

Jan 22, 2026

Version 3

Amplicon sequencing protocol for MLVA genotyping of *Bacillus anthracis* strains on the Nanopore platform V.3

DOI

<https://dx.doi.org/10.17504/protocols.io.3byl4jzozlo5/v3>

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Protocol Citation: Ágnes Nagy 2026. Amplicon sequencing protocol for MLVA genotyping of *Bacillus anthracis* strains on the Nanopore platform. **protocols.io** <https://dx.doi.org/10.17504/protocols.io.3byl4jzozlo5/v3> Version created by **Ágnes Nagy**.

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Protocol status: Working

We use this protocol and it's working

Created: January 22, 2026

Last Modified: January 22, 2026

Protocol Integer ID: 239105

Keywords: Bacillus anthracis MLVA, environmental samples, amplicon sequencing, protocol for bacillus anthracis mlva, bacillus anthracis vntr loci, bacillus anthracis mlva, related bacillus anthraci, bacillus anthraci, protocol on hungarian virulent bacillus anthraci, clear cultures of bacillus anthraci, hungarian virulent bacillus anthraci, 34f2 bacillus anthracis vaccine strain, silico analysis of whole genome sequencing, generating pcr amplicon, clear isolates of typeable strain, whole genome sequencing, capillary electrophoresis of pcr amplicon, protocol for mlva genotyping, pcr amplicon, sequencing protocol, native barcoding of pcr amplicon, equipped high biosafety level laboratory, high biosafety level laboratory, nanopore platform, generating amplicon, typeable strain, mlva genotyping, level subtyping methods for outbreak, sequencing

Funders Acknowledgements:

Hungarian Defence Forces Medical Centre

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 the Hungarian Defence Forces field-deployable laboratory. This version of the protocol describes the preparation and native barcoding of PCR amplicons for 31 *Bacillus anthracis* VNTR loci using the Native Barcoding Kit 96 V14 (SQK-NBD114.96) with flow cell R10.4.1 (FLO-MIN114).

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.

<https://doi.org/10.1371/journal.pntd.0001534>

LINK

Materials

Primers 25nm, desalted, ideally LabReady formulation from IDT



BaMLVA_primers_v3.xlsx 11.8KB

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

Native Barcoding Kit 96 V14 SQK-NBD114.96

R10.4.1 flow cells Nanopore FLO-MIN114



Safety warnings

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

Before start

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_v3.xlsx 11.8KB

- 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

12m 30s

- 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 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  0.2 mL PCR tube for each sample and assemble the following PCR dilution for each sample for final volume of  12.5 μL :

Pool 1 PCR reaction volume ( x μL) containing  45 ng PCR amplicon

Pool 2 PCR reaction volume ( x μL) containing  5 ng PCR amplicon

Nuclease-free water volume ( x μL) to a final volume of  12.5 μL

Total amount of PCR amplicons per sample  50 ng in  12.5 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 the target DNA is <35, it is possible to put the total  50 ng amount of PCR amplicons to  12.5 µL according to our experience. If the Ct value of target DNA is >35, put as much PCR amplicons to  12.5 µL final volume as you can, keeping the 1:9 ratio of Pool 1 : Pool 2 PCR amplicons.

End-prep reaction

1h 49m

- 11 Set up the following reaction for each sample:

Component	Volume
PCR dilution from previous step	 12.5 µL
Ultra II End Prep Reaction Buffer	 1.75 µL
Ultra II End Prep Enzyme Mix	 0.75 µL
Total	 15 µL

- 12 Incubate at room temperature for  00:10:00
- Incubate at  65 °C for  00:10:00
- Incubate on ice for  00:01:00

21m

Native barcode ligation

- 13 In a new 0.2 mL PCR tube set up the following reaction for each sample:

Component	Volume
Previous reaction mixture	 7.2 µL
Native Barcode	 2.5 µL
Ultra II Ligation Master Mix	 10 µL
Ligation Enhancer	 0.3 µL
Total	 20 µL

**Note**

Use one Native Barcode (NB) from the Oxford Nanopore Native barcoding kit version 14 (SQK-NBD114.24 or SQK-NBD114.96) per sample.

14 Incubate at room temperature for  00:20:00

36m

15 Add  2 μL EDTA with blue cap to each sample, mix thoroughly by pipetting.

16 In a new 1.5 ml Eppendorf tube pool all  22 μL barcoded samples together.

17 Add 0.4X AMPure XP Beads (AXP) to the pooled reaction, and mix by pipetting. For example, add  52.8 μL SPRI beads to  132 μL 6-plex pooled barcoded samples.

Note

Vortex AMPure XP Beads (AXP) thoroughly before use to ensure they are well resuspended, the solution should be a homogenous brown colour.

18 Incubate for  00:05:00 at room temperature.

5m

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

2m

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

21 Keep the tube on the magnetic rack and wash the beads with  700 μL of Short Fragment Buffer (SFB) without disturbing the pellet.

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



- 23 Remove supernatant and discard.
- 24 Repeat step 21-23 to perform a second SFB wash.
- 25 Pulse centrifuge and remove any residual SFB.

Note

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

- 26 Resuspend pellet in  30 μL nuclease-free water, mix gently by pipetting, and incubate for  00:10:00 at  37 $^{\circ}\text{C}$ in thermomixer shaking at  400 rpm .
- 27 Place on magnetic rack and transfer sample to a clean  1.5 mL Eppendorf tube ensuring no beads are transferred into this tube.

5m

Adapter ligation

1h 49m

- 28 Set up the following adapter ligation reaction:

Component**Volume**

Barcoded amplicon pool

 30 μL

NEBNext Quick Ligation Reaction Buffer (5X)

 10 μL

Native Adapter (NA)

 5 μL

Quick T4 DNA Ligase

 5 μL **Total** 50 μL

- 29 Incubate at room temperature for  00:20:00 .

20m

- 30 Add  20 μL of AMPure XP Beads (AXP) to the sample tube and mix gently by pipetting.

**Note**

Vortex AMPure XP Beads (AXP) thoroughly before use to ensure they are well resuspended, the solution should be a homogenous brown colour.

- 31 Pulse centrifuge to collect all liquid at the bottom of the tube.
- 32 Incubate for  00:05:00 at room temperature. 5m
- 33 Place on magnetic rack and incubate for  00:02:00 or until the beads have pelleted and the supernatant is completely clear. 2m
- 34 Carefully remove and discard the supernatant, being careful not to touch the bead pellet.
- 35 Add  125 μL SFB and resuspend beads completely by pipette mixing.
- 36 Pulse centrifuge to collect all liquid at the bottom of the tube.
- 37 Place on magnetic rack and incubate until the beads have pelleted and the supernatant is completely clear.
- 38 Remove supernatant and discard.
- 39 Repeat step 35-38. to perform a second SFB wash.
- 40 Pulse centrifuge and remove any residual SFB.

Note

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



- 41 Add  15 μL EB and resuspend beads by pipette mixing.
- 42 Incubate for  00:10:00 at  37 °C in thermomixer shaking at  400 rpm .
- 43 Place on magnetic rack and transfer final library to a clean 1.5mL Eppendorf tube ensuring no beads are transferred into this tube.
- 44 Quantify  1 μL of the final library using the Quantus Fluorometer using the ONE dsDNA assay.

8m

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.

- 45 Prepare your final library in 12 μL of Elution Buffer (EB):
- If the final amount of the library is <25 ng (<1,8 ng/ μL concentration), use the whole amount of library for sequencing;
 - If the final amount of the library is $\geq$ 25 ng ($\geq$ 1,8 ng/ μL concentration), dilute the library to 25 ng in 12 μL with Elution Buffer (EB).

MinION sequencing

46 Prime the flowcell and load 25 ng sequencing library onto the flowcell.

46.1 Thaw the Sequencing Buffer (SB), Library Beads (LIB) or Library Solution (LIS, if using), Flow Cell Tether (FCT) and Flow Cell Flush (FCF) at room temperature. Prepare the flow cell priming mix:

Component	Volume
Flow Cell Flush (FCF)	 1170 µL
Flow Cell Tether (FCT)	 30 µL
Bovine Serum Albumin (BSA) at 50 mg/ml	 5 µL
Total	 1205 µL

Note

Adding BSA to the flow cell priming mix improves sequencing performance and output on MinION R10.4.1 flow cells (FLO-MIN114). However, in case of this amplicon sequencing library it is not necessary. We didn't notice any difference in sequencing performance and output without BSA.

46.2 Before adding priming mix, remove any air bubbles from flow cell by drawing back 20-30 µl buffer via Priming port with a P1000 pipette.

46.3 Load  800 µL of the priming mix into the flow cell via the priming port, avoiding the introduction of air bubbles. Wait for  00:05:00 .

46.4 During this time, prepare the library for loading:

Component	Volume
Sequencing Buffer (SB)	 37.5 µL
Library Beads (LIB)	 25.5 µL
DNA library (25 ng)	 12 µL
Total	 75 µL

**Note**

Mix Library Beads (LiB) immediately before use to ensure homogeneity of beads.

- 46.5 Open the Priming port and the SpotON sample port, and complete the flow cell priming by loading  200 μL priming mix into the Priming port, avoiding the introduction of air bubbles.
- 46.6 Add  75 μL of the prepared library to the flow cell via the SpotON sample port in a dropwise fashion. Ensure each drop flows into the port before adding the next.
- 47 Start the sequencing run using MinKNOW.
- 47.1 If required plug the MinION into the computer and wait for the MinION and flowcell to be detected.
- 47.2 Choose flow cell 'FLO-MIN114' from the drop-down menu.
- 47.3 Then select the flowcell so a tick appears.
- 47.4 Click the 'New Experiment' button in the bottom left of the screen.
- 47.5 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 Native Barcoding Kit 96 V14 (SQK-NBD114.96).

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

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.

Click 'Start run'.

Note

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

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

Citations

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

<https://doi.org/10.1371/journal.pntd.0001534>