Buruli ulcer laboratory network and new external quality assessment programme for PCR-based diagnosis in the WHO African Region

Terms of reference

World Health Organization

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Contents

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The standard operating procedures annexed to this document were written by Dr Sara Eyangoh, Dr Estelle Marion and Numfor Hycenth. They were reviewed by members of the Buruli ulcer laboratory network and authorized by the Advisory Board.

Abbreviations and acronyms

BU-LABNET	Buruli ulcer laboratory network
CPC	Pasteur Center of Cameroon
EQA	external quality assessment
FNA	fine-needle aspiration
IPIN	International Pasteur Institute Network
ITM	Institute of Tropical Medicine
NTD	neglected tropical disease
PCR	polymerase chain reaction
SOP	standard operating procedure
WHO	World Health Organization

1. Background

Since 2009, the Institute of Tropical Medicine (ITM) in Antwerp, Belgium has organized a biennial external quality assessment (EQA) programme to assess the proficiency of laboratories involved in the diagnosis of Buruli ulcer by molecular detection of *Mycobacterium ulcerans* in clinical samples. The fourth round, organized in 2018, was the latest EQA conducted by ITM. During the 12th meeting of the WHO Technical Advisory Group on Buruli ulcer (Geneva, 27 March 2019), ITM announced that it was discontinuing the EQA programme.

In response, the Group recommended that EQA be assumed by an African country in which Buruli ulcer is endemic, for sustainability purposes. In the meantime, data presented by national programme managers at a meeting on Buruli ulcer (Geneva, 25–27 March 2019) demonstrated a continuing poor rate of confirmation of cases by polymerase chain reaction (PCR). WHO therefore considers that there is an urgent need to analyse the impact of the four previous rounds of external evaluation in order to direct future action.

A total of 18 laboratories from 13 countries participated in the four rounds of EQA: 10 laboratories from eight African endemic countries, four of which participated in all four rounds and three in three rounds. The overall results showed that the median performance of these laboratories improved over the four rounds. However, the proportion of laboratories reporting false–positive cases remains high and indicates a problem of specificity probably due to contamination. The proportion of laboratories reporting both false–positive and false–negative results raises the issue of the quality of the data reported by WHO in Africa as well as the results of the studies carried out in these different laboratories in various countries.

As ITM has discontinued the evaluation process, a model should be proposed to improve the performance of laboratories involved in the molecular diagnosis of Buruli ulcer in endemic countries in Africa in order to ensure that patients receive correct diagnostic results and that data recorded by WHO are accurate, reliable and comparable with those of other continents such as Australia.

WHO intends to transfer the EQA programme to a volunteer laboratory in an endemic African country that demonstrated good performance during the four rounds of EQA. The Mycobacteriology service of the Pasteur Center of Cameroon was judged one of the best performing laboratories and has the capacity and the experience to implement and conduct a new EQA programme.

1.1. The Pasteur Center of Cameroon

The Pasteur Center of Cameroon (CPC), a public administrative institution, has been a member of the International Pasteur Institute Network (IPIN) for 60 years. The Center already serves as the WHO national and regional laboratory for many infectious diseases, including poliomyelitis, yellow fever, measles and influenza, and as the national reference laboratory for diseases such as tuberculosis, Buruli ulcer, meningitidis, cholera, HIV, arboviruses and viral haemorrhagic fever.

The Mycobacteriology unit of CPC organized four training sessions on microbiology of *M. ulcerans* (in January 2006, September 2007, November 2009 and November 2011), all of which were supported by WHO, Fondation Raoul Follereau, Sanofi Espoir Foundation and IPIN, to improve technical capacity, facilitate national implementation of *M. ulcerans* molecular detection and improve the quality of clinical diagnosis. Some 50 health personnel, biologists and technicians from 11 countries (Benin, Central

African Republic, Congo, Côte d'Ivoire, Democratic Republic of the Congo, Gabon, Ghana, Guinea Conakry, Nigeria, Uganda and Togo) in addition to Cameroonian personnel were trained. CPC also implemented various research programmes on Buruli ulcer and published many peer-reviewed articles.

The laboratory is led by a senior scientist with recognized expertise in the area, who is a member of the Case Management Regional Programme Review Group of WHO's Regional Office for Africa. It has strong collaboration with the University of Angers (team led by Dr Laurent Marsollier) and supported the proposed activities. Dr Marsollier's team has extensive field experience in diagnosis of Buruli ulcer and also supported the establishment of the PCR laboratory at the Pobé hospital in Benin through a biannual mentorship model. Recently, the laboratory received support from WHO to integrate molecular differential diagnosis of yaws into its PCR platform.

2. Objectives

The main objective is to establish a new EQA programme with a robust network of laboratories conducting high-performance PCR-based diagnosis of Buruli ulcer in endemic African countries.

The specific objectives are:

- to establish a standardized procedure for PCR-based diagnosis taking into consideration the specific environment and the available equipment;
- to formalize a functioning network of laboratories;
- to set up an EQA programme; and
- to provide training and support for laboratories in the subregion.

3. Scope of work

3.1 Harmonization of standard operating procedures

3.1.1 *Purpose*

To propose, adopt and disseminate harmonized standard operating procedures (SOPs) for PCR-based detection of M. ulcerans to ensure proper sample testing, achieve comparable results among countries, improve the quality of data reported by WHO, optimize EQA (organization of a rechecking/retesting systems) and maximize its use for improvement.

3.1.2 *Methods*

To achieve standardization, the Coordinating Centre will:

- investigate and inventorize all procedures used by the 10 laboratories in the eight participating countries by collecting and consolidating, with the support of WHO, existing procedures on:
  - sample collection, storage and transport;
  - DNA extraction and purification protocols;
  - DNA amplification protocols (master-mix, and amplification methods);
  - positive control DNA for standard graphs;
  - detection sensitivity;
- compare procedures and methods on:
  - feasibility,
  - cost,
  - robustness,
  - repeatability,
  - sensitivity and specificity; and
- adopt harmonized SOPs for all laboratories conducting PCR-based diagnosis of Buruli ulcer, at the meeting on Buruli ulcer (Yaoundé, October 2019), and provide the most cost–effective method for EQA, including a list of essential materials to ensure proper sample testing in all laboratories.

3.1.3 *Expected results*

The harmonized SOPs will be adopted for use by all the laboratories to ensure that further EQA samples are analysed comparably as routine samples. Member laboratories will be required to follow the recommended SOPs in order to be involved in the laboratory network and the EQA programme.

3.2 Formalization of the laboratory network

3.2.1 *Purpose*

To establish a functional network of all laboratories involved in diagnosis of Buruli ulcer in all endemic countries in Africa. The role of the Buruli ulcer laboratory network (BU-LABNET) is to provide ongoing technical guidance and leadership in order to ensure proper testing of samples in all laboratories and to optimize EQA for continuous improvement. The network will ensure proper data collection and

reporting to national Buruli ulcer control programmes and WHO. Additionally, it will provide a robust platform from which to advise and help other countries without capacity for case confirmation to:

- designate a coordinating centre;
- use, promote and disseminate the methods validated by BU-LABNET;
- develop reference reagents and materials for BU-LABNET to ensure accuracy and reliability of test results and facilitate the supply process;
- conduct and/or coordinate scientific and technical studies in collaboration with other laboratories;
- provide scientific and technical training for personnel from endemic countries;
- organize and participate in scientific meetings; and
- discuss and propose an integrated strategy for other neglected tropical diseases (NTDs).

3.2.2 *Functioning of the network*

Membership: Laboratories in endemic countries performing PCR-based diagnosis of Buruli ulcer: Benin (two laboratories), Cameroon (Coordinating Centre), Côte d'Ivoire, Democratic Republic of the Congo, Gabon, Ghana (two laboratories), Liberia, Nigeria and Togo.

Network Advisory Group: Representatives from the Coordinating Centre (Cameroon), WHO, external members (with interest in laboratory diagnosis), one representative from BU-LABNET and experts. The Coordinating Centre will be responsible for maintaining the terms of reference of the group.

The Advisory Group will be accountable for:

- fostering collaboration;
- facilitating the successful delivery, adoption and use of the network; and
- maintaining focus on its agreed scope, outcomes and benefits.

Schedule of meetings

- Expert Panel and Coordinating Centre – annual meeting (see details below)
- Advisory Group – Skype meetings as and when necessary, coordinated by the Coordinating Centre
- Network meetings – first meeting in October 2019 (Coordinating Centre, Yaoundé); second meeting probably organized as a side meeting during the next WHO meeting on Buruli ulcer (WHO, Geneva).

Network activities

Composition: The members of the network will be Member laboratories and corresponding Member countries.

Activities

- The Coordinating Centre will develop the terms of reference of the expert panel and the advisory group.
- The first meeting of the network will be organized at CPC in Yaoundé, Cameroon in October 2019. The harmonized SOPs will be shared with all Member laboratories during the meeting.
- The expert panel (selected members) will visit the laboratories to assess the status of the laboratory set up, infrastructure, training requirements and needs for reagents and consumables. Predesigned questionnaires developed by the Coordinating Centre will used to assess performance. Member laboratories will be divided among the expert panel; each expert will visit 1–3 laboratories. **Visits will take place in early 2020**.

- After the visits the expert panel will send the assessments to the Coordinating Centre for dissemination of reports and recommendations to respective Member laboratories.
- Requirements for infrastructure and consumables will be discussed at the expert panel meeting. A report with appropriates details will be submitted to WHO. If supported, WHO/Coordinating Centre will disseminate required materials to the Member laboratories.
- Based on needs, the Coordinating Centre will organize training for staff/technicians at CPC or at any other Member laboratories that it recognizes as being capable of training staff in SOPs. Budgetary needs for training will be submitted by the Coordinating Centre as a proposal to WHO after discussion with the expert panel.
- Thereafter, the Collaborating Centre will develop the necessary questionnaires/forms and send them to the Member laboratories for information on SOPs, consumables and infrastructural changes (if any) once every 3 months. A report will be generated from the updates provided by the Member laboratories and presented to the expert panel each year. The panel will meet once a year, at a venue recommended by the Coordinating Centre, to discuss and recommend improvements. The Coordinating Centre, together with the expert panel, will generate a report on the discussions and outcomes of the meetings and share it with WHO.
- The expert panel and the Coordinating Centre will also recognize research needs identified at expert panel meetings and develop collaborative research proposals as appropriate.
- The Coordinating Centre will communicate to WHO any needs for materials/consumables required by Member laboratories to facilitate procurement.
- The network will also connect with and assist neighbouring countries (not Member countries) in which cases of Buruli ulcer are detected or suspected in order to confirm the diagnosis.

3.2.3 *Essential reagents and consumables – key materials*

Once the protocols for PCR confirmation are harmonized and approved by the network, help will be needed to access and ship key materials. Basic molecular laboratory equipment must be present and functional in order to participate in the programme. Basic equipment includes biosafety cabinets, pipettes, centrifuges, freezers, fridges, thermomixers and real-time PCR instruments. Alternatively, the network could be supported by WHO through nongovernmental organizations to help laboratories access key materials for PCR more easily.

Key materials (to be modified):

- qPCR master-mix
- standard DNA
- IS2404 probes and primers
- purification kit

Based on the data from WHO, the estimated number of samples for PCR from eight countries will be approximately 5000 PCR per year.

3.3 Functioning of the Coordinating Centre

The main objective of the Coordinating Centre is to establish a laboratory network of African countries endemic for Buruli ulcer with high performance in PCR. In this context and to properly coordinate the network, CPC will receive specific funding to cover the cost of a programme manager and a technician.

The programme manager (full-time) will be recruited by the Coordinating Centre and remunerated based on the salary grid of CPC. The incumbent will be responsible for:

- communicating with the network, including circulation of emails from members;
- storing all documents, including forms and information from each member;
- organizing programme activities, including visits, trainings and meetings;
- drafting quarterly reports, describing the activities of each laboratory and other activities of the network;
- organizing the control quality programme, with the help of the technician; and
- drafting the integrated strategy for NTDs.

The technician (part-time 50%) will be recruited by the Coordinating Centre and remunerated based on the salary grid of CPC. The incumbent will be responsible for:

- collaborating with the expert team on supportive visits to the other laboratories of the network;
- preparing EQA panel samples/strains for distribution to the participating laboratories (twice/year);
- analysing EQA panel results obtained from the participating laboratories;
- preparing the feedback report obtained from result analysis; and
- participating in supervising integration of other NTDs in the PCR-based platform for case confirmation.

3.4 Implementation of the new external quality assurance programme

3.4.1 *Purpose*

The new EQA programme will:

- continuously monitor and evaluate the SOPs and infrastructure of its Member laboratories through the laboratory network (see section 3.2); and
- provide support for training and materials and send blinded pretested sample panels to evaluate performance; and
- operate initially for 5 years but may be extended subject to need and availability of grants.

3.4.2 *Functioning*

The Coordinating Centre will:

- design and send blinded, pre-tested sample panels to Member laboratories biannually and collect and comparatively analyse the results;
- develop and distribute protocols for sending/testing sample panels (with associated descriptions if any) to each member laboratory; and
- send an assessment report to each member laboratory and extend support to laboratories that demonstrate suboptimal performance.

3.4.3 *Quality control*

The standard positive DNA control will either be procured from BEI Resources (https://www.beiresources.org/) or prepared (extraction, purification, quality assessment and lyophilization) at the Mycobacterial Research Laboratory (Colorado State University, Fort Collins, USA) with the involvement/direction of the Coordinating Centre/WHO. The same positive DNA control will be shared with the Member laboratories; standard graphs will be developed by each laboratory and compared with those of the Coordinating Centre.

Annexes

Annex 1. Standard operating procedure for collection, transport and storage of samples (SOP1)

A1.1 Purpose

To describe the recommended procedure for collecting, transporting and storing clinical specimens for laboratory confirmation of Buruli ulcer by polymerase chain reaction (PCR). For diagnostic purposes, samples should be collected before treatment. Given the heterogeneous distribution of mycobacteria in lesions, at least two clinical specimens should be collected from each lesion.

A1.2 Application domain

To be applied to all members of the Buruli ulcer laboratory network (BU-LABNET) conducting PCR diagnosis of Buruli ulcer.

A1.3 Associated documents

Worksheet: request for laboratory confirmation of Buruli ulcer cases by PCR.

A1.4 Type of samples

- Swabs for collecting samples from opened lesions with undermined edges.
- Fine-needle aspiration (FNA) for collecting samples from closed lesions or open wounds with closed edges (not undermined).
- **Biopsy is not recommended for laboratory confirmation of Buruli ulcer cases**.

A1.4.1 Collecting swabs

1. Use a sterile unitary swab.
2. Swab under the undermined edge of the ulcer.
3. After swabbing, replace the swab in the original tube; do not add any liquid.
4. A minimum of two swabs are required per lesion; if there are several lesions, take two swabs for each lesion.

A1.4.2 Collecting fine-needle aspirates

1. Transfer 0.5 mL of sterile water in the microtube with a screw-cap or vacutainer blood dry tube (red cap).
2. Alternatively, use physiological water or phosphate-buffered saline; do not inoculate the liquid into the lesion.
3. Using a 23-gauge needle and a syringe of 2 mL, aspirate the liquid from the closed lesion.
4. Put the content of the syringe into the microtube or vacutainer tube containing sterile water.
5. Gently draw some of the liquid into the needle and then expel it back into the microtube. To ensure that all of the sample is transferred, repeat this procedure three times, then close the microtube.

Note: Do not inoculate liquid into the lesion.

6. Repeat the aspiration if lesions are large.

A1.5 Reagents and consumables

See list in Appendix A1.

A1.6 Equipment

Not applicable.

A1.7 Information on patients

All samples should be hermetically closed and identified, using a permanent marker, with the date of the sampling and the surname and first name of the patient.

If two types of samples have been collected from the patient (i.e. FNA and swabs) or the patient has several lesions, consider both samples for analysis.

Shipment should be accompanied by an information sheet with a table summarizing the samples (see Worksheet: request for Buruli ulcer confirmation by PCR).

A1.8 Storage conditions

A1.8.1 Storing samples before transportation to the laboratory

- Samples should be stored at 2–8 °C or otherwise at room temperature in a dry place.
- The maximum waiting time before transportation is 1 week (maximum 2 weeks if countries are populous).

A1.8.2 Storing samples during transportation to the laboratory

- Samples should be shipped at room temperature or in a cool box if available.

A1.8.3 Storing samples at the laboratory

Upon arrival at the laboratory, the samples must be stored at 2–8°C until treatment for PCR analysis. See also Annex 2: Standard operating procedure for registration and treatment of samples before PCR analysis (SOP2).

A1.9 Internal quality control

Not applicable

A1.10 Safety precautions

- Always consider all used materials as infectious and discard them appropriately.
- Discard all needles in a safety box or sharps container

A1.11 Bibliography

Portaels F (editor). Laboratory diagnosis of Buruli ulcer: a WHO manual for health care providers. Geneva: World Health Organization; 2014 (*https://apps.who.int/iris/bitstream/handle/10665/111738/9789241505703_eng.pdf,* accessed May 2020).

A1.12 Reading and understanding list

Name of personnel	Date (dd/mm/yy)	Signature

Appendix A1. List of materials and reagents for collection, transport and storage of samples

Name	Reference	Commentary	Pictures
Swabs: sterile and in a tube	For example: 8150CC (Gauss); 552C (Copan)	Numerous references exist; ensure that swabs are individually packed in a tube	
Needles and syringes	21-gauge to 23-gauge needles; 1 mL to 2 mL syringes		
1.5 mL microtube with screw-cap	For example: 39289 (Dutscher)	Ensure that the cap is attached to the tube	
Vacutainer dry tube (red cap)		If no screw-cap tube available	
Sterile water	Not applicable	Not applicable	Not applicable
Permanent marker	Not applicable	Ball-point tip	Not applicable
Worksheet for BU confirmation request	Use worksheet disseminated by BU-LABNET	Not applicable	Not applicable
Waste container for discarding swabs	Not applicable	Leakproof	Not applicable
Sharps container/ safety box for discarding needles	Not applicable	Leakproof and puncture-resistant (*the picture is an example of a safety container*)	
Transport box (optional)	Not applicable	Tight-fitting lid	Not applicable
Refrigerator to store samples (optional)	Not applicable	2–8 °C	Not applicable
Gloves	Not applicable	Non-powdered	Not applicable

Annex 2. Standard operating procedure for registration and treatment of samples before PCR analysis (SOP2)

A2.1 Purpose

To describe the recommended procedure for registering and treating samples for laboratory diagnosis of cases of Buruli ulcer by polymerase chain reaction (PCR).

A2.2 Application domain

To be applied to all members of the Buruli ulcer laboratory network (BU-LABNET) conducting PCR diagnosis of Buruli ulcer.

A2.3 Associated documents

- BU manual register (form BU 01)
- BU request form (form BU 03)

A2.4 Type of samples

- Swabs for collecting samples from opened lesions with undermined edges.
- Fine-needle aspiration (FNA) for collecting samples from closed lesions or open wounds with closed edges (not undermined).
- **Biopsy is not recommended for laboratory confirmation of Buruli ulcer cases**.

A2.5 Reagents and consumables

See list in Appendix A2.

A2.6 Equipment

See list in Appendix A2.

A2.7 Information on patients

When the laboratory receives a shipment of samples for confirmation of Buruli ulcer, the samples should be registered and treated that day or the day just using the following procedure.

1. To register the samples, complete the manual register (optional: informatics register, but manual register must be present in any case). The manual register contains the following information from the worksheet request:
    a. name of the structure requesting the PCR analysis;
    b. name of the patient;
    c. surname of the patient;
    d. sampling date;
    e. sampling type;
    f. laboratory sample number;
    g. PCR data;
    h. PCR result; and
    i. number of bacilli per mL.
2. Keep the request sheet received with the shipment in a folder.
3. Attribute a “Lab sample number” to each new sample; keep continuity between the samples.

A2.8 Procedure for processing samples

Note: Each tube must be identified with the "Lab sample number".

A2.8.1 Dry swabs

1. Rehydrate swabs in a 15 mL tube containing 2 mL of sterile water, then vortex.
2. Normally, there are at least two swabs per lesion; pool all the swabs from the same lesion into a single 15 mL tube.
3. Wait at least 5 minutes and vortex again.
4. Discard the swabs from the 15 mL tube.
5. Pipet and transfer 400 µL into a 1.5 mL microtube with screw-cap.
6. Transfer the remainder into a 1.5 mL microtube with a screw-cap for use in Ziehl–Neelsen staining; store the rest as a backup at −20 °C.

A2.8.2 Fine-needle aspiration

1. Vortex the tube containing the FNA.
2. Pipet 400 µL and transfer them into a microtube with a screw-cap for DNA extraction.
3. If the volume is < 500 µL, add 500 µL of water before pipetting; store the rest as backup at −20 °C.
4. Keep the tubes containing 400 µL of DNA suspension at room temperature for DNA extraction (see Annex 3A: Extraction and purification of DNA from *Mycobacterium ulcerans* with internal positive control (SOP3A)), or freeze at −20 °C until DNA extraction.

A2.9 Internal quality control

Not applicable

A2.10 Safety precautions

- Always consider all used materials as infectious and discard them appropriately.
- Discard all needles in a safety box or sharps container.

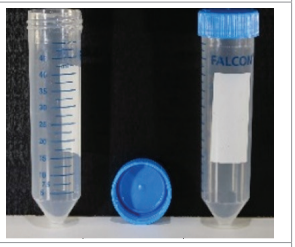
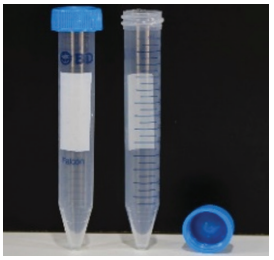
A2.11 Bibliography

Portaels F (editor). Laboratory diagnosis of Buruli ulcer: a WHO manual for health care providers. Geneva: World Health Organization; 2014 (*https://apps.who.int/iris/bitstream/handle/10665/111738/9789241505703_eng.pdf,* accessed May 2020).

A2.12 Reading and understanding list

Name of personnel	Date (dd/mm/yy)	Signature

Appendix A2. List of materials and reagents for registration and treatment of samples before PCR analysis

Name	Reference	Commentary	Pictures
Manual register	Not applicable	Obtained locally	Not applicable
BU request form	Not applicable	Format specific to country	Not applicable
50 mL Falcon tubes (PP, graduated, conical bottom, blue screw-cap, sterile)	Not applicable (e.g. 227 261 Greiner bio one)	Withstand centrifugation	
15ml Falcon tubes (PP, graduated, conical bottom, blue screw cap, sterile)	Not applicable (e.g. 188261 Greiner bio one)	Withstand centrifugation	
Sterile water	Not applicable	Not applicable	Not applicable
1.5 mL microtube with screw-cap	For example: 39289 (Dutscher)	Ensure that cap is attached to the tube	
Storage box	Not applicable	Plastic box preferable	Not applicable
Vortex mixer	Not applicable	Currently used in respective laboratories	
Pipettes	Not applicable	Currently used in respective laboratories	Not applicable
Filter tips	Will be provided by BU-LABNET, based on pipette information by laboratories	Laboratories will maintain currently used pipettes	Not applicable
Gloves	Not applicable	Non-powdered	Not applicable
Disposable laboratory coat	Not applicable	Long sleeves/full length	Not applicable
–20 °C freezer	Not applicable	To store DNA suspension prior to extraction	Not applicable
Waste container	Not applicable	Leakproof	Not applicable

Annex 3A. Standard operating procedure for extraction and purification of DNA from *Mycobacterium ulcerans* with internal positive control (SOP3A)

A3.A1 Purpose

To describe the recommended procedure for extracting DNA and purifying samples for polymerase chain reaction (PCR) targeting *Mycobacterium ulcerans*.

A3.A2 Application domain

To be applied to all members of the Buruli ulcer laboratory network (BU-LABNET) for PCR diagnosis of Buruli ulcer.

A3.A3 Associated documents

None

A3.A4 Type of samples

- Swabs are used for collecting samples from opened undermined lesions.
- Fine-needle aspiration (FNA) is used for collecting samples from closed lesions or opened lesions that are not undermined.
- **Biopsy is not recommended for laboratory confirmation of Buruli ulcer cases**.

A3.A5 Reagents and consumables

See list in Appendix A3.

A3.A6 Equipment

See list in Appendix A3.

A3.A7 Procedure

A3.A7.1 DNA extraction

DNA extraction is performed by bacterial lysis through alkaline lysis using the GenoLyse kit (Ref: 51610, Hain LifeScience).

1. For each sample, use 400 µL of specimen suspension prepared previously and placed in a microtube with screw cap (see Annex 2: Standard operating procedure for registration and treatment of samples before PCR analysis (SOP2)).
2. Centrifuge the tube at 12000 x g for 15 minutes at room temperature.
3. Discard the supernatant using a P1000 filter tip; a pellet may or may not be visible.
4. Resuspend the pellet with 400 µL of water and centrifuge at 12000 x g for 15 minutes at room temperature.
5. Discard the supernatant.
6. Resuspend the pellet in 50 µL of A-LYS buffer.
7. Add 10 µL of IPC DNA (IC, Diagenode, ref: Dia-EIC/DNA-050).
8. Incubate for 10 minutes at 95 °C.
9. Centrifuge the tube for 10 seconds to pellet the suspension.
10. Add 50 µL of buffer A-NB to neutralize.
11. Store the tube at 4 °C if for use during the day for PCR amplification, or at –20 °C.

Note: For swabs and FNA samples, there is no need to perform purification.

A3.A7.2 DNA purification for biopsy samples

DNA purification is recommended for biopsy samples in order to remove traces of inhibitors.

Use the QIAquick PCR purification kit (ref: 28106, QIAGEN) as follows:

1. Add 300 µL of buffer PB (Binding buffer) to each sample and 10 µL of Na Acetate 3M.
2. Load the column with the sample and centrifuge for 1 minute at 5000 x g at room temperature.
3. Wash the column with 750 µL of buffer PE (wash buffer) and centrifuge for 1 minute at 5000 x g at room temperature.
4. Repeat a second centrifugation without adding a buffer in order to remove any trace of ethanol.
5. Eluate DNA by adding 100 µL of buffer EB (elution buffer)and centrifuge for 1 minute at 5000 x g at room temperature.
6. Store at 4 °C for short-term storage or at –20 °C for long-term storage.

A3.A8 Internal quality control

Internal positive control.

A3.A9 Safety precautions

Always consider all used materials as infectious and discard them appropriately.

A3.A10 Bibliography

Portaels F (editor). Laboratory diagnosis of Buruli ulcer: a WHO manual for health care providers. Geneva: World Health Organization; 2014 (*https://apps.who.int/iris/bitstream/handle/10665/111738/9789241505703_eng.pdf,* accessed May 2020).

A3.A11 Reading and understanding list

Name of personnel	Date (dd/mm/yy)	Signature

Annex 3B. Standard operating procedure for extraction and purification of DNA from *Mycobacterium ulcerans* without internal positive control (SOP3B)

A3.B1 Purpose

To describe the recommended procedure for extracting DNA and purifying samples for polymerase chain reaction (PCR) targeting *Mycobacterium ulcerans*.

A3.B2 Application domain

To be applied to all members of the Buruli ulcer laboratory network (BU-LABNET) conducting PCR diagnosis of Buruli ulcer.

A3.B3 Associated documents

None

A3.B4 Type of samples

- Swabs are used for sampling of opened undermined lesions.
- Fine needle aspiration (FNA) is used for sampling of closed lesions or opened but not undermined lesions.
- **Biopsy is not recommended for laboratory confirmation of Buruli ulcer cases**.

A3.B5 Reagents and consumables

See list in Appendix A3.

A3.B6 Equipment

See list in Appendix A3.

A3.B7 Procedure

A3.B7.1 DNA extraction

DNA is extracted by bacterial lysis through alkaline lysis using the GenoLyse kit (Ref: 51610, Hain LifeScience) as follows:

1. For each sample, use 400 µL of specimen suspension prepared previously and placed in a microtube with screw cap (see Annex 2: Standard operating procedure for registration and treatment of samples before PCR analysis (SOP2).
2. Centrifuge the tube at 12000 x g for 15 minutes at room temperature.
3. Discard the supernatant using a P1000 filter tip; a pellet may or may not be visible.
4. Resuspend the pellet with 400 µL of water and centrifuge at 12000 x g for 15 minutes at room temperature.
5. Discard the supernatant.
6. Resuspend the pellet in 50 µL of A-LYS buffer.
7. Incubate for 10 minutes at 95 °C.
8. Centrifuge tube for 10 seconds to pellet the suspension.
9. Add 50 µL of buffer A-NB to neutralize.
10. Keep tube at 4 °C if used during the day for PCR amplification or store at −20 °C.

Note: For swabs and FNA samples, there is no need to perform the purification step.

A3.B.7.2 DNA purification for biopsy samples

Purification of DNA is recommended for biopsy samples to remove traces of inhibitors.

Use the "QIAquick purification kit" (ref. 28106, QIAGEN) as follows:

1. Add 300 µL of PB buffer to each sample and 10 µL of Na Acetate 3M.
2. Load the column with the sample and centrifuge for 1 minutes at 5000 x g at room temperature.
3. Wash the column with 750 µL of PE buffer and centrifuge for 1 minute at 5000 x g at room temperature.
4. Repeat a second centrifugation without adding a buffer in order to remove any trace of ethanol.
5. Eluate DNA by adding 100 µL of EB buffer and centrifuge for 1 minute at 5000 x g at room temperature.
6. Store at 4 °C for short- term storage or at −20°C for long- term storage.

A3.B8 Internal quality control

Not applicable.

A3.B9 Safety precautions

Always consider all used materials as infectious and discard them appropriately.

A3.B10 Bibliography

Portaels F (editor). Laboratory diagnosis of Buruli ulcer: a WHO manual for health care providers. Geneva: World Health Organization; 2014 (*https://apps.who.int/iris/bitstream/handle/10665/111738/9789241505703_eng.pdf,* accessed May 2020).

A3.B11 Reading and understanding list

Name of personnel	Date (dd/mm/yy)	Signature

Appendix A3. List of materials and reagents for extraction and purification of DNA from *M. ulcerans*

Name	Reference	Commentary	Pictures
Manual register	Not applicable	Obtained locally	Not applicable
Sterile water	Not applicable	Not applicable	Not applicable
GenoLyse kit	51610, Hain LifeScience	To be provided by BU-LABNET	Not applicable
QIAquick PCR purification kit	28106, QIAGEN	For biopsy samples; to be provided by BU-LABNET	Not applicable
Vortex	Not applicable	Currently used in respective labs	Not applicable
Pipette	Not applicable	Currently used in respective laboratories	Not applicable
Filter tips	Will be provided by BU-LABNET, based on pipette information by laboratories	Laboratories will maintain currently used pipettes	Not applicable
Gloves	Not applicable	Non-powdered	Not applicable
Disposable laboratory coat	Not applicable	Long sleeves/full length	Not applicable
Centrifuge	Any model that can hold 1.5 mL tubes and spin up to 12000 x g	12000 x g 1.5 mL	Not applicable
Dry water-bath	Not applicable	Currently used in respective laboratories	Not applicable
–20 °C freezer	Not applicable	To store DNA eluate (long-term)	Not applicable
Refrigerator 2–8 °C	Not applicable	To store DNA eluate (short-term)	Not applicable
Waste container	Not applicable	Leakproof	Not applicable

Annex 4A. Standard operating procedure for preparation of real-time quantitative PCR with internal positive control (SOP4A)

A4.A1 Purpose

To describe the recommended procedure for preparing real-time quantitative polymerase chain reaction (qPCR) for detection of *Mycobacterium ulcerans* using an internal positive control.).

A4.A2 Application domain

To be applied to all members of the Buruli ulcer laboratory network (BU-LABNET) conducting PCR diagnosis of Buruli ulcer.

A4.A3 Associated documents

Worksheet: qPCR mix calcul and plate plan.

A4.A4 Type of samples

- Swabs are used for sampling of opened undermined lesions.
- Fine- needle aspiration (FNA) is used for sampling of closed lesions or opened but not undermined lesions.
- **Biopsy is not recommended for laboratory confirmation of Buruli ulcer cases**.

A4.A5 Reagents and consumables

See list in Appendix A4.

A4.A6 Equipment

See list in Appendix A4.

A4.A7 Procedure

A4.A7.1 Prepare the experiment

When the laboratory receives a shipment of samples for confirmation of Buruli ulcer, the samples should be registered and treated that day or the day just using the following procedure.

1. Complete the plate plan of the worksheet for each new amplification with samples.
2. Count the number of amplifications that need to be prepared; importantly, always count three supplemental reactions to have enough mix at the end.
3. Calculate and fill in the table of PCR mix preparation.
4. Switch on the thermocycler and fill in the plate plan.

A4.A7.2 PCR mix preparation

Under a clean PCR hood:

1. Wear gloves and a disposable blouse dedicated to this space.
2. Defrost the following reagents: qPCR master-mix/primers and probe (IS2404)/IPC primers and probe CY5/an aliquot of sterile water.
3. Probe (IS2404) dilution: 10- fold dilution before use:
   - centrifuge the defrosted tube;
   - in a new screw cap tube, add 18 µL of water;
   - add 2 µL of probe;
   - vortex slowly and centrifuge for a few seconds.

4. Primers (IS2404) dilution:
   - centrifuge the two aliquots of primer tubes;
   - add directly 95µL of water into each tube;
   - vortex slowly and centrifuge for a few seconds.

A4.A7.3 PCR mix preparation

1. Follow the quantity of water, master- mix, probe and primers calculated in the worksheet.
2. Prepare a new screw- cap tube and mix the master- mix by reversal.
3. Always start by pipetting water, then the primers/probe (IPC), the two primers and the probe (IS2404). Finish by adding the master- mix.
4. Mix the tube by reversal and centrifuge for a few seconds.
5. Prepare a rack with eight strip tubes.
6. Transfer 20 µL of the mix in each tube.
7. Do not close the tubes.

A4.A7.4 PCR mix and samples

1. On a dedicated bench, bring the rack with the PCR strip, the patient samples and the plasmid.
2. Add 5 µL of patient samples following the plate plan.
3. Close the patient strips.
4. Prepare the standard range of plasmid DNA in screw cap tubes as follows:
   - defrost an aliquot of 10 µL tube at 1E8 bact/mL;
   - add 90 µL of water directly in the tube = first point of the standard curve = 1E7 bact/mL;
   - add 45 µL of water in five new screw cap tubes;
   - perform cascade dilution by pipetting 5 µL of the first tube and mixing it into the 45 µL tube: 1E7 bact/mL, 1E6 bact/mL, 1E5 bact/mL, 1E4 bact/mL, 1E3 bact/mL, 1E2 bact/mL;
   - add 5 µL of plasmid DNA in strip tubes following the plate plan;
   - close the strips.

A4.A7.5 Amplification

1. Centrifuge the strips and place them into the thermocycler.
2. Run the amplification program.

A4.A7.6 PCR analysis

1. Check the two negative controls: extraction and mix preparation.
2. Check the standard curve to ensure that Ct values and R2 are correct.
3. Check the internal control (IPC Diagenode CY5): 27<Ct<35.
4. Check the limit of detection for humans: < 35 cycles.
5. Calculate the number of bacilli/mL for positive samples.
6. Write the result in the manual registration book and in the worksheet for the clinician.
7. Disseminate the results following the procedure for validation in your country (email, WhatsApp, etc.).

A4.A8 Internal quality control

Internal positive control.

A4.A9 Safety precautions

Always consider all used materials as infectious and discard them appropriately.

A4.A10 Bibliography

Portaels F (editor). Laboratory diagnosis of Buruli ulcer: a WHO manual for health care providers. Geneva: World Health Organization; 2014 (*https://apps.who.int/iris/bitstream/handle/10665/111738/9789241505703_eng.pdf*, accessed May 2020).

A4.A11 Reading and understanding list

Name of personnel	Date (dd/mm/yy)	Signature

Annex 4B. Standard operating procedure for preparation of real-time PCR without internal positive control (SOP4B)

A4.B1 Purpose

To describe the recommended procedure for preparing real-time polymerase chain reaction (qPCR) for identification of *Mycobacterium ulcerans* without the internal positive control.

A4.B2 Application domain

To be applied to all members of the Buruli ulcer laboratory network (BU-LABNET) conducting PCR diagnosis of Buruli ulcer.

A4.B3 Associated documents

Worksheet: qPCR mix calcul and plate plan.

A4.B4 Type of samples

- Swabs are used for sampling of opened lesions.
- Fine-needle aspiration (FNA) is used for sampling of closed lesions.
- **Biopsy is not recommended for laboratory confirmation of Buruli ulcer cases**.

A4.B5 Reagents and consumables

See list in Appendix A4.

A4.B6 Equipment

See list in Appendix A4.

A4.B7 Procedure

A2.B7.1 Prepare the experiment

1. Complete the plate plan of the worksheet for each new amplification with samples.
2. Count the number of amplifications that needed to be prepared. Importantly, always count three supplemental reactions to have enough mix at the end.
3. Calculate and fill in the table of PCR mix preparation.
4. Switch on the thermocycler and fill in the plate plan.

A4.B7.2 PCR mix preparation

Under a clean PCR hood:

1. Wear gloves and a disposable blouse dedicated to this space.
2. Defrost the following reagents:

a. qPCR master-mix/primers and probe/an aliquot of sterile water

3. Probe dilution: 10-fold dilution before using:
    a. centrifuge the defrost tube;
    b. in a new screw cap tube, add 18 µL of water;
    c. add 2 µL of probe;
    d. vortex slowly and centrifuge for a few seconds;
4. Primers dilution:
    a. centrifuge the two aliquots of primer tubes;
    b. add directly 95 µL of water into each tube;
    c. vortex slowly and centrifuge for a few seconds.

A4.B7.3 PCR mix preparation

1. Follow the quantity of water, master- mix, probe and primers calculated in the worksheet.
2. Prepare a new screw- cap tube and mix the master- mix by reversal.
3. Always start by pipetting water, then the primers/probe (IPC), the two primers and the probe (IS2404). Finish by adding the master- mix.
4. Mix the tube by reversal and centrifuge for a few seconds.
5. Prepare a rack with eight strip tubes.
6. Transfer 20 µL of the mix in each tube.
7. Do not close the tubes.

A4A.B7.4 PCR mix and samples

1. On a dedicated bench, bring the rack with the PCR strip, the patient samples and plasmid.
2. Add 5 µL of patient samples following the plate plan.
3. Close the patient strips.
4. Prepare the standard range of plasmid DNA in screw-cap tubes:
    a. defrost an aliquot of 10 µL tube at 1E8 bact/mL;
    b. add 90 µL of water directly into the tube = first point of the standard curve = 1E7 bact/mL;
    c. add 45 µL of water in five new screw-cap tubes;
    d. perform cascade dilution by pipetting 5 µL of the first tube and mixing it into the 45 µL tube: 1E7 bact/mL, 1E6 bact/mL, 1E5 bact/mL, 1E4 bact/mL, 1E3 bact/mL, 1E2 bact/mL;
    e. add 5 µL of plasmid DNA in strip tubes following the plate plan;
    f. close the strips.

A4.B7.5 Amplification

1. Centrifuge the strips and place them into the thermocycler.
2. Run the amplification program.

A4.B7.6 PCR analysis

1. Check the two negative controls: extraction and mix preparation.
2. Check the standard curve to ensure that the Ct values and R2 are correct.
3. Check the limit of detection for human: < 35 cycles.
4. Calculate the number of bacilli/mL for positive samples.
5. Write the result in the manual registration book and in the worksheet for the clinician.
6. Disseminate the results following the procedure for validation in your country (email, WhatsApp, etc.).

A4.B8 Internal quality control

No internal positive control

A4.B9 Safety precautions

Always consider all used materials as infectious and discard them appropriately.

A4.B10 Bibliography

Portaels F (editor). Laboratory diagnosis of Buruli ulcer: a WHO manual for health care providers. Geneva: World Health Organization; 2014 (*https://apps.who.int/iris/bitstream/handle/10665/111738/9789241505703_eng.pdf,* accessed May 2020).

A4.B11 Reading and understanding list

Name of personnel	Date (dd/mm/yy)	Signature

Appendix A4. List of materials and reagents for real-time PCR without internal positive control

Name	Reference	Commentary	Pictures
QPCR mix calcul and plate plan	(BU-FRM-01, version 1.0)	Provided by BU-LABNET	Not applicable
qPCR master-mix	HOT FIREPol Probe qPCR Mix Plus (e.g. Solis BioDyne, new mix candidate, by Dutscher)	To be provided by BU-LABNET	Not applicable
Primers and probe	New primers and probes will be used; to be provided by BU-LABNET	To be provided by BU-LABNET	Not applicable
Is2404 plasmid	(IS2404) n° 30-8606-01 (GenExpress)	To be provided by BU-LABNET	Not applicable
Sterile water	Not applicable	Not applicable	Not applicable
8 strip-tubes and caps	Not applicable	Currently used in respective laboratories	Not applicable
1.5 mL microtube with screw-cap	For example: 39289 (Dutscher)	Ensure that cap is attached to the tube	
Vortex mixer	Not applicable	Currently used in respective laboratories	
Pipette	Not applicable	Currently used in respective laboratories	Not applicable
Filter tips	Will be provided by BU-LABNET based on pipette information by laboratories	Laboratories will maintain currently used pipettes	Not applicable
Gloves	Not applicable	Non-powdered	Not applicable
Disposable laboratory coat	Not applicable	Long sleeves/full length	Not applicable
qPCR machine	Not applicable	qPCR machine currently used in respective laboratories	Not applicable
Waste container	Not applicable	Leakproof	Not applicable

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