STANDARD OPERATING PROCEDURE

Truenat™ MTB Plus Assay using a Truelab Duo Analyzer (Version 1.0)

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IDDS

Definitions & abbreviations

PCR	Polymerase Chain Reaction
DNA	Deoxyribonucleic Acid
MTB	Mycobacterium tuberculosis complex
IPC	Internal Positive Control
Ct	Cycle threshold
LCD	Liquid-crystal Display
ECT	Elute Collection Tube

Scope

This SOP describes the use of the Truenat™ MTB Plus assay, a chip-based Real Time Polymerase Chain Reaction (PCR) test, for the semi-quantitative, detection and diagnosis of Mycobacterium tuberculosis complex bacteria (MTBC) in human sputum samples.

Education & Training

All lab staff performing this procedure must have successfully completed training in the following areas: potential risks to health (symptoms of TB disease and transmission), precautions to be taken to minimize aerosol formation and prevent exposure, hygiene requirements, wearing and use of protective equipment and clothing, handling of potentially infectious materials, prevention of incidents and steps to be taken by workers in the case of incidents (biohazard incidents, chemical, electrical, post exposure prophylaxes and fire hazards), good laboratory practice and good microbiological techniques, organization of work flow from clean to dirty areas, use of chemical and biological indicators, waste management, use of equipment (operation, identification of malfunctions, maintenance).

The training is given before a staff member takes up his/her post, strictly supervised, adapted to take account of new or changed conditions

Table of Contents

1. Procedure

1.1. Principle

The Truenat™ MTB Plus works on the principle of Real Time Polymerase Chain Reaction. A sputum specimen is first liquefied and lysed using the Trueprep™ AUTO MTB Sample Pre-treatment Pack. The DNA from the sample is then extracted using the Trueprep™ AUTO v2 Universal Cartridge Based Sample Prep Device and Trueprep™ AUTO v2 Universal Cartridge Based Sample Prep Kit. The extracted DNA is then amplified by the Truelab Real Time micro-PCR analyzer. The Truenat™ MTB Plus chip is placed on the chip tray of the Truelab™ Real Time micro PCR Analyzer. Six (6) µL of the purified DNA is then dispensed into the reaction well of the Truenat™ MTB Plus chip and the test is started.

1.2. Sample Type

Sputum samples

1.3. Equipment and Materials

1.3.1. Extraction of DNA

- Trueprep AUTO v2 Universal Cartridge Based Sample Prep Device
- Trueprep AUTO MTB Sample Pre-treatment Pack
- Trueprep AUTO v2 Universal Cartridge Based Sample Prep Kit

1.3.2. Amplification of purified DNA

- Truelab Duo Real Time Quantitative micro PCR Analyzer
- Truenat™ MTB Plus micro PCR chip
- Truelab™ micro PCR printer
- Truepet SPA fixed volume (6 µl) Precision micropipette
- DNase and RNase-free pipette tips with filter barrier

1.3.3. Others

- Truenat™ Positive Control Kit - Panel I
- Powder free disposable gloves
- Two waste disposal containers, with lids, containing bleach solutions
- Timer
- Two waste bags
- Microtube Stand
- Cartridge Holder
- Two cryovial racks

1.4. Reagents and Solutions

- Liquefaction buffer
- Lysis buffer
- Conc. bleach and 70% alcohol

1.5. Detailed procedure

1.5.1. Specimen collection

Spot and morning sputum samples (before eating or drink anything) are collected from each patient.

1.5.2. Sample storage and transportation

Samples collected for testing on the Truenat instruments should be stored in the fridge between 2°C to 8°C and transported to the testing lab. During transportation, the samples should be well parceled in a sample flask/sample transportation box at 2°C to 8°C containing ice packs.

1.5.3. Installation of Trueprep AUTO v2 Universal Cartridge Based Sample Prep Kit Reagent Pack

1. Connect a new reagent pack to the Trueprep Auto v2 device by inserting the Plug-in Connector into the slot provided **(Figure 1)**.

2. One reagent pack is sufficient to conduct 50 extractions.

3. When the instrument is off, press the "Power" button to switch on the Trueprep™ AUTO v2 device. Power in use indicator LED glows red. Note:
    - **Trueprep™ AUTO v2 device will not let you begin a run if the battery is low.**
    - For charging or to use Trueprep™ AUTO v2 on direct electricity line, connect the AC Adapter to the charging port on the left side of the back panel of the device and the other end to the lightning protected distributor.
4. When new buffer is loaded using a new Reagent Pack of color-coded reagent bottles, perform a buffer count reset. When prompted to change the Reagent Pack and reset, press 'start' and 'eject' simultaneously to reset.

Figure 1

Procedure to change Trueprep AUTO v2 Reagent Pack after completion of 50 extractions

a. After completion of 50 extractions, the Trueprep AUTO v2 device will prompt the user to change the reagent pack and reset the buffer count.

b. Disconnect the used reagent pack by removing the Plug-in connector.

c. Take a new reagent pack. Hold the reagent pack's connector and remove the cap.

d. Plug in the connector into the socket of Trueprep AUTO v2.

e. Press Eject button to open the cartridge holder and gently pull out the door.

f. Insert the Reagent card as shown and gently push to close the cartridge holder.

g. Press start button.

h. It will display New Reagent Pack Registered and Ejects the Reagent Reset Card.

i. Remove Reagent Reset card and proceed with further testing

1.5.4. Sample Processing procedure

Prior to sample processing on the Truelab instrument, the sputum sample should be homogenized and pipettable.

1. Put on personal protective equipment.
2. Clean the working surfaces with freshly prepared 10% bleach then with 70% alcohol.
3. Clean the instruments with paper towel wet with 70% alcohol.
4. Empty the two liquid waste containers and fill the two waste containers ½ way with concentrated bleach solution.
5. Open an Trueprep AUTO MTB sample pre-treatment kit, which contains a graduated 1 mL transfer pipette, lysis buffer bottle and liquefaction buffer bottle. Bring all refrigerated samples or reagents to room temperature before using
6. Arrange the items needed to run a complete batch of 2 samples.
    I. Liquefaction buffer bottle
    II. Graduated 1ml and 3ml transfer pipette
    III. Lysis buffer bottle. Visually check for any damage and that the volume is 2.5ml. If the volume is less than 2.5ml due to damage, do not use that lysis buffer.
    IV. Cartridge pouch and the cartridge holder.
    V. Result register
7. Ensure the Trueprep AUTO v2 sample prep device is ON.
8. Arrange the cryovials containing the 0.5ml pipettable sputum in ascending order of sample numbers on the sample rack.
9. Record sample information (serial number, sample number, date of test) in the Truenat register.
10. Label lysis buffer bottle with corresponding sample number and date of extraction.
11. Place labelled lysis buffer bottle in front of the corresponding sample.
12. Add 2 drops of liquefaction buffer to sputum container containing 1st sample in the batch.
13. Swirl container to allow buffer to mix with sample.
14. Incubate for 10 minutes at room temperature. If sample is not pipettable after 10 minutes, incubate for another 5 minutes with swirling at 2 minutes intervals.
15. Transfer 0.5 ml of liquefied sputum sample from the sample container to the corresponding lysis buffer bottle using the 1ml graduated transfer pipette provided.
16. Dispose the transfer pipette into the container filled with concentrated bleach.
17. Add 2 drops of liquefaction buffer into the lysis buffer bottle.
    Note: To avoid cross contamination, DO NOT bring the nozzle of the liquefaction buffer bottle near the sample container.
18. Swirl gently to mix and incubate lysis buffer bottle at room temperature for 3-5 minutes and observe to ensure that the sample has completely liquified.
    Note: Sputum may be stored in lysis buffer for up to 1 week at 30°C with no degradation of DNA

1.5.5. Nucleic acid extraction

19. While incubating the sputum in the lysis buffer, tear open the cartridge pouch. Each pouch contains a cartridge, an eluate collection tube (ECT) and a transfer pipette.
20. Take out the cartridge and place on the cartridge stand and keep the transfer pipette and the ECT in the pouch for later use.
21. Observe the sample chamber and visually confirm that the reddish IPC is present. If absent, discard that cartridge and take a different one and report this issue.
22. Label the cartridge pouch with the patient number.
23. Label the cartridge with number of the sample and the date of test.

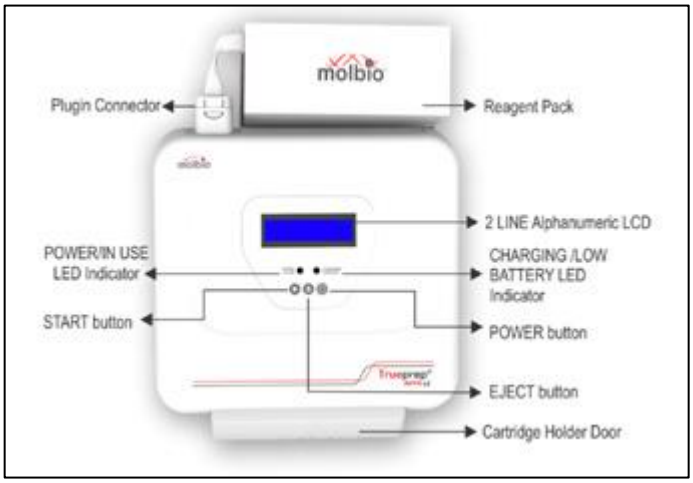


Figure 2

24. Open the sample chamber of the cartridge by gently pulling the black cap upward.
25. Transfer **ALL** the contents of the lysis buffer bottle (3 ml) into the sample chamber of the cartridge using the 3ml transfer pipette provided.
    Note: Sample should not be stored inside the cartridge. Therefore, only load the cartridge when ready to run the test.
26. Dispose the transfer pipette and the used lysis buffer bottle into the waste container filled with concentrated bleach.
27. Recap the cartridge sample chamber with the black cap and put on a fresh pair of gloves.
28. Press "Eject" button to eject cartridge holder **(Figure 2)**.
29. Gently pull out after ejection and insert the cartridge into the cartridge holder of the instrument **(Figure 3)**.
    Note: The orientation of the cartridge is to be placed as in Figure 3. Ensure that the site containing the sample is to the right, when looking at the front of the instrument.
30. Gently push to close the cartridge holder. You will hear 2 **clicking sounds** when the cartridge is properly loaded in the instrument.
    Caution: Placing the cartridge in the wrong orientation will cause the cartridge holder to remain open, and the cartridge will not be inserted in the device.

Figure 3

31. Press the "Start" button to begin the DNA extraction process.
32. The reagents from the bottles in the back will be automatically added to the cartridge based on the pre-programmed protocol.
33. While the test is in progress, prepare the next sample in the batch as in steps 1 to 12.

34. After 18-20 minutes, the device will give a beeping sound indicating completion of the extraction process with a displayed message.
35. The device will automatically eject the cartridge holder.
36. **IMPORTANT:** To avoid the elute from being evaporated by heat generated during the extraction process, remove the cartridge as soon as the cartridge holder is ejected.
37. Lift the cartridge up and place on the cartridge stand.
38. Inspect the tray in the cartridge holder for any spilled liquid.
    Note: In the event of a spill, dispose of the tray in container filled with concentrated bleach for 30 minutes and spray the cartridge holder with 70% isopropyl alcohol. After 5 minutes, place a new tray in the cartridge holder.
39. Take out the ECT tube from the test pouch and label the tube using the sticker provided in the pouch with patient number, age, sex, and date.
40. With the precision and filter barrier pipette tip, pierce the covering of elute compartment in the cartridge, and aspirate the entire amount of the elute.
41. Dispense the eluate into the labelled ECT tube and close the ECT tube cap tightly.
42. Put the ECT tube in the labelled ECT holder.
43. If eluate is not to be amplified immediately, store in a fridge at 4°C for up to 24 hours or at -20°C for up to 1 year.
44. Dispose the pipette tip and used cartridge into the container filled with concentrated bleach.
45. Dispose your gloves in dedicated waste container.
46. Load the next sample in the batch into the Trueprep instrument.
47. Transfer the ECT tube containing the eluate to the Truelab micro-PCR analyzer for amplification.

1.5.6. Nucleic acid amplification

Important note: The Truelab Duo device can run a maximum of 2 tests at a time.

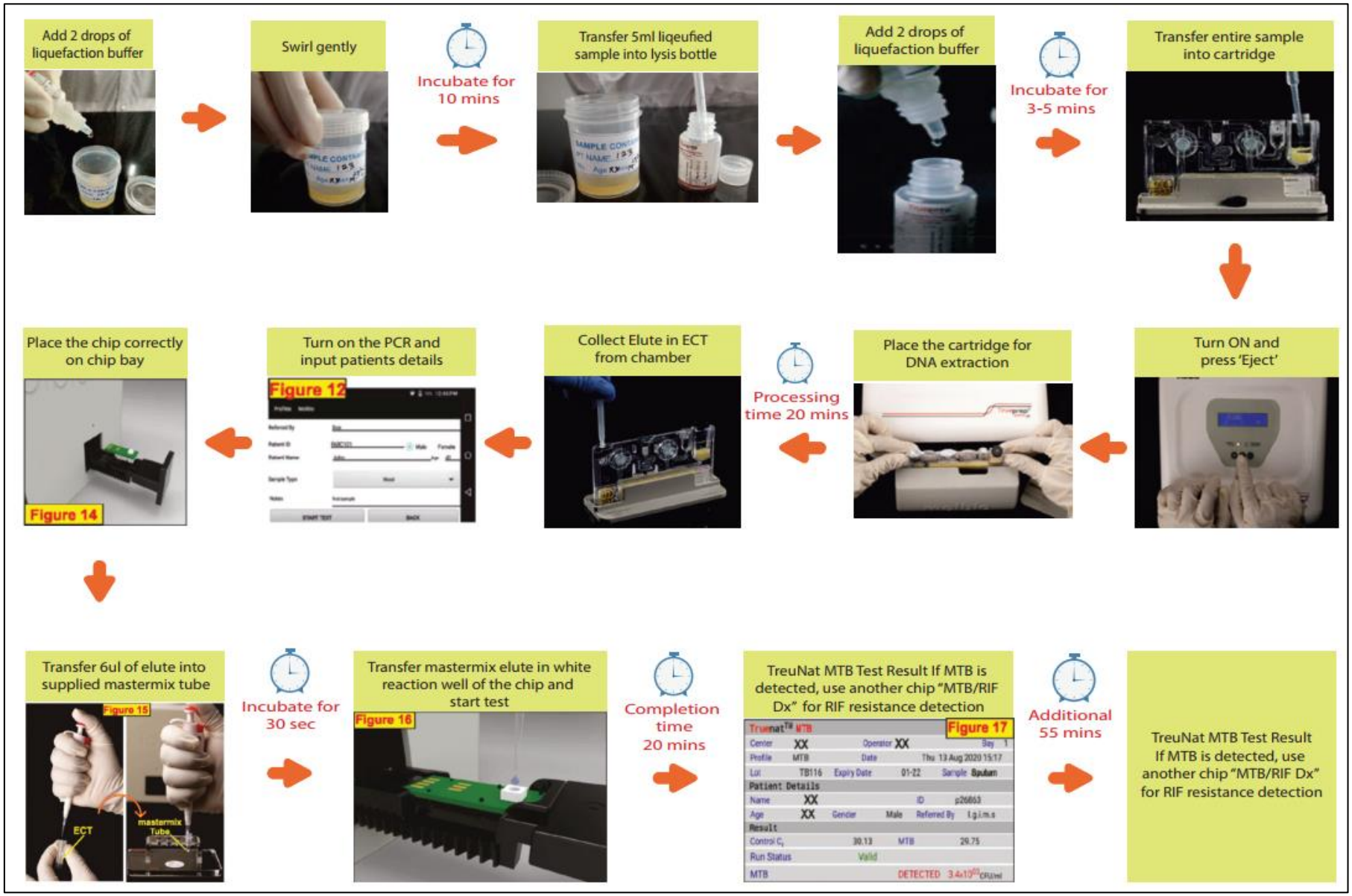
1. Clean the working surfaces with 10% beach followed by 70% alcohol.
2. Clean the instruments with paper towel wet with 70% alcohol.
3. Put on a fresh pair of gloves
4. Before starting the amplification process for a maximum of 2 samples, ensure you have the following arranged on your workstation:
    I. PCR chip set which contains the chip, micro tube with freeze dried PCR reagent micropipette tip and a blue desiccant
    II. The white, fixed-volume micropipette
    III. The microtube stands to hold the microtubes
    IV. ECT tubes containing the extracted DNA
5. Switch on the Truelab Duo device by pressing the red button in the back left corner for 2 seconds. The power/ in use indicator will glow green. In 30-50 seconds, the boot screen will appear followed by the home screen. Ignore the insert sim pop up message.
6. Click on Molbio and select a username from the drop-down menu.
7. Tap on the password text box to pull up the on-screen keyboard.
8. Enter password and press "sign in "to log in" the selected user.
9. Inspect the two-chip bay to ensure there are no used chip in the instrument by clicking on each bay. Select 'open/close' to close the bay.
10. Choose any of the test bay 1 or 2 and select MTB plus.
11. A pop up will appear. Confirm selection by pressing "PROCEED".

12. Enter the information required (referred by, patient ID, patient name, age and gender).
13. Select the sample type SPUTUM.
14. Press 'start test' and the cartridge bay selected will automatically open.
    Note: When the "please load sample" prompt appears, DO NOT press "YES" until the chip is loaded.
15. Tear open the chip pouch.
16. Pull out the desiccant pouch and confirm that it is blue.
    Note: If the desiccant pouch is white or pink in color, do not use the contents of that pouch. Take another one. (This means the chip has been exposed to excess moisture.)
17. Pull out the chip enclosed in the chip sleeve.
    Note:
    - NEVER touch the white reagent well.
    - Minimize the exposure of the chip to light by preparing and running the test immediately after opening
18. Label the chip with the participant ID using a marker at the space provided on the back side of the chip. Avoid writing on the QR code.
19. Place the chip on the tray by aligning the registration holes with the tray pins.
    Note: The white reaction well should face upward and away from the device
20. Take out the microtube containing the freeze-dried PCR reagent and remove the lid
21. Place the microtube containing the freeze-dried PCR reagents in the microtube stand provided.
22. Inspect to be sure the PCR reagent is at the bottom of the tube.
23. Take the 6µl precision micropipette and attach the micropipette filter barrier tip enclosed in the chip pouch.
24. Pipette out 6µl of purified DNA from the ECT and put into the microtube. Confirm visually that the pipetted solution is 6µl.
    Note: DO NOT mix it by tapping, shaking or by reverse pipetting.
25. Do not dispose the pipette tip. Keep it attached to the fixed volume micropipette but ensure the tip is retain into the sleeve.
26. Cap the remaining extracted DNA in the ECT tube and move it one step behind.
27. Allow mixture of eluate and PCR reagent to stand for 30 to 60 seconds to get a clear solution.
28. Pipette out 6µl of treated DNA from the microtube and put into the reaction well of the chip.
    Note: DO NOT spill eluate on the outsides of the well. Take care not to scratch the internal well surface
29. On the instrument screen, select "YES" when the "please load sample" pops up.
30. Chip tray will close automatically, and reaction will start.
31. Dispose the microtube, microtip and used gloves into waste container containing concentrated bleach.
32. Truelab will verify chip and commence test.
    Note:
    - If desired, press "PLOT" to view test progress in real time. No user intervention or interpretation is required. Amplification profile is visible in "optical" view.
    - DO NOT touch or shake the instrument while the test is in progress
33. Follow steps 9-29 to load the second sample in the batch but this time selecting the other test bay.
34. At the end of the run (35 minutes), press "RESULT" to go to the result screen.
35. Possible result:

I. Valid/invalid and
II. MTB result Detected/not detected/Errors

36. Record the result in the lab register.
37. If result is MTB detected, test same eluate for rifampin resistance using MTB-RIF chip. In this case, select the MTB Rif assay.
38. If test gives an invalid or error result, record result and repeat the amplification using the same extracted DNA and a different chip. If valid result cannot still be obtained, run test with a different sample and eluate.
39. Press "print" to print results.
    Note: Test results are automatically stored and can be retrieved any time later.
40. Lift the chip from the instrument tray and directly dispose into waste container filled with concentrated bleach.
    Caution! DO NOT put the chip down on table or any other place. Do not discard chip anywhere else. The amplicons may contaminate another test and give a false positive result.
41. Dispose your gloves in the dedicated waste container.
42. Switch off the Truelab analyzer and Trueprep device at the end day.
43. Cover each of these instruments with the instrument plastic covers.
44. Clean the surfaces using bleach, at the end of the day

Truenat steps flowchart:



1.5. Reading and interpretation results

At the end of the test run, the result screen will display "DETECTED" for Positive result or "NOT DETECTED" for Negative results. The result screen will also display the MTB load as "HIGH", "MEDIUM", "LOW" or "VERY LOW" for positive specimens. The result screen also displays the validity of the test run as "VALID" or "INVALID".

1. Two amplification curves are displayed on the Truelab Analyzer screen when optical plot is selected to indicate the progress of the test.
2. The target and the internal positive control (IPC)* curves will take a steep, exponential path when the fluorescence crosses the threshold value in case of POSITIVE samples.
3. The target curve will remain horizontal throughout the test duration and the IPC curve will take an exponential path in case of NEGATIVE samples.
4. If the IPC curve remains horizontal in a negative sample, the test is considered as INVALID. This may be due to inhibitors in the sample or issues with the reagents used. Tests with an Invalid result should be repeated using a fresh specimen and processed starting with the sample preparation step.
   Note: IPC will co-amplify in most positive cases. In some specimens having a high target load, the IPC may not amplify, however the test run is still considered valid.

1.6. Storage of DNA

Store the rest of the eluate after extraction and amplification in the ECT tube at -20$_{o}$C.

1.7. Quality control

To ensure that the Truelab Analyzer is working accurately, positive and negative controls may be run one time per month. The Truenat Positive Control Kit- Panel I containing Positive Control and Negative Control may be used in running these controls; alternatively, PBS may be used as a negative control and a known positive sample (e.g., from culture) as a positive control.

- Quality control will also be performed if the temperature of the storage area falls outside of 2-30$_{o}$C.
  Acceptable criteria: The result will be acceptable if the positive controls give positive results while negative controls give negative results.
  Corrective action: Repeat the control and/or inform the Lab Supervisor.
  Documentation: Controls should be recorded in the result register.

2. Waste management and other safety precautions

- Submerge the used cartridges, replaceable trays, reagent bottles and other consumables in freshly prepared 10% bleach for 30 minutes before disposal as per the standard medical waste disposal guidelines.
- Samples and reagents of human origin, as well as contaminated materials, disposables, neutralized acids and other waste materials must be discarded after decontamination by immersion in a freshly prepared 0.5% of sodium hypochlorite for 30 minutes (1 volume of 5% sodium hypochlorite for 10 volumes of contaminated fluid or water).
- Do not autoclave materials or solutions containing bleach.
- Chemicals should be handled in accordance with good laboratory practice and disposed according to the Biosafety Manual.

- Check all packages before using the kit. Damage to the packaging does not prevent the contents of the kit from being used. However, if the outer packaging is damaged the user must confirm that individual components of the kit are intact before using them.
- Do not perform the test in the presence of reactive vapours (e.g., from sodium hypochlorite, acids, alkalis or aldehydes) or dust.
- While retrieving the Truenat™ MTB micro-PCR chip and the DNase & RNase free pipette tip from the pouch, ensure that neither bare hands nor gloves that have been used for previous tests run are used.
- All pipetting steps should be performed with utmost care and accuracy in order to prevent cross-contamination between reagents and samples which may lead to invalid results

3. Recording and Reporting

Utilize following templates for record keeping and necessary reporting:

- Laboratory register
- TB 05 (request form)
- DRTB 06 (request form)
- Stock register
- Maintenance log
- Monthly reporting form

4. References

- Truenat MTB Plus package insert version 5.
- The Trueprep™ AUTO v2 Universal Cartridge Based Sample Prep Device user manual.
- TBRL Bamenda Biosafety manual, Version 4.0, section 10.
- Truenat™-A Point-of-care Real Time PCR Test for Tuberculosis, video by Molbio available at https://youtu.be/ydR2I5S2v3c