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ARTIC-like Bacillus anthracis MLVA amplicon sequencing protocol for MinION

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ABSTRACT

Multiple-Locus Variable Number of Tandem Repeats (VNTR) Analysis (MLVA) is one of the gold standard strain-level subtyping methods for outbreak-related Bacillus anthracis strains. The repeat numbers of 31 VNTR loci can be determined by capillary electrophoresis of PCR amplicons spanning repeat regions or in silico analysis of whole genome sequencing (WGS) data. However these methods require clear isolates of typeable strains and can be performed in fixed well-equipped high biosafety level laboratories.

We developed field-applicable amplicon sequencing protocol for Bacillus anthracis MLVA typing directly from environmental samples without isolating clear cultures of Bacillus anthracis strains. 62 primers were used for generating PCR amplicons for 31 Bacillus anthracis VNTR loci, according to MLVA31 typing scheme described by Beyer et al. 2012. The primers generating amplicons longer than 200 bp were used from MLVA31 typing scheme (44 primers for 22 VNTR loci). For amplicons shorter than 200 bp, primers were redesigned to generate longer amplicons (between 300-700 bp) suitable for MinION sequencing.

We optimized and tested this protocol on hungarian virulent Bacillus anthracis strains, a 34F2 Bacillus anthracis vaccine strain, and on spiked environmental samples in Hungarian Defence Forces field-deployable laboratory.

OPEN ACCESS

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Protocol status: In development
We are still developing and optimizing this protocol

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CITATION

Beyer W, Bellan S, Eberle G, Ganz HH, Getz WM, Haumacher R, Hilss KA, Kilian W, Lazak J, Turner WC, Turnbull PC (2012). Distribution and molecular evolution of bacillus anthracis genotypes in Namibia.. PLoS neglected tropical diseases.

LINK
https://doi.org/10.1371/journal.pntd.0001534

MATERIALS

Primers 25nm, desalted, ideally LabReady formulation from IDT BaMLVA_primers.xlsx
Qiagen DNeasy Blood&Tissue kit Qiagen 69504
Q5 Hot Start HF Polymerase NEB M0493S
NEBNext Ultra II End Repair/dA-Tailing Module NEB E7546S
NEBNext Ultra II Ligation Module NEB E7595S
NEBNext Quick Ligation Module NEB E6056S
QuantiFluor ONE dsDNA System, 100rxn Promega E4871
Agencourt AMPure XP Beckman Coulter A63880
Native Barcoding Expansion 1-12 Nanopore EXP-NBD104
Native Barcoding Expansion 13-24 Nanopore EXP-NBD114
Native Barcoding Expansion Kit 1-96 Nanopore EXP-NBD196
Ligation Sequencing Kit Nanopore SQK-LSK109
Sequencing Auxiliary Vials Nanopore EXP-AUX001
Adapter Mix II Expansion Nanopore EXP-AMII001
Short Fragment Buffer Expansion kit Nanopore EXP-SFB001
Flow Cell Priming Kit Nanopore EXP-FLP002
R9.4.1 flow cells Nanopore FLO-MIN106

SAFETY WARNINGS

All procedures and manipulation with samples containing virulent B. anthracis spores should be performed in a biosafety level 3 laboratory.

BEFORE START INSTRUCTIONS

Isolate DNA from environmental samples suspected to contain Bacillus anthracis spores with Qiagen DNeasy Blood&Tissue kit or similar suitable for DNA isolation from Gram positive bacteria. It is recommended to apply an extra mechanical lysis step (for ex. bead beating) before DNA isolation to increase the effectiveness of spore disruption.
Before MLVA analysis check the isolated DNA with Bacillus anthracis-specific real-time PCR assay for Bacillus anthracis DNA content.

Primer pool preparation

1 If required resuspend lyophilised primers at a concentration of 100µM each. Primer names, characteristics, concentrations and volumes required for primer stocks are listed in the table below.
BaMLVA_primers.xlsx

2 Generate 500 µL primer Pool 1 stock by adding 7 µL, 13.5 µL or 15.5 µL of each primer to a 1.5 mL Eppendorf labelled “Pool 1 (stock)”, following the table above.

Note

Primers should be diluted and pooled in the **mastermix cabinet** which should be cleaned with decontamination wipes and UV sterilised before and after use.

3 Dilute primer Pool 1 stock 1:10 in molecular grade water, to generate Pool 1 working solution.

4 Generate 100 µL primer Pool 2 stock by adding 5 µL of each odd region primer to a 1.5 mL Eppendorf labelled “Pool 2”, and adjust final volume to 100 µL with molecular grade water.

Note

It is recommend that multiple aliquots of each primer pool are made to in case of degradation or contamination.

Multiplex PCR

5 In the mastermix hood set up the multiplex PCR reactions as follows in 0.2 mL 8-strip PCR tubes:

Component	**Pool 1**	**Pool 2**
5X Q5 Reaction Buffer	5 µL	5 µL
10 mM dNTPs	0.5 µL	0.5 µL
Q5 Hot Start DNA Polymerase	0.25 µL	0.25 µL
BaMLVA Primer Pool 1 working solution or Pool 2	4.3 µL	1.0 µL
Nuclease-free water	12.45 µL	15.75 µL
Total	22.5 µL	22.5 µL

Note

A PCR mastermix for each pool should be made up in the **mastermix cabinet** and aliquoted into PCR strip tubes. Tubes should be wiped down when entering and leaving the mastermix cabinet.

6 In the extraction and sample addition cabinet add 2.5 µL DNA to each tube and mix well by pipetting.

Note

The **extraction and sample addition cabinet** should should be cleaned with decontamination wipes and UV sterilised before and after use.

7 Pulse centrifuge the tubes to collect the contents at the bottom of the tube.

8 Set-up the following programs on a gradient thermal cycler, or a thermal cycler suitable for running 2 or more different PCR cycles in one time, or 2 thermal cyclers: 12m 30s

Program for Pool 1 PCR:

Step	Temperature	Time	Cycles
Heat Activation	98 °C	00:00:30	1
Denaturation	98 °C	00:00:15	45
Annealing	65 °C	00:05:30	45
Hold	4 °C	Indefinite	1

Program for Pool 2 PCR:

Step	Temperature	Time	Cycles
Heat Activation	98 °C	00:00:30	1
Denaturation	98 °C	00:00:15	45
Annealing	63 °C	00:05:30	45
Hold	4 °C	Indefinite	1

Equipment

Veriti 96-Well Thermal Cycler	NAME
Applied Biosystems	BRAND
4375786	SKU
https://www.thermofisher.com/order/catalog/product/4375786#/4375786	LINK

Quantification and normalisation

9 Quantify 1 µL PCR product using the Quantus Fluorometer using the ONE dsDNA assay.

Protocol

NAME

DNA quantification using the Quantus fluorometer

CREATED BY

Josh Quick

PREVIEW

Equipment

Quantus	NAME
Fluorometer	TYPE
Promega	BRAND
E6150	SKU
https://www.promega.co.uk/products/microplate-readers-fluorometers-luminometers/fluorometers/quantus-fluorometer	LINK

9.1 Remove Lambda DNA 400 ng/µL standard from the freezer and leave on ice to thaw. Remove ONE dsDNA dye solution from the fridge and allow to come to room temperature.

QuantiFluor(R) ONE dsDNA System, 500rxn **Promega Catalog #E4870**

9.2 Set up two 0.5 mL tubes for the calibration and label them 'Blank' and 'Standard'

9.3 Add 200 µL ONE dsDNA Dye solution to each tube.

9.4 Mix the Lambda DNA standard 400 ng/µL standard by pipetting then add 1 µL to one of the standard tube.

9.5 Mix each sample vigorously by vortexing for 00:00:05 and pulse centrifuge to collect the liquid.

9.6 Allow both tubes to incubate at room temperature for 00:02:00 before proceeding.

9.7 Selection 'Calibrate' then 'ONE DNA' then place the blank sample in the reader then select 'Read Blank'. Now place the standard in the reader and select 'Read Std'.

9.8 Set up the required number of 0.5 mL tubes for the number of DNA samples to be quantified.

> **Note**
>
> Use only thin-wall, clear, 0.5mL PCR tubes such as Axygen #PCR-05-C

9.9 Label the tubes on the lids, avoid marking the sides of the tube as this could interfere with the sample reading.

9.10 Add 199 µL ONE dsDNA dye solution to each tube.

9.11 Add 1 µL of each user sample to the appropriate tube.

> Note
>
> Use a P2 pipette for highest accuracy.

9.12 Mix each sample vigorously by vortexing for 00:00:05 and pulse centrifuge to collect the liquid.

9.13 Allow all tubes to incubate at room temperature for 00:02:00 before proceeding.

9.14 On the Home screen of the Quantus Fluorometer, select `Protocol`, then select `ONE DNA` as the assay type.

> Note
>
> If you have already performed a calibration for the selected assay you can continue, there is no need to perform repeat calibrations when using ONE DNA pre diluted dye solution. If you want to use the previous calibration, skip to step 11. Otherwise, continue with step 9.

9.15 On the home screen navigate to 'Sample Volume' and set it to 1 µL then 'Units' and set it to ng/µL.

9.16 Load the first sample into the reader and close the lid. The sample concentration is automatically read when you close the lid.

9.17 Repeat step 16 until all samples have been read.

9.18 The value displayed on the screen is the dsDNA concentration in ng/µL, carefully record all results in a spreadsheet or laboratory notebook.

10 Label a 1.5 mL Eppendorf tube for each sample and assemble the following PCR dilution for each sample for final volume of 10 µL:

Pool 1 PCR reaction volume (x µL) containing 196 ng PCR amplicon
Pool 2 PCR reaction volume (x µL) containing 21 ng PCR amlicon
Nuclease-free water volume (x µL) to a final volume of 10 µL

Total amount of PCR amplicons per sample 217 ng in 10 µL

Note

Input from Pool 1 and Pool 2 PCR reactions will vary depending on the starting amount of target DNA. If the Ct value of of the target DNA is <30, it is possible to put the total 217 ng amount of PCR amplicons to 10 µL according to our experiences. If the Ct value of target DNA is >30, put as much PCR amplicons to 10 µL final volume as you can, keeping the 1:10 ratio of Pool 1 : Pool 2 PCR amplicons.

11 Dilute PCR amplicon pool of each sample 1:10 adding 90 µL molecular grade water, and mix well by pipetting.

12 Label a 0.2 mL PCR tube for each sample.

13 Use 10 µL input for the One-pot native barcoding reaction to give a total of 21.7 ng per sample.

Note

Input to the one-pot native barcoding reaction will vary depending on the amplicon length but we have determined 21.7 ng is the correct input for efficient barcoding of this amplicon length. Process at least 6 samples per native barcoded library in order to have sufficient material at the end.

Native barcoding

14 Barcode the amplicon pools using the one-pot native barcoding approach.

Note

We developed native barcoding protocol with modifications of Josh Quick 2020. One-pot native barcoding of amplicons v2. **protocols.io** https://dx.doi.org/10.17504/protocols.io.bdp8i5rw

14.1 Set up the following reaction for each sample:

Component	Volume
PCR dilution from previous step	10 µL
Nuclease-free water	2.5 µL
Ultra II End Prep Reaction Buffer	1.75 µL
Ultra II End Prep Enzyme Mix	0.75 µL
Total	15 µL

14.2 Incubate at room temperature for 00:10:00 21m
Incubate at 65 °C for 00:10:00
Incubate on ice for 00:01:00

14.3 In a new 0.2 mL PCR tube set up the following reaction:

Component	Volume
Previous reaction mixture	3.5 µL
Nuclease-free water	3.7 µL
NBXX barcode	2.5 µL
Ultra II Ligation Master Mix	10 µL
Ligation Enhancer	0.3 µL
Total	20 µL

Note

Use one native barcode from the EXP-NBD104 (1-12) or EXP-NBD114 (13-24) or EXP-NBD196 (1-96) per

sample. The minimum use of 6 barcodes is sufficient for effective application of R9 flow cells. Under these sample numbers the cost effectivity of this method is highly decreased due to the low yield of overall data.

14.4 Incubate at room temperature for 00:20:00 36m
Incubate at 65 °C for 00:15:00
Incubate on ice for 00:01:00

Note

The 65°C incubation is to inactivate the DNA ligase to prevent barcode cross-ligation when reactions are pooled in the next step.

14.5 In a new 1.5 ml Eppendorf tube pool all 20 µL one-pot barcoding reactions together.

Note

It is recommended to pool maximum 6 one-pot barcoding reactions together in one tube. If more than 6 reactions are pooled into one tube, the next amplicon-cleaning step will take very long time due to the slow drying of high amount of SPRI beads.

14.6 Add 1.8x volume of SPRI beads to the sample tube and mix gently by either flicking or pipetting. For example add 216 µL SPRI beads to 120 µL 6-plex pooled one-pot native barcoding reactions.

Note

1.8x volume of SPRI will bind the shortest 200 bp amplicons in the presence of ligation buffer as in a one-pot reaction. It is recommended to use 1.8x volume of SPRI beads to not lose short amplicons even though this will result in excessive native barcode carryover.

Note

Vortex SPRI beads thoroughly before use to ensure they are well resuspended, the solution should be a homogenous brown colour.

14.7 Pulse centrifuge to collect all liquid at the bottom of the tube.

14.8 Incubate for 00:05:00 at room temperature. 5m

14.9 Place on magnetic rack and incubate for 00:02:00 or until the beads have pelleted and the supernatant is completely clear. 2m

14.10 Carefully remove and discard the supernatant, being careful not to touch the bead pellet.

14.11 Add 500 µL SFB and resuspend beads completely by pipette mixing.

14.12 Pulse centrifuge to collect all liquid at the bottom of the tube.

14.13 Place on magnetic rack and incubate until the beads have pelleted and the supernatant is completely clear.

14.14 Remove supernatant and discard.

14.15 Pulse centrifuge and remove any residual SFB.

Note

You do not need to allow to air dry with SFB washes.

14.16 Bath the pellet in 500 µL of room-temperature 75% volume ethanol without resuspending the beads.

14.17 Carefully remove and discard ethanol, being careful not to touch the bead pellet.

14.18 Pulse centrifuge to collect all liquid at the bottom of the tube and carefully remove as much residual ethanol as possible using a P10 pipette.

14.19 With the tube lid open incubate for 00:05:00 or until the pellet loses it's shine (if the pellet dries completely it will crack and become difficult to resuspend). 5m

14.20 Resuspend pellet in 30 µL nuclease-free water, mix gently by either flicking or pipetting and incubate for 00:05:00 . 5m

14.21 Place on magnetic rack and transfer sample to a clean 1.5 mL Eppendorf tube ensuring no beads are transferred into this tube.

Note

If the barcoding reactions were pooled in 2 tubes (more than 6-plex), resuspend each pellet in 30-30 ul nuclease-free water, and pool into one tube the cleaned barcoded amplicon pools after incubation.

15 Set up the following AMII adapter ligation and clean-up with SFB.

Note

We developed adaper ligation protocol with modifications of Josh Quick 2020. Adapter ligation with AMII. **protocols.io** https://dx.doi.org/10.17504/protocols.io.bdp9i5r6

15.1 Set up the following AMII adapter ligation reaction:

Component	Volume
Barcoded amplicon pool	30 µL
NEBNext Quick Ligation Reaction Buffer (5X)	10 µL
Adapter Mix (AMII)	5 µL
Quick T4 DNA Ligase	5 µL
Total	50 µL

Note

If the volume of barcoded amplicon pool is 60 µL, double each component of the adapter ligation reaction, thus final volume will be 100 µL.

15.2 Incubate at room temperature for 00:20:00. 20m

15.3 Add 1x volume of SPRI beads to the sample tube (1:1 ratio of beads to sample volume) and mix gently by either flicking or pipetting. For example add 50 µL SPRI beads to 50 µL adapter ligation reaction.

Note

Vortex SPRI beads thoroughly before use to ensure they are well resuspended, the solution should be a homogenous brown colour.

15.4 Pulse centrifuge to collect all liquid at the bottom of the tube.

15.5 Incubate for 00:05:00 at room temperature. 5m

15.6 Place on magnetic rack and incubate for 00:02:00 or until the beads have pelleted and the supernatant is completely clear. 2m

15.7 Carefully remove and discard the supernatant, being careful not to touch the bead pellet.

15.8 Add 200 µL SFB and resuspend beads completely by pipette mixing.

> **Note**
>
> SFB will remove excess adapter without damaging the adapter-protein complexes. Do not use 70% ethanol as in early clean-ups.

15.9 Pulse centrifuge to collect all liquid at the bottom of the tube.

15.10 Place on magnetic rack and incubate until the beads have pelleted and the supernatant is completely clear.

15.11 Remove supernatant and discard.

15.12 Repeat step 15.8-15.11. to perform a second SFB wash.

15.13 Pulse centrifuge and remove any residual SFB.

> **Note**
>
> You do not need to allow to air dry with SFB washes.

15.14 Add 15 µL EB and resuspend beads by pipette mixing.

15.15 Incubate at 37 °C for 00:08:00 . 8m

> **Note**
>
> The longer incubation at 37°C helps to eluate shorter amplicons.

15.16 Place on magnetic rack and transfer final library to a clean 1.5mL Eppendorf tube ensuring no beads are transferred into this tube.

16 Quantify 1 µL of the final library using the Quantus Fluorometer using the ONE dsDNA assay.

> **Protocol**
>
> NAME
>
> **DNA quantification using the Quantus fluorometer**
>
> CREATED BY
>
> Josh Quick
>
> PREVIEW

> **Note**
>
> Final library can be now be stored at 4°C for up to a week if needed otherwise proceed directly to MinION

sequencing.

MinION sequencing

17 Prime the flowcell and load 20-25 ng sequencing library onto the flowcell.

Protocol

NAME

Priming and loading a MinION flowcell

CREATED BY

Josh Quick

PREVIEW

17.1 Thaw the following reagents at room temperature before placing on ice:

Sequencing buffer (SQB)
Loading beads (LB)
Flush buffer (FLB)
Flush tether (FLT)

17.2 Add 30 µL FLT to the FLB tube and mix well by vortexing.

17.3 If required place a new MinION flowcell onto the MinION by flipping open the lip and pushing one end of the flowcell under the clip and pushing down gently.

17.4 Rotate the inlet port cover clockwise by 90° so that the priming port is visible.

17.5 Take a P1000 pipette and tip and set the volume to 800 µL. Place the tip in the inlet port and holding perpendicularly to the plane of the flowell remove any air from the inlet port by turning the volume dial anti-clockwise.

Note

Be careful not to remove so much volume that air is introduced onto the rectangular array via the outlet.

17.6 Load 800 µL of FLB (plus FLT) into the flow cell via the inlet port, dispense slowly and smoothly trying to avoid the introduction of any air bubbles.

17.7 Wait for 00:05:00 .

17.8 Gently lift the SpotON cover to open the SpotON port.

17.9 Load another 200 µL of FLB (plus FLT) into the flow cell via the inlet port, this will initiate a siphon at the SpotON port to allow you to load the library dilution.

17.10 In a new tube prepare the library dilution for sequencing:

Component	Volume
SQB	37.5 µL
LB	25.5 µL
Final library	12 µL
Total	75 µL

Note

Mix LB immediately before use as they settle quickly.

Dilute library in EB if required.

17.11 Mix the prepared library gently by pipetting up and down just prior to loading.

17.12 Add the 75 µL library dilution to the flow cell via the SpotON sample port in a dropwise fashion. Ensure each drop siphons into the port before adding the next.

17.13 Gently replace the SpotON sample port cover, making sure the bung enters the SpotON port, close the inlet port and close the MinION lid.

18 Start the sequencing run using MinKNOW.

18.1 If required plug the MinION into the computer and wait for the MinION and flowcell to ben detected.

18.2 Choose flow cell 'FLO-MIN106' from the drop-down menu.

18.3 Then select the flowcell so a tick appears.

18.4 Click the 'New Experiment' button in the bottom left of the screen.

18.5 On the New experiment popup screen, select the running parameters for your experiment from the individual tabs:

Experiment: Name the run in the experiment field, leave the sample field blank.

Kit: Selection: Select LSK109 and Native barcoding kit (EXP-NBD104 or EXP-NBD114 or EXP-NBD196).

Run Options: Set the run length to minimum 24 hours (you can stop the run once sufficient data has been collected as determined using RAMPART).

Basecalling: Leave basecalling turned but select 'superaccurate basecalling'.

Barcoding: Leave barcoding turned, turn on trim barcodes, but turn off Barcode both ends option.

Output: The number of files that MinKNOW will write to a single folder. By default this is set to 4000 but can be reduced to make RAMPART update more frequently.

Click 'Start run'.

Note

In case of using GridION or MinION with "high peformance" computer, superaccurate basecalling and barcoding are recommended.

18.6 Monitor the progress of the run using the MinKNOW interface.