LifeArc

TB-MBLA User Manual

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Purpose

The purpose of this user manual is to provide guidance and recommendations on the RNA extraction, RT-qPCR and data analysis procedures to be used in conjunction with LifeArc TB-MBLA (Tuberculosis Molecular Bacterial Load Assay).

The protocols listed within this manual have been rigorously tested to ensure that the highest possible accuracy and precision is achieved when testing pre-clinical or clinical samples for the presence of Mycobacteria complex RNA, with the purpose of monitoring load over time.

Scope

The scope of this user manual covers the verified procedures regarding TB-MBLA kit storage, training, RNA extraction and RT-qPCR. Additionally, a troubleshooting section is included to address common issues that may be encountered. It may not be necessary for all users to complete every section in this manual and this will be determined on a case-by-case basis.

Out of Scope Methods & Equipment

Throughout this manual, boxes with out of scope sections are included to advise the user when deviating from recommended protocols. TB-MBLA can be used in combination with various RNA extraction methods and qPCR instruments, however, it should be noted that if users generate data while deviating from the recommended protocols, they will be responsible for ensuring the processes are suitable and reliable.

Table of Acronyms & Definitions

Acronym/Term	Definition
Assay Transfer	Transfer of TB-MBLA processes to a collaborator laboratory Beta mercaptoethanol
BCG	Mycobacterium Bovis var. Bacillus Calmette-Guérin
β-ME	Beta-Mercaptoethanol
BSC	Biological Safety Cabinet
BSL-3	Bio-safety Level 3
Cq	Quantification Cycle
DNA	Deoxyribonucleic Acid
EC	Extraction Control
GTC	Guanidine Thiocyanate
MTB	Mycobacterium Tuberculosis
NTC	No-template Control
PCR	Polymerase Chain Reaction
PPE	Personal Protective Qquipment
QC	Quality Control
qPCR	Quantitative Polymerase Chain Reaction
RNA	Ribonucleic Acid
RT	Reverse Transcriptase
RT-qPCR	Reverse Transcriptase Quantitative Polymerase Chain Reaction
SDS	Safety Data Sheet
SQ	Starting Quantity
TB-MBLA	Mycobacterium Tuberculosis Molecular Bacterial Load Assay

1 Kit Storage

1.1 Purpose

The purpose of this section is to describe the procedure for receipt and storage of the TB-MBLA kits.

1.2 Equipment & Materials

- Freezer (-20°C).
- Low temperature freezer (-80°C).

1.3 Safety Precautions & Waste Disposal Procedure

- Personal protective equipment (PPE) must be worn.
- Additional PPE such as insulated gloves should be used while working with dry ice and in low temperature environments.
- Disposal of dry ice should be carried out according to local policy, or it may be kept for further use if stored in an appropriate ice box.

1.4 Procedure

Procedure

1.4.1 Receipt of Package(s)

- Immediately on receiving the package, visually inspect to ensure there is no damage to the outer box.
- All TB-MBLA kits will be shipped on dry ice. Open the box carefully and inspect the level of dry ice; there should be enough dry ice remaining to ensure the contents are still frozen on arrival. Do not use kit if it is not frozen on arrival.
- Check the inner packaging to ensure no damage.

1.4.2 Storage of TB-MBLA Kits

- The TB-MBLA kit is comprised of two cardboard boxes, each to be stored at a different temperature: one at -20°C and the other at -80°C. **Table 1** details the TB-MBLA kit reagents and their appropriate storage temperatures.
- Store kits at the appropriate temperature until required for use. Storage temperatures are indicated on the outside of each box.

Table 1: TB-MBLA Kit Components & Storage Conditions

Reagent	Box No.	Tubes Per Box	StorageTemp
TB-MBLA qPCR Master Mix	1	2	-20°C
TB-MBLA Reverse Transcriptase		1	
TB-MBLA Assay Mix			
Nuclease-Free Water			
TB-MBLA RNA Standard	2	2	-80°C
TB-MBLA Extraction Control			
Nuclease-Free Water		1	

2 TB-MBLA Training

2.1 Scope

This section describes the training procedure for prospective users of the TB-MBLA which they must pass prior to processing clinical samples.

Out of Scope Methods & Equipment

Note: Users that generate data while deviating from the recommended protocols are responsible for ensuring the processes are suitable and reliable.

- If using a sample preservation method other than GTC e.g. TRIzol. LifeArc may be able to provide alternative mock sample types for training.
- LifeArc must approve all protocols if deviating from those recommended.
- The pass criteria cited in this section is only applicable when using the Bio-Rad CFX96 thermal cycler. If using another instrument, discuss criteria with LifeArc.
- It should be noted that in certain clinical trials, final competency to perform TB-MBLA will be determined by the trial sponsor.

2.2 TB-MBLA Practical Training & Assessment

2.2.1 Practical Session 1: Sample to result processing using TB-MBLA Kit (4 samples)

Trainees will perform the TB-MBLA protocol supervised by the TB-MBLA trainer processing a small number of artificial sputum (mock) samples in the first instance, as follows:

1. **Extraction of 4 artificial sputum (mock) samples provided by LifeArc, 2 positive 2 negative**

a. Complete RNA Extraction according to the protocol described in section 5 of this manual.

2. **RT-qPCR of RNA extracts**

a. Complete TB-MBLA RT-qPCR Procedure according to the protocol described in section 6 of this manual.

3. Analysis of results

a. Training can be provided on evaluating results and identifying issues.

b. Common issues are listed in **Section 7** Troubleshooting.

2.2.2 **Practical Session 2: Sample to result processing using TB-MBLA Kit (12 samples)**

Trainees to repeat the above steps as in Practical Session 1 but handling/processing a larger number (12, 6 positive and 6 negatives) of artificial sputum (mock) samples provided by LifeArc.

Note: Practical competency assessment pass criteria includes a trainee having completed and passed all practical training (as deemed a pass by LifeArc) and at a minimum reading and understanding the procedures referenced within this user manual.

2.2.3 **TB-MBLA Training Pass Criteria**

Results from practical sessions will be deemed a pass based on the following criteria:

- Mean Cq of S1 from RNA standard falls within the acceptable range of 14 - 17.
- Mtb Standard curve efficiency falls within the acceptable range of 90 – 110%.
- Mean Cq of each positive mock samples fall within acceptable range of 16.8 – 26.8.
- Negative mock samples are negative (NaN).

3 Quality Control Check

3.1 **Scope**

This section describes the quality control (QC) procedure undertaken upon receipt of a new shipment of TB-MBLA kits.

Out of Scope Methods & Equipment

Note: Users that generate data while deviating from the recommended protocols are responsible for ensuring the processes are suitable and reliable.

- If using a qPCR instrument other than the recommended Bio-Rad CFX96, ensure that setup is completed using compatible consumables while following the manufacturer's instructions.
- Thermal cycling parameters must match those stated in **Table 4** of this section.
- If using an alternative analysis software, ensure accurate efficiency calculations from the standard curve are completed according to the manufacturer's instructions.
- The EC pass criteria will not be applicable if using an instrument other than the CFX96. Discuss criteria with LifeArc.

3.2 **Equipment & Materials**

- Chemical waste discard containers.
- Ice box, or cold blocks.
- Racks for 1.5mL or 1.7mL micro-centrifuge tubes.
- Vortex, for 1.5mL micro-centrifuge tubes.
- Centrifuge for 1.5mL or 1.7mL tubes micro-centrifuge tubes.
- Bio-Rad Hard-shell 96-well PCR plate, low profile, thin wall, skirted, white/clear; HSP9601, HSP9601B.
- Bio-Rad Microseal B PCR plate sealing film, adhesive, optical; MSB1001.
- Plate seals (generic); for transfer of plates from clean area to template area **(Section 2.5.2, Step 7).**
- 96-well plate centrifuge.
- 96-well plate vortex (use standard vortex if no plate vortex is available).

- CFX96.
- CFX Manager or Maestro.
- TB-MBLA CFX96 cycling template (TB-MBLA cycling.prcl) - Provided by LifeArc.
- TB-MBLA CFX96 kit QC template (TB-MBLA Kit QC Template.pltd) - Provided by LifeArc.
- Calibrated pipettes and matching sterile filtered pipette tips, DNase and RNase-free, range: P1000, P200, P100 (optional), P20, P10.
- The following components of the Mycobacterium tuberculosis Molecular Bacterial Load Assay (TB-MBLA) kit (LifeArc): TB-MBLA qPCR Master Mix, TB-MBLA Reverse Transcriptase, TB-MBLA Assay Mix, RNase-Free Water and TB-MBLA RNA Standard, TB-MBLA Extraction Control.
- Starlab 1.5ml TubeOne Microcentrifuge tube; S1615-5510 or Starlab Crystal Clear (non-sterile) tubes (E1415-1500) for preparation of control RNA and PCR reaction mixes.

3.3 **Safety Precautions**

- Refer to the relevant TB-MBLA kit material safety data sheets (MSDS).
- Personal protective equipment (PPE) must be worn.
- All work must be carried out in an appropriate PCR workstation where possible.
- Follow decontamination procedures prior to, and after use.

3.4 **Method**

Presented below is the methodology for performing quality control (QC) assessment of TB-MBLA kits.

3.4.1 **Test Design**

1. Testing should be performed on one kit (Box 1 and 2).
2. Testing should be performed using one TB-MBLA RNA Standard and one TB-MBLA Extraction Control (EC) per kit.

3.4.2 **The Plate Plan to be used per kit is included in Figure 2. Reaction Mix Preparation**

1. Remove TB-MBLA qPCR Master Mix, TB-MBLA Assay Mix and RNase-Free Water (contained in Box 1) from the freezer and thaw (If not proceeding immediately place reagents in a refrigerator/on ice).
2. At this stage, TB-MBLA RNA Standard, TB-MBLA EC, and RNase-free water (contained in box 2) can be removed from the freezer and thawed on ice.
3. Prepare the reaction mixes as Table 2 and 3, excluding the Reverse Transcriptase (RT). The plate has 21 wells with RT+ reaction mix and 3 wells with RT- reaction mix.
4. Remove the TB-MBLA Reverse Transcriptase (RT) from the freezer, and immediately add to the reaction mix. Return the RT to a freezer; if this process cannot be followed, then the RT should be placed on ice when not in use.

Table 2: RT Positive Reaction Mix (units are µL)

Reagent	**x 1**	**+ 10%**	**x 21 (Includes 10% excess)**
TB-MBLA qPCR Master Mix	10	11	231
RNase-Free Water	4.8	5.28	110.88
TB-MBLA Assay Mix	1.0	1.1	23.1
TB-MBLA Reverse Transcriptase	0.2	0.22	4.62

Table 3: RT Negative Reaction Mix (units are µL)

Reagent	**x 1**	**+ 10%**	**x 3 (Includes 10% excess)**
TB-MBLA qPCR Master Mix	10	11	33
RNase-Free Water	5.0	5.5	16.5
TB-MBLA Assay Mix	1.0	1.1	3.3

5. Add 16 µl of RT negative reaction mix to the appropriate wells based on the plate plan in **Figure 2.**
6. Add 16 µl of RT positive reaction mix to the appropriate wells based on the plate plan in **Figure 2.**
7. To NTC reactions, add 4 µl of RNase-free water (provided as part of the LifeArc TB-MBLA kit). Cover the plate and move to the positive control lab/area.

3.4.3 **Standard Preparation**

1. If not already done, remove the TB-MBLA RNA Standard and EC from the freezer and defrost. Once defrosted, place on ice.
2. Label six clean 1.5 mL microcentrifuge tubes (TubeOne or Crystal Clear) 'S1-S6' and add 90 µL of RNase-free water to each.
3. Vortex the TB-MBLA RNA Standard to mix, then take 10 µL and add to the S1 tube. Close the tube, vortex for approximately 5 seconds, and briefly centrifuge to collect.
4. Take 10 µL of this S1 dilution and add to the S2 tube. Close the tube, vortex well, and briefly centrifuge to collect. Repeat to tube S6. A schematic representation of steps 2 to 4 is shown in **Figure 1.**

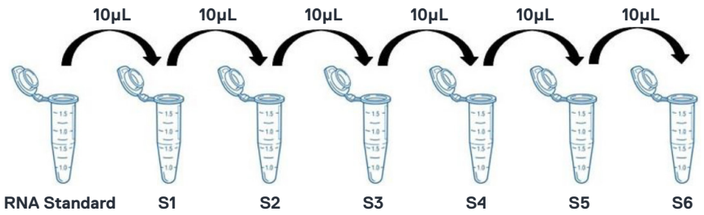


Figure 1: Schematic representation of serial dilution of the TB-MBLA Standard

5. Add 4 µL of each standard to the appropriate reactions according to the plate plan.
6. Add 4 µL of the TB-MBLA EC to the appropriate reactions according to the plate plan.
7. Apply the Microseal B plate seal, vortex to mix, and briefly centrifuge to ensure all reaction components are at the bottom of each well and there are no large bubbles.
8. Place plate in CFX96.

3.4.4 **Thermal Cycling & Data Collection**

1. On the computer connected to the CFX96, open the **CFX Manager** or **Maestro software.**
2. In the **Start-up Wizard** dialog box click **User defined** under **Select run type.**
3. In the **Run Setup** window click **Select Existing.**
4. In the subsequent window, navigate to the location of the **TB-MBLA cycling template,** highlight and click open.
5. The cycling conditions should match those presented in **Table 4,** with a **20 µL** reaction volume and the camera icon 📷 present in the fourth column (annealing/extension) which indicates data acquisition will occur here.

Table 4: TB-MBLA Assay CFX96 Thermal Cycling Conditions

Step	Temperature °C	Time	Cycles
Reverse Transcription	50	30 minutes	N/A
Activation	95	15 minutes	N/A
Amplification	94	15 seconds	40
	60	30 seconds	

6. Click Next, moving to the **Plate** view, and click **Select Existing.**
7. In the subsequent window, navigate to the location of the **TB-MBLA Kit QC Template,** highlight and click open.
8. A window will be presented that displays the well positions of each reaction based on the file that was imported.
9. Click **OK** to close this window when all information has been checked. Click **OK** in the Plate Editor window.
10. In **Plate** view, click **Next** for the Start Run view.
11. In the Start Run view, select the CFX96 that will be used (by clicking on it), and click **Start Run.**
12. When prompted to save, **save file.**

3.4.5 **Data Analysis**

1. Open the CFX96 output file.
2. Selecting one channel at a time by ensuring **FAM™** or **HEX™** is ticked under the amplification plot and set the threshold at 100 RFU. This can be done either by right clicking on the amplification plot then **Baseline Threshold,** or **Settings > Baseline Threshold.** In the subsequent window, under Single Threshold ensure **User Defined** is selected and enter **100** in the box, then click **OK.**

3.4.6 **Kit QC Pass Criteria**

1. Report the **Efficiency** from the MTB-component standard curve (FAM™) provided by the CFX Manger/Maestro software (the efficiency value (E) can be found in the legend box under standard curve plot). **The Efficiency should be between 90-110%.**
2. Calculate the mean Cq values (from the triplicate sets) of the TB-MBLA EC. **The Mean EC Cq should be <20.0 Cq.**
3. RT positive and negative NTC reactions should have no amplification. The plate will be considered a fail if more than 1 of 3 replicates in the NTCs have positive amplification.
4. **If kits do not meet specification, inform LifeArc immediately.**

	1	2	3	4	5	6	7	8	9	10	11	12
A	S1	S1	S1	S2	S2	S2	S3	S3	S3	S4	S4	S4
B	S5	S5	S5	S6	S6	S6	NTC RT+	NTC RT+	NTC RT+	NTC RT-	NTC RT-	NTC RT-
C	EC	EC	EC									
D												
E												
F												
G												
H												

Figure 2: Plate plan template for kit QC. Presented are reverse transcriptase positive (RT+, turquoise) and reverse transcriptase negative (RT-, yellow) reactions. Standard (S) and extraction control (EC) are also shown.

4 Sample preservation with Guanidine Thiocyanate (GTC)

4.1 **Scope**

This section describes the procedures required for the collection and preservation of human sputum samples with guanidine thiocyanate (GTC).

Out of Scope Methods & Equipment

Note: Users that generate data while deviating from the recommended protocols are responsible for ensuring the processes are suitable and reliable.

- If using a sample preservation method other than GTC e.g., TRIzol. LifeArc may be able to provide alternative mock sample types for training.
- TB-MBLA quantifies 16S RNA from whole cells. If using an RNA preservative with cell lysing capabilities (e.g., TRIzol) spin down sputum sample to pellet cells, discard supernatant and resuspend in preservative for storage.

4.2 **Equipment & Materials**

- Biological waste discard containers
- Chemical waste discard containers
- Fume hood
- Laboratory scales
- Weighing boats
- 500 mL Duran bottle, with lid
- Measuring cylinder
- Incubator, 37°C
- 15 mL centrifuge tubes
- 50 mL centrifuge tubes
- 2mL Eppendorf tubes
- Distilled water
- Guanidine thiocyanate (GTC); V2791, Promega
- 1 M Tris-HCl pH 7.4; 93313, Sigma
- β-mercaptoethanol; 63689, Sigma
- Molecular grade water (RNase free); W4502, Sigma

4.3 Safety Precautions

- Work in a Bio-safety Level 3 (BSL-3) laboratory with restricted and controlled access.
- Personal Protective Equipment (PPE) must be worn.
- Work should be carried out in an appropriate Class II Biological Safety Cabinet (BSC).
- A fume hood is recommended for the handling of β-mercaptoethanol and guanidine thiocyanate powder.

4.4 Preparation of Guanidine Thiocyanate (GTC) Solution

This section details the preparation of 400 mL of GTC preservative solution. The reagents required and their volumes are detailed in **Table 5. Steps 1 - 6** of the below methodology should be performed in a fume hood.

Table 5: Reagents required for reparation of GTC Solution

Reagent	Amounts for 400 mL
Guanidine Thiocyanate (GTC)	200 g
1M Tris-HCl pH 7.5	40 mL
β-mercaptoethanol	4 mL
Molecular Grade Water	Up to 400 mL

1. Transfer 200 g GTC powder into a 500 mL glass bottle (with lid).
2. Measure 120 mL molecular grade water and add to the GTC.
3. Firmly seal the container with the lid; shake thoroughly and incubate overnight at 37°C to dissolve the GTC.
4. **Note:** The next day you may need to shake again and incubate for another 30 minutes to allow any remaining GTC powder to dissolve.
5. After removing the GTC from the incubator, add 40 mL 1 M Tris-HCl pH 7.4 and adjust the total volume to 396 mL with molecular grade water.
6. When the GTC solution is completely clear, add 4 mL β-mercaptoethanol and mix well by shaking.
7. Aliquot 4 mL into labelled 15 mL centrifuge tubes. Do not leave for more than 2 hours on the bench.
8. Store any aliquots not immediately required at -80°C.

4.5 Sample Preservation Method

The sputum samples intended for TB-MBLA testing should be stored in the presence of a guanidine thiocyanate (GTC) based preservative solution. Per 1 mL of sputum, 4 mL of GTC preservative is required. **Steps 1 - 4** below should be performed in a BSL-3 laboratory. **Figure 3** provides a general overview of the below process.

1. Remove one pre-prepared 4 mL aliquot of GTC per sample from -80°C and thaw on ice. **Note:** Once GTC has been defrosted, refreeze within 2 hours.
2. Transfer 1 mL of untreated sputum into the GTC tube.
3. Close lid firmly and vortex the sample well for approximately 10 seconds to mix.
4. Transfer to a -80°C freezer for at least one overnight period prior to RNA extraction **(Section 5)** and testing **(Section 6).**

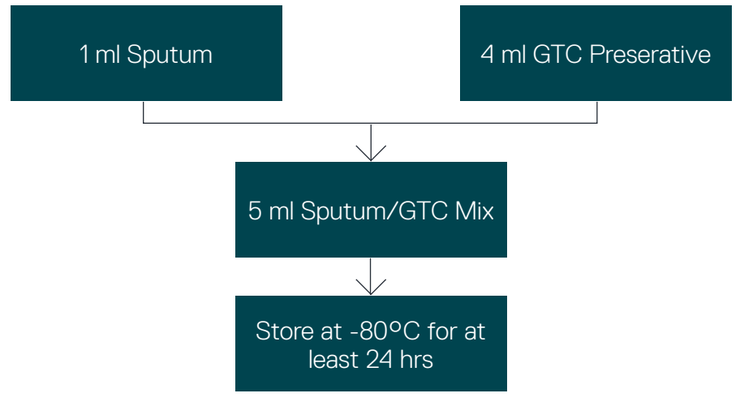


Figure 3: TB-MBLA Sample Peservation Workflow

5 RNA Extraction

5.1 Scope

This section describes the isolation of total RNA and removal of DNA from GTC preserved human sputum samples (or mock samples when performing training). This recommended protocol uses mechanical lysis (bead beating), the Invitrogen PureLink™ RNA Mini Kit and Qiagen RNase-Free DNase Set.

Out of Scope Methods & Equipment

Note: Users that generate data while deviating from the recommended protocols are responsible for ensuring the processes are suitable and reliable.

- If using an alternative RNA extraction method, ensure extractions are carried out according to the manufacturer's instructions.
- DNA removal step must be performed, ensuring DNA removal method does not create inhibition in qPCR.
- RNA extraction and DNA removal methods must be consistent between runs/operators/sites.
- If still using the Lysing matrix B tubes, ensure a minimum of 700 µL of lysis buffer is used to account for loss of liquid amongst beads.

5.2 Equipment & Materials

- Biological waste discard containers.
- Biological waste discard containers.
- Chemical waste discard containers.
- Fume hood.
- Ice box, or cold blocks.
- Timer.
- Measuring cylinder, 50 mL.
- RNase-free needle and syringe (1 mL).
- Racks for 1.5 mL and 2 mL tubes and for 15 mL tubes; chemical-resistant and autoclavable.
- Vortex, for 1.5 mL and 2 mL micro-centrifuge tubes and 15 mL centrifuge tubes.
- Benchtop centrifuge (with sealed removable buckets for 15 mL centrifuge tubes), for speeds of 3000 x g for 30 minutes.
- Benchtop centrifuge (for 2 mL lysing matrix tubes and spin columns), for speeds at least 12,000 x g for 5 minutes.
- Micro-centrifuge for 1.5 mL tubes.
- Precellys 24 Tissue Homogenizer (Bertin); setting 6000rpm, 40 seconds.
- Calibrated pipettes and matching sterile filtered pipette tips, DNase and RNase-free: P1000, P200, P100 (optional).
- Ethanol (EtOH), absolute 96-100%.
- β-mercaptoethanol; 63689, Sigma.
- Molecular grade water (RNase free).
- Invitrogen PureLink™ RNA Mini Kit (12183018A or 12183025).
- RNase-Free DNase Set (50); cat: 79354 (Qiagen).
- Lysing Matrix B tubes (50); cat: 116911050 (MP Biomedicals).
- Micro-centrifuge tubes (1.5ml or 1.7ml) for lysate transfers **(Step 10)** and RNA collection.
- TB-MBLA Extraction Control (EC) from TB-MBLA Kit (LifeArc).

5.3 Safety Precautions

- Personal protective equipment (PPE) must be worn.
- All work should be carried out in an appropriate Class II Biological Safety Cabinet (BSC).
- A fume hood is recommended for the handling of β-mercaptoethanol.
- Follow decontamination procedures prior to and after use.
- Refer to all relevant local guidelines and risk assessments prior to commencing work.

To isolate total RNA and remove DNA from the sputum samples, the below protocol should be followed. This includes use of the Invitrogen PureLink™ RNA Mini kit, and Qiagen RNase-free DNase set. For a general overview of the RNA extraction workflow, see **Figure 4.**

5.4 Method

5.4.1 Preparation of Materials

When using new Invitrogen PureLink™ RNA Mini Kit and/or a Qiagen RNase-free DNase Set the below instructions should be followed to prepare the indicated reagents. Prepared reagents are to be used as part of one kit and should not be used with a new kit.

Wash Buffer II – When opening a new Invitrogen PureLink™ RNA Mini kit only

Wash Buffer II is supplied as a concentrate. Before using for the first time, add 60 mL of absolute ethanol (96-100%) for Cat. no. 12183018A, or 300 mL for Cat. no. 12183025, as indicated on the bottle to obtain a working solution.

Preparation of 70% ethanol

For 100 mL of 70% ethanol, add 30 mL of molecular grade water to 70 mL of absolute 96-100% ethanol.

Preparation of DNase I – When opening a new Qiagen RNase-free DNase-set only

Prepare DNase I stock solution before using the RNase-Free DNase Set for the first time. Dissolve the lyophilized DNase I in 550 µL of the RNase-free water provided. To avoid loss of DNase I, do not open the vial. Inject RNase-free water into the vial using an RNase-free needle and syringe. Mix gently by inverting the vial. Do not vortex. Freeze aliquots at -20°C if not being used immediately.

5.4.2 Extraction of RNA & Removal of DNA Procedure

1. Remove sputum samples in GTC and TB-MBLA Extraction Control (EC) from -80°C freezer and allow to defrost on ice.
2. Vortex each sample for approximately 10 seconds.
3. Put sample tubes into centrifuge buckets, secure lids.
4. Centrifuge for 30 minutes at 3000 x g and gently pour off supernatant, discard in accordance with local regulations.
5. Prepare Lysis Buffer and β-mercaptoethanol (β-ME) mix; 10 µL β-ME per 1 mL Lysis Buffer is required, and 700 µL is required per sample. Prepare sufficient mix for all samples with an excess for pipetting error to the nearest mL, e.g., for 18 samples: 18 x 700 µL = 12.6 mL, prepare 13 mL. See **Table 6.**

Table 6: Preparation of RLT Buffer

Reagent	x 1	x 13mL
Lysis Buffer	1mL	13mL
β-Mercaptoethanol	10µL	130µL

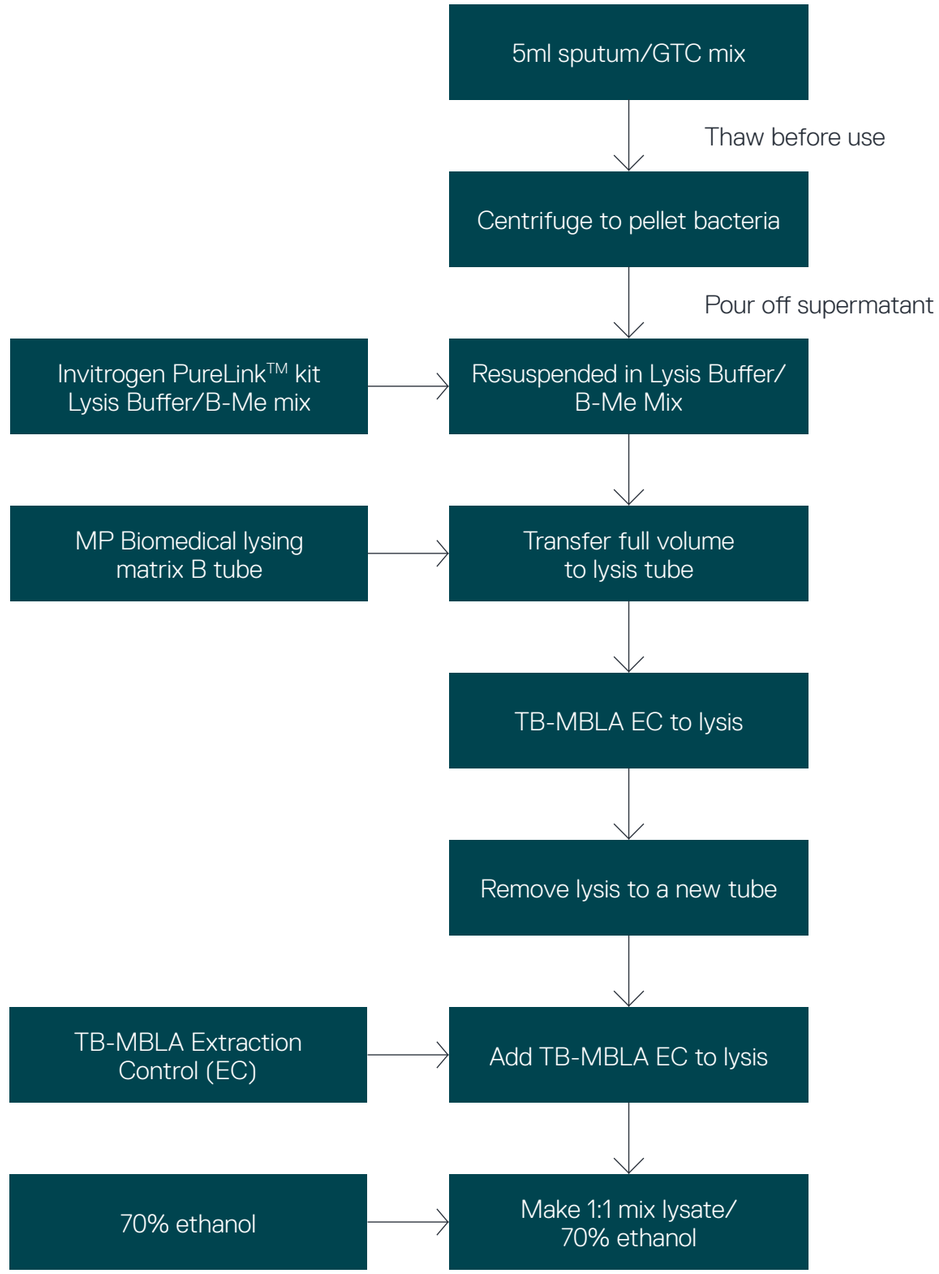
6. Add 700 µL of Lysis Buffer (containing β-ME) to each sample tube and vortex for approximately 10 seconds.
7. Transfer the full volume of each sample into MP Biomedical Lysis Matrix B tubes. Ensure tubes are labelled clearly.
8. Place in Precellys 24 instrument and process sample for 40 seconds at 6000 rpm.
9. Transfer lysis tubes to a centrifuge. Centrifuge for 5 minutes at 12,000 x g, then transfer the samples into a tube stand and incubate at room temperature for 5 minutes.
10. Using a P1000 pipette, transfer 520 µL lysate (avoid beads) into a clean 1.5 mL microcentrifuge tube.
11. Vortex mix EC briefly prior to use.
12. Add 10 µL of EC into each lysate for the RNA extraction procedure and vortex mix.
13. Add 1 volume of 70% ethanol to the lysate/EC mix (e.g., 530 µL lysate/EC mix + 530 µL 70%ethanol). **Note:** Do not vortex the lysate/ethanol mix, instead use pipetting or mix by inversion.
14. Add 700 µL of lysate to an Invitrogen PureLink™ spin column and centrifuge for 15 seconds at 12,000 x g. Discard flow through. Up to 700 µL of lysate can be added to each PureLink™ spin column at a time. Repeat step until all sample is processed.
15. Add 350 µL Wash Buffer I to the spin column, and centrifuge for 15 seconds at 12,000 x g. Discard flow through.

16. Prepare the DNase Incubation Mix (include excess) by mixing 10 µL of DNase I with 70 µL of Buffer RDD per sample. **Table 7** presents example sufficient for 18 samples (including excess of an additional sample volume). **Note:** Do not vortex the DNase or Incubation mix, these should only be mixed by pipetting.

Table 7: Preparation of DNase Mix

Reagent	x 1	x 19
DNase I	10µL	190µL
RDD	70µL	1,330µL

17. Add 80 µl DNase I Incubation Mix to the centre of the column membrane (avoid touching the membrane with pipette tip) and incubate at room temperature for 15 minutes.
18. Add 350 µL Wash Buffer I to the spin column, and centrifuge for 15 seconds at 12,000 x g. Discard flow through. Place spin column into a new collection tube.
19. Add 500 µL Wash Buffer II to the spin column, and centrifuge for 15 seconds at 12,000 x g. Discard flow through.
    Note: If using a new kit, see section 5.4.1 for preparation of Wash Buffer II.
20. Add 500 µL Wash Buffer II to the spin column, and centrifuge for 15 seconds at 12,000 x g. Discard flow through.
21. Centrifuge the spin column at 12,000 x g for 2 minutes to eliminate any carryover of Wash Buffer II.
22. Place column into a new 1.5 mL collection tube (provided in the Invitrogen PureLink™ RNA kit) and add 50 µL of RNase-free water to the membrane. Incubate for 1 minute at room temperature.
23. Centrifuge for 2 minutes at 12,000 x g to elute the RNA.
24. Discard the column and store the eluted RNA at -80°C or proceed to RT-qPCR process.



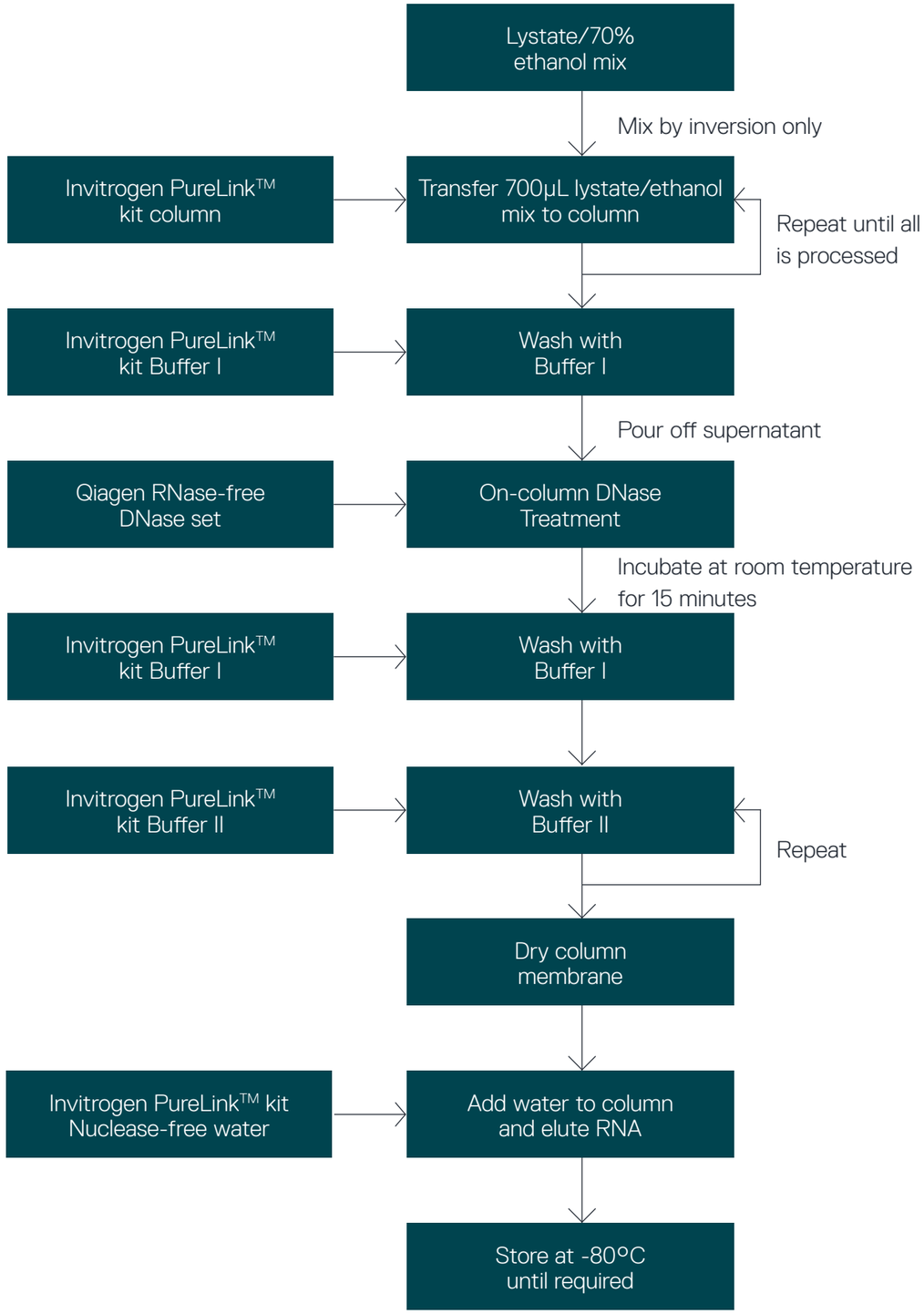


Figure 4: RNA Extraction Workflow Overview

6 RT-qPCR (using CFX96)

6.1 Scope

This section describes the reverse transcriptase (RT)-qPCR procedure. This recommended protocol uses the CFX96, the operation of which is detailed in **sections 6.4.5 and 6.4.6.** The set-up of the RT-qPCR should be carried out as follows regardless of qPCR cycler used.

Out of Scope Methods & Equipment

Note: Users that generate data while deviating from the recommended protocols are responsible for ensuring the processes are suitable and reliable.

- It should be noted that the TB-MBLA master mix does not contain any passive dyes such as ROX. If using an alternative qPCR instrument, either add ROX to the reaction mixture or ensure that this option is disabled.
- If using a qPCR instrument other than the recommended Bio-Rad CFX96, ensure that setup is completed using compatible consumables while following the manufacturer's instructions.
- Thermal cycling parameters must match those stated in Table 10 of this section.
- The threshold must be set at a consistent point across all plates for both FAM™ and HEX™/VIC™ channels. This does not mean FAM™ and HEX™/VIC™ need to be the same as each other. LifeArc can assist with determining an appropriate setting once the Assay Transfer process has been completed.
- A minimum of 4 RNA standards must be used to calculate standard curve efficiency.
- A standard curve which is from the same kit lot number can be imported from a previous run if the qPCR cycler has that capability.
- When recording results, use the average concentration reported in the FAM™ channel as the TB 16S copies per reaction which have been converted using the standard curve.

6.2 Equipment & Materials

- Chemical waste discard containers.
- Ice box, or cold blocks.
- Racks for 1.5ml or 1.7mL micro-centrifuge tubes.
- Vortex, for 1.5mL micro-centrifuge tubes.
- Centrifuge for 1.5ml or 1.7mL micro-centrifuge tubes.
- Bio-Rad Hard-shell 96-well PCR plate, low profile, thin wall, skirted, white/clear; HSP9601, HSP9601B.
- Bio-Rad Microseal B PCR plate sealing film, adhesive, optical; MSB1001.
- Plate seals (generic); for transfer of plates from clean area to template area (Section 5.2, step 5).
- 96-well plate centrifuge.
- 96-well plate vortex.
- CFX96 (Bio-Rad).
- CFX Manager or Maestro software.
- TB-MBLA CFX96 cycling template (TB-MBLA cycling.prcl).
- TB-MBLA CFX96 plate template (TB-MBLA Plate.pltd).
- TB-MBLA CFX96 import template (TB-MBLA Import Template.csv).
- Calibrated pipettes and matching sterile filtered pipette tips, DNase and RNase-free, range: P1000, P200, P100 (optional), P20, P10.
- The following components of the Mycobacterium tuberculosis Molecular Bacterial Load Assay (TB-MBLA) kit (LifeArc): TB-MBLA qPCR Master Mix, TB-MBLA Reverse Transcriptase Mix, TB-MBLA Assay Mix, RNase-Free Water and TB-MBLA RNA Standard.
- Starlab 1.5ml TubeOne Microcentrifuge tube; S1615-5510 or Starlab Crystal Clear (non-sterile) tubes (E1415-1500) for preparation of control RNA and PCR reaction mixes.

6.3 Safety Precautions

- Refer to the relevant TB-MBLA kit material safety data sheets (MSDS).
- Personal protective equipment (PPE) must be worn.
- All work should be carried out in an appropriate PCR workstation where possible.
- Follow decontamination procedures prior and after use.

6.4 Method

Presented below is the methodology for performing the TB-MBLA RT-qPCR assay. The information is provided in sections for ease of interpretation. **Figure 8** of this manual provides a general overview of the qPCR set-up procedure.

6.4.1 Experiment Design

1. A plate plan for testing 18 sample extracts is included in **Figure 5** of this manual. If not testing 18 sample extracts, it should be noted that each sample requires three RT positive (RT+), and one RT negative (RT-) reactions. If using a CFX96, templates for sample upload and cycling conditions can be provided.
2. If not using the reaction mix volumes presented in **Tables 8 and 9,** calculate the individual reagent volumes for the number of reactions required.

6.4.2 Reaction Mix Preparation

1. Remove TB-MBLA qPCR Master Mix, TB-MBLA Assay Mix and RNase-Free Water from the freezer and thaw. If not proceeding immediately thaw reagents in a refrigerator/on ice.
2. Prepare the reaction mixes (excluding the RT). Prepare as below or using the volumes calculated.
3. Remove the TB-MBLA Reverse Transcriptase (RT) from the freezer, and immediately add to the reaction mix. Return RT to a freezer; if this process cannot be followed, then the RT should be placed on ice when not in use.

Note: LifeArc recommends that each TB-MBLA qPCR Master Mix tube is designed to run one full plate to avoid freeze/thaw. All other reagents in Box 1 should be used no more than twice. Any reactions carried out following more than the recommended number of freeze thaw cycles is done so at the user's risk.

Table 8: RT Positive Reaction Mix (units are µL)

Reagent	x 1	+ 10%	x 75 (Includes 10% excess)
TB-MBLA qPCR Master Mix	10	11	825
RNase-Free Water	4.8	5.28	396
TB-MBLA Assay Mix	1.0	1.1	82.5
TB-MBLA Reverse Transcriptase	0.2	0.22	16.5

Table 9: RT Negative Reaction Mix (units are µL)

Reagent	x 1	+ 10%	x 21 (Includes 10% excess)
TB-MBLA qPCR Master Mix	10	11	231
RNase-Free Water	5.0	5.5	115.5
TB-MBLA Assay Mix	1.0	1.1	23.1

4. Add 16 µl of **RT negative** reaction mix to the appropriate wells based on the plate plan in **Figure 5.**
5. Add 16 µl of **RT positive** reaction mix to the appropriate wells based on the plate plan in **Figure 5.**
6. To NTC reactions, add 4 µl of RNase-free water (provided as part of the LifeArc TB-MBLA kit). Cover the plate and move to the template addition lab/area.

	1	2	3	4	5	6	7	8	9	10	11	12
A	S1	S1	S1	S2	S2	S2	S3	S3	S3	S4	S4	S4
B	S5	S5	S5	S6	S6	S6	NTC RT+	NTC RT+	NTC RT+	NTC RT-	NTC RT-	NTC RT-
C	Sample 1 RT +	Sample 1 RT +	Sample 1 RT +	Sample 1 RT -	Sample 2 RT +	Sample 2 RT +	Sample 2 RT +	Sample 2 RT -	Sample 3 RT +	Sample 3 RT +	Sample 3 RT +	Sample 3 RT -
D	Sample 4 RT +	Sample 4 RT +	Sample 4 RT +	Sample 4 RT -	Sample 5 RT +	Sample 5 RT +	Sample 5 RT +	Sample 5 RT -	Sample 6 RT +	Sample 6 RT +	Sample 6 RT +	Sample 6 RT -
E	Sample 7 RT +	Sample 7 RT +	Sample 7 RT +	Sample 7 RT -	Sample 8 RT +	Sample 8 RT +	Sample 8 RT +	Sample 8 RT -	Sample 9 RT +	Sample 9 RT +	Sample 9 RT +	Sample 9 RT -
F	Sample 10 RT +	Sample 10 RT +	Sample 10 RT +	Sample 10 RT -	Sample 11 RT +	Sample 11 RT +	Sample 11 RT +	Sample 11 RT -	Sample 12 RT +	Sample 12 RT +	Sample 12 RT +	Sample 12 RT -
G	Sample 13 RT +	Sample 13 RT +	Sample 13 RT +	Sample 14 RT -	Sample 14 RT +	Sample 14 RT +	Sample 14 RT +	Sample 14 RT -	Sample 15 RT +	Sample 15 RT +	Sample 15 RT +	Sample 15 RT -
H	Sample 16 RT +	Sample 16 RT +	Sample 16 RT +	Sample 16 RT -	Sample 17 RT +	Sample 17 RT +	Sample 17 RT +	Sample 17 RT -	Sample 18 RT +	Sample 18 RT +	Sample 18 RT +	Sample 18 RT -

Figure 4: Plate plan for 18 samples, wells in yellow indicate reverse transcriptase negative (RT-) reaction mix.

6.4.3 Template RNA Addition

1. Remove the required RNA samples from the freezer and thaw. Once defrosted place on ice before use.
2. Immediately before adding to the plate, vortex mix each RNA sample and briefly centrifuge to collect.
3. Add 4 µL of each RNA sample to the appropriate wells as per the plate plan. The remaining sample RNA should be kept and stored in -80 °C freezer.
4. When all samples have been added cover the plate and remove to the positive control lab/area (if applicable).

6.4.4 Standard Preparation & Addition

1. Remove the TB-MBLA RNA Standard and water from Box 2 from the freezer and defrost. Once defrosted place on ice.
2. Label six clean 1.5 mL microcentrifuge tubes ‘S1-S6’ and add 90 µL of RNase free water to each.
3. Vortex TB-MBLA RNA Standard to mix, then take 10 µL and add to the S1 tube. Close the tube, vortex for approximately 5 seconds, and briefly centrifuge to collect.
4. Take 10 µL of this S1 dilution and add to the S2 tube. Close the tube, vortex well, and briefly centrifuge to collect. Repeat to tube S6. A schematic representation of steps 2 to 4 is shown in **Figure 6.**

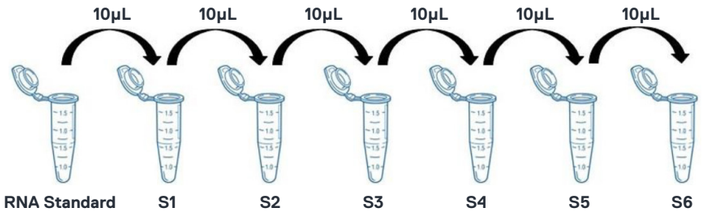


Figure 6: Schematic representation of serial dilution of the TB-MBLA Standards.

5. Add 4 µL of each standard to the appropriate reactions according to the plate plan.
6. Apply the plate seal vortex to mix, and briefly centrifuge to ensure all reaction components are at the bottom of each well and there are no large bubbles.

Note: TB-MBLA RNA Standard is designed to be used in a single run. LifeArc recommends that after one use any remaining standard should not be used in subsequent plates to avoid using this reagent with a freeze-thaw cycle. Box 2 water should not be used more than twice.

6.4.5 Thermal Cycling & Data Collection

Note: This section describes the recommended procedure when using the Bio-Rad CFX96 thermal cycler and accompanying software. If using another thermal cycler, then ensure that cycling conditions match those presented in **Table 10** and follow set up and data collection according to the manufacturer's instructions.

1. On the computer connected to the CFX96, open the **CFX Manager** or **Maestro** software.
2. In the **Startup Wizard** dialog box click **User defined** under **Select run type.**
3. In the **Run Setup** window click **Select Existing.**
4. In the subsequent window, navigate to the location of the TB-MBLA cycling template, highlight and click open.
5. The cycling conditions should match those presented in Table 10, with a 20 µL reaction volume and the camera icon 📷 present in the fourth column (annealing/extension) which indicates data acquisition will occur here.

Table 10: TTB-MBLA thermal cycling conditions:

Step	Temp (°C)	Time	Cycles
Reverse Transcription	50	30 minutes	n/a
Activation	95	15 minutes	n/a
Amplification	94	15 seconds	40
	60	30 seconds	

6. Click **Next,** moving to the **Plate** view, and click **Select Existing.**
7. In the subsequent window, navigate to the location of the TB-MBLA plate template, highlight and click open.
8. In the **Plate** view, click **Edit Selection.**
9. In the **Plate Editor** window, click **Spreadsheet View/ Importer,** and in the next window enter the samples names manually or click **Import.** This will allow import of sample information via the TB-MBLA CFX96 import template which can be prepared in advance (see section 6.4.1).
10. Navigate to the prepared template file, highlight, and click **Open.**
11. A window will be presented that displays the well positions of each sample based on the file that was imported. This should be checked for accuracy, and any errors in sample name can be corrected by editing the appropriate entry in the Sample Name column. **Note: This will not update your template file, and this should be corrected also.**
12. Click **OK** to close this window when all information has been checked. Click **OK** in the **Plate Editor** window.
13. In **Plate** view, click **Next** for the **Start Run** view.
14. In the **Start Run** view, select the CFX96 that will be used (by clicking on it), and click **Start Run.**
15. Save your run file according to your naming convention and begin the run.

6.4.6 **Data Analysis**

1. Open the CFX96 **Bio-Rad Optical file.**
2. Selecting one channel at a time by ensuring **FAM™** or **HEX™** is ticked under the amplification plot and set the threshold at **100 RFU**. This can be done either by right clicking on the amplification plot then **Baseline Threshold,** or **Settings > Baseline Threshold.** In the subsequent window, under Single Threshold ensure **User Defined** is selected and enter **100** in the box, then click **OK.**

Note: If using another thermal cycler and accompanying software, ensure that the threshold intersects the amplification curve at the exponential portion. This threshold must be kept consistent between runs.

3. In the standard curve plot, **check the efficiency of the FAM™ channel is between 90-110%.** If acceptable go to next step. If the efficiency is not between 90-110% this will be classed as an invalid plate. It should also be noted that a minimum of 4 standards should be included for efficiency calculations.
4. Review curve shape of samples (and NTCs). If atypical shapes are observed, consult the troubleshooting section **(section 7)** for common causes and solutions.
5. Review RT positive NTCs. No more than 1/3 replicates should be positive with a Cq greater than 35. If 2/3 replicates are positive, or 1/3 has a Cq less than 35 this will be classed as invalid.
6. Using the definitions in **Table 11,** assess the validity of each sample.

Table 11: Reaction Status Definitions

Status	Criteria
Positive	MTB amplification in 2/3 replicates, no EC amplification required.
	MTB 1/3 below 128 c/rxn with 2/3 EC positives.
Negative	No MTB amplification with at least 2/3 EC replicates positive.
Invalid	No amplification for MTB and EC.
	MTB 1/3 amplification above 128 c/rxn.

7. When amplification is seen in participant RT negative reactions, the delta Cq (ΔCq) should be calculated for these samples (ΔCq = Cq RT negative – Cq RT positive) and there should be a minimum of 5 Cq difference between the RT negative and RT positive reactions or the RT negative should be below 128 c/rxn.
8. For downstream data processing a .csv file of all valid data may be required. If a whole plate has been deemed invalid this step is not required as the data will not be analysed further. To generate a .csv file, highlight all the wells (click the square in the top left corner of the plate layout image) and ensure both the FAM™ and HEX™ channels are selected. Next, right click anywhere on the data table to the right of the plate image and click **Export to CSV.** In the subsequent window navigate to the appropriate save location, do not change the default file name, and click **Save.**
9. Save changes made to the Bio-Rad optical file before closing.

6.5 Results & Trending TB-MBLA data

1. The result required for trending is found in the SQ (Starting Quantity) column on the CFX96 software. This number is the copies of TB 16S per reaction (c/rxn).
2. Calculate the average c/rxn of the 3 RT+ replicates. This number can be trended over time to monitor the individual's progress, as per example shown in **Figure 7.** The EC Cq value can also be plotted to monitor the extraction method.

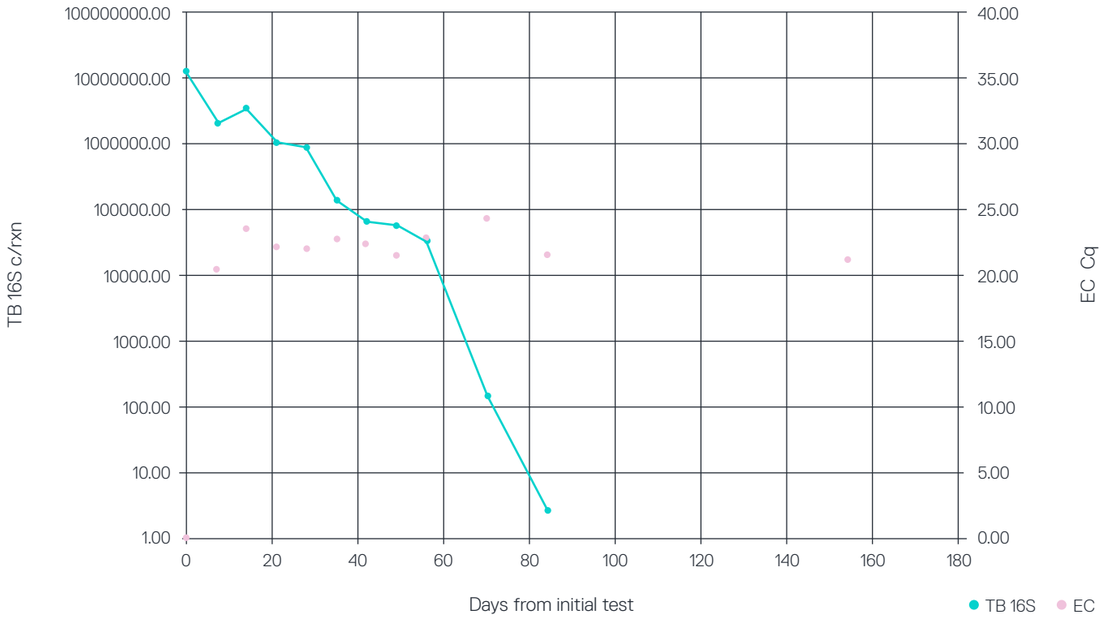


Figure 7: TB 16S copies per reaction plotted over time log10 scale (left axis) and corresponding EC Cq (right axis)

6.6 Actions for Invalid Results

1. **Positive NTC Reactions:**
   This is likely the result of contamination during PCR set up, either of the reagents or the plate. A repeat should be performed after a thorough clean, and/or fresh reagents.
2. **Poor amplification efficiency:**
   PCR efficiencies outside the accepted values (90 – 110%) can be caused by a range of issues, e.g. pipettes, reagents, plasticware, PCR machine or operator error. This should be investigated based on any evidence available (e.g., have other runs on this PCR machine failed recently). If no issues have been observed/identified the PCR procedure should be repeated with a new LifeArc TB-MBLA kit.
3. **Poor distinction between RT positive and negative:**
   This is likely the result of poor or no DNase activity during the nucleic acid extraction process. A repeat extraction should be performed.
4. **EC failure:**
   EC failure in the absence of high levels of MTB amplification may be caused by the presence of PCR inhibitors or poor nucleic acid extraction efficiency. In the first instance the PCR should be repeated.

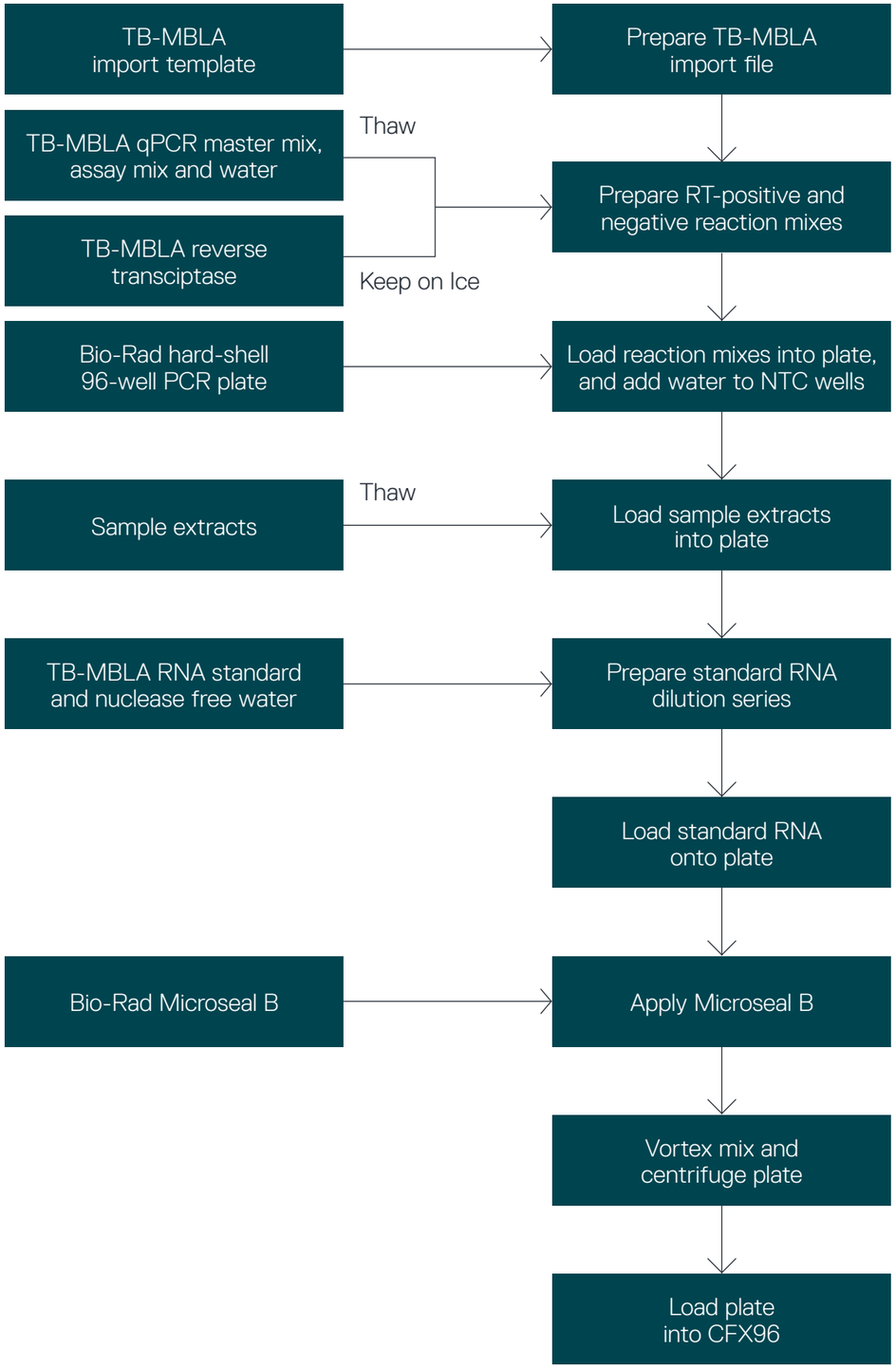


Figure 8: RT-qPCR General Workflow

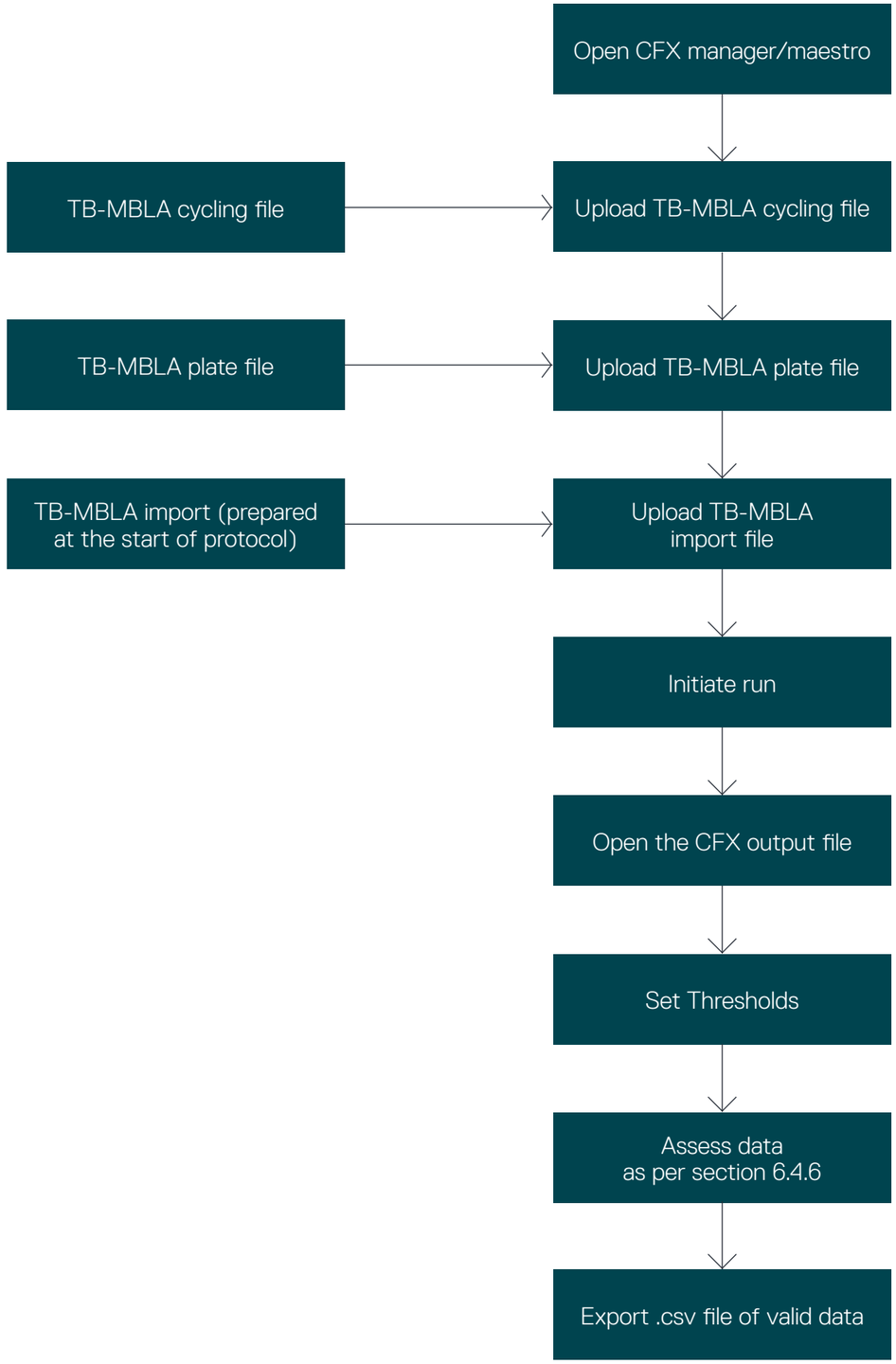
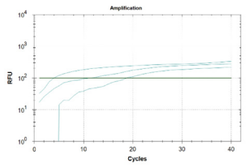
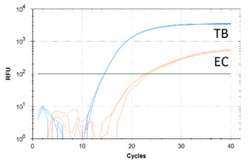
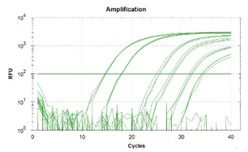
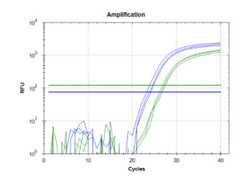
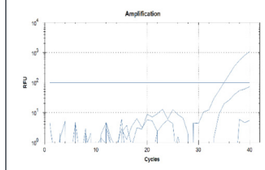
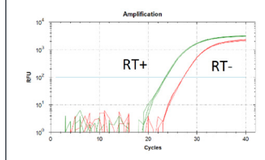
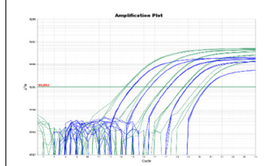


Figure 9: Data Analysis General Workflow

7 Troubleshooting

Problem	Cause	Example	Solution
Atypical profile of amplification curve.	Incorrect baseline applied.		Set baseline subtraction to an appropriate level so that amplification curves show the expected profile.
Low EC amplification in high burden TB samples.	Competitive inhibition of reverse transcriptase and DNA polymerase enzymes by TB target sequence.		Competitive inhibition cannot be avoided. User must be aware of this issue when analysing high burden TB samples.
Non-uniform increase in Cq/Ct between standard concentrations.	RNA adsorption to plastic reaction tube walls.		Repeat plate or exclude affected standards. A minimum of 4 standard concentrations are recommended per plate.
Inconsistent Cq or Ct values for standards with same concentrations.	Custom threshold between runs/ operators/sites is not consistent.		The custom threshold level should intercept at the exponential portion of the amplification curves. This value should be consistent between runs/ operators/sites included for analysis.
High level of variation in Extraction control between samples/runs. LifeArc recommends that range in extraction control Cq/Ct values should not exceed 5.0 when testing clinical samples.	Inconsistent RNA extraction methods. Extraction control spiked at incorrect step.	N/A	Ensure that a consistent RNA extraction control method is used between runs/sites/ operators e.g., phenol/ chloroform, silica column. Ensure that 10µL of extraction control is spiked into lysate prior to 70% ethanol addition.

Problem	Cause	Example	Solution
Positive amplification detected in NTC reactions.	Contamination of equipment or reagents with target RNA/DNA.		Switch to using fresh, unopened reagents. Deep clean ‘problem areas’ of equipment e.g., centrifuge rotors. Safeguard against contamination during plate set-up e.g., separate ‘clean’ and ‘template’ areas.
Genomic DNA present in sample. Less than 5.0 Cq/Ct between RT positive and RT negative reactions.	Ineffective DNase I treatment step.		Prepare fresh DNase solution. Avoid vortex mixing of DNase solution in RDD buffer. If using on-column DNase treatment - ensure careful pipetting onto column membrane.
Reduction in Extraction Control channel fluorescence when using qPCR instruments from alternative manufacturers. Results in minor Cq/Ct shift.	Alternative chromatic filters may be required e.g. HEX™ channel for CFX96 vs VIC™ channel for QuantStudio.		Acknowledge when analysing data that MTB and EC target channels may require different thresholds. LifeArc can advise if necessary.

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