contamination.

## Deconvolution Individual Testing Following Positive Pools

LC-aNAAT platforms typically generate the following result categories:

- MTB not detected
- MTB detected (with semi-quantitative categories where applicable, including trace)
- Error, invalid or no result (non-valid test results)

If the pooled test result is MTB not detected, no additional molecular testing of those specimens is required and all individuals who provided those samples within that pool are reported to have a negative test.

If the pooled test result is MTB detected (including trace), each individual specimen included in the pool must undergo deconvolution individual testing:

1. Retrieve all processed individual specimens that contributed to the pool.
2. Mix each specimen again briefly to re-homogenise.
3. Perform individual LC-aNAAT testing following the standard testing procedure.

### Additional Considerations

Because sputum specimens are mixed with the manufacturer's sample reagent during molecular test preparation, the processed specimen is not suitable for mycobacterial culture. If culture or additional microbiological testing is required, a separate sputum specimen should be collected according to routine procedures.

Rifampicin resistance information generated during pooled testing cannot be interpreted for clinical decision-making because pooled samples contain multiple specimens and resistance cannot be attributed to a specific individual. Individual testing of all specimens in the pool is required to determine whether rifampicin resistance is present and in which specimen.

## 8. Interpretation of Results

This section describes how results generated during pooled sputum testing should be interpreted within the laboratory workflow. LC-aNAAT output should be interpreted according to the standard result categories provided by the diagnostic platform and NTP guidance. For pooled sputum testing, results are interpreted as follows:

**MTB not detected (pooled test):**

If a pooled test result is MTB not detected, all specimens included in that pool are interpreted as negative according to the molecular test result, as if each specimen had produced a negative individual LC-aNAAT result, even though individual testing is not performed.

**MTB detected (including trace) in pooled test:**

If the pooled test result is *MTB* detected, including trace, this indicates that at least one specimen in the pool contains detectable *MTB* DNA. In this situation, all individual specimens included in the pool must undergo deconvolution individual testing (refer to **Deconvolution Individual Testing Following Positive Pools in Section 7**). The results obtained from these individual tests determine the final TB status of each specimen and should be used for clinical interpretation and patient management in accordance with NTP guidance for routine individual LC-aNAAT results.

### Handling Trace Results

If the pooled test result is MTB detected (trace), all individual specimens included in the pool must undergo deconvolution individual testing. Because pooled testing dilutes the original specimens, the semi-quantitative category observed in the pooled result may not reflect the bacillary load of the individual specimen responsible for the signal. For example, a pooled result reported as trace may originate from an individual specimen that would yield a higher semi-quantitative category when tested individually.

For this reason, pooled trace results should not be interpreted in the same way as trace results from individual testing. Individual testing of all specimens in the pool with trace result is required to determine the final diagnostic result.

Interpretation of trace results obtained during individual testing should follow NTP guidance and diagnostic algorithms, particularly in populations where trace results require specific clinical consideration.

### Handling Error, Invalid, or No-Result Outcomes

If the pooled test result is **error, invalid, or no result**, laboratories should follow the same procedures used for routine individual LC-aNAAT testing.

Where sufficient specimen volume remains:

1. Repeat the pooled test using the remaining pooled specimen material.
2. If repeat testing is not possible or the result remains non-valid, individual testing of the specimens may be performed according to laboratory procedures.

All repeat testing and non-valid outcomes should be documented according to laboratory quality assurance procedures.

### Summary of Result Interpretation

| Pooled Result | Action Required | Final Interpretation |
|---|---|---|
| **MTB not detected** | No further testing required | All individuals considered negative according to the molecular test result |
| **MTB detected** | Perform individual testing of all specimens in the pool | Individual results determine final diagnosis |
| **MTB detected (trace)** | Perform individual testing of all specimens in the pool | Individual results determine final diagnosis |
| **Error / invalid / no result** | Repeat pooled test or proceed to individual testing according to laboratory procedures | Based on repeat or individual results |

## 9. Quality Assurance and Quality Control

Quality assurance and quality control measures for pooled sputum testing should be integrated into the existing quality management system used for individual LC-aNAAT testing. Because pooled testing introduces additional specimen handling and sample mixing steps, particular attention should be given to specimen identification, aliquoting accuracy, and prevention of cross contamination.

### Internal Quality Control

Routine internal quality control procedures required for LC-aNAAT platforms must be followed. These include:

- Verification that the instrument internal controls are valid for each run
- Use of manufacturer recommended control materials where applicable
- Routine instrument maintenance and calibration according to platform guidance
- Monitoring of non-valid (error, invalid, or no result) rates

Any unusual increase in non-valid results should prompt review of specimen preparation procedures, pipetting technique, reagent handling, and instrument performance.

## Verification of Negative Pools

To ensure that pooled testing procedures remain reliable, laboratories may periodically verify the performance of pooled testing by randomly selecting a small proportion of negative pools for individual testing of the constituent specimens.

As a practical guide, approximately 1 to 5% of negative pools, or at least one negative pool per testing batch or testing day, may be selected for verification testing.

This verification helps confirm that pooled testing procedures are being performed correctly and that negative pools do not contain missed positive specimens.

## Monitoring Unexpected Results

Laboratories should monitor for unusual testing patterns that may indicate procedural errors or contamination. These may include:

- Rate of false positive pools, defined as pooled tests that are MTB detected but where all individual specimens test MTB not detected after deconvolution testing
- Trends in pooled test positivity compared with expected epidemiological positivity rates
- Agreement of Ct values between pooled and individual positive specimens, where available, to assess consistency of detection
- Increased frequency of non-valid result outcomes
- Failure to perform deconvolution individual testing for all specimens in a positive pool

Such events should trigger review of the pooled testing procedure, including specimen identification, pipetting technique, workspace organisation, and adherence to the SOP.

## Handling Positive Pools with No Individual MTB Detected Results

In rare situations, a pooled test may be positive but none of the individual specimens tested during deconvolution testing show an MTB detected result.

If this occurs:

- Collect a second specimen from each individual included in the original pool.
- Test each new specimen individually using routine LC-aNAAT procedures.
- If any repeat specimen shows MTB detected, report the result according to routine procedures.
- If all repeat specimens show MTB not detected, report the results for all individuals as MTB not detected.

All individuals from the original positive pool should be recorded in the patient follow-up log and monitored clinically, with additional testing performed if clinically indicated.

## Documentation of Non-Valid Test Results and Corrective Actions

All non-valid test results, operational errors, and corrective actions should be documented in laboratory registers or quality management records.

Corrective actions may include:

- Repeating tests when sufficient specimen volume remains
- Reviewing specimen preparation and pooled testing procedures
- Retraining staff on aliquoting technique and specimen identification
- Reviewing instrument maintenance and calibration records

Significant deviations from expected performance should be reported to laboratory supervisors and addressed according to the laboratory quality management system.

### External Quality Assurance

Where external quality assessment (EQA) schemes exist for LC-aNAAT testing, laboratories performing pooled sputum testing should continue to participate in these programmes. Laboratories should liaise with EQA providers to ensure that the EQA provider has sufficient understanding of pooled testing procedures and is able to appropriately support or adapt quality assurance activities where feasible.

Pooled testing procedures can be incorporated into existing external quality assurance activities, including:

- national or regional proficiency testing schemes
- supervisory visits and laboratory audits
- NTP laboratory quality monitoring systems

Integration of pooled sputum testing into existing quality assurance systems helps ensure that diagnostic performance remains consistent with routine individual molecular testing.

## 10. Recording and Reporting of Results


Accurate recording of pooled and individual test results is essential to maintain clear linkage between specimens, pools, and any individual deconvolution tests performed. Laboratory records must allow each specimen included in pooled testing to be traced throughout the testing process.

### Pool and Specimen Identification and Traceability

Each specimen must have a unique individual identifier, and each pool must be assigned a unique pool identifier (Pool ID). These identifiers must be clearly linked so that every specimen included in a pool can be traced and individually retested if required. For example:

**Pool ID format = [Site or laboratory code] – [Date] – [Pool number] = LIV-20260309-P01**

Laboratories must maintain a pool to individual specimen mapping system documenting which specimens are included in each pool. At a minimum, records should link:

- Individual specimen ID
- Pool ID
- Pooled test result
- Results of any deconvolution individual testing

Example:

| Pool ID | Specimen ID 1 | Specimen ID 2 | Specimen ID 3 | Specimen ID 4 |
|---|---|---|---|---|
| **LIV-20260309-P01** | TB-001245 | TB-001246 | TB-001247 | TB-001248 |

The Pool ID should appear on:

- the pooled sample container
- the laboratory register or electronic record
- the LC-aNAAT instrument entry or result file

Traceability may be maintained using paper registers or electronic systems such as laboratory information systems, barcode systems, or spreadsheet tracking tools. Regardless of the system used, Pool IDs and specimen IDs must be recorded consistently across all testing steps.

## Recording of Pooled Test Results

All pooled tests must be recorded in the laboratory register or electronic laboratory information system used for routine LC-aNAAT testing.

At a minimum, the following information should be documented:

- Pool ID
- Date of testing
- Pool size plus documentation of why pool size not the “usual” that that lab/setting uses (i.e. why deviated)
- Individual specimen IDs included in the pool
- Pooled test result (MTB detected, MTB not detected, trace, error, invalid, or no result)

Where laboratory registers can be modified, an additional Pool ID column may be added to link individual specimens to the corresponding pooled test.

In laboratories using fixed paper registers that cannot be modified, pooled testing information may be recorded on a separate pooled testing log sheet or tracking form. The final individual test results should then be entered in the official laboratory register according to routine reporting procedures.

Other indicators routinely documented for individual LC-aNAAT testing should be maintained (cartridge serial numbers, instrument identifiers, operator information...)

## Reporting of results

For clinical management, final results should be communicated at the individual specimen level as described in **Section 8: Interpretation of Results**.

Countries may determine how much information about the pooled testing procedure is included in laboratory reports. In some settings, laboratories may report the result simply as MTB not detected for the individual specimen, without reference to the pooled testing procedure. In these situations, the result is interpreted and reported as an individual negative molecular test result, even though individual testing was not performed because the specimen was part of a pooled test that was negative.

In all cases, pooled testing is a laboratory workflow strategy, and the result communicated to clinicians should correspond to the final individual interpretation used for clinical decision making, in accordance with NTP guidance.

# 11. Waste Management

Waste management procedures for pooled sputum testing follow the same biosafety and waste disposal practices used for routine individual LC-aNAAT testing.

## Disposal of biological waste and cartridges

All biological waste generated during pooled testing should be handled and disposed of according to national biosafety guidance and routine laboratory procedures for molecular TB testing.

This includes:

- used sputum containers
- pipettes and pipette tips
- absorbent bench pads
- sample preparation materials
- pooled sample containers or tubes
- used LC-aNAAT cartridges

## Disposal of specimen reagent remnants

After pooled testing and any required individual deconvolution testing have been completed, remaining specimen reagent mixtures may be discarded according to routine biological waste disposal procedures.

Specimen containers and pooled sample containers should be closed securely prior to disposal and handled in accordance with local laboratory biosafety protocols.

## Surface decontamination

Routine surface decontamination procedures used for LC-aNAAT testing should be followed. Work surfaces should be disinfected regularly using appropriate laboratory disinfectants, particularly after specimen handling and pool preparation, to minimise the risk of cross contamination.

Laboratories should follow the same cleaning and decontamination protocols used for routine molecular TB testing platforms.

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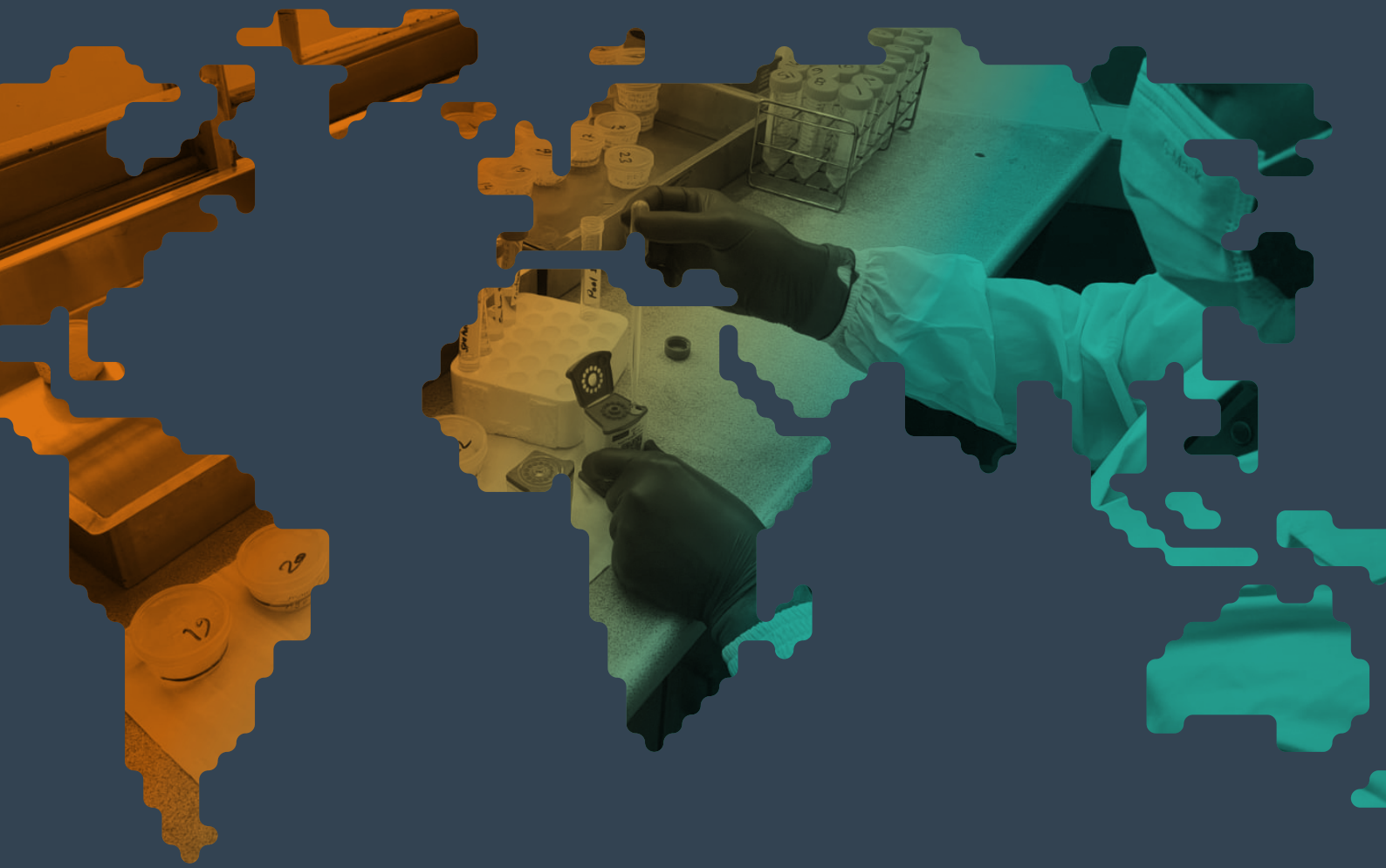
Pooling Toolkit

# Frequently Asked Questions (FAQs)

On pooled sputum testing for TB diagnosis



Start4All

**This section provides answers to common questions from National TB Programmes, clinicians, laboratory staff, and implementers regarding the introduction and use of pooled sputum testing for TB diagnosis.**

# 1. General questions about pooled sputum testing

### *What is pooled sputum testing?*

Pooled sputum testing is a laboratory approach in which small aliquots from several individual sputum specimens are combined and tested together in a single molecular assay. If the pooled test result is negative, all specimens included in that pool are interpreted as negative. If the pooled test result is positive, each specimen in the pool is tested individually to identify the positive sample(s).

### *Why is pooled sputum testing used?*

Pooled testing improves testing efficiency by allowing more people to be tested with the same number of molecular cartridges. This can help expand access to molecular testing, particularly in settings where TB positivity rates are relatively low.

### *In which settings is pooled testing most useful?*

Pooled testing is most useful in high volume testing settings where many specimens are expected to be negative. Examples include community-based case finding (CBCF) activities and large outpatient screening programmes. Pooled testing should be applied in line with current WHO guidance for individuals with symptoms, and with caution in CBCF settings where sensitivity may be lower.

### *Does pooled testing affect diagnostic accuracy?*

Pooled testing introduces dilution of specimens, which may slightly reduce sensitivity for specimens with very low bacterial load (i.e. trace). However, studies have shown that pooled sputum testing maintains high overall agreement with individual molecular testing. Some studies have reported lower sensitivity when specimens are collected through community-based case finding, which is reflected in WHO guidance, highlighting the need for careful implementation in such settings.

### *Does pooled testing require new laboratory equipment?*

No major new equipment is required. Pooled testing can be implemented in laboratories already performing molecular TB testing using LC-aNAAT platforms.

### *Can pooled testing be implemented in mobile or outreach laboratories?*

Yes. Pooled testing can be implemented in mobile laboratories or outreach settings provided that standard molecular testing procedures, biosafety practices, and specimen traceability systems are maintained.

# 2. Operational questions for laboratories

### *Which specimens can be used for pooled testing?*

Pooled testing has been evaluated using sputum specimens tested on LC-aNAAT platforms such as Xpert MTB/RIF Ultra but the technique can be applied equally to other molecular testing platforms.

### *Can raw sputum be pooled directly?*

No. Each specimen must first be processed individually with the manufacturer's sample reagent according to routine molecular testing procedures. Pools are then created using aliquots from the processed sputum reagent mixtures.

### *What is the minimum sputum volume required for pooling?*

As a general guide, at least 1 mL of raw sputum is recommended to ensure that sufficient processed specimen remains available for both pooled testing and potential individual deconvolution testing.

### *What should be done if a specimen has insufficient volume for pooled testing?*

If sputum volume is insufficient, pooled testing should not be performed and the specimen should be managed according to routine laboratory procedures, such as individual testing or requesting a new specimen according to national guidance.

### *How many specimens can be included in a pool?*

Pools commonly contain between two and four specimens. The optimal pool size may depend on local TB positivity rates and operational considerations. Larger pools may increase efficiency in low positivity settings but can reduce sensitivity due to dilution, while smaller pools are more appropriate where positivity is higher or where maintaining sensitivity is critical.

### *What should laboratories do if there are not enough specimens to form a full pool?*

Laboratories may create smaller pools rather than delaying testing or perform individual testing according to local laboratory procedures.

### *How long can processed specimens be stored before testing?*

After mixing sputum with sample reagent, specimens should be tested within four hours if kept at room temperature. If testing cannot be performed within this timeframe, specimens may be stored at 2 to 8°C and tested within 24 hours according to platform guidance. Where delays are anticipated due to laboratory operating hours, weekends, or public holidays, sample grouping and pool size may be adapted to ensure testing is completed within recommended timeframes.

### *What happens if a pooled test result is positive?*

If the pooled result is MTB detected, including trace, each specimen included in the pool must undergo individual deconvolution testing using the remaining processed sputum reagent mixture.

### *How should trace results be handled in pooled testing?*

If a pooled test result is trace, individual testing of all specimens in the pool is required. Interpretation of trace results obtained during individual testing should follow National TB Programme guidance.

### *Can rifampicin resistance be interpreted from a pooled test result?*

No. Rifampicin resistance signals detected in pooled tests cannot be attributed to a specific specimen. Individual deconvolution testing is required to determine whether resistance is present and in which specimen. However, in settings with a high burden of drug-resistant TB, a resistance signal in a pooled test may still provide an early alert, prompting careful and timely follow up testing of individual specimens.

## 3. Troubleshooting and common operational issues

### *What should be done if a pooled test is positive but all individual tests are negative?*

This may occur due to contamination, specimen mix up, or assay variability near the limit of detection. A second specimen should be collected from each individual and tested individually. If any repeat specimen is MTB detected, report accordingly; if all are MTB not detected, report all individuals as MTB not detected. Individuals should be recorded and followed up clinically as appropriate.

### *What should be done if pooled testing produces frequent error, invalid, or no result outcomes?*

Laboratories should review pipetting technique, reagent handling, and instrument maintenance, and repeat testing according to standard LC-aNAAT procedures.

### *What if TB positivity rates are higher than expected?*

Pooled testing is most efficient when TB positivity rates are relatively low. If many pools become positive, laboratories may consider reducing pool size or temporarily returning to individual testing.

## 4. Programme and communication considerations

### *What if staff are concerned that pooled testing could miss TB?*

Pooled testing may slightly reduce sensitivity for specimens with very low bacterial load. However, pooled testing can allow many more people to access molecular testing using the same resources.

### *What if clinicians are unfamiliar with pooled testing?*

Laboratories may explain that pooled testing is a laboratory workflow used to improve testing efficiency and that final diagnostic decisions are always based on individual test results. National TB Programmes (NTPs) and implementing institutions should ensure that clinicians and laboratory staff are trained in pooled testing procedures and in how to interpret results. Early engagement of healthcare providers supports understanding and professional buy-in.

### *How should pooled testing be explained to people undergoing testing?*

The level of information provided about pooled testing may be determined by NTPs. In some settings, pooled testing may not be routinely explained, but where needed, pooled testing can be explained simply as testing multiple samples together to improve efficiency, with individual testing performed if required.

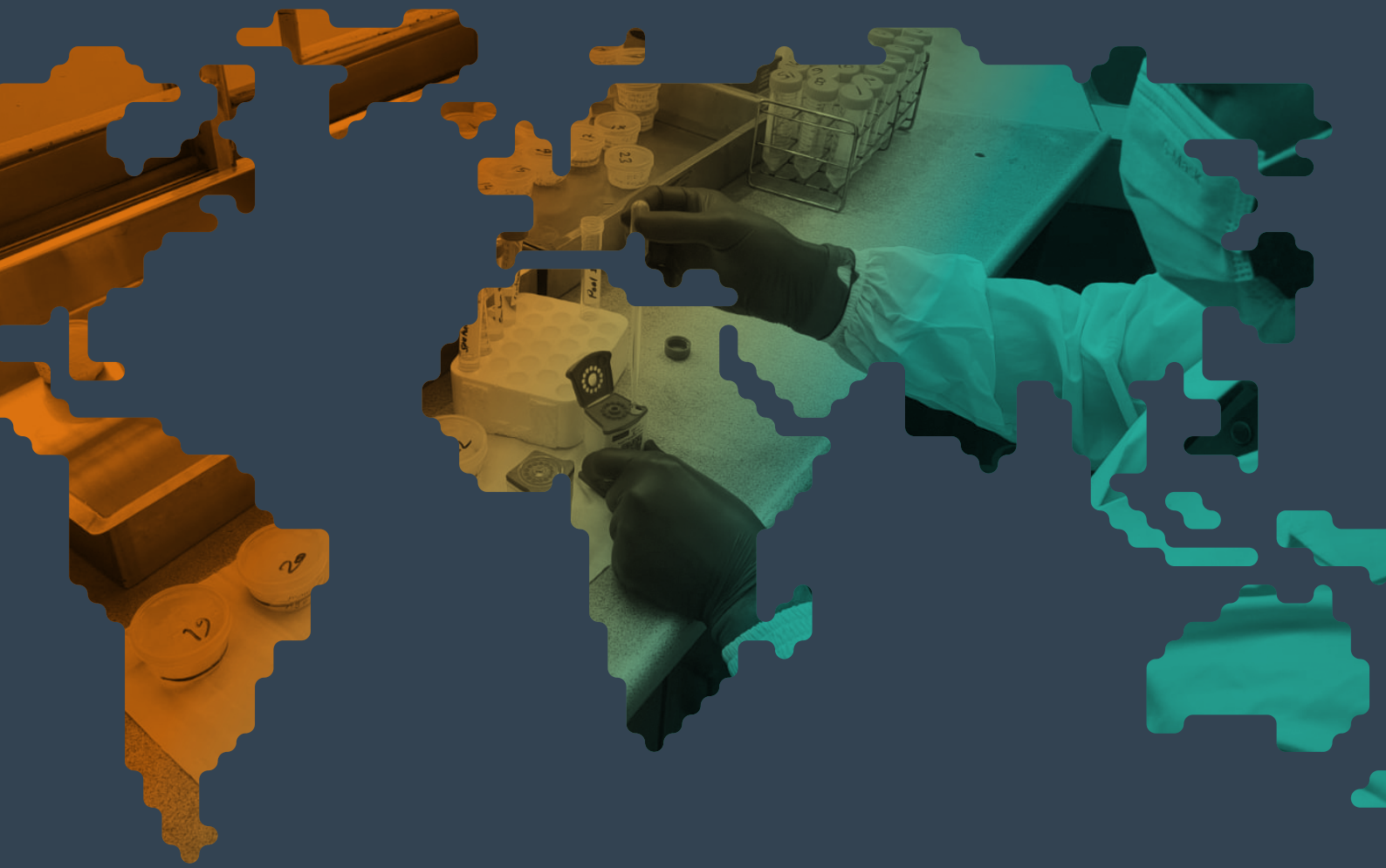
### *How are results from pooled testing reported to individuals?*

Results are reported at the individual level for clinical management, typically as MTB detected or MTB not detected. In many settings, results may be reported without reference to pooled testing. The level of detail provided should be determined by the NTP.

Pooling Toolkit

# Site readiness checklist

For implementing pooled sputum testing



Start4All

# Site readiness checklist for implementing pooled sputum testing

**This checklist outlines the minimum requirements to assess whether a laboratory site is ready to implement pooled sputum testing using LC-aNAAT platforms. National TB Programmes and implementing institutions may define additional requirements based on local context and programme needs.**

## How to use this checklist

- **Site ready for pooled testing implementation:** All items are marked "**Yes**".
- **Site not ready for pooled testing implementation:** One or more items are marked "**No**". All gaps should be addressed before implementation.

---

## 1. Laboratory Infrastructure

| Item | Yes | No |
|---|---|---|
| Laboratory already performs routine LC-aNAAT testing for TB | ☐ | ☐ |
| Adequate bench space for specimen handling and pooled testing | ☐ | ☐ |
| Clean, well-ventilated laboratory workspace | ☐ | ☐ |
| Hand hygiene and infection prevention facilities available | ☐ | ☐ |
| Biohazard waste disposal system in place | ☐ | ☐ |

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## 2. Equipment and Supplies

| Item | Yes | No |
|---|---|---|
| Functional LC-aNAAT platform (e.g. GeneXpert or equivalent) | ☐ | ☐ |
| Adequate supply of molecular test cartridges | ☐ | ☐ |
| Sample reagent and testing consumables available | ☐ | ☐ |
| Transfer pipettes or micropipettes available | ☐ | ☐ |
| Sufficient pipette tips or disposable transfer pipettes | ☐ | ☐ |
| Containers or tubes available for pooled samples | ☐ | ☐ |
| Rack or tray system to organise pooled specimens (e.g. muffin tray or rack) | ☐ | ☐ |

### 3. Staff Capacity and Training

| Item | Yes | No |
|---|---|---|
| Laboratory staff trained in routine LC-aNAAT testing | ☐ | ☐ |
| Staff trained on pooled sputum testing procedures | ☐ | ☐ |
| Staff understand specimen traceability and pool mapping | ☐ | ☐ |

---

### 4. Specimen Management and Workflow

| Item | Yes | No |
|---|---|---|
| Specimen labelling system in place | ☐ | ☐ |
| System to link individual specimens to pools (pool ID system) | ☐ | ☐ |
| Laboratory register or electronic system adapted to record pool IDs | ☐ | ☐ |
| Workflow allows storage of processed specimens until pooled results are available | ☐ | ☐ |
| Procedures in place for deconvolution individual testing of positive pools | ☐ | ☐ |

---

### 5. Programme Context and Testing Volume

| Item | Yes | No |
|---|---|---|
| Testing volume sufficient to support pooled testing | ☐ | ☐ |
| TB positivity rate appropriate for pooled testing | ☐ | ☐ |
| Specimen transport systems functioning for receiving samples | ☐ | ☐ |
| National TB Programme approval for pooled testing | ☐ | ☐ |

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### 6. Quality Assurance and Supervision

| Item | Yes | No |
|---|---|---|
| Laboratory participates in routine LC-aNAAT quality assurance systems | ☐ | ☐ |
| Procedures for monitoring pooled testing performance available | ☐ | ☐ |
| System for documenting procedural errors and corrective actions | ☐ | ☐ |
| Supervisor responsible for monitoring pooled testing implementation | ☐ | ☐ |

---

### 7. Overall Site Readiness Assessment

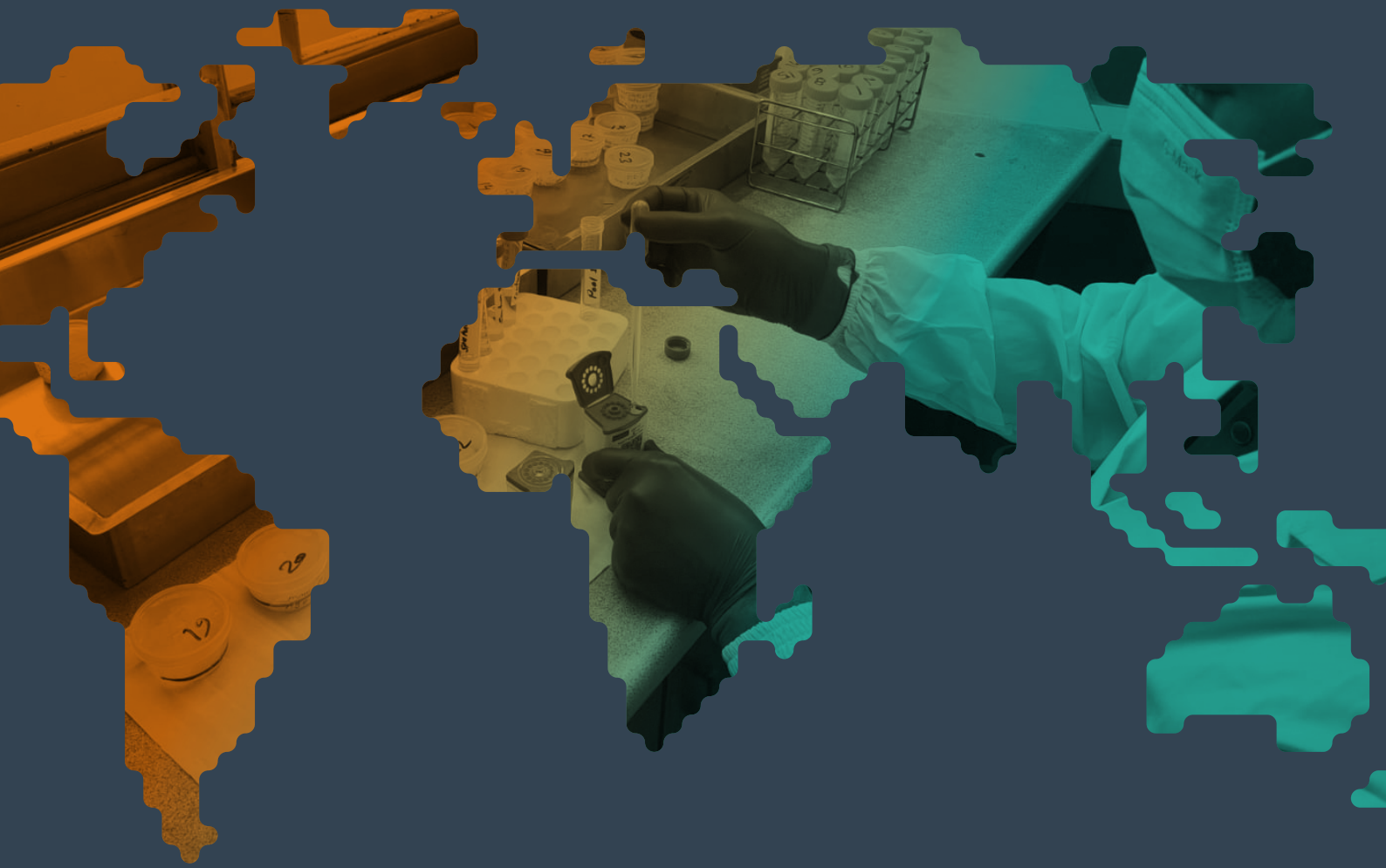
| Assessment | Yes | No |
|---|---|---|
| Site ready for pooled testing implementation | ☐ | ☐ |

Notes:

Pooling Toolkit

# When and why to use pooled testing

## Case Study 1



Start4All

# 1. Purpose and background

This implementation case study provides guidance on key questions related to pooled testing within routine TB diagnostic services using low complexity automated nucleic acid amplification tests (LC-aNAAT). The case study is primarily intended for Ministry of Health, National TB Programmes (NTPs), laboratory teams, supervisors, implementers, and researchers.

Pooled testing is not new. It has been used in other areas of disease, including syphilis[1] and SARS-CoV-2[2], to reduce costs and efficiently expand laboratory capacity. For this case study, we draw on experience from "Start Taking Action for Tuberculosis Diagnosis" (Start4All - **lstmed.ac.uk/start4all**).

SCAN ME

Start4All is a seven country Unitaid-funded research consortium that works with National TB Programmes (NTPs) to assess the diagnostic performance, costs and cost effectiveness, operational feasibility and acceptability of new tools and approaches. The tools and approaches incorporated included computer-aided detection chest x-ray (CAD-CXR), C-reactive protein (CRP) on fingerprick blood tests, urinary third-generation lipoarabinomannan assays (LAM), and pooling of sputum samples. All of the nearly 15,000 participants recruited to Start4All received each of these tests and, regardless of the results of any of these tests, an individual sputum Xpert Ultra. Each participant also provided a sputum for mycobacterial culture as the microbiological reference standard.

Our analyses of these approaches cut across performance, health economic evaluation, and feasibility and acceptability. Of note, our health economic evaluation considered two complementary economic perspectives for pooled samples: the cost effectiveness of pooled testing compared with individual testing; and the potential test savings achieved when pooled testing is applied.

In this case study, we will focus on pooled testing for TB diagnosis using nucleic acid amplification tests (LC-aNAAT). We will use our Start4All experience to describe when to use pooled testing, what pooled testing is, who is best placed to use pooling, and why it is a practical, useful, and cost-saving strategy.

# 2. Use cases (WHEN to use pooled testing)

Pooled testing can help NTPs address three practical questions:

- How to expand molecular testing coverage with the same number of tests and staff
- How to maintain coverage at a lower cost
- How to use existing laboratory capacity more efficiently without compromising diagnostic accuracy

Therefore, pooled testing is most relevant and applicable in specific contexts. The points below outline the conditions, settings and populations where pooling is appropriate and likely to function most effectively and cost-effectively.

## 2.1 Conditions that make pooled testing appropriate

- Low to moderate pooled test positivity rate (the main driver of efficiency) up to 30%[3]. Where positivity is close to the upper end of this range, actual cartridge savings may vary because they depend on how the positive sputum samples from people with TB are distributed across pools.
- Adequate sputum quality (i.e. sputum rather than saliva) and volume
- Ability to track samples and manage "deconvolution" individual testing of samples in positive pools
- Laboratory capacity to handle pooled testing workflows

## 2.2 Settings where pooled testing works well

- Routine outpatient clinics
- Community or outreach mass screening where many samples are collected in a short period of time
- Hub laboratories processing large batches of specimens from multiple facilities

### 2.3 Populations suitable for pooled testing

- Individuals being screened in low-risk or general population groups
- Individuals undergoing periodic screening for employment, school entry, or other routine requirements

## 3. Testing algorithms (WHAT, at high level, the pooled testing workflow is)

Pooled testing can be used with LC-aNAAT such as Xpert MTB/RIF Ultra[4] where most evidence currently exists[5], but can also be applied to other molecular test platforms including, for example, Truenat MTB[6]. The general workflow is similar across platforms and does not require changes to the processes and procedures or the assay itself.

### 3.1 Pool size choices

The choice of pool size depends mainly on expected TB positivity rate. Smaller pools may be more appropriate in higher-yield settings and populations; larger pools where yield is low and cartridge savings are likely. Studies to date have typically used pool sizes between 2 and 4. In practice, pool of 4 is most commonly applied, although larger pools are feasible where specimen quality, workflows, and expected yield allow.

### 3.2 Deconvolution testing for positive pools

The simplest implementation of pooled testing involves combining equal volumes from multiple individuals' samples and testing them together using a single test. If the pooled test result is negative, all individuals are presumed not to have the disease. If positive, each sample is retested individually to identify who has the disease[7].

Pooled testing could also be considered for resistance testing among individuals with confirmed TB, especially in low-resource settings with higher burdens of drug-resistance. However, the accuracy of pool - rather than individual-level resistance testing has not been clearly described and predictive value will vary depending on the population and pre-test probability of drug-resistance.

## 4. Evidence from Start4All (WHY pooled testing is valuable)

### 4.1 Diagnostic performance

Across Start4All sites, pooled testing of sputum samples using Xpert Ultra showed minimal loss of accuracy compared with individual Xpert Ultra testing. When both were compared with culture, pooled testing had a sensitivity of 84.8% (CI 82.4%–87.0%) and a specificity of 98.3% (CI 98.0–98.5%) while individual testing had a sensitivity of 88.0% (CI 85.8%–89.9%) and a specificity of 97.7% (CI 97.4%–97.9%). This pattern appeared consistent across adults aged 15 years and above (~14,000 participants), children and young adolescents aged 1 to 14 years (~800 participants), and people with and without HIV. However, interpretation requires caution. Current WHO guidance does not recommend pooled testing for children or people living with HIV due to limited evidence. In addition, consistent with WHO evidence, diagnostic sensitivity may be reduced in community-based case finding (CBCF) settings, likely reflecting lower bacillary load and variability in specimen quality.

For the minority of pools that were negative despite an individual who provided a sample for the pool testing positive by individual Xpert and/or the microbiological reference standard, these were linked to samples with very low bacillary load or trace level results in individual testing, particularly in community-based screening activities.

### 4.2 Cost implications

Pooled testing can reduce cartridge use, save staff time, and lower overall testing costs, especially where most samples are negative. Evidence from Start4All showed that pooled testing could help stretch available resources without compromising the testing accuracy. Our aggregate analysis for all individuals, irrespective of HIV status, indicated that pooled testing could more than half cartridge use and were associated with a reduction in overall commodity costs of 31% at primary health care facilities and 17% at district hospital level.

### 4.3 Operational and efficiency findings

Findings from Start4All, including qualitative feedback from people undergoing testing and healthcare workers delivering the testing, showed that pooled testing was perceived to meaningfully support large screening activities. As noted above, pooled testing led to a 51.1% reduction in the number of cartridges required, which could ease pressure on laboratory capacity and resources, and free-up tests and resources for use elsewhere. Healthcare and laboratory workers we interviewed acknowledged that the pooling approach introduced some additional steps to workflows. However, they also noted that, when sample labelling and transport were well organised, adequate training was offered, and existing workflows were appropriately refined, pooling did not slow turnaround time.

## 5. Readiness considerations (WHO can implement pooled testing)

Pooled testing is easiest to introduce where LC-aNAAT tests are already in routine use because the essential infrastructure and skills are in place. Before adopting pooled testing, NTPs should confirm that:

- sputum transport and storage systems are reliable;
- staff are comfortable with routine LC-aNAAT workflows; and
- laboratories can track pool IDs and deconvolution tests in their data systems.

Pooled testing is straightforward to introduce where these elements already exist, but NTPs can also strengthen them progressively as they expand molecular testing. This is particularly important because the optimal opportunity gap for pooled testing is in districts that currently lack LC-aNAAT access and rely on smear microscopy, where pooled testing can make molecular diagnosis feasible by reducing cartridge consumption and lowering the overall cost per person tested. In these contexts, NTPs often face a practical trade-off. In the short term, pooled testing may represent a small increase in cost compared with sputum smear. However, pooled testing enables far broader access to molecular testing at a cost that many NTPs would consider acceptable and achieves greater efficiency in cartridge use compared with testing all samples individually with LC-aNAAT cartridges.

## 6. Monitoring and evaluation (HOW to assess impact)

For planning purposes, NTPs mainly need to confirm that pooled testing maintains diagnostic reliability and offers a meaningful benefit to the testing system. Some key high-level “signals” for NTPs to consider in assessments of pooled testing following implementation include:

- Diagnostic signals, such as whether positive pools consistently lead to identification of individuals with TB
- Throughput signals, for example whether more samples can be processed with existing platforms
- Operational signals, including timely deconvolution testing and manageable workflow
- Cost signals, such as reductions in cartridge use per person tested where pooled testing is applied

Detailed indicators and measurement approaches, including distinctions between research and routine NTP monitoring, are provided in more detail in the linked case study in this manual. See **Case Study 2: How to implement and evaluate pooled testing**.

# 7. Lessons for national planning

Pooled testing set-up and performance will be optimal when certain conditions are in place at subnational or national level. Early consideration of these conditions during planning phases can guide NTPs on where and how to introduce pooled testing.

## 7.1 Where pooled testing fits well

- High throughput activities such as community screening or district hub testing
- Low to moderate positivity (up to 30%) settings where most pools will be negative
- Existing basic LC-aNAAT infrastructure, even if limited to a number of hubs
- Reliable sputum transport from peripheral or community sites

## 7.2 Integrating pooled testing into routine NTP practice and activities

Pooled testing can be targeted rather than nationwide. NTPs may vary:

- Pool size depending on positivity rates with larger pools (up to 4) most efficient in low-prevalence settings, while smaller pool sizes or individual testing may be more appropriate as positivity increases;
- Workflow location, such as sputum collection at peripheral sites, pooled testing at hubs; and
- Testing strategy, for example individual LC-aNAAT in high yield populations or settings, pooled testing in low yield populations or settings.

## 7.3 Common pooled testing misconceptions can be addressed with clear communication[7]

- Sensitivity loss is minimal and performance is comparable to individual testing when pooled testing is used appropriately.
- Turnaround time does not increase when training is adequate, and deconvolution testing pathways and workflows are well organised.
- People seeking care do not need repeated clinic visits solely to provide an additional specimen; deconvolution testing can be managed using the original sputum sample.
- Results from positive pools are not shared with individuals, only their individual sample result following that positive people, so uncertainty or anxiety is reduced amongst those being screened using pooled testing.

## 7.4 Implications for NTPs guidance

NTPs may consider incorporating the following elements into national or regional guidance:

- Eligibility criteria outlining when pooled testing is appropriate including its use among individuals with TB symptoms or those who screen positive, in line with WHO guidance. NTPs may prioritise individual testing for specific groups, such as PLHIV, children, or individuals with previous episodes of TB, where evidence for pooled testing remains limited or concerns about reduced sensitivity and equity apply.
- Updated LC-aNAAT algorithms showing where pooled testing fits within existing diagnostic pathways
- Clear deconvolution testing steps for managing positive or trace pools
- Adjustments to training, supervision, and quality assurance systems to ensure consistent practice

Pooled testing can also support the gradual extension of molecular testing into areas that still rely on smear microscopy, allowing NTPs to broaden access without a proportional increase in cartridges or instruments.

## 7.5 Addressing knowledge gaps in pooled testing

During Start4All, we have learnt much about the performance, cost-effectiveness, feasibility and acceptability of pooled testing strategies both alone and in combination with other tests such as CAD-CXR. However, knowledge gaps remain.

Potential areas for further NTP evaluation and/or research investigation include:

- Performance and cost savings of pooled testing to non-sputum near point-of-care nucleic acid amplification tests (NPOC-NAAT) specimens, such as tongue swabs

- Assessing resistance detection strategies in pooled samples and optimal deconvolution approaches to identify individuals with drug-sensitive and drug-resistant TB
- Conducting operational modelling to guide pool size, placement, cost effectiveness and equity of access across diverse epidemiological landscapes
- Undertaking implementation and scale up feasibility studies to understand how pooled testing performs when integrated into routine NTP activities
- Exploring alternative pooled testing methods, such as matrix (non-hierarchical) pooled testing

## 8. A real-world example of pooled testing for TB in Cameroon from 2020-2025

### Case study from Dr Valerie Donkeng of Centre Pasteur Du Cameroun, Cameroon

Below, to complete this case study, is a real-world example of the scale-up and integration of pooled testing into routine practice in collaboration with the Cameroon NTP. It details the planning, implementation, scale-up, performance and cost saving results (including by site and positivity rate), and lessons learned from one of the world's first large-scale programmatic introductions of pooled testing for TB.

### Background

In Cameroon, as in many low-income high TB burden settings, access to molecular tests, and in particular Xpert MTB/RIF Ultra tests, is challenged by multiple interlinked barriers.

These barriers including: the high cost of cartridges and equipment; infrastructure and maintenance requirements; and shortages, stock-outs, and disrupted supply chains of Xpert MTB/RIF Ultra cartridges. Additionally, GeneXpert modules are often shared with other programmes, such as HIV programmes where the modules are used for viral load monitoring, which can create competition for machine time and lead to delays in TB diagnosis and treatment initiation.

These barriers were exacerbated by the COVID-19 pandemic.

### Intervention and level of integration

In Cameroon, between 2020 and 2022, pooled testing was initially implemented at two reference laboratories in response to TB test cartridge shortages brought about due to the COVID-19 pandemic.

From 2023-2025, following a positive initial experience at reference laboratories, the intervention was scaled up to 18 Xpert labs in four of 10 regions of the country as part of a TB REACH Wave 10 project. These Xpert labs received specimens referred for molecular testing from a network of primary healthcare centres and district hospitals.

Pooled testing for TB on the Xpert MTB/RIF and Xpert MTB/RIF Ultra cartridges was conducted in collaboration with the NTP, regional tuberculosis programs and delegations of health, and public and private health facilities of Cameroon.

### Population and coverage

People attending 183 health facilities (both primary care centers and district hospitals) who were identified as having presumptive TB including TB symptoms (cough, fever, night sweats, weight loss) and/or had risk factors for TB (diabetes, at risk of undernutrition or with undernutrition, smoking, alcohol use disorders, people living with HIV not on ART) were identified as suitable for the pooled testing strategy.

### Xpert laboratory technicians: Training, testing and results reporting

Pooled testing was conducted by Xpert laboratory technicians that already had experience performing individual Xpert testing for TB. Laboratory technicians were trained on pooled testing in a one-day workshop, that also included site TB clinicians and NTP coordinators. Training materials included a **job aid** on how to perform pooled testing and a **5-minute video** demonstration of pooled testing. Individual patient results, whether from individual testing or pooled testing, were recorded in an **Xpert laboratory register** and in the NTP laboratory register. Summary testing data were recorded in a 1-page **Xpert lab monthly indicator summary sheet** that was submitted to the data team each month, together with the Xpert testing data exported from the Xpert instrument at least monthly.

### Monitoring results and ongoing feedback

Xpert lab technicians, reference laboratory personnel, and the data monitoring and evaluation team participated in monthly virtual meetings where summary results were discussed, with monitoring of lab level data including TB positivity rates, testing efficiency (cartridges used per result produced), and number of cartridges used and saved. These implementers also participated in a WhatsApp group where questions were raised and addressed, and best practices shared.

### Deciding whether to test individually or in a pool, and pool size determination

Pooled testing was performed on a pragmatic basis, with laboratory personnel at each Xpert lab deciding whether to test each specimen individually or in a pool, and the size of the pool to use. This decision was informed by factors including the TB microscopy result of the specimen (if available), the operational characteristics of the lab at the time of the test (including the laboratory positivity rate, number of specimens received for testing on that day, the availability of Xpert MTB/RIF Ultra cartridges and the functionality and availability of GeneXpert modules for testing), and the characteristics of the person tested (including the HIV-status and ART-status and if the specimen was from a child).

**Indications for individual testing include** (typically, assuming sufficient testing cartridges and instrument modules are available):

- Positive smear microscopy result (from one of 63 microscopy labs that refer specimens for testing to the 18 Xpert testing labs); in this case, Xpert testing was performed for rifampin resistance testing
- Individual client characteristics, including: person living with HIV not on ART or a child
- Specimen characteristics, including: extrapulmonary specimen or sputum with volume < 1 mL

**Factors contributing to pool size determination:**

- Historical TB positivity rate at laboratory (e.g. If a laboratory has a historical positivity rate of 13% of specimens positive for TB, they might use pools of 3 samples, while a lab with a historical rate of 7% might use pools of 4 samples)[2]
- Number of specimens available for testing at the laboratory in the turnaround time window for the client (e.g. If the lab typically performs pools of 4, but only 3 specimens are available in the turnaround time window – a weekend or evening closure of facility - and people are waiting for results, then a pool of 3 would be used)

## Results of scale up in 18 Xpert laboratories, from 2023-2025

Over an 18-month period, 73,188 specimens from people with presumptive TB were tested at 18 GeneXpert labs across four regions of Cameroon, including 12,863 specimens tested individually and 60,325 specimens tested in pools of size 2-8.

By using pooled testing, an estimated **39,191 additional people had molecular testing results for TB** as compared to 21,134 if only individual testing had been used.

The efficiency of specimens tested in pools, with 3.3% of pools with MTB detected (1,999/60,325), was 0.35 cartridges used per specimen with result. This meant that the pooled testing strategy **saved 65% of Ultra cartridges compared to individual testing**, with better efficiency as pool size increased and positivity rate decreased.

The average instrument time to result for specimens tested with pooled tested was **23.7 minutes per result as compared to 66.2 minutes for specimens tested individually**, saving 65% of instrument time. This is predominantly driven by the high proportion of pools that were negative and did not require re-testing.

**The average test cost per result of specimens tested in pools was $2.79 as compared to $7.97** for specimens tested individually (65% cost reduction). This is comparable to current reported prices for individual NPOC swab-based testing. To provide results for the 60,325 specimens in pools during this work **the overall test cost was $168,438 as compared to $480,790 that would have been needed for individual testing**.

## Experience of three representative laboratories

Summary indicators of three laboratories that adopted pooled testing are shown below:

| Laboratory indicator | Lab A | Lab B | Lab C |
|---|---|---|---|
| % TB positivity | **13%** | **9%** | **3%** |
| Median pool size | **3** | **4** | **4** |
| Efficiency (# cartridges used / # results delivered) | **0.70** | **0.49** | **0.38** |
| % of cartridges saved (vs. individual testing) | **30%** | **51%** | **62%** |
| Average number of specimens tested per day | **11** | **8** | **21** |
| % of specimens pooled (vs. tested individually) | **71%** | **79%** | **97%** |

Lab A had a TB positivity rate of 13% as compared to Lab B with 9% and Lab C with 3%. Lab A used primarily pools of 3, while labs B and C typically used pools of 4. Due to TB positivity and pool size differences, the percentage of cartridges saved with pooled testing was 30% in Lab A as compared to 62% in Lab C. Although the optimal pool size for a positivity rate of 3% is 6, as for Lab C, significant cartridge savings were still achieved with pools of 4.

The proportion of invalid results following a pooled test was 3.2% (505/15,593), which was similar to the proportion of invalid results following an individual test (5.0%, 675/13,358) in this work.

Overall, different laboratories with different TB positivity rates and workloads (from 8 to >30 specimens tested per day), were able to successfully implement pooled testing and increase testing efficiency to provide molecular TB test results for many more people than would have been possible with individual testing alone.

## Key considerations / Lessons learned

**Pooled testing is highly accurate for TB diagnosis:**

- Pooled testing leads to a slight reduction of sensitivity overall (-3.2% in Start4All); specimens with low numbers of bacteria, leading to individual results of MTB detected very low or trace are more likely to be missed on pooled testing. This is particularly relevant in CBCF settings and is consistent with WHO evidence showing reduced sensitivity in these contexts.
- Some of the variations in pooled testing can also be attributed to the inherent variability in the assay (multiple individual tests on the same person also can produce discrepant results, especially for trace and very low semi quantitative results); this variability is also a consideration in individual testing
- As with all diagnostic testing, which is never 100% sensitive, patient follow-up after a negative test is critical, including consideration of clinical diagnosis and/or additional diagnostic testing as indicated.

**Documentation:**

- In this intervention, the laboratories used a system for recording each individual patient result following pooled testing directly in the Xpert instrument (using a combination of a pool ID number and specimen ID number); results were also recorded in a paper **Xpert laboratory register** that includes one line for each pool and one line for each individual specimen in the pool.
- As pooled testing is scaled up programmatically, it will be useful to have a system to record the result of each pool and the result of each specimen, for tracking patient results and also cartridge usage. Ideally, this could be added as an option into the connectivity solution (e.g. Xpert LIS tracker) as well as in the NTP laboratory register.

**Materials:**

- Pooled testing requires some additional supplies including sputum mugs (1 per pool) and disposable pipettes (approximately 30-70% additional depending on testing efficiency). These were procured locally at $0.18 per sputum mug and $0.14 per disposable pipet.
- The Xpert Sample Reagent (SR) provided with the Xpert cartridges was used for all specimen processing; one SR bottle can be used to process more than one specimen by using the pipets provided with the cartridges (or procured locally) to aseptically add the SR to the specimen (rather than by pouring as described in the packet insert).

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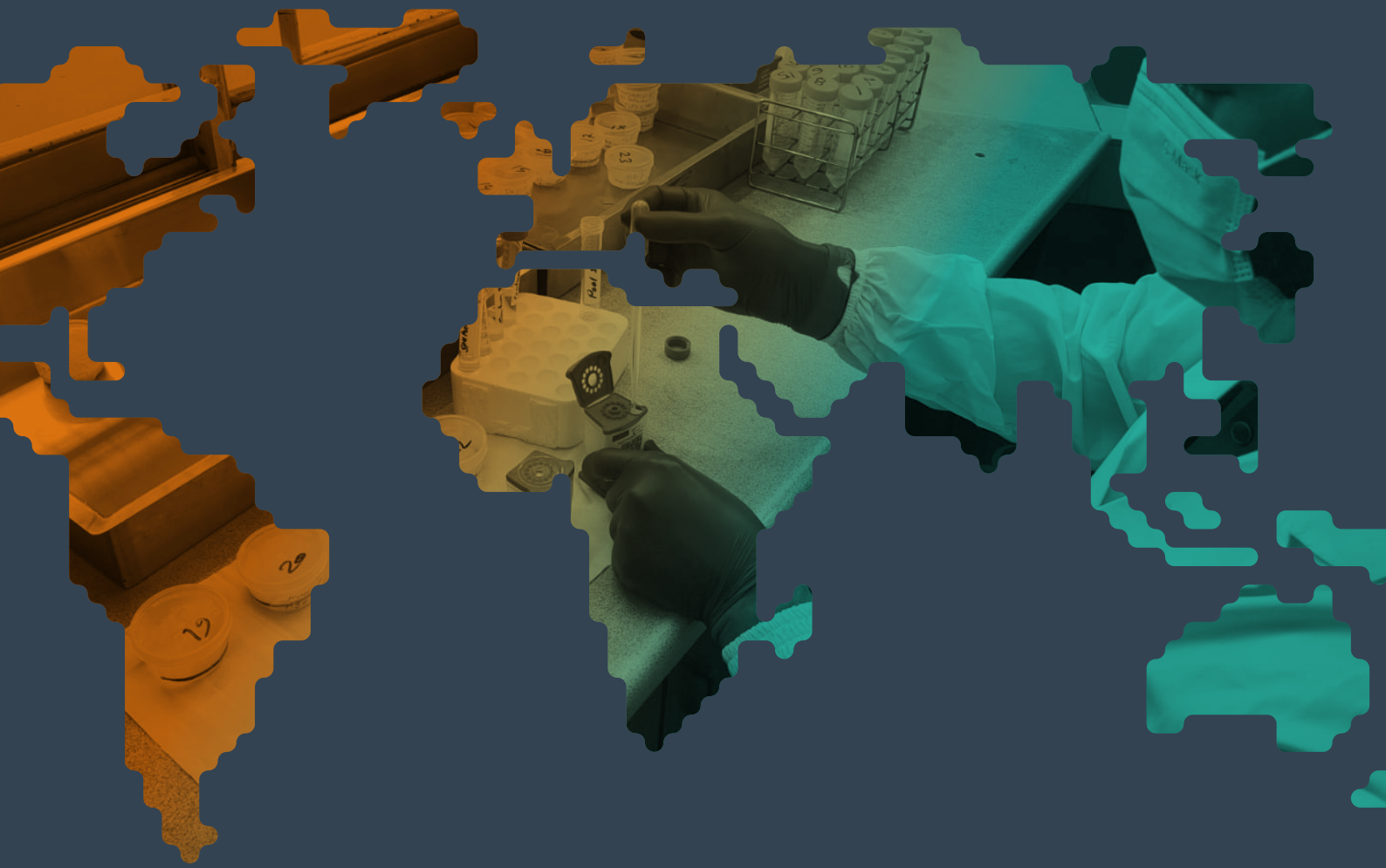
Pooling Toolkit

# How to implement and evaluate pooled testing

## Case Study 2



Start4All

# 1. Purpose

This second implementation case study provides practical guidance on how to introduce and run pooled testing within routine TB diagnostic services using low complexity automated nucleic acid amplification tests (LC-aNAAT). It outlines the operational steps, tools, and quality measures needed to carry out pooled testing safely and consistently.

The document is intended for Ministry of Health, National TB Programmes (NTPs), laboratory teams, supervisors, implementers, and researchers, and helps bridge programme indicators used for routine monitoring with the more detailed research indicators that support deeper evaluation.

# 2. Site preparation, equipment, and supplies

Pooled testing can be conducted in any laboratory already performing LC-aNAAT, as the infrastructure requirements are the same including but not limited to minimum biosafety, workflow space, and staff skills required (see the WHO Tuberculosis Biosafety Manual and Infection Prevention and Control guidance). Pooled testing should take place in a clean, well-ventilated area with access to hand hygiene, waste disposal, and clear separation between specimen handling and administrative space.

## 2.1 Biosafety and infection prevention and control (IPC) considerations

- Standard LC-aNAAT biosafety practices apply, including gloves, gowns, masks, and surface decontamination.
- Pools are formed with sputum mixed with sample reagent, which substantially reduces infectiousness, but does not make samples non-infectious. Standard precautions must therefore be followed.
- A simple bench layout (pipette area, mixing area, waste bin, clean area for test) helps maintain IPC and reduces cross-contamination risk.

*Photo from the Start4All study showing vehicle-based pooled testing facilities during community-based case findings activities needs set-ups.*

## 2.2 Equipment and consumables needed

### 2.2.1 Core items for standard individual LC-aNAAT testing:

- LC-aNAAT platform
- LC-aNAAT tests with sample reagents
- Calibrated transfer pipettes or micropipettes
- Sputum containers with secure caps
- Racks, timers, absorbent bench pads
- Waste containers for biohazard disposal
- PPE (gloves, lab coat/gown, mask, eye protection)
- Sites may use paper-based or electronic systems depending on capacity:
  - Core option: handwritten labels, ID stickers, paper registers
  - Enhanced option: preprinted barcodes, electronic logbooks, simple pool-to-individual mapping in existing laboratory information system (LIS)

### 2.2.2 Additional materials needed specifically for pooled testing:

- Extra transfer pipettes or tips (for combining aliquots into pools)
- Pool containers or tubes
- Low cost pot holders such as a repurposed or adapted “muffin baking tin” or a simple 3D printed rack to organise samples belonging to the same pool, provide clearer visual grouping, make aliquoting easier, and reduce the risk of mixing samples.

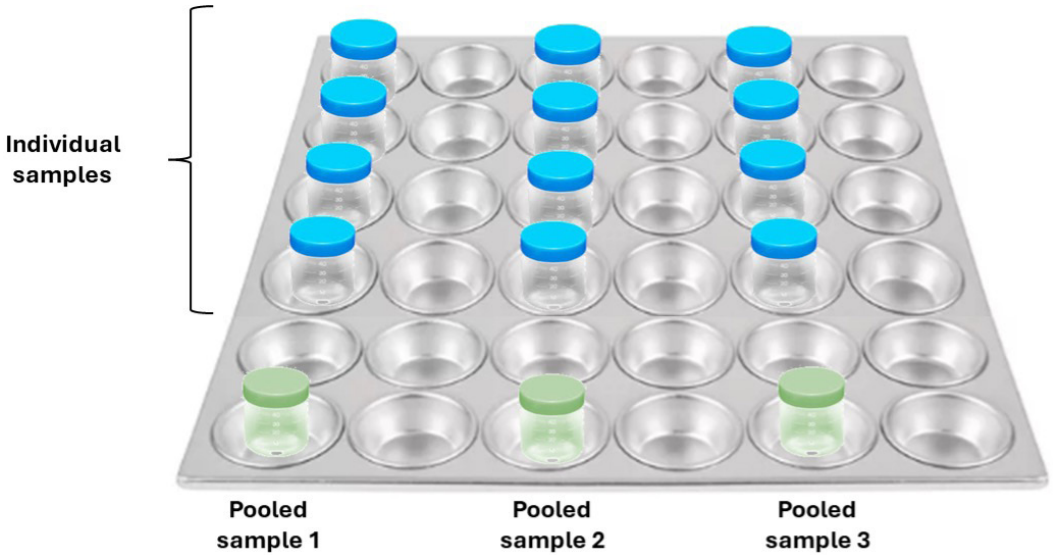



*Figure 1. An example of “muffin baking tin” used in the Start4All study, containing 3 pooled samples (green pots) from 4 individual samples (blue pots).*

- Stickers or labels that distinguish pools from individual specimens
- A simple pool-to-individual mapping system
  - Core option: paper-based mapping sheet
  - Enhanced option: barcode-based or LIS-supported mapping
  - In both approaches, each pool and each constituent sample need a unique, traceable ID to ensure correct deconvolution testing and reporting.

# 3. Step by step workflow

The pooled testing workflow follows the same principles as individual LC-aNAAT testing for TB, but with additional steps for preparing and identifying pools. The sequence below reflects procedures used across all Start4All sites, during which we tested sputum samples of nearly 15,000 people with both pooled and individual Xpert Ultra (see **Case Study 1: when and why to use pooled testing** for more details about the Start4All project).

## 3.1 Receiving and preparing specimens

- **Log each specimen on arrival:** enter the specimen details into the lab register or electronic log with a unique individual ID.
- **Check basic suitability:** confirm correct label, adequate sputum quality, adequate sputum volume (to allow both pooled and individual testing if needed), and container integrity.
- **Apply pooled testing eligibility rules:** mark which specimens are eligible for pooled testing according to NTP guidelines.
- **Prepare for processing:** place eligible specimens together in a designated area for sample reagent processing.

## 3.2 Sample processing and preparation

Follow your standard individual testing procedure for the chosen LC-aNAAT platform. Once each specimen has been processed according to routine practice, the processed samples will be ready for pooled or individual testing.

## 3.3 Forming pools from processed samples (pool size, mixing, aliquoting)

- Confirm pool size according to NTP (for example, pools of 3 or pools of 4).
- Assign and label a pool tube with a unique pool ID.
- For each processed specimen to be included in the pool:
  - Mix the individual tube briefly (vortex or inversion) to re homogenise.
  - Aliquot an equal volume from each processed sample (for example, X µl per sample) using a sterile disposable pipette.
- Combine aliquots into the pool container.
- Mix the pool tube gently (vortex or inversion) so the pooled sample is uniform.
- Place the pooled tube in a rack, ready for loading onto the instrument.

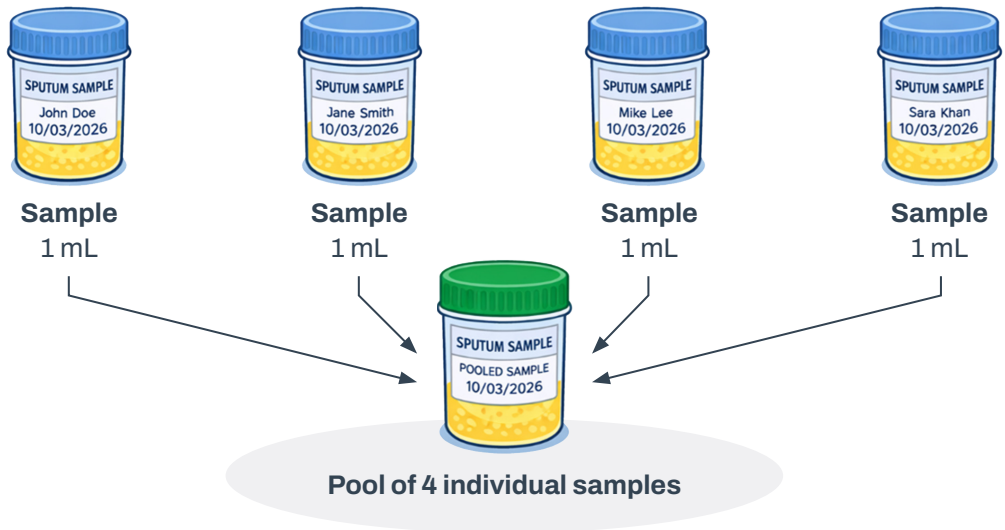



*Figure 2. Example of preparing a ~4 mL pool of four samples.*

### 3.4 Running pooled tests and deconvolution testing (load the pooled sample)

- Using a sterile pipette, transfer the required volume of the pooled sample into the LC-aNAAT test tube or cartridge.
- Load into the instrument and start the run using the standard software protocol.

### 3.5 Interpret the pool result according to platform guidance

- If the pool is MTB-negative: all individuals in that pool are considered negative for TB.
- If the pool is MTB-positive or trace, proceed with individual testing of all individual processed specimens that contributed to that pool:
  - Homogenise each individual sample again.
  - Load individual aliquots into separate LC-aNAAT test/cartridge.
  - Run tests and record individual results.
  - Follow national guidelines for clinical management based on individual results

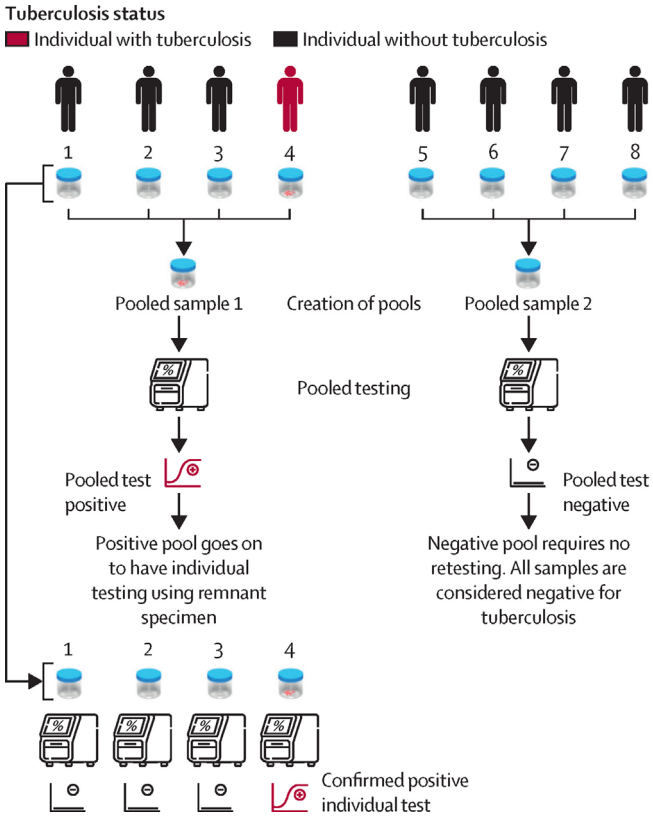



*Figure 3. Pooled testing algorithm using sputum specimens adapted from Iem et al, Lancet Resp Med 2025.*

### 3.6 Managing storage and transport of specimens

- Unprocessed sputum before sample reagent addition: store according to local LC-aNAAT policy (usually at 2–8°C if not processed the same day).
- Processed individual samples awaiting pooled testing or deconvolution testing: can generally be kept at room temperature for a limited period as per platform guidance, or at 2–8°C if delayed (up to 24 hours).
- Specimens from peripheral sites
  - Ship raw sputum to the pooled testing lab using existing transport pathways.
  - Sample processing and pooled testing are done at the receiving laboratory, not at peripheral sites.

## 3.7 Preventing and managing cross-contamination

Although the basic LC-aNAAT process remains unchanged, pooled testing introduces more specimen handling. Key practices include:

- Use a new pipette for each individual specimen.
- Mix tubes carefully and, when not in use, keep containers closed.
- Refresh absorbent bench pads and disinfect surfaces between batches.

If contamination is suspected (for example, positive pool with negative individuals), repeat testing and a short procedural review can help identify the root cause.

## 3.8 Potential issues and how to avoid them

| Issue | How to avoid |
|---|---|
| **Mislabelled or wrongly assigned samples** | Use pre-labelled containers or assign pool IDs before aliquoting |
| **Uneven sample volumes in pools** | Always aliquot equal volumes; avoid “eyeballing” |
| **Contamination leading to false-positive pools** | Change pipettes between samples; disinfect bench surfaces between batches |
| **Delayed deconvolution testing of individual samples in a positive pool** | Pre-sort individual samples and store them together immediately after pooled testing |
| **High error/invalid/no results rates** | Re-train on pipetting, ensure correct reagent ratios, check instrument maintenance. |

### 3.8.1 Do

- Use clear and consistent pool IDs to avoid mislinking specimens.
- Mix each individual processed sample well before aliquoting.
- Keep pool formation and cartridge loading areas physically separated.
- Run deconvolution testing as soon as possible after a positive or trace pool.
- Review error/invalid/no results rates regularly to detect workflow drift early.

### 3.8.2 Don’t

- Don’t pool raw sputum; always add sample reagent to individual samples first.
- Don’t share or report pool-level results to clients; only final individual results are meaningful.
- Don’t increase pool size solely for efficiency if positivity rises.

# 4. Quality assurance

Quality assurance (QA) in pooled testing includes both laboratory process checks and data accuracy checks, ensuring that results remain reliable and traceable throughout pooled testing and deconvolution testing.

## 4.1 Laboratory QA checks and indicators

- **Non-valid results (error, invalid, no result):** track the frequency of non-valid results. Increases may signal issues with aliquoting, reagent handling, or equipment performance.
- **Unexpected positive pools:** occasionally, a pool may test positive while all individuals test negative upon deconvolution. This typically indicates cross-contamination or mislabelling during pooled testing and should trigger a procedural review.
- **Completeness of deconvolution testing:** for every positive or trace pool, confirm that all individual samples in that pool undergo deconvolution testing and that none are missed.
- **Periodic checks of negative pools:** at set intervals, retest a small number of negative pools to confirm correct pooled testing and detect any missed positives due to dilution or handling errors.

These checks help identify operational drift and ensure that pooled testing maintains diagnostic performance.

## 4.2 Data QA (linking laboratory steps with correct reporting)

Accurate linkage between individuals, pools, and deconvolution results is central to pooled testing. Data management QA should ensure that all required fields have appropriate validation rules, and that a structured data query and reconciliation process is in place to accurately link each individual test ID to its corresponding pool ID. This includes verifying that every individual ID appears in one and only one pool, that no IDs are duplicated or missing, and that each pool contains only the intended constituent samples. Audit trails should be maintained so any discrepancies identified can be traced and resolved.

**Data QA should confirm:**

- Correct pool–individual mapping: all specimens assigned to the correct pool, with no duplicates or omissions.
- Deconvolution testing completeness: each positive/trace pool has a corresponding set of individual results.
- Accurate transcription or data entry: whether using paper or electronic systems, verify that pool results and individual results are correctly recorded and linked.
- Consistency across systems: laboratory registers, LC-aNAAT result files, and reporting forms should match.

These data checks prevent misclassification, lost results, or reporting errors and are essential for programme-level reliability.

# 5. Recording and reporting

Accurate record keeping and reporting is essential for pooled testing because each pool must be correctly linked to its constituent individual samples and any subsequent deconvolution tests. This can be done using paper-based registers, electronic systems, or a combination of both, depending on site capacity.

Records should maintain an audit trail showing when data were entered or modified and by whom, and any changes to sample IDs, pool assignments, or test results should be clearly documented.

Procedures should be in place to identify and resolve discrepancies between individual samples and pool IDs, including guidance on corrective actions or re-testing if errors are discovered.

All records must be stored securely, ensuring participant confidentiality, and recording practices should facilitate timely reporting, monitoring, and data extraction for analysis.

## 5.1 Pool IDs and linkage to individuals

Each individual sample should have a unique identifier that is consistently used across all records, and each pool must also have a unique pool ID linked to the IDs of its constituent samples. Minimum required elements include:

- Unique individual sample ID
- Assigned pool ID
- Date and time the pool was created
- Deconvolution testing status for all samples in that pool (if applicable)

A simple one-line entry for each individual is sufficient, if the pool ID links them together. Pool IDs should appear on:

- the pooled testing tube
- the laboratory register
- the LC-aNAAT result printout or electronic file

This ensures correct traceability through the entire testing pathway.

## 5.2 Modifications to registers and forms

Routine LC-aNAAT registers can be adapted using one additional column for Pool ID, without requiring major format changes. A typical register layout includes:

| Individual ID | Pool ID | Date received | Date pooled | Pool result | Deconvolution required (Y/N) | Individual result | Comments |
|---|---|---|---|---|---|---|---|
| | | | | | | | |

Where electronic systems are not available, permanent marker, or simple paper stickers or colour coding can help avoid mislinking individuals and pools.

## 5.3 Electronic tools (core vs enhanced options)

Electronic recording can simplify linkage between pool and individual results, but sites vary widely in capacity. Options include:

### 5.3.1 Core (minimum) electronic approach

- Manual entry of pool IDs into existing GeneXpert software fields
- Simple Excel or Google Sheets template to track pool–individual relationships
- Scanned copies of paper registers stored centrally

This approach requires no new software and works in low-connectivity settings.

### 5.3.2 Enhanced electronic approach

- Use of barcoded labels that auto-populate pool and individual IDs
- Linking LC-aNAAT result files to electronic registers for automatic pool result import
- Integration with national diagnostic connectivity systems (e.g., GxAlert, DataToCare) where available

In both cases, the primary requirement is that every individual result can be traced back to its pool, and every positive pool is linked to complete deconvolution testing.

# 6. Training and supervision

The success of implementation and integration of pooled testing into routine practice depends on staff understanding their roles, consistently applying procedures, and receiving appropriate support as the workflow is introduced.

## 6.1 Staff roles and responsibilities

Clear task assignment helps minimise errors. Typical roles include:

- **Sample intake staff:** receiving specimens, verifying IDs, and determining pooled testing eligibility.
- **Laboratory technician:** processing individual specimens, forming pools, aliquoting, and preparing cartridges.
- **LC-aNAAT operator:** running pooled and deconvolution tests on the platform and reviewing internal controls.
- **Supervisor or team lead:** verifying correct pool–individual linkage, authorising deconvolution testing, and reviewing any incidents or discordances.

Roles may be combined or shared in smaller laboratories, provided responsibilities remain clearly documented.

## 6.2 Training, mentorship, and refresher support

Training should include the specific procedural differences introduced by pooled testing, including:

- Correct formation of pools from sample reagent processed specimens
- Accurate identification, labelling, and tracking
- Handling deconvolution testing without delays
- Recognising common pitfalls (pipetting errors, mislabelling, inadequate mixing)

Mentorship from experienced sites has proven extremely valuable. Regional or south–south and high-burden to high-burden learning exchanges, whether their TB epidemics and health systems are similar or contrasting, can accelerate adoption and strengthen confidence in implementation.

Refresher training may be needed after staff rotation or when workload changes.

## 6.3 Competency assessment

Competency can be assessed through a combination of:

- Direct observation of pooled testing and loading steps
- Review of register completeness and correct pool–individual mapping
- Error/invalid/No result rates monitoring over time
- Short written or practical assessments included in routine supervision visits

Sites should be encouraged to document competency assessments using a simple checklist.

## 6.4 SOPs and training materials

Selected SOPs, training slides, videos, and job aids used in Start4All can be accessed through the Start4All LSTM portal here (link to be added). These resources can be referenced directly by NTPs seeking to introduce pooled testing or strengthen staff training.

# 7. Practical implementation tips and common pitfalls

This section summarises practical insights and feedback collected during Start4All qualitative research. This research included focus group discussions (FGD) and key informant interviews (KII) with stakeholders including implementation team members, people with TB, NTP staff, and policymakers across the seven partner countries and others from international organisations. Their perspectives clearly show that the success of pooled testing depends not only on the technical workflow but also on the broader laboratory and health system around it.

Reference: Iem V, Vo L, Nguyen Q, Codlin A, et al, *Feasibility and Acceptability of Pooled Sputum Testing for Tuberculosis: A Multi-Country Qualitative Study of the Start4All Project*. Available at SSRN: https://ssrn.com/abstract=6334397 or http://dx.doi.org/10.2139/ssrn.6334397

## 7.1 System readiness and infrastructure

Countries should ensure basic transport, LC-aNAAT capacity, and simple data systems are functioning before starting pooled testing. Weak data linkage, poor sample labelling, or inconsistent biosafety practices could be source of errors and should be addressed early.

> ***"In the facility, we have infrastructure already there... But in the community setting, a lot of things need to be in place."***
>
> Malawi KII3, Male, TB technical advisor

## 7.2 Strategic and operational value

Pooled testing adds value only in low-positivity or high-volume contexts. Teams warned that applying pooled testing in high-positivity settings reduces efficiency and can create unnecessary workload. Countries should assess local yield carefully before choosing pool sizes.

> ***"If most turn out negative, it is good for us. So, we save a lot of cartridges... it is something that is worth trying. Because with the experience [of Start4All], we have realised our positivity is also low."***
>
> Kenya KII2, Male, Laboratory staff

## 7.3 Workforce preparedness and procedural precision

Training and clear steps are essential. Errors could come from staff turnover, rushed work, or gaps in refresher training. Countries should plan ongoing supervision, not just one-off training.

> ***"Wouldn't they sit down later and take 20 samples and mix them? So, all this boils down to good training. You have to train your staff well. Because we know what happens afterwards. 'I'm even tired today' and all that."***
>
> Cameroon KII5, Female, Government Hospital TB focal point

### 7.4 Evidence, confidence, and implementation boundaries

Demonstration across partner countries of minimal sensitivity loss helped build trust in pooled testing at a local level, especially in country settings in which a pooled testing strategy had not previously been used. However, concerns remained around the use of pooled testing in specific groups including children, PLHIV, and people with very low bacillary load or likely trace results. Countries should set clear eligibility rules and avoid pooled testing for groups where sensitivity could be a concern.

> ***"If you have a region with a large number of these people (children, people living with HIV, and individuals with a history of TB treatment), and you dilute the sputum, it seems like we're going backwards… I'm just not sure pooling is the best approach."***
>
> International KII1, Female, Market access specialist

### 7.5 Stakeholder and community engagement

Misunderstandings about "mixing samples" or fear of shared results created hesitation in some sites. Clear explanation that pool results are never communicated to individuals and that one sample is generally sufficient helps avoid confusion.

> ***"If [healthcare providers] explain it very clearly, people would support it."***
>
> Brazil FGD with people living with TB

## 8. Analysis considerations: Core vs ideal approaches for evaluating pooled testing

The evaluation of pooled testing can be done across a spectrum from light-touch to in-depth depending on the resources and mandate of the NTPs. Most programmes will focus on core, pragmatic indicators, while funded studies or WHO GDG evaluations require a more detailed, research-grade assessment.

### 8.1 Performance

NTPs usually need to understand whether pooled testing performs consistently when compared with their existing diagnostic pathway. Research studies, in contrast, assess full diagnostic accuracy and cost-effectiveness.

#### 8.1.1 Routine real-world approach

- Comparator is the current standard of care, usually individual LC-aNAAT.
- Track simple signals such as:
  - concordance between pool results and deconvolution individual results
  - positivity patterns by site or activity
  - trends in errors/invalids/no results rates
  - unexpected positive pools (for procedural review)

### 8.1.2 Research approach

- Comparator is culture as the microbiological reference standard.
- Full analysis of sensitivity, specificity, and subgroup performance (HIV status, age groups, community vs facility samples).
- Examination of trace results, low-burden samples, and any discordances.

## 8.2 Cost and cost-effectiveness

For NTP planning, a small set of practical cost indicators is often sufficient. Research studies can explore full economic impact.

### 8.2.1 Routine real-world approach

- Cartridges used per person tested
- Extra consumables for pooled testing (tips, tubes)
- Additional staff time required for pooled testing and deconvolution testing
- Incremental cost per extra person tested or screened

### 8.2.2 Research approach

- Full costing including overheads, equipment amortisation, and energy use
- Sample transport and workflow costs
- Cost per person with TB detected and per TB treated
- Budget impact modelling for different pool sizes and positivity rates
- Full analysis of costs and cost-effectiveness including not only cartridge savings but broader costs such as other consumables and equipment, staff costs and time, laboratory costs, and overheads.
- Potential for modelling studies to estimate the impact of diagnostic and treatment coverage, and cost savings at scale, including consideration of alternative use of resources freed-up by pooled testing.

## 8.3 Feasibility and acceptability

Routine programmes may not have the capacity to conduct interviews or focus group discussions (FGDs), but simple tools can still capture essential implementation feedback.

### 8.3.1 Routine real-world approach

- Short, structured instruments such as Intervention Appropriateness Measure, Acceptability of Intervention Measure, and Feasibility of Intervention Measure to assess acceptability and feasibility
- Routine supervision checklists
- Simple staff feedback on ease of use and workflow impact
- Basic indicators such as the proportion of eligible samples successfully pooled

### 8.3.2 Research approach

- Mixed-methods participatory research to understand multisectoral stakeholders' perceptions of pooling prior to implementation including qualitative interviews and FGDs with laboratory staff, implementers, and people accessing services
- Workflow observation or time-motion and patient simulation modelling studies
- Detailed analysis using established implementation science frameworks
- Quasi-experimental stepped wedge interventional cohort studies and/or cluster randomized controlled trials that evaluate individual-level and population-level health and socioeconomic outcomes related to pooled testing, from linkage to appropriate treatment and care, to treatment success rates, to TB notification and prevalence.
- Political economy analyses and health systems readiness assessments for pooled testing.

Notes:

# Pooled Testing Visual Tools

**This toolkit includes additional visual resources designed to support learning and strengthen the practical implementation of pooled testing.**

## Pooled Sputum Testing for Tuberculosis Diagnosis Training Presentation:

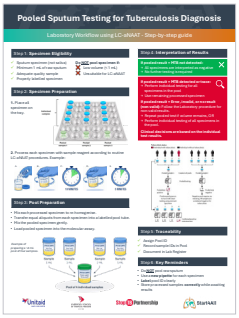
This PowerPoint training package is intended for laboratory staff and screening teams involved in sputum sample processing, pooled LC-aNAAT testing, result interpretation, and documentation.

Scan the QR code or visit:
**lstmed.ac.uk/start4all/toolkits**


## Pooled Sputum Testing for Tuberculosis Diagnosis Poster:

This poster provides a step-by-step workflow for preparing, pooling, testing, and interpreting sputum specimens for TB diagnosis using LC-aNAAT.

Scan the QR code or visit:
**lstmed.ac.uk/start4all/toolkits**




## Films:

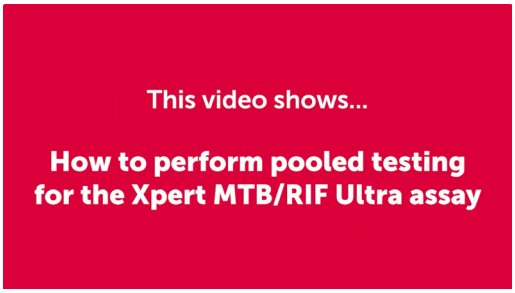
These short StopTB Partnership films introduce the concept of pooled sputum testing and provide clear, step-by-step guidance on how to perform the method. They are ideal for training staff and supporting consistent implementation across teams. These videos will also be available on our website: **lstmed.ac.uk/start4all/toolkits**

### What is pooled testing and why it's relevant


Scan the QR code or visit:
**youtube.com/watch?v=WsMLjcf2MgQ**

### How to perform pooled testing for TB

Available in 3 languages:




**English**
Scan the QR code or visit:
**youtu.be/gQdQ60PbAhQ**

**Spanish**
Scan the QR code or visit:
**youtu.be/4MFM0-9Jfs4**

**French**
Scan the QR code or visit:
**youtu.be/t7B_MggbgAU**

# Acknowledgements


## Leadership and coordination

This toolkit was developed under the Start4All initiative, funded by Unitaid, with technical leadership from the Liverpool School of Tropical Medicine (LSTM).

- **Overall supervision:** Dr Tom Wingfield (LSTM)
- **Technical oversight and toolkit development:** Dr Vibol Iem (LSTM)

## Technical contributors

The following experts contributed technical input and operational insights during the development of the pooled testing toolkit:

- Dr Rachel Byrne (LSTM)
- Dr Ana Cubas Atienzar (LSTM)
- Dr Jacob Creswell (STP)
- Ms Nadia Kontogianni (LSTM)
- Dr Tushar Garg (STP)

## Reviewers

The toolkit was reviewed by experts in tuberculosis diagnostics, laboratory systems, and implementation science:

- Dr Victor Santana Santos, Universidade Federal de Sergipe (UFS), Brazil
- Dr Valerie Donkeng, Centre Pasteur Du Cameroun, Cameroon
- Dr Melissa Sander, Centre for Health Promotion and Research, Cameroon
- Dr Comfort Vuchas, Center for Health Promotion and Research, Cameroon
- Teyim Pride, Tuberculosis Reference Laboratory Douala, Cameroon
- Etiendem Asonganyi, Chifu Nadege, Neh Angela and Cyrille Mbuli - Centre for Health Promotion and Research, Cameroon

Their feedback helped ensure the clarity, technical accuracy, and operational relevance of the materials.

## Implementing partners

This toolkit draws on implementation experiences from the Start4All consortium and investigators in:

- **Bangladesh:** International Centre for Diarrheal Disease Research, Bangladesh (icddr,b)
- **Brazil:** Federal University of Sergipe (FUS)
- **Cameroon:** The Centre for Health Promotion and Research (CHPR)
- **Kenya:** Kenya Medical Research Institute (KEMRI)
- **Malawi:** Malawi Liverpool Wellcome Programme (MLW)
- **Nigeria:** Janna Health Foundation (JHF) and Zankli Research Centre (ZRC)
- **Viet Nam:** Friends for International TB Relief (FIT)

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This work is dedicated to Professor Luis Cuevas whose life mission was to bring TB diagnostics closer to those who need them. The world is less without him but better because of him.

## Programme coordination and project management support

- Ms Jude Jones (LSTM ADAPT)

## Photography

Pages 1, 21, 25, 29, 39, (seen within map), and page 4, Photo credit: Md. Sohag Mia, Research Officer of Mycobacteriology Laboratory, icddr,b. Image description: Under Start4All study, sputum samples were collected from individuals with presumptive pulmonary TB at the informal settlement areas of Dhaka metropolitan city during the chest X-ray campaigning. Healthcare worker tested the samples individually and by pooling with Xpert® MTB/RIF Ultra at the nearest Tuberculosis Screening and Testing Centers (TBSTCs).
Pages 11, 12 & 16 photo credit: Emily Pinna for the Stop TB Partnership and TB REACH in Cameroon.
Page 40 Photo credit: Center for Health Promotion and Research (CHPR), Cameroon.

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# Start4All

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LSTM
LIVERPOOL SCHOOL OF TROPICAL MEDICINE

Stop TB Partnership

Zankli Research Center

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KENYA MEDICAL RESEARCH INSTITUTE
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RESEARCH PROGRAMME

JANNA HEALTH FOUNDATION

CDC
U.S. Department of Health and Human Services
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