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Technical report:
Technical Protocol for NGS Sequencing of Tick-Borne Encephalitis Virus (TBEV)

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Executive Summary

This technical protocol provides a comprehensive, laboratory-ready workflow for near-complete **whole-genome amplicon sequencing of the Tick-borne encephalitis virus, European subtype (TBEV-Eu)**, using both **Illumina and Oxford Nanopore Technologies (ONT)** sequencing platforms. Designed for high-quality genomic surveillance, the protocol enables reliable generation of TBEV-Eu genome data directly from a wide range of **clinical and field-derived specimens**, including human and rodent samples, as well as individual or pooled ticks.

TBEV represents one of the most significant tick-borne viral pathogens in Europe, causing disease outcomes that range from mild febrile illness to severe neurological manifestations. The European subtype (TBEV-Eu), *Orthoflavivirus neudoerflense*, predominates across Europe and is associated with a case fatality rate of up to 1%. Rapid and accurate genomic characterization is essential for monitoring viral evolution, understanding transmission patterns, supporting public health responses, and guiding research into viral pathogenicity and epidemiology.

This protocol outlines the required reagents, equipment, and step-by-step laboratory procedures for amplifying and sequencing the ~11 kb TBEV-Eu genome. The workflow is optimized for sensitivity, allowing direct sequencing from diverse biological matrices, without prior virus isolation or culture. Importantly, while the protocol is validated for TBEV-Eu, its performance with other TBEV subtypes (Siberian and Far Eastern) has not been assessed.

Overall, this document provides a standardized, reproducible method intended to support laboratories engaged in **molecular diagnostics, genomic surveillance, and research applications** related to TBEV-Eu, ensuring harmonized data generation across projects, institutions, and sequencing platforms.

Tick-borne encephalitis virus-European subtype whole genome amplicon sequencing protocol

V.1 (2026-03-05)

1 Introduction

Tick-borne encephalitis (TBE) is the most significant tick-borne viral infection in Europe and parts of Asia. In Central Europe, it is a seasonal disease, with most cases occurring between April and November. It is caused by tick-borne encephalitis virus (TBEV) (*Orthoflavivirus neudorfelense*), which is most commonly transmitted by the bite of an infected tick, although other routes are possible, such as consumption of unpasteurised dairy products, transmission via solid organ transplantation, and laboratory infection. The viral genome is an approximately 11 kb single-stranded positive-sense RNA molecule that encodes three structural proteins (C, PrM, and E) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5). TBEV is phylogenetically divided into three major subtypes: European (TBEV-Eu), Siberian (TBEV-Sib), and Far Eastern (TBEV-FE). The TBEV-Eu subtype is the most common in Europe, whereas TBEV-Sib and TBEV-FE are mainly found in Asia, but can also be detected in north-eastern Europe. TBEV-Eu is associated with various disease courses, from febrile illness to neurological symptoms such as meningitis, meningoencephalitis, and meningoencephalomyelitis. The case fatality rate of TBEV-Eu is up to 1%.

2 Intended use

This protocol describes all laboratory procedures, reagents and equipment needed for near-complete genome sequencing of TBEV-Eu directly from clinical samples. It is suitable for biological samples collected from humans and rodents as well as whole ticks or pooled ticks and is compatible with Illumina and Oxford Nanopore Technologies instruments. We performed a bioinformatic *in silico* analysis to assess the cross-reactivity of the developed oligonucleotides for possible amplification of TBEV-Sib and TBEV-FE subtypes, which showed a total of 11/50 and 6/50 amplicons (29% and 18% coverage) might form for TBEV-Sib and TBEV-FE, respectively. Wet-lab performance for non-European TBEV subtypes was not further determined.

3 Samples, reagents and consumables

3.1 Suitable samples:

Human: EDTA blood or serum, cerebrospinal fluid, urine, brain tissue samples

Rodents: tissue samples

Tick: supernatant from homogenised whole tick or pooled ticks

3.2 Nucleic acid isolation

This protocol has been successfully used with total nucleic acids isolated with automated systems EZ1&2 Virus Mini Kit v2.0 (Qiagen; 955134) on an EZ2 instrument (Qiagen); TANBead OptiPure Viral Auto Plate (TANBead Inc.; W665A46/301224) on a TANBead Maelstrom™ 9600 instrument (TANBead Inc.), and manual RNA isolation kits Direct-zol RNA Miniprep (Zymo Research; R2051), and the QIAamp Viral RNA Mini (Qiagen; 52904). Other nucleic acid isolation methods could be used but suitability has to be tested by the user.

3.3 General reagents

Component	Supplier	Cat. Number
AMPure XP beads	Beckman Coulter	A63882
Qubit DNA HS Assay Kit	Thermo Fisher Scientific	Q32851
Qubit™ Assay Tubes	Thermo Fisher Scientific	Q32856
Ethanol 100 % without denaturing compounds	various	NA
Nuclease-free water	various	NA

3.4 cDNA synthesis and amplification reagents

Component	Supplier	Cat. Number
Primers 25nm, desalted	IDT or equal	NA
SuperScript IV (50 rxn)	Thermo Fisher Scientific	18090050
dNTP mix (10 mM each)	Thermo Fisher Scientific	R0192
Random Hexamers (50 µM)	Thermo Fisher Scientific	N8080127
RNase OUT (125 rxn)	Thermo Fisher Scientific	10777019
Q5 Hot Start HF Polymerase	New England Biolabs	M0493S
AMPure XP beads	Beckman Coulter	A63882
Qubit DNA HS Assay Kit	Thermo Fisher Scientific	Q32851
Qubit™ Assay Tubes	Thermo Fisher Scientific	Q32856

3.5 NGS libraries preparation reagents

3.5.1 Illumina

Component	Supplier	Cat. Number
Nextera XT DNA Library Preparation Kit (96rxn)	Illumina	FC-131-1096
NextSeq HighOutput Kit v2.5 (300 cycles)	Illumina	20024908
MiSeq Reagent Kit v3 (600 cycles)	Illumina	MS-102-3003
Illumina® DNA/RNA UD Indexes Set A/B/C/D	Illumina	20091654 20091656 20091658 20091660
200 mM Tris-HCl, pH 7.0	Various	NA
1.0 N NaOH, molecular biology grade	Various	NA

3.5.2 Oxford Nanopore Technologies

Component	Supplier	Cat. Number
NEBNext Ultra II End Repair/dA-tailing module	New England Biolabs	E7546
Blunt/TA Ligase Master Mix	New England Biolabs	M0367S
Native Barcoding Kit 96 V14	Oxford Nanopore Technologies	SQK-NBD114.96
NEBNext Quick Ligation Module	Oxford Nanopore Technologies	E6056S
MinION/GridION Flow Cell	Oxford Nanopore Technologies	FLO-MIN114
PromethION Flow Cell	Oxford Nanopore Technologies	FLO-PRO114M
Flow Cell Wash Kit (optional)	Oxford Nanopore Technologies	EXP-WSH004

3.6 Instruments and equipment

Component	Supplier	Cat. Number
Pipettes (P1000,P200,P20,P10, multichannel)	various	NA
Thermal cycler	various	NA
Qubit fluorometer (or equivalent)	Thermo Fisher Scientific	Q33238
MixMate or equivalent	Eppendorf	5353000529
Hulamixer or equivalent	various	NA
Bioanalyzer 2100/ TapeStation or equivalent	Agilent Technologies	G2991BA
Microplate centrifuge	various	NA
Magnetic separation rack for tubes (like DynaMag)	Thermo Fisher Scientific	12321D
Magnetic separation rack for 96-well PCR plates	various	NA

3.7 Interactive excel protocol (optional)

As an option, users can use the **TBEV-Eur_amplicon_WGS_interactive protocol.xlsx** which is a streamlined step by step protocol that also automatically performs calculations for reagents and mastermixes. In the first tab users enter their samples names, number of samples and number of rows (red fields). Users then follow the suitable combination of tabs depending on the sequencing platform they will use. For nanopore, automatic calculation of required concentrations and dilution instructions is also performed (enter Qubit concentrations in red fields). For Illumina molarity and dilutions need to be calculated by the user.

4 TABLE 1: TBEV-Eu oligos

#	Oligo ID	Sequence (5' -> 3')	[°C]	Pool
1	TBE_LJ_1_LEFT	TAGCAGCGGTTGGTTTGAAAGA	60.93	1
2	TBE_LJ_1_RIGHT	CTGACCTCCTTTTCCCACGTTT	60.93	1
3	TBE_LJ_2_LEFT	CGTTCTGGAACTCAGTCCCTCT	61.59	2
4	TBE_LJ_2_RIGHT	GTTTCCCACAGCGTCCATACTC	61.43	2
5	TBE_LJ_3_LEFT	AGATCAAGGAGAAGAGCCTGTTG	60.31	1
6	TBE_LJ_3_RIGHT	TCCCCTGAGTACCAGTCACAAA	60.88	1
7	TBE_LJ_4_LEFT	TTGTGTGGTTGACCCTGGAGAG	62.32	2
8	TBE_LJ_4_RIGHT	TCCAAACAGTCCACAGTGGTTG	61.13	2
9	TBE_LJ_5_LEFT	CACTTTGGCTGAGGAACACCAG	61.83	1
10	TBE_LJ_5_L_alt_1	CACCCTGGCTGAAGAACACCAG	60.31	1
11	TBE_LJ_5_RIGHT	TGTTCCACTGTCTTGTCAAGCT	60.28	1
12	TBE_LJ_6_LEFT	GGAGATGTGTCCTTGTTGTGCA	61.25	2
13	TBE_LJ_6_L_alt_1	GGAGATGTGTCTTTGTTGTGCA	60.53	2
14	TBE_LJ_6_RIGHT	GCTCTCTTCCATGTGAACTTTGTT	60.04	2
15	TBE_LJ_7_LEFT	CTGCGAAGTGGGACTGGAAAAA	61.51	1
16	TBE_LJ_7_RIGHT	CGTGCTCTCCTATCACTGTCAG	60.40	1
17	TBE_LJ_8_LEFT	GTTCCAAAAAGGGAGCAGCATC	60.53	2
18	TBE_LJ_8_RIGHT	TTCTGAGACCTCTCTCCACACG	61.32	2
19	TBE_LJ_9_LEFT	ATGAGCTTCCTCTTGGCTGGAG	62.26	1
20	TBE_LJ_9_L_alt_1	ATGAGCTTTCTCTTGGCTGGAG	60.40	1
21	TBE_LJ_9_RIGHT	CCAGGAGACTTTTATGTCCTTTCCT	60.67	1
22	TBE_LJ_10_LEFT	CAGTGGTGGTGGACAAGTTTGA	61.13	2
23	TBE_LJ_10_RIGHT	GTCAGTGACCAAAAGTTCAACTATGT	60.40	2
24	TBE_LJ_11_LEFT	ACATGAGTGTGACACAGGAGTG	60.40	1
25	TBE_LJ_11_RIGHT	TCTGGGATAACCTTGCCACTCT	61.02	1
26	TBE_LJ_12_LEFT	CCGAGTCATCAGAGAGGAGTGT	61.13	2
27	TBE_LJ_12_RIGHT	ACATAGCGCACCAGACTCTCTA	60.87	2
28	TBE_LJ_12_R_alt_1	ACATAGCGCACCAGGCTCTCTA	60.87	2
29	TBE_LJ_13_LEFT	GTGGCATTGTTTGTGGTCCTTG	60.98	1
30	TBE_LJ_13_RIGHT	CCGTGCAAGCCCTGAATATCAG	61.82	1
31	TBE_LJ_14_LEFT	TTTTGCTGGTCCTGGAATTGGG	61.53	2
32	TBE_LJ_14_L_alt_1	TTCTGCTGGTCCTGGAATTGGG	61.53	2
33	TBE_LJ_14_RIGHT	ACTGGCCAGTGTTAACATGACC	61.00	2
34	TBE_LJ_14_R_alt_1	GCTGGCCAGTGTTAGCATGACT	60.53	2
35	TBE_LJ_15_LEFT	CTGTCAGCTCACAGAGGAAGAC	60.53	1

36	TBE_LJ_15_RIGHT	CACAGTCCCATCACACCAAGAA	60.67	1
37	TBE_LJ_16_LEFT	AATGATGGCTTTTTGGCTGCTG	60.80	2
38	TBE_LJ_16_L_alt_1	AATGATGGCTTTTTGGCTGCTT	59.74	2
39	TBE_LJ_16_RIGHT	TTTTTCCTCCAGACTCCAAGCT	59.74	2
40	TBE_LJ_16_R_alt_1	TTTTTCCTCCAGACTCCAGGCT	59.74	2
41	TBE_LJ_17_LEFT	GTCTATTGATGACGCTGTGGCC	61.80	1
42	TBE_LJ_17_RIGHT	GATCTGACCCTTTGCTGTCCAG	61.12	1
43	TBE_LJ_18_LEFT	GAGACCTACGTCAGCAGCATTG	61.48	2
44	TBE_LJ_18_RIGHT	CCAATGGGCCTCATCCATGATT	60.94	2
45	TBE_LJ_19_LEFT	CAACCTATGTCAACAGACGGCT	60.79	1
46	TBE_LJ_19_RIGHT	CCTTTTCAAAGGTTTTGCTGTTCAA	59.97	1
47	TBE_LJ_20_LEFT	CAAAGGGTGGTGCTATAGCTCG	59.74	2
48	TBE_LJ_20_L_alt_1	CAAAAGGTGGTGCCATAGCTCG	59.74	2
49	TBE_LJ_20_L_alt_2	CAAAAGGTGGTGCTATAGCTCG	59.74	2
50	TBE_LJ_20_RIGHT	CGCAAGGTTGTTATGTTGTCAAGA	60.58	2
51	TBE_LJ_20_R_alt_1	CGCAAGGTTGTTATGTTGTCAAGG	60.58	2
52	TBE_LJ_21_LEFT	TGGACAGTGTGATGATGATGACA	59.99	1
53	TBE_LJ_21_RIGHT	TCACGTCCCTCTTTGAACATGC	61.31	1
54	TBE_LJ_22_LEFT	GATCGAAGCTGGACATGGGAAG	61.24	2
55	TBE_LJ_22_RIGHT	GACGACGAAACACCAGATGACA	61.04	2
56	TBE_LJ_23_LEFT	CCCCAGAGGCCTTTCTGACTAT	61.42	1
57	TBE_LJ_23_RIGHT	ATTCGTCCATTCACTCCATGGC	61.19	1
58	TBE_LJ_24_LEFT	TTCTGGAGAAGACCAAGGCAGA	61.21	2
59	TBE_LJ_24_L_alt_1	TTCTGGAGAAGACTAAGGCAGA	61.21	2
60	TBE_LJ_24_L_alt_2	TTCTGGAGAAGACTAAAGCAGA	61.21	2
61	TBE_LJ_24_RIGHT	AGACCTTGTGAGCTCTCTGTGT	61.20	2
62	TBE_LJ_24_R_alt_1	AGACCTTATGGGCTCTCTGTGT	61.20	2
63	TBE_LJ_25_LEFT	CCAACAACTTGTCAACAGTGCC	60.91	1
64	TBE_LJ_25_RIGHT	CTGTTATGGAGGCCACTGTTCG	61.43	1
65	TBE_LJ_25_R_alt_1	CTGTTATGGAGGCCACCGTTCG	61.43	1
66	TBE_LJ_25_R_alt_2	CTGTCATGGAGGCCACCGTTCG	61.43	1
67	TBE_LJ_26_LEFT	AGGAAAATGAGTCTGGTGTTAGCC	61.13	2
68	TBE_LJ_26_L_alt_1	AGGAAAATGAGTCTGGTGTTGGCC	61.13	2
69	TBE_LJ_26_RIGHT	TTGGTCTCTCCTCTTCTGAGCA	60.68	2
70	TBE_LJ_27_LEFT	CACCAGGGAGGAATTCTTCGTG	61.12	1
71	TBE_LJ_27_L_alt_1	CACCAGGGAGGAATTCTTTGTG	61.12	1
72	TBE_LJ_27_RIGHT	GCTCTCTCCGATGTCACACATG	61.23	1

73	TBE_LJ_28_LEFT	CAAGCCTGGGTTGGAACTTGAT	61.27	2
74	TBE_LJ_28_RIGHT	CTGGTCTCCAAACCGAGCTAAG	60.85	2
75	TBE_LJ_28_R_alt_1	CTGGTCTCCAAACCGGGCCAAA	60.85	2
76	TBE_LJ_28_R_alt_2	CTGGTCTCCAAACCGAGCCAAG	60.85	2
77	TBE_LJ_28_R_alt_3	CTGGTCTCCAAACCGAGCCAAA	60.85	2
78	TBE_LJ_29_LEFT	TACTCAACAGCTGTCACTGGGA	61.20	1
79	TBE_LJ_29_RIGHT	TTTGAACACTCTCTGCTGTCCG	60.98	1
80	TBE_LJ_30_LEFT	AACTTCTCAGCTGGCCATGGAA	62.41	2
81	TBE_LJ_30_RIGHT	CCCAGTTTCTTCTCTCTCTTGCC	61.12	2
82	TBE_LJ_31_LEFT	GAGAGAAAGGCACCTTATGGGG	60.61	1
83	TBE_LJ_31_RIGHT	TGGTTGCCAATTGTTTGTGCTC	60.92	1
84	TBE_LJ_32_LEFT	GCTGGGACACGAAAGTTACCAA	61.25	2
85	TBE_LJ_32_RIGHT	TGCCAAATCTGTCATCCAAGGG	61.07	2
86	TBE_LJ_33_LEFT	AATCCGAATGATGGAGGGGGAA	60.85	1
87	TBE_LJ_33_L_alt_1	AATCCGAATGATGGAAGGGGAA	60.85	1
88	TBE_LJ_33_RIGHT	CTTTTGAAAGGCAGGCCGTCTC	62.45	1
89	TBE_LJ_33_R_alt_1	CTTTTGAGAGACAAGCCGTCTC	62.45	1
90	TBE_LJ_34_LEFT	CCAAGATGAACTCGTTGGGAGG	61.12	2
91	TBE_LJ_34_RIGHT	TCTTGGCCCATTCTGCTCTTTC	61.06	2
92	TBE_LJ_35_LEFT	TGTTCCGTACCTCCCTAAAGCT	61.01	1
93	TBE_LJ_35_L_alt1	TGTTCCATACCTCCCTAAAGCT	61.01	1
94	TBE_LJ_35_RIGHT	AGCTCAGCCTATTTGCTTTGTCA	61.07	1
95	TBE_LJ_36_LEFT	AGCCAGAATTGAGCTGAACCTG	61.06	2
96	TBE_LJ_36_RIGHT	TTCGGCCTTATCATGATGCAGG	60.99	2
97	TBE_LJ_36_R_alt_1	AGCTCAGCCTCTTTGCTTTGTCA	60.99	2
98	TBE_LJ_36_R_alt_2	AGCTAAGCCTCTTTGCTTTGTCA	60.99	2
99	TBE_LJ_37_LEFT	CGAAGCCACAGATCATGGAATG	59.89	1
100	TBE_LJ_37_RIGHT	GTGGCTCAGGGAGAACAAGAAC	61.31	1

5 Procedure

5.1 Primer pool preparation

1. Resuspend lyophilised primers at a concentration of 100 µM each according to the manufacturer's instructions.
2. Prepare primer pool stocks by adding 5 µL of each primer to a 1.5 mL LoBind tube labelled Pool 1 Stock (100 µM), Pool 2 Stock (100 µM), according to the table with oligo sequence information.

3. Dilute each primer pool stock 1:10 with molecular grade water to generate 10 µM working primer pools.

5.2 cDNA preparation

1. Mix the following components in a 0.2mL 8 tubes-strip or 96 well plate

Component	Volume
50µM random hexamers	1 µL
10mM dNTPs mix (10mM each)	1 µL
Template RNA	11 µL
Total	**13 µL**

Note: When preparing multiple samples, first prepare the HEX master mix (random hexamers and 10mM dNTPs mix), then aliquot it into a PCR strip or PCR 96-well plate.

2. Gently mix by pipetting, then briefly centrifuge the tube to collect the liquid at the bottom. 96-well plate can be mixed on a shaker like Eppendorf MixMate at 1600 RPM for 1 minute and then briefly centrifuged to collect the liquid at the bottom.
3. Incubate the reaction in a PCR cycler at 65°C for 5 minutes, then immediately place it on ice for 1 minute.
4. Prepare the cDNA master mix for cDNA synthesis as follows:

Component	Volume
SSIV Buffer	4 µL
100mM DTT	1 µL
RNaseOUT RNase Inhibitor	1 µL
SSIV Reverse Transcriptase	1 µL
Total	**20 µL**

5. Gently mix by pipetting, then briefly centrifuge the tube to collect the liquid at the bottom.
6. Incubate the reaction in a PCR cycler using the FSS (First strand synthesis) programme:

42° C	50 minutes
70°C	10 minutes
5°C	hold

➢ SAFE STOPPING POINT (cDNA can be stored at –20°C for further use.)

5.3 Multiplex PCR

1. In the master mix room prepare multiplex PCR solutions as follows:

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
Primer Pool 1/2/3/4	4.0 µL	4.0 µL

Nuclease-free water	12.75 µL	12.75 µL
Total	22.5 µL	22.5 µL

2. In the library-prep room (or a separate cabinet), add 2.5 µL cDNA to each tube or well and mix by pipetting.
3. Briefly centrifuge the tubes to collect the contents at the bottom.
4. Incubate the reaction in a thermal cycler using the following PCR programme:

Step	Temperature	Time	Cycles
Heat Activation	98 °C	00:00:30	1
Denaturation	98 °C	00:00:15	35
Annealing	63 °C	00:05:00	
Hold	10 °C	Indefinite	1

➢ SAFE STOPPING POINT (PCR can be run overnight. Plate can be stored at 4°C for up to 2 days.)

5.4 PCR clean-up

1. Bring AMPure XP beads to room temperature.
2. Prepare fresh 80% ethanol (420µL per sample).
3. Combine the products of both reactions for the same sample into one tube or well (total volume 50 µL).
4. Add an equal volume of resuspended AMPure XP beads (50 µL).
5. Mix gently by flicking or pipetting. Briefly centrifuge to collect all liquid at the bottom of the tube.
6. Incubate for 5 minutes at room temperature.
7. Place the tube on a magnetic rack and incubate for 2 minutes or until the beads have pelleted and the supernatant is completely clear.
8. Carefully remove and discard the supernatant, taking care not to disturb the bead pellet.
9. Add 200 µL of room-temperature, freshly prepared 80% ethanol to the pellet. Do not disturb the pellet.
10. Carefully remove and discard ethanol, ensuring the bead pellet is not disturbed.
11. Repeat steps ethanol wash one more time without drying the beads.
12. Pulse centrifuge to collect all liquid at the bottom of the tube, then carefully remove as much residual ethanol as possible using a P10 pipette.
13. With the tube lid open, incubate for 1 minute or until the pellet loses its shine (if the pellet dries completely, it will crack and become difficult to resuspend).
14. Resuspend the pellet in 25 µL molecular grade water, mix gently by flicking or pipetting, and incubate for 2 minutes.
15. Place on the magnet for 2 minutes or until the beads have pelleted and the supernatant is completely clear.

16. Transfer the sample to a clean 1.5 mL LoBind tube, ensuring no beads are transferred.
17. Quantify 1 µL of PCR product with a Qubit or equivalent instrument to ensure there is sufficient material for NGS library preparation.

➢ SAFE STOPPING POINT (Amplicons can be stored at 4°for 2 days or at -20°C for further use.)

6 NGS library preparation

6.1 Illumina Nextera XT

6.1.1 Tagment Genomic DNA

1. Prepare reagents
    a. Thaw ATM (Amplicon Tagment Mix) on ice and invert 3–5 times to mix.
    b. Thaw TD (Tagment DNA Buffer) on ice and invert 3–5 times to mix.
    c. Prepare NT (Neutralize Tagment Buffer) by inverting or vortexing.

   For the next step (NGS library amplification), prepare the following:
    a. Thaw Index adapters at room temperature.
    b. Thaw NPM (Nextera PCR Master Mix) on ice.
2. Prepare the thermal programme TAG on the thermal cycler.

Preheat lid option at 100°C and reaction volume 50 µL

Step	Temperature	Time	Cycles
Incubation	55°C	00:05:00	1
Hold	10 °C	Indefinite	1

3. Dilute amplicons to 1 ng in a total volume of 5 µL using molecular-grade water (final concentration 0.2 ng/µL).
4. For each sample, add the following to a 96-well PCR plate:
   10 µL TD
   5 µL diluted TBEV amplicons (containing a total of 1 ng amplicons at 0.2 ng/µL)
5. Pipette to mix.
6. Add 5 µL ATM to each well.
7. Pipette up and down 10 times to mix, then seal the plate.
8. Centrifuge at 280 × g at 20°C for 1 minute.
9. Place the plate in a thermal cycler and run the preprogramed TAG programme. When the programme reaches 10°C, immediately proceed to step 10 as the transposome remains active.
10. Add 5 µL NT to each well.
11. Pipette up and down 10 times to mix, then seal the plate.
12. Centrifuge at 280 × g at 20°C for 1 minute.
13. Incubate at room temperature for 5 minutes.

6.1.2 Amplify Libraries

1. Prepare the thermal programme NXT on the thermal cycler:
   Set the preheat lid option to 100°C and the reaction volume to 50 µL.

Step	Temperature	Time	Cycles
Heat Activation 1	72°C	00:03:00	1
Heat Activation 2	95°C	00:00:30	1
Denaturation	95 °C	00:00:10	12
Annealing	55°C	00:00:30	
Elongation	72°C	00:00:30	
Final Elongation	72°C	00:05:00	1
Hold	10 °C	Indefinite	1

2. Add 10 µL index adapter per sample from the Index Adapter Plate to the tagmented TBEV amplicons.
3. Add 15 µL NPM to each well.
4. Pipette up and down 10 times to mix, then seal the plate.
5. Centrifuge at 280 × g at 20°C for 1 minute.
6. Place the plate on the pre-programmed thermal cycler and run the NXT PCR programme.

➢ SAFE STOPPING POINT (A sealed plate can be stored at 2°C to 8°C for up to 2 days. Alternatively, leave the plate on the thermal cycler overnight.)

6.1.3 Clean Up Libraries

1. Thaw RBS (Resuspension Buffer) and vortex before use.
2. Bring AMPure XP beads to room temperature and vortex before use.
3. Centrifuge the plate containing amplified libraries at 280 × g at 20°C for 1 minute to collect the contents at the bottom of the wells.
4. Add 90 µL of resuspended AMPure XP beads equilibrated to room temperature.
5. Seal the plate, then use a plate shaker at 1800 rpm for 2 minutes.
6. Incubate at room temperature for 5 minutes.
7. Place the plate on the magnetic stand for 2 minutes or until the liquid is clear.
8. Without disturbing the beads, remove and discard all supernatant.
9. Wash twice with 200 µL 80% ethanol as follows:
   a. With the plate on the magnetic stand, add 200 µL fresh 80% ethanol without mixing.
   b. Incubate for 30 seconds.
   c. Without disturbing the beads, remove and discard all supernatant.
10. Use a 10 µL pipette to remove and discard residual ethanol.
11. Air-dry on the magnet until the beads lose their shine (approximately 1–2 minutes, depending on humidity).
12. Remove from the magnetic stand and add 52.5 µL RSB.

13. Seal the plate and use a plate shaker at 1800 rpm for 2 minutes. If the beads do not resuspend during shaking, resuspend them by pipetting.
14. Incubate at room temperature for 2 minutes.
15. Place on the magnetic stand and wait until the liquid is clear (approximately 2 minutes).
16. Transfer 50 µL supernatant to a new 96-well PCR plate.

➢ SAFE STOPPING POINT (A sealed plate can be stored at -25°C to -15°C for up to 7 days).

6.1.4 Check library quality

1. Run 1 µL undiluted library on an Agilent Technologies 2100 Bioanalyzer or equivalent using a High Sensitivity DNA kit. The average expected library size after tagmentation is between 400 bp and 1.2 kb.
2. Use 1 µL to measure concentration with Qubit dsDNA HS or an equivalent method.

6.1.5 Library manual normalization

1. Calculate the molarity of the library or pooled libraries using the following formula:
    a. When using a Bioanalyzer or another trace instrument, use the average size obtained for the library.
    b. Use the library concentration as measured with Qubit.

$$\frac{concentration\ \left[\frac{ng}{\mu L}\right]}{660\left[\frac{g}{mol}\right]\ \cdot\ average\ library\ size\ [bp]}\cdot\ 10^6\ =\ Molarity\ [nM]$$

2. Dilute NGS libraries to 4 nM with water.
3. Combine all NGS libraries by adding 10 µL of each to a new low-binding tube.
4. Proceed with the denaturation protocol suitable for the Illumina sequencing system being used.

6.2 <u>Oxford Nanopore Technologies - Ligation sequencing amplicons using Native Barcoding Kit 96 V14 (SQK-NBD114.96)</u>

6.2.1 End prep

2. Thaw NEBNext Ultra II End Repair / dA-Tailing Module reagents on ice. Vortex the NEBNext Ultra II End Prep Reaction Buffer. Spin down the NEBNext Ultra II End Prep Reaction Buffer and Enzyme Mix.
3. In a clean 96-well plate, aliquot 250 fmol of DNA per sample (approximately 80 ng for 500 bp amplicons) in 12.5 µL of nuclease-free water.
4. For each sample, add the following in order to a well of a new 96-well PCR plate:
    a. 12.5 µL (250 fmol amplicons)

Prepare end-prep master mix by adding:

b. 1.75 µL Ultra II End-prep Reaction Buffer
c. 0.75 µL Ultra II End-prep Enzyme Mix

Add 2.5 µL end-prep master mix to each well.

Ensure the components are thoroughly mixed by pipetting and briefly spin down.

Incubate at 20°C for 5 minutes followed by 65°C for 5 minutes in a thermal cycler.

6.2.2 Native barcode ligation

1. Prepare reagents:
    a. Thaw the NEB Blunt/TA Ligase Master Mix at room temperature, spin down the reagent tube, mix by pipetting, and store on ice.
    b. Thaw the AMPure XP Beads at room temperature and mix by vortexing. Keep the beads at room temperature.
    c. Thaw the EDTA at room temperature and mix by vortexing. Then spin down and place on ice.
    d. Thaw the Short Fragment Buffer (SFB) at room temperature and mix by vortexing. Place on ice. Instead of SFB, freshly prepared 80% ethanol can be used for washing the barcoded libraries.
    e. Thaw the required Native Barcodes (NB01–96) for your number of samples at room temperature. Mix each barcode individually by pipetting, spin down, and place on ice.
2. In a new 96-well PCR plate, add the reagents to each well in the following order, mixing well by pipetting between each addition:
    a. 3 µL nuclease-free water
    b. 0.75 µL end-prepped DNA
    c. 1.25 µL Native Barcode (NB01–96)
    d. 5 µL Blunt/TA Ligase Master Mix
3. Thoroughly mix the reaction by gently pipetting and briefly spinning down.
4. Incubate for 20 minutes at room temperature.
5. Add 2 µL EDTA (blue cap) to each well, mix thoroughly by pipetting, and spin down briefly.
6. Collect all barcoded samples into a new low-binding 1.5 mL tube.
7. Resuspend the AMPure XP Beads by vortexing.
8. Add volume AMPure XP Beads that corresponds to 0.4× pooled samples volume to the and mix by pipetting until completely homogenous.
9. Incubate on a Hula mixer (rotator mixer) for 10 minutes at room temperature, or place on a shaker such as the Eppendorf MixMate and shake at 800 rpm for 10 minutes, inverting every 2 minutes to mix to keep the beads in suspension. After 10 minutes, perform a quick spin.

10. Spin down the sample and pellet it on a magnet for up to 10 minutes or until completely clear. Keep the plate on the magnetic rack and remove the supernatant without disturbing the pellet.
11. Wash the beads with 700 µL of 80% freshly prepared ethanol. Flick the beads to resuspend, spin down, then return the sample to the magnetic rack and allow the beads to pellet. Remove the buffer with a pipette and discard it.
12. Repeat step 11.
13. Spin down and place the tube back on the magnetic rack. Pipette off any residual buffer.
14. Dry the pellet for 1 minute or until the pellet loses its shine (if the pellet dries completely it will crack and become difficult to resuspend).
15. Remove the tube from the magnetic rack and resuspend the pellet in 35 µL nuclease-free water by gently flicking.
16. Incubate for 10 minutes at room temperature. Every 2 minutes, agitate the sample by gently flicking for 10 seconds to promote DNA elution.
17. Pellet the beads on the magnetic rack until the eluate is clear and colourless.
18. Transfer and retain 35 µL of eluate in a clean 1.5 ml DNA low-binding.
19. Quantify 1 µL of the eluted sample using a Qubit fluorometer.

➢ SAFE STOPPING POINT (4°C, overnight)

6.2.3 Adapter ligation and clean-up

1. Prepare reagents:
    a. Thaw Elution Buffer (EB) at room temperature, mix by vortexing, then spin down and place on ice.
    b. Spin down the Native Adapter (NA) and Quick T4 DNA Ligase, mix by pipetting, and place on ice.
    c. Thaw Short Fragment Buffer (SFB) at room temperature, mix by vortexing, then spin down and keep at room temperature.
    d. Thaw NEBNext Quick Ligation Reaction Buffer (5X) at room temperature, mix by vortexing, then spin down and place on ice.
2. In a 1.5 ml DNA low binding tube, mix in the following order:
    a. 30 µL pooled barcoded sample
    b. 5 µL Native Adapter (NA)
    c. 10 µL NEBNext Quick Ligation Reaction Buffer (5X)
    d. 5 µL Quick T4 DNA Ligase

    Mix by pipetting 10–20 times and centrifuge briefly.
3. Incubate the reaction for 20 minutes at room temperature.
4. Resuspend the AMPure XP Beads by vortexing.

5. Add 20 µL of resuspended AMPure XP Beads to the reaction and mix by pipetting until homogenised.
6. Incubate on a Hula mixer (rotator mixer) for 10 minutes at room temperature, or place on a shaker such as the Eppendorf MixMate and shake at 800 rpm for 10 minutes. Invert to mix every 2 minutes. After 10 minutes, perform a quick spin.
7. Pellet the beads on the magnetic rack. Keep the tube on the magnet and remove the supernatant by pipetting.
8. Wash the beads by adding 125 µL Short Fragment Buffer (SFB). Flick the beads to resuspend, spin down, then return the tube to the magnetic rack and allow the beads to pellet. Remove and discard the supernatant using a pipette.
9. Repeat step 8.
10. Spin down and place the tube back on the magnet. Pipette off any residual supernatant.
11. Remove the tube from the magnetic rack and resuspend the pellet in 15 µL Elution Buffer (EB).
12. Spin down and incubate for 10 minutes at room temperature. Every 2 minutes, agitate the sample by gently flicking for 10 seconds to encourage DNA elution.
13. Pellet the beads on the magnet until the eluate is clear and colourless, for at least 1 minute.
14. Remove and retain 15 µL of eluate containing the DNA library in a clean 1.5 ml DNA low binding tube. Dispose of the pelleted beads.
15. Quantify 1 µL of the eluted sample using a Qubit fluorometer.

6.2.4 Priming and loading the MinION and GridION Flow cell

1. Dilute the NGS libraries to 120 fmol in 12 µL Elution Buffer (EB).
2. Thaw the Sequencing Buffer (SB), Library Beads (LIB), Flow Cell Tether (FCT), and Flow Cell Flush (FCF) at room temperature. Mix by vortexing, then spin down and store on ice.
3. Prepare the low cell priming mix by combining:
    a. 1,170 µL Flow Cell Flush (FCF)
    b. 5 µL Bovine Serum Albumin (BSA) at 50 mg/mL
    c. 30 µL Flow Cell Tether (FCT)

To a final volume of 1,205 µL.

4. Open the MinION or GridION device lid and slide the flow cell under the clip. Press down firmly on the priming port cover to ensure correct thermal and electrical contact.
5. Complete a flow cell check.
6. Slide the flow cell priming port cover clockwise to open the priming port.

7. Set a P1000 pipette to 200 µL and insert the tip into the priming port. Turn the wheel until the dial shows 220–230 µL to draw back 20–30 µL, or until you see a small volume of buffer entering the pipette tip.
8. Load 800 µL of the priming mix into the flow cell via the priming port, avoiding the introduction of air bubbles. Wait for five minutes. During this time, prepare the library for loading.
9. Preparation of the library for loading – mix in a new 1.5 ml DNA low binding tube:
    a. 37.5 µL Sequencing Buffer (SB)
    b. 25.5 µL Library Beads (LIB), mixed immediately before use
    c. 12 µL DNA library
10. Complete the flow cell priming by gently lifting the SpotON sample port cover to make the SpotON sample port accessible. Load 200 µL of the priming mix into the flow cell priming port (not the SpotON sample port), avoiding the introduction of air bubbles.
11. Mix the prepared library gently by pipetting up and down just before loading.
12. Add 75 µL of the prepared library to the flow cell via the SpotON sample port, dropwise. Ensure each drop flows into the port before adding the next.
13. Close the SpotON sample port and the priming port.
14. Install the light shield.
15. Start sequencing.

6.2.5 Priming and loading the PromethION Flow Cell

1. Dilute the NGS libraries to 120 fmol in 32 µL Elution Buffer (EB).
2. Thaw the Sequencing Buffer (SB), Library Beads (LIB), Flow Cell Tether (FCT), and Flow Cell Flush (FCF) at room temperature, then mix by vortexing. Spin down and store on ice.
3. Prepare the low cell priming mix by combining:
    a. 1,170 µL Flow Cell Flush (FCF)
    b. 30 µL Flow Cell Tether (FCT)

To a final volume of 1,200 µL.

4. Load the flow cell into the instrument.
5. Complete a flow cell check.
6. Slide the inlet port cover clockwise to open.
7. Set a P1000 pipette to 200 µL and insert the tip into the inlet port.
8. Turn the wheel until the dial shows 220–230 µL to draw back 20–30 µL, or until a small volume of buffer is visible entering the pipette tip.
9. Load 500 µL of the priming mix into the flow cell via the inlet port, avoiding the introduction of air bubbles. Wait five minutes.

10. Prepare the library for loading by mixing the following in a new 1.5 ml DNA low binding tube:
    a. 100 µL Sequencing Buffer (SB)
    b. 68 µL Library Beads (LIB), thoroughly mixed before use
    c. 32 µL DNA library
11. Complete the flow cell priming by slowly loading 500 µL of the remaining priming mix into the inlet port.
12. Mix the prepared library gently by pipetting up and down just before loading.
13. Load 200 µL of the library into the inlet port using a P1000 pipette.
14. Close the valve to seal the inlet port.
15. Install the light shield.
16. Wait 10 minutes after loading the flow cell. This help to increase the sequencing output.
17. Start sequencing.

7 Bioinformatic Analysis

After sequencing apply bioinformatic tools to demultiplex the sequencing data, trim adapters and barcodes, perform QC and filtering (see details in Zakotnik et al., 2022). Proceed with mapping to reference TBEV-Eu sequence (NC_001672.1), remove primer sequences from reads ends and obtain near-full genome sequences. As an option, users can analyse the data by using the bioinformatics pipeline refmap (https://github.com/NGS-bioinf/refmap).

8 References

(1) Zakotnik S, Knap N, Bogovič P, Zorec TM, Poljak M, Strle F, Avšič-Županc T, Korva M. Complete Genome Sequencing of Tick-Borne Encephalitis Virus Directly from Clinical Samples: Comparison of Shotgun Metagenomic and Targeted Amplicon-Based Sequencing. Viruses. 2022 Jun 10;14(6):1267. doi: 10.3390/v14061267.
(2) Lindquist L, Vapalahti O. Tick-borne encephalitis. Lancet. 2008 May 31;371(9627):1861-71. doi: 10.1016/S0140-6736(08)60800-4.
(3) Oxford Nanopore Technologies. Ligation sequencing amplicons - Native Barcoding Kit 96 V14 (SQK-NBD114.96) protocol. (NBA_9170_v114_revR_03Oct2025)
(4) Illumina. Nextera XT DNA library Prep Product documentation (Document # 15031942 v07)

9 Annexes

TBEV-Eur_amplicon_WGS_interactive protocol.xlsx - An Excel template that streamlines lab planning by automatically calculating reagent volumes, workflow steps, and required materials based on sample inputs, ensuring accurate and user-friendly setup for laboratory procedures.

10 Contact

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11 Suggested citation:

European Union Reference Laboratory for Public Health on Vector-borne Viral pathogens, ZAKOTNIK S., LUŠTREK M., KOGOJ R., KNAP N and KORVA M., Technical Protocol for NGS Sequencing of Tick-Borne Encephalitis Virus (TBEV), University of Ljubljana, 2026.

The technical protocol provides a detailed description of the methodology presented in the referenced publication, which can be cited as recommended:

Zakotnik S, Knap N, Bogovič P, Zorec TM, Poljak M, Strle F, Avšič-Županc T, Korva M. Complete Genome Sequencing of Tick-Borne Encephalitis Virus Directly from Clinical Samples: Comparison of Shotgun Metagenomic and Targeted Amplicon-Based Sequencing. Viruses. 2022 Jun 10;14(6):1267. doi: 10.3390/v14061267.