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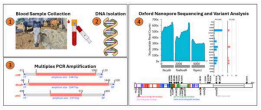
Protocol for Identification of Genetic Variation in Genes Linked to Buparvaquone Resistance in Theileria spp. infecting dairy cattle in India



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Abstract

Buparvaquone (BQO) is the primary drug used to treat bovine theileriosis, a tick-borne disease caused by *T. annulata* and *T. orientalis*. This protocol establishes a sensitive and rapid multiplex PCR-based method, combined with Oxford nanopore sequencing, for species detection and BQO resistance profiling of *T. annulata* and *T. orientalis* by targeting three key genes: *cytochrome b* (*cytb*), *dihydroorotate dehydrogenase* (*dhodh*), and *peptidyl-prolyl isomerase* (*pin1*), Monitoring and establishing the link between genetic variations in these genes and BQO resistance is crucial for understanding emerging resistance patterns and improving treatment strategies for bovine theileriosis.

Guidelines

You must read, understand, and follow all health and safety instructions before performing the protocol.

- **Sample Collection:** Use sterile EDTA or heparin tubes to collect blood via jugular or tail vein puncture using a sterile needle and
  syringe. Ensure proper animal restraint by trained personnel to minimize stress and injury.
- **Transportation and Storage:** Transport at 4°C in cooler boxes with ice packs and store at -80°C until further processing. Ensure tubes are sealed properly to prevent spillage and contamination.
- **DNA Isolation:** Follow manufacturer protocols for DNA extraction using kits or automated instruments. Label samples consistently and store isolated DNA at -20°C for short-term use.
- **DNA Quantification:** Use Nanodrop to assess DNA quality and concentration. Ensure 260/280 and 260/230 ratios are within
  acceptable limits before sequencing.
- **PCR Setup:** Maintain primer pools and DNA samples at -20°C to prevent degradation. Prepare reactions in a clean,
  nuclease-free environment to avoid contamination.
- **Bead Purification:** Use fresh 80% ethanol for bead washing and mix by gentle pipetting to prevent DNA shear. Keep samples on a magnetic stand during washing and elution steps.
- **Oxford Nanopore Sequencing:** Use at least 50 ng of high-quality DNA for sequencing. Avoid introducing air bubbles during flow cell priming and sample loading to ensure sequencing accuracy.
- **Sequenicng Analysis:** Process raw sequencing data using our in-house pipeline for quality control, alignment, variant calling, annotation, and resistance profiling using custom scripts and bioinformatics tools.

Following these guidelines ensures a reliable and reproducible protocol for detecting and buparvaquone resistance profiling of *T. annulata* and *T. orientalis* using Oxford Nanopore Sequencing.

Materials

1. MagNA Pure 96 DNA and Viral NA LV KitRocheCatalog #06 374 891 001
2. Nuclease-Free WaterQiagenCatalog #129115
3. GoTaq Green Master MixPromegaCatalog #M7122
4. RepliQa HiFi ToughMix‰ VWR InternationalCatalog #95200-500
5. 1X TAE Buffer
6. Ethidium bromide 10 mg/mlMerck MilliporeSigma (Sigma-Aldrich)Catalog #E1510
7. Invitrogen‰ UltraPure‰ AgaroseInvitrogen - Thermo FisherCatalog #16500100
8. GeneRuler 100 bp Plus DNA Ladder, ready-to-useThermo Fisher ScientificCatalog #SM0323
9. AMPure XPBechman CoulterCatalog #A63882
10. Rapid Barcoding Kit 96 V14 (SQK- RBK114.96) Oxford Nanopore TechnologiesCatalog #SQK- RBK114.96
11. Nanopore Flow Cell R10.4.1Oxford Nanopore TechnologiesCatalog #FLO-MIN114

Protocol materials

- Nuclease Free Water **Qiagen** **Catalog #**129115
- Nuclease-Free Water **Qiagen** **Catalog #**129115
- GoTaq Green Master Mix **Promega** **Catalog #**M7122
- Invitrogen™ UltraPure™ Agarose **Invitrogen - Thermo Fisher** **Catalog #**16500100
- GeneRuler 1 kb DNA Ladder **Thermo Fisher Scientific** **Catalog #**SM0311
- Ethidium bromide 10 mg/ml **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**E1510
- 1X TAE Buffer
- Ampure XP beads **Beckman Coulter** **Catalog #**A63881
- Rapid Barcoding Kit 96 V14 **Oxford Nanopore Technologies** **Catalog #**SQK-RBK114.96
- Nanopore Flow Cell R10.4.1 **Oxford Nanopore Technologies** **Catalog #**FLO-MIN114
- RepliQa HiFi ToughMix® **VWR International (Avantor)** **Catalog #**95200-500
- MagNA Pure 96 DNA and Viral NA Small Volume Kit **Roche** **Catalog #**06543588001

Safety warnings

This protocol does not involve hazardous substances or infectious agents but includes DNA extracted from cattle blood samples.

- **Personal Safety Measures:** Always wear lab coats, gloves, and safety goggles when handling biological samples and reagents. Do not remove DNA samples from the laboratory, and follow strict hygiene practices, including handwashing before and after work.
- **Biohazard Risk:** Samples from infected animals may pose a biohazard risk. Use appropriate PPE, such as gloves, lab coats, and masks, to prevent exposure and contamination.
- **Cross-Contamination:** Change gloves between samples and work aseptically to prevent contamination. Maintain a clean working environment during sample collection, processing, and PCR setup.
- **Sample Integrity:** Improper storage or temperature fluctuations can degrade DNA quality. Transport samples at 4°C and store them at -80°C until further processing.
- **DNA Quality Check:** Ensure the A260/280 ratio is around 1.8–2.0 and A260/A230 is around 2 to confirm DNA purity before PCR or sequencing. Use Nanodrop for quantification to prevent inaccurate downstream results.
- **Primer Handling:** Prevent cross-contamination between outer and inner primer pools. Thaw primers on ice, gently vortex, and store them at -20°C when not in use.
- **Bead Purification Precautions:** Avoid over-drying AMPure XP beads, as this can lead to DNA loss. Pipette gently to prevent DNA shearing and ensure optimal sequencing performance.
- **Flow Cell Handling:** Introducing air bubbles during flow cell priming can reduce sequencing efficiency. Follow pipetting instructions carefully and use slow, controlled loading techniques.
- **Chemical Safety:** Handle staining agents like ethidium bromide with care, as they are toxic and mutagenic. Dispose of hazardous materials according to lab safety guidelines.
- **Instrument Usage:** Strictly follow the manufacturer's protocols for all equipment, including thermal cyclers, fluorometers, and sequencers. Regularly calibrate instruments to ensure accuracy.

Adhering to these safety measures will enhance the reliability and reproducibility of the protocol.

Ethics statement

The study protocol was approved by the Institutional Animal Ethics Committee (IAEC) of BAIF Development Research Foundation, Pune, Maharashtra, India (V-11011(13)/13/2023-CPCSEA-DADF). All activities were carried out in compliance with the guidelines set forth by the Committee for Control and Supervision of Experiments on Animals (CPCSEA). Collection of all field samples was conducted with the explicit consent of cattle owners.

Before start

- **Personal Protective Equipment (PPE):** Ensure all personnel wear lab coats, gloves, masks, and eye protection to maintain biosafety standards and prevent contamination. Disinfect work surfaces and equipment before and after use.
- **Sample Collection Materials:** Prepare sterile syringes, EDTA tubes for blood collection. Label tubes with cryo-resistant markers or barcoded stickers and keep samples on ice for transportation at 4°C.
- **DNA Isolation Equipment & Reagents:** Set up the DNA extraction system MagNA Kit following the manufacturer's instructions. Ensure that alternative DNA isolation kits are available.
- **PCR Setup Materials:** Prepare primer pools for multiplex PCR and store them at -20°C until use. Thaw and mix reagents on ice before use, ensure Nuclease Free Water , GoTaq Mater Mix and repliQa HiFi ToughMix are available.
- **DNA Quantification & Quality Check:** Calibrate nanodrop for accurate measurements and confirm all quantification reagents are ready.
- **Gel Electrophoresis Setup:** Prepare 1% of agarose and 1X TAE buffer, have a DNA ladder and electrophoresis power supply set up for fragment size verification.
- **Bead Purification Materials:** Ensure AMPure XP beads and freshly prepared 80% ethanol are available. Set up a magnetic stand for bead purification and have pipettes calibrated for precise handling.
- **Sequencing Kit & Flow Cells:** Confirm the availability of Rapid Barcoding kit and Flow cell (R10.4 or latest version). and verify that MinKNOW software is updated.
- **Computational Setup for Analysis:** Ensure sequencing analysis software is installed and functional. Verify reference genome availability and confirm the in-house pipeline is ready for downstream analysis.
- **General Laboratory Readiness:** Clean and disinfect all workstations to minimize contamination risk. Arrange all reagents, pipettes, tubes, and instruments in a sterile environment, and confirm equipment calibration before starting.

Having all these preparations in place ensures a smooth workflow and minimizes the risk of errors during the protocol execution for *Theileria* detection and buparvaquone resistance profiling.

Sample Collection and Storage

10m

1 To collect and store blood samples from cattle, including both symptomatic and asymptomatic animals, for the detection and resistance profiling of *Theileria* infections in a laboratory setting.

1.1 **For Blood Sample Collection:** 10m

- Collect blood from the jugular vein.
- Restrain the animal properly to minimize stress and movement.
- Clean the site with an alcohol swab using an aseptic technique.
- Use a sterile gauge needle with a syringe.
- Insert the needle at a 30-45° angle into the vein.
- Collect the required volume of blood (e.g., 5 mL to 10 mL) into a vacutainer tube (e.g., EDTA tube for molecular analysis or plain tube for serum).
- Gently invert anticoagulant tubes to mix the sample properly.

Precautions:

- Use gloves and appropriate protective clothing (e.g., lab coat, mask, and shoe covers).
- Change or clean your gloves with [M] 70 % (v/v) ethanol after each sample collection to avoid cross-contamination between samples.
- Apply pressure to the puncture site with gauze or cotton to stop any bleeding.
- Dispose of needles and other sharps in a designated sharps container.
- Label the tubes with relevant information.

Safety information

Animal has to be properly restrained with help from trained personnel for blood sample collection.

1.2 **Transportation and Storage:**

The samples must be transported to the laboratory in a cooler box, maintaining 4 °C conditions. Upon arrival, until processed for DNA isolation, store the samples at 4 °C for short-term storage but freezing at lower temperatures is recommended for long-term preservation.

Precautions:

- Transport samples at 4 °C in a cooler box to prevent degradation.
- Ensure tubes are tightly sealed to prevent spillage and cross-contamination.

- Clearly label and mark samples with necessary details.

DNA Isolation, Quantification and Storage

3h 45m

2

Note

Multiple equipment and kits are available for DNA isolation from the blood samples. The method and protocol given here have been optimised using Roche MagNA Pure 96 Instrument and compatible kits.

2.1 **Isolation of DNA:**

3h

MagNA Pure 96 DNA and Viral NA Small Volume Kit **Roche Catalog #**06543588001

as per manufacturer's protocol of MagNA Pure 96 Instrument.

Equipment

MagNA Pure 96 Instrument	NAME
Automated nucleic acid purification	TYPE
Roche Molecular Systems, Inc	BRAND
06541089001	SKU

Precautions:

- Ensure the tubes are properly labelled and sealing of sample tubes to prevent contamination.
- Store and prepare reagents at recommended temperatures.

2.2 **Qualitative and Quantitative Analysis of DNA:**

45m

A. DNA quantification using a Nanodrop Instrument:
Purity and integrity of DNA samples can be determined using a Nanodrop instrument as given below;

Equipment

NAME	BRAND	SKU
Nanodrop	Thermo Scientific	NND-1 ND-LITE

Step 1: Set up the instrument by opening the nanodrop software and select the appropriate application (e.g., "nucleic acid" mode for DNA/RNA measurement).
Step 2: Pipette 2 µL of Buffer (used to elute the DNA) onto the lower pedestal, repeat the blank reading twice to ensure the instrument is accurately calibrated.
Step 3: Pipette 1 µL of your DNA sample onto the lower pedestal and measure the concentration (ng/ul), A260/A280 and A260/A230 ratio.

- The integrity of DNA samples is assessed by running a [M] 0.8 Mass / % volume to [M] 1 Mass / % volume agarose gel and loading 2 µL of genomic DNA.

Expected result

- Concentration: Ensure the DNA concentration is above [M] 10 Mass Percent .
- A260/A280: Ratios closer to 1.8 are ideal for DNA. Lower values may indicate protein contamination.
- A260/A230: Ratios closer to 2.0–2.2 are ideal. Lower values may indicate contamination by organic compounds or salts.
- Intact DNA appears as a high-molecular-weight band, while degradation results in smearing.

B. DNA quantification using Qubit:
Reagents used for DNA quantification using the Qubit method
Qubit™ dsDNA HS Assay Kit Invitrogen - Thermo Fisher Catalog #Q32851
Qubit Fluorometer Invitrogen - Thermo Fisher Catalog #Q32866
Qubit™ Assay Tubes Invitrogen - Thermo Fisher Catalog #Q32856

DNA quantification steps -
NOTE: Select the option for DNA quantification in the fluorometer

- Take the amount of 190 µL of HS buffer and the amount 10 µL of standards 1 and 2 in separate assay tubes labelled as S1 and S2 respectively
- For each sample, take an amount 199 µL of HS buffer in an assay tube and add amount 1 µL of the sample to be quantified
- The assay tubes must be vortexed to mix the contents and readings are taken using the fluorometer

- # NOTE: DNA concentration of 10ng/µL or above is desirable for further steps

Note

If the isolated DNA does not match the expected results, the Isolation process can be repeated to obtain better-quality DNA.

3 **Storage of DNA:**

After DNA isolation and quantification, store the samples at -20 °C until further use and at 4°C for short-term use to prevent degradation.

Precautions:

- Avoid repeated freeze-thaw cycles to prevent DNA degradation; aliquot samples if needed.

Screening of *Theileria spp.* by 18S rRNA PCR

2h 30m

4
- Objective: Detection of *Theileria* species in cattle using 18S rRNA PCR.
- Target Gene: 18S rRNA, a conserved region among *Theileria* spp. for reliable detection.

4.1 ***Theileria* 18S rRNA Primers:**

- The PCR primers for *Theileria* 18S rRNA amplification were used as per previously published primers (PMID: **28501503**), based on the *Theileria annulata* 18S ribosomal RNA gene (GenBank: **EU083801.1**), a partial sequence of 1758 bp.

	A	B	C	D	E	F
	Primer ID	**Primer sequence (5' -3')**	**Size (bp)**	**GC content (%)**	**Tm (°C)**	**Amplicon size (bp)**
	BTH_18S_FP	GTGAAACTGCGAATGGCTCATTAC	24	46	58	*T. annulata- 1609 bp , T. orientalis- 1616 bp*

	A	B	C	D	E	F
	BTH_18S_RP	AAGTGATAAGGTTCACAAAACTTCCC	26	38	57	

Table 1: Primer details of *Theileria* 18S rRNA.

4.2 **Dilutions of Primers :**

10m

Step 1: Reconstitute primers by adding appropriate amount of Nuclease Free Water **Qiagen Catalog #**129115 to a stock concentration of [M] 100 % (v/v) .

Step 2: Prepare a working concentration of [M] 10 micromolar (µM) by diluting the stock (1:10) using Nuclease Free Water **Qiagen Catalog #**129115 .

Precautions:

- Use sterile, nuclease-free materials and clean workspaces to maintain primer integrity.
- Avoid cross-contamination between outer and inner primers while pipetting.
- Always thaw primers on ice after taking them out from -20 °C to prevent degradation.
- Ensure proper labelling of primers to avoid confusion or mix-ups.

Note

Primers can be obtained from commercial companies, which come in ready- to-use lyophilized form and can be reconstituted.

4.3 **Setting up of *Theileria* 18S rRNA PCR:**

2h 20m

- The *Theileria* 18S rRNA PCR was set up using GoTaq Green Master Mix **Promega Catalog #**M7122 2X Master mix.
- Ensure to use [M] 10 micromolar (µM) primers prepared as a working solution go to step #5.2 .

	A	B	C
	Component	**Final Concentration**	**Amount**
	DNA Template	~50 ng	1 µl

	A	B	C
	BTH_18s_FP (10 µM)	0.5 µM	1.25 µl
	BTH_18s_RP (10 µM)	0.5 µM	1.25 µl
	2X Master mix	1X	12.5 µl
	Nuclease Free Water	-	9 µl
	Total	-	25 µl

Table 2: PCR conditions for *Theileria* 18S rRNA

	A	B	C	D
	Step	**Temperature**	**Time**	**Cycles**
	Initial Denaturation	94°C	4 mins	1 X
	Denaturation	94 °C	45 secs	35 X
	Annealing	57 °C	1 min	
	Extension	72 °C	2 mins	
	Final Extension	72 °C	5 mins	1 X
	Hold	4°C	∞	

Table 3: Thermocycling conditions for *Theileria* 18S rRNA PCR

Precautions:

- Ensure proper primer design and optimize concentrations to minimize non-specific amplification.
- Store primers, and enzymes at recommended temperatures (-20 °C or lower) to maintain efficiency.
- Keep the PCR master mix and reagents on ice to maintain enzyme stability and avoid degradation.
- Use high-quality, intact DNA and avoid multiple freeze-thaw cycles.
- Use separate aliquots of reagents and change pipette tips between samples to avoid cross contamination.
- Ensure the correct cycling conditions are programmed to prevent PCR failure.

Multiplexed PCR amplification of *T. annulata cytochrome b (cytb)*, *dihydroorotate dehydrogenase (dhodh)*, and *peptidyl-prolyl isomerase pin1 (pin1)* gene locus

3h 15m

5 Objective: Detection of specific genes of *T. annulata* in cattle using multiplex PCR.
Target Genes: *cytb, dhodh,* and *pin1* among *T. annulata* for reliable detection.
Primer Selection: Species-specific primers designed for *T. annulata*.

5.1 Designing of *T. annulata* Multiplex PCR Primers:

Note

Samples that tested positive for *Theileria* 18S rRNA PCR are considered for *T. annulata* specific multiplex PCR

- Multiplex PCR primers were designed based on *T. annulata* genes, including *cytochrome b* **Tap370b08.q2ca38.03c**, 1092 bp, *dihydroorotate dehydrogenase* (*dhodh*) **TA11695**, 1449 bp, and *pin1* **TA18945**, 572 bp. The primers were designed considering flanking regions to achieve an amplicon size of approximately 1500 bp using **Primerscheme**.

	A	B	C	D	E	F	G
	Primer ID	**Gene**	**Primer sequence (5'-3')**	**Size (bp)**	**GC (%)**	**Tm (°C)**	**Amplicon size (bp)**
	TACB_FP	*cytb*	CGGCGTTCTTAACCCAACTCA	21	52.38	60.98	1443
	TACB_RP		GCGGTTAATCTTTCCTATTCCTTACG	26	42.31	60.5	
	TADH_FP	*dhodh*	CTCGCAAATCAACCAAAATCGCA	23	43.48	61.4	1647
	TADH_RP		GGCGGTCACATTATGGTCACAA	22	50	61.38	
	TAPN1_FP	*pin1*	CAGCCTATGTTCAGAAGTTCAAACG	25	44	60.99	1474
	TAPN1_RP		GGCGCTGAGAATAAAAGTGAACG	23	47.83	60.97	

Table 4: Primer details of *T. annulata* Multiplex PCR.

5.2 **Dilutions and Pooling of Primers for Multiplex PCR:**

15m

Step 1: Reconstitute primers by adding appropriate amount of Nuclease Free Water **Qiagen Catalog #**129115 to a stock concentration of [M] 100 % (v/v) .

Step 2: Mix 10 µL of each forward and reverse primer stock in 1.5 mL tube to prepare the primer pool stock.

Step 3: During setting up the PCR reaction, dilute the primer pool stock to working concentration of [M] 10 micromolar (µM) by diluting the stock (1:10) using Nuclease-Free Water **Qiagen Catalog #**129115 .

Precautions:

- Use sterile, nuclease-free materials and clean workspaces to maintain primer integrity.
- Avoid cross-contamination between outer and inner primers while pipetting.
- Always thaw primers on ice after taking them out from -20 °C to prevent degradation.
- Ensure proper labelling of primers to avoid confusion or mix-ups.

Note

Primer pools can be obtained from commercial companies, which come in ready- to-use lyophilized form and can be reconstituted.

5.3 **Setting up of *T. annulata* Multiplex PCR:**

3h

- The *T. annulata* multiplex PCR was set up using RepliQa HiFi ToughMix® **VWR International (Avantor) Catalog #**95200-500 2X Master mix.
- Ensure to use [M] 10 micromolar (µM) primer pool prepared as a working solution go to step #5.2 .

	A	B	C
	Compoents	**Final concentration**	**Amount**
	DNA template	50 ng	1 µl
	Primer Pool (10 µM)	1.4 µM	3.5 µl

	A	B	C
	2X Master mix	1X	12.5 ul
	Nuclease Free Water	-	8 ul
	Total	-	25 ul

Table 5: PCR conditions for *T. annulata* Multiplex PCR.

	A	B	C	D
	Step	**Temperature**	**Time**	**Cycles**
	Initial Denaturation	98°C	30 secs	1 X
	Denaturation	98 °C	15 secs	35 X
	Annealing + Extension	65 °C	5 min	
	Hold	4° C	∞	

Table 6: Thermocycling conditions for *T. annulata* Multiplex PCR.

Precautions:

- Ensure proper primer design and optimize concentrations to minimize non-specific amplification.
- Store primers, and enzymes at recommended temperatures (-20 °C or lower) to maintain efficiency.
- Keep the PCR master mix and reagents on ice to maintain enzyme stability and avoid degradation.
- Use high-quality, intact DNA and avoid multiple freeze-thaw cycles.
- Use separate aliquots of reagents and change pipette tips between samples to avoid cross contamination.
- Ensure the correct cycling conditions are programmed to prevent PCR failure.

Multiplexed PCR amplification of *T. orientalis cytochrome b (cytb)*, *dihydroorotate dehydrogenase (dhodh)*, and *peptidyl-prolyl isomerase pin1 (pin1)* gene locus

3h 15m

6 Objective: Detection of specific genes of *T. orientalis* in cattle using multiplex PCR.
Target Genes: *cytb, dhodh,* and *pin1* among *T. orientalis* for reliable detection.
Primer Selection: Species-specific primers designed for *T. orientalis.*

6.1 **Designing of *T. orientalis* Multiplex PCR Primers:**

Note

Samples that tested positive for *Theileria* 18S rRNA PCR are considered for *T. orientalis* specific multiplex PCR

- Multiplex PCR primers were designed based on *T. orientalis* genes, including *cytochrome b* **MACJ_004198**, 1125 bp, *dihydroorotate dehydrogenase* (*dhodh*) **TOT_020000157**, 1651 bp, and *pin1* **TOT_010000107**, 351 bp. The primers were designed considering flanking regions to achieve an amplicon size of approximately 1500 bp using **Primerscheme**.

	A	B	C	D	E	F	G
	Primer ID	**Gene**	**Primer sequence (5'-3')**	**Size (bp)**	**GC (%)**	**Tm (°C)**	**Amplicon size (bp)**
	TOFC_CB_FP	*cytb*	CCTCCCGACGTTTTTAACCCAA	22	50	61.25	1464
	TOFC_CB_RP		TAACTGGCCCTGTTCGGTATTG	22	50	60.54	
	TOSH_DH_FP	*dhodh*	GTCTGGAAGCCTGCGGATATTT	22	50	60.93	1731
	TOSH_DH_RP		TTTCATGTGAGCTGCTCCGATC	22	50	61.18	
	TOSH_PIN1_FP	*pin1*	GACTGAGAATAGTTACCTCGAGCAG	25	48	60.88	1535
	TOSH_PIN1_RP		AACAAGTGTGACGAGTCTACGC	22	50	61.03	

Table 7: Primer details of *T. orientalis* Multiplex PCR.

6.2 **Dilutions and Pooling of Primers for Multiplex PCR:**

15m

go to step #5.2

6.3 **Setting up of *T. annulata* Multiplex PCR:**

3h

go to step #5.3

Confirmation of PCR by Gel Electrophoresis

45m

7 The PCR amplification was confirmed by agarose gel electrophoresis, where the expected amplicon size was observed, validating the successful amplification.

7.1 **Analysis of PCR Product using 1% agarose gel:**

45m

Reagents required are:

- Invitrogen™ UltraPure™ Agarose **Invitrogen - Thermo Fisher Catalog #**16500100
- GeneRuler 1 kb DNA Ladder **Thermo Fisher Scientific Catalog #**SM0311
- Ethidium bromide 10 mg/ml **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**E1510
- 1X TAE Buffer

- A total of 4 µL of the PCR product was loaded onto an agarose gel and runned at 120 V.
- The gel was then visualised using Gel Doc system (Mentioned below) to confirm the presence of the expected amplicon.

Equipment

Gel Doc XR+ Gel Documentation System	NAME
Gel Documentation System	TYPE
Bio-rad Laboratories	BRAND
1708195	SKU
https://www.bio-rad.com/en-us/product/gel-doc-xr-gel-documentation-system?ID=O494WJE8Z	LINK

PCR Clean up for Oxford Nanopore Sequencing (optional)

45m

8

Note

- After confirming the PCR positivity through gel electrophoresis, the samples can be considered for sequencing.
- However, This is an optional step but is highly recommended if primer dimers or other contaminants are detected in the PCR product.

8.1 **Bead Purification of PCR Products:**

40m

Step 1: Add Ampure XP beads **Beckman Coulter Catalog #**A63881 bead slurry to each sample in a 1:1 volumetric ratio.

Step 2: Incubate at room temperature for 00:15:00 to allow DNA binding to the beads.

Step 3: Place the sample tubes on a magnetic stand to separate beads bound to DNA.

Step 4: Carefully remove the supernatant without disturbing the beads.

Step 5: Wash the beads twice with 150 µL of freshly prepared 80 % ethanol, ensuring the beads remain undisturbed.

Note

Ensure that the beads remain fully immersed in ethanol during the washing steps.

Step 6: Allow the beads to dry at room temperature for 00:00:30 to 00:01:00 to remove excess ethanol.

Note

Do not leave the beads for an extended period and ensure they do not dry completely.

Step 7: Remove the tube from the magnetic stand and add 25 µL of Nuclease Free Water **Qiagen Catalog #**129115 , Mix the beads properly by pipetting.

Step 8: Incubate at Room temperature for 00:15:00 to elute the DNA from the beads.

Step 9: Place the tube back on the magnetic stand and wait for the beads to separate.

Step 10: Carefully recover the eluate 20 µL containing the purified DNA into a fresh tube.

Precautions:

- Vortex the AMPure XP bead suspension well before use to ensure even distribution.
- While removing the supernatant and during ethanol washes, avoid disturbing the bead pellet to prevent DNA loss.
- After placing the tube on the magnetic stand, allow beads to fully separate before collecting the eluate.
- Ensure thorough mixing of beads with Nuclease Free Water **Qiagen Catalog #**129115 to elute DNA efficiently

8.2 **Qualitative and Quantitative Analysis of Purified PCR Product:** 5m

- DNA quantification is carried out using Nanodrop as shown in go to step #2.2 .
- The purified PCR products with a minimum concentration of 50 ng are considered suitable for further sequencing.

Oxford Nanopore Sequencing Steps Using Rapid Barcoding Kit SQK-RBK114.96 Kit 1h 20m

9 If bead purification (Step 7.1) is omitted, proceed directly to the barcoding step (Step 8.1).

The sequencing kit Rapid Barcoding Kit 96 V14 **Oxford Nanopore Technologies Catalog #**SQK-RBK114.96 workflow includes DNA barcoding, where each PCR product is labeled with a unique barcode and pooled together. This is followed by sample pooling and clean-up to remove contaminants, and adapter ligation to prepare the DNA for sequencing. Finally, the flow cell is primed and barcoded and adapter ligated DNA is loaded onto the flow cell for nanopore sequencing.

9.1 **DNA Barcoding:** 10m

Step 1: Take 50 ng to 100 ng of each purified PCR product in a maximum volume of 9 µL .

If needed, adjust the volume with Nuclease Free Water **Qiagen Catalog #**129115 .

Note

If bead purification was skipped, directly use 9 µL of the PCR product in this step.

Step 2: Add 1 µL of sequencing rapid barcode (RB01-96) provided in the kit and mix thoroughly by pipetting at least for 10 times.

Step 3: Incubate the sample as follows:

- 30 °C for 00:02:00
- 80 °C for 00:02:00
- 4 °C or on ice for 00:05:00

Precautions:

- Ensure that the barcode reagents and buffers are stored correctly and used before expiration.
- Clearly label all tubes to avoid mix-ups between barcoded samples.

9.2 **Sample Pooling and Clean-up:**

30m

Step 1: Pool all barcoded samples into a single 1.5 mL microfuge tube.
Proceed with all further steps using the pooled barcoded sample.
Step 2: Add Ampure XP beads **Beckman Coulter Catalog #**A63881 bead slurry to each sample in a 1:1 volumetric ratio.
Step 3: Incubate at room temperature for 00:15:00 to allow DNA binding to the beads.
Step 4: Place the sample tubes on a magnetic stand and wait for the beads to separate.
Step 5: Carefully remove the supernatant without disturbing the beads.
Step 6: Wash the beads twice with 1 mL of freshly prepared 70% ethanol without disturbing them.
Step 7: Allow the beads to dry at room temperature for 00:00:30 to 00:01:00
Do not let the beads dry completely.
Step 8: Remove the tube from the magnetic stand and add 20 µL of Elution Buffer (EB) from the kit.
Step 9: Mix the beads properly by pipetting and incubate at Room temperature for 00:15:00 to release the DNA.
Step 10: Place the tube back on the magnetic stand and wait for beads to separate.
Step 11: Recover the eluate 15 µL containing the barcoded library into a fresh tube.

Note

The barcoded library can be stored at 4°C for up to 1 day or at -20°C for up to 1 month for long-term storage.

Precautions:

- Vortex the AMPure XP bead suspension well before use to ensure even distribution.
- While removing the supernatant and during ethanol washes, avoid disturbing the bead pellet to prevent DNA loss.
- After placing the tube on the magnetic stand, allow beads to fully separate before collecting the eluate.
- Ensure thorough mixing of beads with Nuclease Free Water **Qiagen Catalog #**129115 to elute DNA efficiently

9.3 **Adapter Ligation of Barcoded Library:** 10m

Step 1: Take 11 µL of the barcoded library and add 1 µL of the diluted rapid adapter i.e, 1.5 µL Rapid Adapter (RA) + 3.5 µL Adapted Buffer (ADB) (provided in the kit).

Step 2: Mix gently and incubate at 37 °C for 00:10:00 .

Immediately proceed with loading the prepared library onto the flow cell for sequencing.

Note

The sequencing library is compatible with version 10 flow cells (FLOMIN_114) from Oxford Nanopore Technology.
Please refer to the manufacturer's website for the latest updates on flow cell compatibility and recommended kits.

9.4 **Priming the flow cell** 15m

Note

This step can be performed during the incubation period for adapter ligation (Step No. 8.3).

Step 1: Perform a pore scan of the Nanopore Flow Cell R10.4.1 **Oxford Nanopore Technologies Catalog #**FLO-MIN114 followed by flow cell priming according to the ONT protocol.

Step 2: Prepare the priming mix by adding 30 µL Flow Cell Tether (FCT) to 1170 µL Flow Cell Flush (FCF) and mix thoroughly by vortexing (provided in the kit).
Step 3: If needed, remove waste from the waste port (Ensure that both the spot-on port and priming port remain closed during this step).
Step 4: Set a 1000 µL pipette to 200 µL, then open the priming port and carefully suck out air by increasing the pipette volume to 220 µL to 230 µL to prevent air bubble entry. (Keep the spot-on port closed).
Step 5: Add 800 µL of the priming mix via the priming port, following the method carefully. (Keep the spot-on port closed).
Step 6: Incubate at room temperature for 00:10:00
Step 7: Perform a second priming by adding 200 µL of the priming mix. (Ensure that the spot-on port is opened during this step).

Precautions:

- Ensure no air bubbles enter the flow cell during priming, as they can lead to pore loss and reduced sequencing efficiency.
- Always check whether the spot-on port and priming port should be open or closed at each step. Incorrect handling can introduce errors in flow cell performance.
- If removing waste from the flow cell, do so carefully to prevent damage to the pores.

9.5 **Sample preparation and Loading on Flow cell:** 5m

Step 1: Add 37.5 µL of Sequencing Buffer (SB) and 25.5 µL of Library Beads (LIB) to the 12 µL adapter-ligated sequencing library.
Step 2: Open the spot-on port and carefully add the prepared sample using a 100 µL pipette.
(Strictly avoid air bubble entry into the spot-on port).
Step 3: Close both the spot-on and priming ports.
Step 4: Remove any remaining solution from the waste port to prevent blockages.

Precautions:

- Avoid air bubbles while loading, as they can cause pore loss and affect sequencing efficiency.
- Ensure no liquid remains in the waste port to prevent blockages that may impact flow cell reuse.

9.6 **Starting the sequencing- MInKNOW Software:** 10m

Step 1: Open MinKNOW Software – Launch the MinKNOW software on your system.
Step 2: Select Start – Click on the left panel and choose Start.
Step 3: Start Sequencing – From the menu, select Start Sequencing.
Step 4: Enter Experiment Details – Choose the flow cell position, enter the experiment

name, and provide the sample ID.
Step 5: Kit Selection – Click on kit selection and choose Rapid Barcoding Kit 96 V14 (SQK-RBK114.96).
Step 6: Adjust Parameters – Set the sequencing parameters as required.
Step 7: Start Sequencing – Click Start to initiate the sequencing process.

Precautions:
- Ensure the correct flow cell and kit are selected.
- Verify pore health before starting to avoid failed runs.
- Keep the system stable (no interruptions or disconnections).
- Monitor sequencing real-time in MinKNOW to check for errors.

Sequencing Data Analysis

2h 10m

10
- The sequencing results is used to confirm the presence of *T. annulata* and *T. orientalis* using targeted multiplex PCR-based nanopore sequencing. The detection will also be validated using 18S rRNA sequencing for further confirmation of species identification.
- The objective is to identify genetic variants in the target genes (*Cytb*, *Dhodh*, and *Pin1*) of *T. annulata* and *T. orientalis* to investigate their potential association with buparvaquone resistance.

10.1 **Read Assignment, Mapping, and Phylogenetic Analysis in Real Time (RAMPART) Workflows for Multiplex PCR-Based Nanopore Sequencing of *Theileria* Species:** 10m

- These GitHub repositories contain workflows for multiplex PCR-based nanopore sequencing of *T. annulata* (**RAMPART Workflow**)and *T. orientalis (***RAMPART Workflow**), focusing on the *cytb*, *dhodh*, and *pin1* genes.
- The workflows are designed for real-time pathogen detection using nanopore sequencing technology.

10.2 **Amplicon Reference Guided Assembly and Variant Calling Pipeline for Nanopore Sequencing:** 2h

- The **AmpAssem** repository provides a pipeline for reference-based assembly for amplicon sequencing
- The **AmpVarPro** repository provides a pipeline for variant calling from amplicon-based nanopore sequencing data.

These two In-house scripts are likely designed to process amplicon sequencing reads, align them to a reference genome, and detect genetic variations such as SNPs and indels. It may incorporate tools for quality filtering, alignment, error correction, and variant annotation, making it useful for applications in pathogen detection, genotyping, and phylogenetic studies.

- Minimap2 for aligning sequencing reads to the reference genome.
- Bedtools for depth masking to improve variant detection accuracy.
- Bcftools for variant mapping and filtering.
- SnpEff for variant annotation, identifying potential functional impacts on genes.

This workflow ensures the accurate detection of genetic variations linked to buparvaquone resistance and species confirmation through multiplex PCR-based nanopore sequencing.

10.3 Phylogenetic Tree and Multiple Alignments Using *Theileria* 18S rRNA

- **MAFFT** – For multiple sequence alignment.
- **MEGA 11** – For phylogenetic tree construction using the Neighbor-Joining method. A bootstrap test (1000 replicates) was performed to assess the reliability of the tree branches
- **Interactive Tree of Life (iTOL)** – For visualization and annotation of the phylogenetic tree.
- **NCBI GenBank** – To retrieve reference sequences.

This approach helped identify genetic relationships and clustering patterns among *Theileria* species, allowing confirmation of species identification and potential genetic variations.

Protocol references

1. Hacilarlioglu S, Bilgic HB, Bakirci S, Tait A, Weir W, Shiels B, Karagenc T. Selection of genotypes harbouring mutations in the cytochrome b gene of Theileria annulata is associated with resistance to buparvaquone. PLoS One. 2023 Jan 4;18(1):e0279925.
PMID: 36598898.

2. Kamyingkird K, Cao S, Masatani T, Moumouni PF, Vudriko P, Mousa AA, Terkawi MA, Nishikawa Y, Igarashi I, Xuan X. Babesia bovis dihydroorotate dehydrogenase (BboDHODH) is a novel molecular target of drug for bovine babesiosis. J Vet Med Sci.
2014 Mar;76(3):323-30. PMID: 24189582.

3. Krishnamoorthy P, Akshata LG, Jacob SS, Suresh KP, Roy P. Theileriosis prevalence status in cattle and buffaloes in India established by systematic review and meta-analysis. Indian J Anim Sci. 2021;91:56–62.

4. Masatani T, Hayashi K, Andoh M, Tateno M, Endo Y, Asada M, Kusakisako K, Tanaka T,Gokuden M, Hozumi N, Nakadohzono F, Matsuo T. Detection and molecular characterization of Babesia, Theileria, and Hepatozoon species in hard ticks collected from Kagoshima, the southern region in Japan. Ticks Tick Borne Dis. 2017 Jun;8(4):581-679 587. PMID: 28501503.

5. Morrison WI. The aetiology, pathogenesis and control of theileriosis in domestic animals. Rev Sci Tech. 2015 Aug;34(2):599-611. PMID: 26601460.

6. Salim B, Chatanga E, Jannot G, Mossaad E, Nakao R, Weitzman JB. Mutations in the TaPIN1 peptidyl prolyl isomerase gene in Theileria annulata parasites isolated in Sudan. Int J Parasitol Drugs Drug Resist. 2019 Dec;11:101-105. Epub 2019 Nov 26. PMID:
31794951.

7. Sharifiyazdi H, Namazi F, Oryan A, Shahriari R, Razavi M. Point mutations in the Theileria annulata cytochrome b gene is associated with buparvaquone treatment failure. Vet Parasitol. 2012 Jul 6;187(3-4):431-5. PMID: 22305656.