Schistosoma Species and Hybrid Genotyping With a Field Deployable Multi-Marker Amplicon Panel

Oluwaremilekun Grace Ajakaye[1,3] and Michael E. Grigg[2]

[1]Laboratory of Molecular Parasitology and Genomics of Neglected Tropical Diseases, Department of Animal and Environment Biology, Adekunle Ajasin University, Nigeria
[2]Molecular Parasitology Section, Laboratory of Parasitic Diseases, NIAID, National Institutes of Health, Bethesda Maryland
[3]Corresponding author: *Oluwaremilekun.ajakaye@aaua.edu.ng*

Published in the Microbiology section

Schistosomes are parasitic trematode worms of the genus *Schistosoma*, responsible for causing urogenital and intestinal schistosomiasis. Six primary species infect humans: *S. mansoni*, *S. japonicum*, *S. mekongi*, *S. intercalatum*, *S. guineensis*, and *S. haematobium*. In addition, several species including *S. bovis*, *S. curassoni*, and *S. mattheei* primarily infect animals, particularly cattle. Hybridization events have been documented both between human-infecting species and between human- and animal-infecting species of *Schistosoma*. Current methods for detecting hybrids rely on genotyping mitochondrial cytochrome oxidase I (cox1) and the ribosomal internal transcribed spacer (ITS), or whole-genome sequencing. This protocol describes a multi-locus, multiplex amplicon sequencing approach using Nanopore technology, referred to as NMAS-Seq, for species-level genotyping and hybrid detection. It includes comprehensive steps for multiplex nested PCR, amplicon quantification and pooling, library preparation, and sequencing. The workflow is adaptable for molecular typing of other parasitic species. © 2026 The Author(s). *Current Protocols* published by Wiley Periodicals LLC.

Basic Protocol 1: DNA extraction and multiplex nested PCR
Basic Protocol 2: Amplicon quantification and pooling
Basic Protocol 3: Amplicon library preparation and Nanopore sequencing

Keywords: genotyping • hybrids • NMAS-Seq • ONT • *Schistosoma*

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How to cite this article:
Ajakaye, O. G., & Grigg, M. E. (2026). *Schistosoma* species and hybrid genotyping with a field deployable multi-marker amplicon panel. *Current Protocols*, *6,* e70333. doi: 10.1002/cpz1.70333

INTRODUCTION

According to the World Health Organization (WHO), schistosomiasis affects an estimated 240 million people worldwide, with an additional 700 million at risk in endemic areas (WHO, 2025a). The disease primarily impacts school-aged children in low-resource communities lacking access to clean water and adequate sanitation, particularly in

CURRENT PROTOCOLS
A Wiley Brand

Current Protocols e70333, Volume 6
Published in Wiley Online Library (wileyonlinelibrary.com).
doi: 10.1002/cpz1.70333
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tropical and subtropical regions (WHO, 2025b). In line with its global health agenda, the WHO has set a target to eliminate schistosomiasis as a public health problem by 2030 (WHO, 2020). However, significant gaps in epidemiological data present challenges in accurately mapping disease distribution and implementing effective intervention strategies.

Historically, *Schistosoma* species were considered to be host specific. Increasing evidence now shows that human-infecting schistosomes can hybridize with other human- or animal-infecting species, resulting in viable hybrids capable of infecting humans, animals, or both (Leger & Webster, 2017; Rey et al., 2021). In Africa, frequent natural intra- and inter-clade hybridization among *Schistosoma* species has reshaped the epidemiological landscape, bringing new attention to the zoonotic potential of these parasites (Leger & Webster, 2017; Panzner, 2021). Hybridization can give rise to novel genotypes, potentially altering parasite virulence, host specificity, transmission dynamics, and drug susceptibility (King et al., 2015; Panzner, 2021, Zacharia et al., 2022). Currently, hybrid detection relies mainly on mitochondrial cytochrome c oxidase subunit 1 (CO1) and the nuclear ribosomal internal transcribed spacer (ITS) markers. While useful, these markers have inherent limitations (King et al., 2015; Leger & Webster, 2017; Panzner, 2021; Rey et al., 2021). CO1 is maternally inherited, it is only useful in the detection of hybrids when the mitochondrial haplotype differs from at least one nuclear marker. Likewise, the ITS marker is relatively conserved across *Schistosoma* species within the same clade, reducing its resolution for differentiating between closely related species. Similarly, existing microsatellite panels often depend on species-specific primers, which fail in hybrids due to primer dropout (Lund et al., 2022). Although whole genome sequencing (WGS) offers the highest resolution for identifying hybrids, it remains expensive, technically demanding, and largely inaccessible in endemic settings.

Multi-locus sequence typing (MLST), originally developed for bacterial genotyping, has successfully been applied to explore the population genetic structure and evolutionary history of various parasites (Cacciò et al., 2016; Costache et al., 2020; Gilchrist, 2014; Lauthier et al., 2012; Wang et al., 2012; Yeo et al., 2011). The method generates genetic polymorphism data from multiple chromosomal regions, which are unlikely to be inherited together through a single genetic event. The increasing availability of reference genomes for parasitic species has made it easier to identify informative loci for MLST studies (Urwin & Maiden, 2003). When coupled with next-generation sequencing technologies, MLST becomes a scalable and cost-effective tool for genotyping and the detection of hybrids because it is capable of analyzing multiple loci and samples in parallel (Levitt et al., 2017).

This article introduces an alternative genotyping strategy called NMAS-Seq, a multi-locus, multiplex amplicon sequencing approach using Oxford Nanopore Technologies (ONT). NMAS-Seq provides a low-cost, high-resolution alternative to conventional mitochondrial and nuclear marker genotyping strategies, enabling the ability to screen large numbers of schistosome miracidia with enhanced sensitivity for detecting species diversity and hybridization. The method utilizes 10 newly developed and validated pan-genus markers, each containing single nucleotide polymorphisms (SNPs) that distinguish among human as well as animal *Schistosoma* species. The marker panel is particularly suited for resolving complex hybrid profiles, including distinguishing first-generation hybrids from later-generation recombinants where species contributions are no longer evenly split.

Basic Protocol 1 explains the process of DNA extraction and the preparation of primer mixes for multiplex-nested PCR. Basic Protocol 2 describes the quantification of multiple amplicons, the calculation needed to pool concentrations of amplicons for multi-locus

sequencing success. Basic Protocol 3 describes the library preparation procedure utilized for Nanopore MinION sequencing.

CAUTION: Schistosoma spp. are human and animal pathogens. Live cercariae can penetrate skin of humans and animals causing infection. The organism is categorized as a Biosafety Level 2 (BSL-2) pathogen. All handling of pathogenic organisms should follow appropriate guidelines and regulations.

DNA EXTRACTION AND MULTIPLEX NESTED PCR

BASIC PROTOCOL 1

This protocol outlines the procedures for DNA extraction and two rounds of PCR amplification for multilocus genotyping. The first round consists of a multiplex PCR using two external primer mixes (PMIX 1 and PMIX 2). The second round involves nested PCR, where individual gene markers are amplified using their respective internal primers.

Strategic planning

This protocol outlines the procedures for parasite storage, selection of gene markers for multilocus sequence typing (MLST), and the grouping of these markers for multiplex PCR applications.

Storage of parasite

This method enables multilocus genotyping of *Schistosoma* larval stages directly from field-collected samples, such as individual *Schistosoma* eggs hatched into miracidia or cercariae stored on Whatman FTA cards (Gower et al., 2013). The same approach is also compatible with parasite stages preserved in RNA Later or 70% ethanol.

Gene marker selection

This technique involves the amplification and sequencing of multiple enzyme-coding, housekeeping, and protein-coding genes. In total, nine nuclear genes and one mitochondrial gene were selected, in addition to the routinely used mitochondrial cytochrome oxidase subunit 1 (CO1) and the nuclear internal transcribed spacer (ITS) region. The selection criteria included: (a) single-copy gene status, (b) easy sequence alignment across multiple species, (c) feasibility for pan-genus primer design, (d) amplification efficiency, (e) informative sequence variation, and (f) orthology (Lauthier et al., 2012; Wang et al., 2012). The ten selected gene targets span six chromosomes in the *S. haematobium* reference genome. The nonsynonymous to synonymous substitution ratio (dN/dS) was calculated using the Nei-Gojobori method via SNAP software (*http://www.hiv.lanl.gov*) (Nei & Gojobori, 1986). Primers were designed to amplify fragments ranging from 279 to 600 bp. Detailed information on marker genes, chromosomal locations, primer sequences, and fragment lengths is provided in Table 1.

Grouping of gene markers

To facilitate multiplex PCR, gene markers were divided into two groups based on fragment size and external primer sequence dissimilarity.

Group 1 includes primers (purchased from Integrated DNA Technologies) targeting the following genes:

Sel-1-like protein
ATP-binding cassette
T-complex protein 1 subunit gamma
NADH dehydrogenase subunit 5

Table 1 Selected Gene Markers

Chr[a]	Gene	Gene ID	External primers	Sequence	Length (Ext)	Internal primers	Sequence	Length (Int)
1	Sel-1-like protein	*SP*	SPEXF	YGATGATCCGGAAACTGTCG	753	SPINF	AACTGCGAACGATTTTCCGG	453
			SPEXR	TAGACAGCTCTTCGAACGCA		SPINR	AACCTCCAAGCAATGTCAGC	
1	ATP-binding cassette	*AT*	ATEXF	CCCTTCTGGCGTGGAAATTT	456	ATINF	AAAACGTGGTCAGTGGTGGT	279
			ATEXR	GAATCCAGCCAACACACCAG		ATINR	CCTCGGAGTATGTTTGCACC	
3	DUF2040 domain-containing protein	*DUF*	DUFEXF	GCGGTAAGGTGTACGGYCTT	596	DUFINF	GAACTTGTAGGGGTTTCATCGT	371
			DUFEXR	TCTCCTCTTCTTCCTTGCGT		DUFINR	TTCCTTCTGAGCCTTCCGTT	
4	T-complex protein 1 subunit gamma	*TSG*	TSGEXF	GYTTAGGWCCWAGGGCAATG	700	TSGINF	TGCTCATGGATCCTATGGGT	554
			TSGEXR	AACGCCATCAAGAACAAYYG		TSGINR	CCGTCGGTTTCCACTGTTAC	
5	Transaldolase	*T*	TEXF	TYYCAAATCTTTCAGTYMCTTC	633	TINF	GTAGCGGACACTGGAGATTT	363
			TEXR	GCRGCTTTTATACCTTCCCATGT		TINR	ACCGAGCATCCACTTCAGTA	
7	Bravo_FIGEY domain-containing protein	*BF*	BFEXF	TGTATCACGCTGGCCATACT	712	BFINF	ACTAGATGGCAGATACGGACC	408
			BFEXR	CCACCTGCCATCAAACTCAC		BFINR	TAGTCCCCTTGAGGTTGTCG	
7	Putative tropomyosin	*TY*	TYEXF	TCCAGTGGAATTACTCGGACC	730	TYINF	TCGATGACAACTCTGCTATGGA	456
			TYEXR	TCATCAGCAGCTTTACTCGC		TYINR	TCTCTTCTGTAGTGAGGCGAC	
ZW	ADP/ATP translocase	*ATP*	ATPEXF	ACACATGAACGTTTACTAGAGGT	729	ATPINF	GCAAAAGCAGCCGAACTTCT	565
			ATPEXR	GGTCAATTTCTGCTTGTTCATCA		ATPINR	CCAAACGCATGCATTCAAATCC	
ZW	60S ribosomal protein L8	*RPL*	RPLEXF	GGTTATTCGWAGYCARCGTA	550	RPLINF	GGAGGTGTTTTCAAAGCGCA	502
			RPLEXR	CTGACTGTGGGCCAACAATG		RPLINR	ACAAGACCTACCATGGCACG	

(*Continued*)

Table 1 Selected Gene Markers, *continued*

Chr[a]	Gene	Gene ID	External primers	Sequence	Length (Ext)	Internal primers	Sequence	Length (Int)
ML	Internal transcribed spacer 2	*ITS*	ITSEXF	GTGCAGCCAACTGTGTGAAT	600	ITSINF	CATATTGCGGCTACGGGATA	398
			ITSEXR	TTGGGCTAATCCCTGTTCAC		ITSINR	AAGTTCAGCGGGTAATCACG	
MG	Cytochrome oxidase subunit 1	*CO*	COEXF	TGGTTGTGGTGTAGGATGAACA	598	COINF	GCGGGTGTATCTAGACTAGTTGG	530
			COEXR	ATACCAGTAACACCACCTATCGT		COINR	ACACGAGACCCACAGCTTTT	
MG	NADH dehydrogenase subunit 5	*ND*	NDEXF	GGGTAAAAGTTGGAATTTGAGGG	625	NDINF	GTCTTATCGTGGTGGAGTGGT	459
			NDEXR	CGCTTTAACCATCTGACCACC		NDINR	TCCACTCTCCCCATAACCATCA	

[a] ML = multiple loci, MG = mitochondrial genome.

DUF2040 domain-containing protein
ADP/ATP translocase.

Group 2 includes primers for:

Putative tropomyosin
Bravo_FIGEY domain-containing protein
Transaldolase
Cytochrome oxidase subunit 1 (CO1)
60S ribosomal protein L8
Internal transcribed spacer 2 (ITS2).

Preparation of external primer stocks for multiplex PCR

Centrifuge all external primers briefly.
Label two microcentrifuge tubes as PMIX 1 (Group 1) and PMIX 2 (Group 2).
For PMIX 1: From 100 µM stock solutions, prepare a 2 µM multiplex mix by combining 10 µl each of the forward and reverse primers for all six Group 1 genes (12 primers total), and add 380 µl of nuclease-free water.
For PMIX 2, repeat the same procedure using the Group 2 primers.
Mix thoroughly by pipetting and briefly centrifuge to collect contents.
Store both primer mixes at –20°C.

Additional planning

Label PCR tubes with both sample ID and primer name to ensure traceability.
Limit processing to no more than 20 samples at a time to reduce the risk of pipetting or labeling errors.
Use multichannel pipettes where possible to streamline workflows and reduce pipetting time.
Include a negative control (no template DNA) in both PCR rounds to monitor for contamination.

Materials

Whatman FTA cards (Sigma, WHAWB120205 or WHA10534612) or worms/eggs stored in RNA*later* stabilization solution (Thermo Fisher, AM7020) or 70% ethanol
DNeasy Blood and Tissue Kit (Qiagen, 69506) containing:
  Buffer ATL
  Proteinase K
  Buffer AL
  Buffer AW1 concentrate
  Buffer AW2 concentrate
  Mini Spin columns
  2-ml collection tubes
100% ethanol
H_2O, Ambion nuclease-free (Thermo Fisher, AM9937)
PMIX 1 (see Strategic planning above)
PMIX 2 (see Strategic planning above)
5× KAPA HiFi buffer (Roche, 07958846001):
  10 mM KAPA dNTP mix
  1 U/µl KAPA HiFi DNA ploymerase
Gel electrophoresis reagents:
  UltraPure agarose powder (Thermo Fisher, 16500-900)
  TAE/TBE (Apex Bioresearch, 20-194)

GelRed stain (VWR, 89139-142)
Bromophenol blue tracking dye (Quality Biological, 351-028-661)

Unicore punch kit, 2.0-mm (Qiagen, WB100029)
1.5-ml Maxymum Recovery Snaplock microcentrifuge tubes (Axygen, MCT-150-L-C)
Pipettes (Gilson and Rainin brands)
10-, 200-, and 1000-µl filtered pipette tips (PurePoint extra-long, FT1010; PurePoint, FT1200; and CellTreat low retention extended length, 229022)
Vortex mixer
Incubator
Eppendorf centrifuges 5810R and 5430R
TempAssure 0.2-ml PCR 8-tube strips with individual caps attached (USA Scientific, 1402-4700)
Eppendorf Mastercycler nexus thermal cycler
Gel electrophoresis equipment
UV or blue-light transilluminator
Multichannel pipettes (Thermo Fisher, Finnpipette)

DNA extraction

Genomic DNA is extracted directly from Whatman FTA cards using the Qiagen DNeasy Blood and Tissue Kit following the manufacturer's instructions. Extracted DNA can be used immediately or stored at –20°C for later use.

1. Prepare samples. Using a 2-mm punch, cut out individual samples from Whatman FTA cards and place each into a 1.5-ml microcentrifuge tube.
2. Lysis step. Add 180 µl Buffer ATL and 20 µl proteinase K to the tube. Mix thoroughly using a vortex mixer, then incubate at 56°C for 2 hr.
3. Cell disruption and preparation for binding. Vortex the tube for 15 s and add 200 µl Buffer AL and mix thoroughly by vortexing. Add 200 µl of 100% ethanol and mix again by vortexing.
4. DNA binding. Transfer the entire mixture (including any precipitate) to a DNeasy Mini spin column placed in a 2-ml collection tube. Centrifuge 1 min at $\geq$6000 $\times$ *g*, room temperature. Discard the flowthrough along with the collection tube.
5. Wash Step 1. Place the spin column into a new 2-ml collection tube. Add 500 µl Buffer AW1, centrifuge 1 min at $\geq$6000 $\times$ *g*, room temperature, and discard the flowthrough and tube.
6. Wash Step 2. Place the Mini spin column into a fresh 2-ml collection tube. Add 500 µl Buffer AW2, centrifuge 3 min at $\geq$20000 $\times$ *g*, room temperature, and discard the flowthrough.
7. Dry spin. Return the spin column to the same collection tube and centrifuge 1 min at $\geq$20000 $\times$ *g*, room temperature, to remove residual wash buffer.
8. Elution. Transfer the Mini spin column to a clean 1.5-ml microcentrifuge tube. Add 10 µl of nuclease-free water directly to the membrane, incubate at room temperature for 1 min, and centrifuge 1 min at $\geq$6000 $\times$ *g*, room temperature. Repeat the elution step with an additional 5 µl of nuclease-free water to obtain a total volume of 15 µl of eluted DNA.
9. Store DNA samples at 4°C for short-term use or at –20°C for long-term storage.

Table 2 PCR Reagents for 25 µl Single Reaction

Reagent	Volume (µl)	Final concentration
Nuclease-free water	13	
KAPA HiFi Buffer	5	1×
1 U/µl KAPA HiFi DNA polymerase	0.5 µl	0.5 U
10 mM KAPA dNTP Mix	0.75	3 mM
Primer	3.75	0.5 µM
Template DNA	2ul	

Table 3 PCR Cycling Conditions

No. of cycles	Time	Temperature	Purpose
1 cycle	3 min	95°C	Initial denaturation
35 cycles	20 s	98°C	Denaturation
	15 s	58°C	Annealing
	30 s	72°C	Extension
1 cycle	10 min	72°C	Final extension

PCR 1: Multiplex PCR using PMIX1 and PMIX2 primers

For each DNA sample, perform two separate multiplex PCR reactions: one with PMIX1 and one with PMIX2 primer mixes. Follow the steps below to prepare reactions and run the thermal cycling protocol.

10. Prepare master mixes. Prepare separate master mixes for PMIX1 and PMIX2 reactions according to the reagent volumes listed in Table 2.

    Multiply the volumes by the number of reactions and include at least 10% extra volume to compensate for pipetting loss.

11. Aliquot master mix. Dispense 23 µl of the appropriate master mix into each labeled PCR tube (label with sample ID and primer group: PMIX1 or PMIX2).

12. Add template DNA. Add 2 µl of extracted genomic DNA to each tube to bring the total reaction volume to 25 µl.

13. Thermal cycling. Load the PCR tubes into a thermal cycler and run the PCR using the cycling conditions specified in Table 3.

    PCR-strip tubes or 96-well plates are recommended for the PCR reactions.

Gel electrophoresis (optional)

This optional step provides a brief overview of gel electrophoresis to check PCR amplification (Fig. 1). Note that bands may not be visible for all loci.

14. Prepare agarose gel. Dissolve 3 g agarose in 100 ml of 1× TBE or TAE buffer to make a 0.3% agarose gel. Heat until fully dissolved.

15. Add DNA stain. Once the agarose has cooled to ∼60°C, add 4 µl of GelRed (or an equivalent nucleic acid stain), and mix thoroughly before pouring into the gel tray with combs in place.

16. Prepare samples for loading. Mix 4 µl of PCR product with 1 µl loading dye. Load into wells of the solidified gel.

17. Run gel. Run the gel at 110 V for 30 to 35 min in 1× TBE or TAE running buffer.

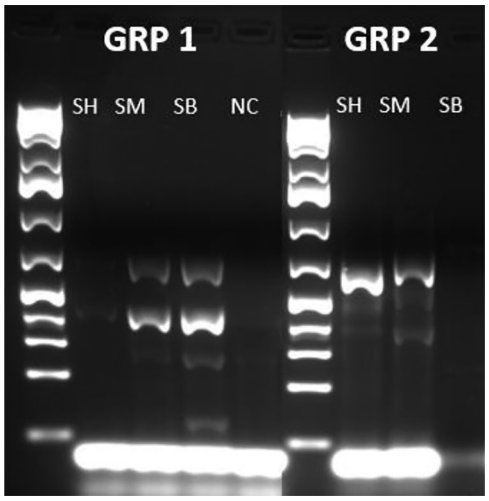

Figure 1 Gel Image for multiplex PCR performed on DNA extracted from schistosome ova-positive animal and patient samples. SH = *S. haematobium*, SM = *S. mansoni*, SB = *S. bovis*, NC = negative control.

Table 4 PCR Reagents for 25 µl Single Reaction

Reagent	Volume (µl)	Final concentration
Nuclease-free water	15.25	
KAPA HiFi Buffer	5	1×
1 U/µl KAPA HiFi DNA polymerase	0.5 µl	0.5 U
10 mM KAPA dNTP Mix	0.75	3 mM
Forward Primer	1.25	
Reverse Primer	1.25	0.5 µM
Amplified DNA (from PCR 1)	1	

18. Visualize DNA bands using a UV or blue-light transilluminator.

PCR II: Nested PCR using individual internal primers

For each DNA sample, perform independent PCR reactions with each of the 12 internal primer pairs to amplify specific gene targets. This step follows the initial multiplex PCR (PCR I) and uses the product from that reaction as template.

19. Prepare master mixes. For each internal primer pair, prepare a separate master mix based on the reagent volumes listed in Table 4.

    Multiply the volumes by the number of reactions and include at least 10% extra volume to compensate for pipetting loss.

20. Aliquot master mix. Dispense 24 µl of the appropriate master mix into labeled PCR tubes (include sample ID and primer name).

    Use a multichannel pipette, if possible, to speed up processing.

21. Add PCR template. Add 1 µl of the corresponding PCR I product (from PMIX1 or PMIX2) to each tube, bringing the final volume to 25 µl.

22. Thermal cycling. Load tubes into the thermal cycler and run the PCR using the cycling conditions specified in Table 3.

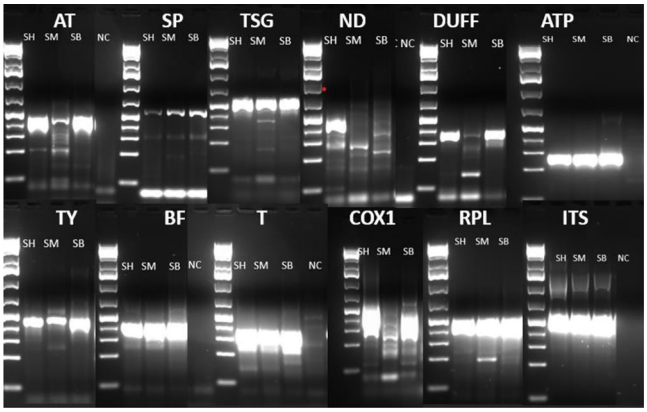

Figure 2 Gel Image for singleplex PCR for all markers using genomic DNA. Although the bands for CO1 and ND were less prominent for *S. mansoni*, the PCR worked well even in field samples. SH = *S. haematobium*, SM = *S. mansoni*, SB = *S. bovis*, NC = negative control.

Gel electrophoresis

This step provides a brief overview of gel electrophoresis to check PCR amplification. Conduct workflow as described above after PCR 1. Note that bands should be visible for all loci (Fig. 2).

BASIC PROTOCOL 2

AMPLICON QUANTIFICATION AND POOLING

This protocol outlines the steps for quantifying PCR amplicons and pooling gene loci for each sample in preparation for sequencing.

Strategic planning

Organize PCR products according to their respective gene loci in preparation for barcoding and sequencing. Refer to the Oxford Nanopore Technologies Native Barcoding Kit 24 V14 (SQK-NBD114.24) or Native Barcoding Kit 96 V14 (SQK-NBD114.96) protocol for detailed instructions on barcoding and library preparation. ONT recommends using 200 fmol of DNA per sample for barcoding. For 1 kb amplicons, this corresponds to ∼130 ng of DNA. To determine the DNA mass equivalent to 200 fmol for each amplicon based on fragment length, calculations were performed using the Promega Biomath Calculator (see Internet Resources). The required DNA quantities for each gene locus are summarized in Table 5.

CAUTION: Do not use a NanoDrop spectrophotometer for DNA quantification, as it lacks the sensitivity and accuracy required for low-concentration amplicons. Use a fluorometric method (e.g., Qubit, or Promega QuantiFluor dsDNA System) for reliable results.

Additional Materials (also see Basic Protocol 1)

Qubit dsDNA BR assay kits (Thermo Fisher, Q32853) or QuantiFluor ONE dsDNA System (Promega, E4870)

Fluorometer, e.g., Qubit 4 Fluorometer (Invitrogen, Q33226) or Quantus (Promega, E6150)
Qubit 0.5-ml assay tubes (Thermo Fisher, Q32856)
Mini centrifuge

1. Using a fluorometer, assay tubes, and the Qubit dsDNA BR assay kits or QuantiFluor ONE dsDNA System assay kits, quantify five randomly selected samples for each gene locus.

Table 5 DNA Concentration (ng) Equivalent to 200 fmol for Each Gene

Chr[a]	Gene ID	Length (Int)	Concentration (ng)
1	SP	453	60
	ATP	279	37
3	DUFF	371	49
4	TSG	554	73
5	T	363	48
7	BF	408	54
	TY	456	60
ZW	AT	565	75
	RPL	502	66
ML	ITS	398	53
MGMG	ND5COX	459530	6170
	Total Size	5338 bp	

[a] ML = multiple loci, MG = mitochondrial genome.

Selective random quantification is recommended to manage workload when processing a large number of samples.

2. Calculate the average DNA concentration for each gene locus and determine the volume of amplified DNA needed for pooling.

   For example, for the gene marker SP amplified with primers SPINF and SPINR, 60 ng of DNA corresponds to 200 fmol. If the average DNA concentration from step 1 is 90 ng/µl, the volume needed for pooling is ∼0.7 µl (calculated as 60 ng ÷ 90 ng/µl).

3. Repeat steps 1 and 2 for all gene loci to determine the required pooling volume for each locus.

4. Sum the individual volumes to calculate the total expected volume for each sample pool.

5. Label new PCR tubes clearly with the corresponding sample IDs.

6. Pool the calculated volumes of all gene loci for each individual sample into the corresponding labeled tube.

7. Mix gently by pipetting up and down, then briefly spin down using a mini centrifuge at maximum speed, to collect the liquid at the bottom of the tube.

8. The total number of pools depends on the Oxford Nanopore Technologies Native Barcoding Kit used.

   For example, with a 24-barcode kit, pool 22 samples along with two negative controls.

AMPLICON LIBRARY PREPARATION AND SEQUENCING

BASIC PROTOCOL 3

This protocol outlines the workflow for library preparation and multiplexed amplicon sequencing using Oxford Nanopore Technologies. Sequencing will be performed on the MinION platform following ONT's standard procedures.

Strategic planning

Determine read number for sequencing coverage

Calculate the minimum number of reads per barcode required to achieve the desired sequencing coverage. Consider important factors, such as the total size of the pooled loci (in base pairs), read filtering efficiency, and mapping efficiency. This calculation is essential for estimating the duration of the sequencing run. The number of reads needed can be estimated using the formula:

$$\text{Number of reads} = \frac{\text{Average read length}}{\text{Target coverage} \times \text{Amplicon length}}$$

For example, Average read length = 5338 bp, Target coverage = 100×, and Amplicon length = 5338 bp.

$$\text{Number of reads} = \frac{5338}{100 \times 5338} = 100 \text{ reads per barcode}$$

Assume 40% uneven barcode distribution (i.e., effective reads = 60%), 50% of reads filtered out during quality control, and 50% mapping efficiency.

$$\text{Number of reads} = \frac{100}{0.6 \times 0.5 \times 0.5} = 667 \text{ reads per barcode}$$

Thus, a minimum of 667 reads per barcode is required to achieve ∼100× coverage.

Determine pool concentration

Calculate the concentration of your pooled amplicon sample using:

$$\text{Pool concentration (y)} = \frac{\text{Total concentration}}{\text{Total volume (x)}} = \frac{706 \text{ ng}}{\text{x } \mu\text{l}}$$

The total volume × is obtained from Basic Protocol 2, step 4.

Determine volume for sequencing

Average gene length ∼445 bp. Using the Promega Biomath calculator (see Internet Resources), the DNA mass corresponding to 200 fmol for 445 bp is ∼58 ng. Calculate the volume v of pooled DNA to use for sequencing based on pool concentration y.

$$\text{Volume for sequencing (v)} = \frac{58 \text{ ng}}{\text{y}}$$

Additional Materials (also see Basic Protocol 1)

Oxford Nanopore Technologies Native Barcoding Kit 24 V14 (SQK-NBD114.24) or Native Barcoding Kit 96 V14 (SQK-NBD114.96):
- Native Barcode plate (NB01-24 or NB01-96)
- DNA Control Sample (DCS)
- Native Adapter (NA)
- Sequencing Buffer (SB)
- Library Beads (LIB)
- Library Solution (LIS)
- Elution Buffer (EB)
- AMPure XP Beads (AXP)
- Long Fragment Buffer (LFB)
- Short Fragment Buffer (SFB)
- EDTA
- Flow Cell Flush (FCF)
- Flow Cell Tether (FCT)

New England Biolabs (NEB) specific reagents for Oxford Nanopore Technologies:

NEBNext Ultra II End Repair/dA-Tailing module (NEB, E7546)
NEB Blunt/TA Ligase master mix (NEB, M0367)
NEBNext Quick Ligation module (NEB, E6056)
200 fmol (130 ng for 1 kb amplicons) DNA per sample to be barcoded
Qubit dsDNA HS assay kit (Thermo Fisher, Q32851)

Ice bucket with ice
Mini centrifuge
0.2-ml non-skirted low profile 96-well PCR plate (Thermo Scientific, AB-700)
Microseal "B" seals (BioRad, MSB1001)
Microplate centrifuge
HulaMixer, i.e., gentle rotator mixer
Invitrogen DynaMag-96 Side (Thermo Fisher, 12331D)
Qubit assay tubes (Invitrogen, Q32856)
MinION Mk1B (Oxford Nanopore Technologies)
R10.4.1 flow cells (Oxford Nanopore Technologies, FLO-MIN114)

End-prep

This step will prepare the DNA ends for attachment of sequencing adapter.

1. Thaw reagents. Thaw the DNA Control Sample (DCS) at room temperature. Mix thoroughly by vortexing, then place on ice. Thaw the NEBNext Ultra II End Repair reagents on ice. Mix gently by pipetting and briefly spin down using a mini centrifuge at maximum speed at room temperature to collect contents.
2. Dilute the Control DNA. Add 105 µl Elution Buffer (EB) directly to one tube of the DNA Control Sample (DCS). Mix gently by pipetting and spin down at room temperature. Keep on ice until use.
3. Aliquot sample DNA. In a clean 96-well plate, aliquot the calculated volume of DNA for each sample (refer to Strategic planning above for guidance). Adjust each sample to a final volume of 11.5 µl using nuclease-free water. Mix gently by pipetting and spin down at room temperature.
4. Prepare End-Prep master mix. Prepare a master mix based on Table 6, scaling to the number of samples being processed. Include extra volume to account for pipetting loss.
5. Add reagents to samples. Add 2.5 µl of End-Prep master mix to each well. Add 1 µl of the diluted DNA Control Sample (DCS) to each sample well. Mix thoroughly by pipetting, seal the plate and briefly spin down in a microplate centrifuge at room temperature.
6. Incubation. Place the plate or tubes in a thermal cycler and incubate at 20°C for 5 min and 65°C for 5 min.
7. Hold at 4°C or place on ice until proceeding to adapter ligation.

Table 6 Preparation of End-Prep Master Mix

Reagent	Volume (µl)
Ultra II End-prep reaction buffer	1.75
Ultra II End-prep enzyme mix	0.75
Total	2.5

Native barcode ligation

This step describes the ligation of native barcodes to end-prepped DNA using the Oxford Nanopore Technologies barcoding workflow. Each sample receives a unique barcode to allow for multiplexed sequencing.

8. Thaw the following reagents at room temperature:

   NEB Blunt/TA Ligase Master Mix
   AMPure XP Beads
   EDTA
   Native Barcodes (thaw only the number needed, one per sample).

   Up to 96 samples can be barcoded and pooled in a single run.

9. Mix the NEB Blunt/TA Ligase master mix. Spin down 5 s at room temperature. Mix thoroughly by pipetting up and down 10 times using the full volume.
10. Vortex the AMPure XP Beads and keep at room temperature.
11. Vortex the EDTA, spin down, and place on ice.
12. Mix each barcode (selected for the samples) by pipetting, spin down briefly, and place on ice.
13. In a clean 96-well plate, add the reagents in the order listed to each well, mixing gently by pipetting after each addition (Table 7)
14. Mix thoroughly by pipetting and spin down briefly.
15. Incubate at room temperature for 20 min.
16. Add 1 to 2 µl EDTA to each well to stop the ligation reaction.

    Refer to the barcode kit manual to confirm the recommended EDTA volume.

17. Mix thoroughly and spin down briefly.
18. Pool all barcoded samples into a single 1.5-ml microcentrifuge tube.

    Check the base of the plate to ensure complete transfer.

19. Resuspend the AMPure XP Beads by vortexing.
20. Add 0.4× AMPure XP Beads to the pooled sample and mix thoroughly by pipetting.
21. Incubate on a HulaMixer at room temperature for 10 min.
22. Spin down briefly, and place the tube on a magnetic rack for 5 min.
23. Once the solution becomes clear and colorless, carefully remove and discard the supernatant.

Table 7 Preparation of Adapter Ligation Reaction

Reagent	Volume (µl)
Nuclease-free water	3
End-prepped DNA	0.75
Native Barcode	1.25
Blunt/TA Ligase Master Mix	5
Total	10

24. With the tube still on the magnetic rack, wash the beads with 700 µl of freshly prepared 80% ethanol without disturbing the pellet.
25. Remove the ethanol carefully using a pipette.

    If the pellet is disturbed, wait for it to re-pellet before proceeding.
26. Repeat the ethanol wash (steps 23 to 24).
27. Spin down briefly, and return the tube to the magnetic rack.
28. Remove any residual ethanol with a fine pipette tip and let the pellet air-dry for ~30 s.

    Do not over-dry the pellet to avoid cracking of the pellets.
29. Remove the tube from the magnetic rack and resuspend the pellet in 35 µl of nuclease-free water by gently flicking the tube.
30. Incubate at 37°C for 10 min, gently flicking the tube every 2 min for 10 s to encourage elution.
31. Return the tube to the magnetic rack and wait until the eluate is clear and colorless.
32. Carefully transfer 35 µl of the eluate into a clean 1.5-ml microcentrifuge tube.
33. Quantify 1 µl of the eluted DNA using a fluorometer.

Adapter ligation and clean-up

34. Thaw the NEBNext Quick Ligation Reaction Buffer, Quick T4 DNA Ligase, and Native Adapter at room temperature.

    *Do **NOT** vortex the Quick T4 DNA Ligase.*
35. Spin down briefly, then mix by pipetting and place the reagents on ice.

    If the Quick Ligation Reaction Buffer appears precipitated, pipette up and down several times to dissolve the precipitate, then vortex briefly to ensure thorough mixing.
36. Thaw the Elution Buffer at room temperature. Vortex briefly, spin down, and place on ice.
37. Thaw the Short Fragment Buffer at room temperature. Vortex briefly and spin down.

    The Short Fragment Buffer retains DNA fragments of all sizes.
38. Prepare the adapter ligation reaction in a 1.5-ml microcentrifuge tube (Table 8). Mix thoroughly by pipetting 10 to 20 times between each addition.
39. Mix the final reaction gently by pipetting and spin down briefly.
40. Incubate the reaction for 20 min at room temperature.

Table 8 Preparation of Adapter Ligation Reaction

Reagents	Volume (µl)
Pooled barcoded sample	30
Native Adapter	5
NEBNext Quick Ligation reaction buffer	10
Quick T4 DNA Ligase	5
Total	50

41. Resuspend the AMPure XP Beads by vortexing.
42. Add 20 µl of resuspended AMPure XP Beads to the ligation reaction and mix by pipetting.
43. Incubate on a HulaMixer for 10 min at room temperature.
44. Spin down, and place the tube on a magnetic rack until the beads are fully pelleted.
45. While the tube remains on the magnet, carefully remove and discard the supernatant.
46. Wash the beads by adding 125 µl of Short Fragment Buffer.
47. Flick the tube to resuspend the beads, spin down, and place it back on the magnetic rack to pellet the beads.
48. Remove and discard the supernatant.
49. Repeat the steps 46 to 48 for a second wash.
50. Spin down, and return it to the magnetic rack.
51. Remove any residual supernatant with a pipette.
52. Remove the tube from the magnetic rack and resuspend the bead pellet in 15 µl of Elution Buffer.
53. Spin down, and incubate for 10 min at 37°C, gently flicking or agitating every 2 min.
54. Place the tube on the magnetic rack and allow the beads to pellet. Wait until the eluate is clear and colorless (at least 1 min).
55. Carefully transfer 15 µl of the eluate (which contains the DNA library) to a clean 1.5-ml microcentrifuge tube.
56. Dispose of the pelleted beads.
57. Quantify 1 µl of eluted sample using a Qubit fluorometer.
58. Prepare the final DNA library, aiming for 35 to 50 fmol (120 to 180 ng) in 12 µl of Elution Buffer.
59. Store the library on ice or at 4°C until ready to load.
60. Follow the appropriate preparation for priming and loading the SpotON flow cell.

COMMENTARY

Background Information

Schistosomiasis is a neglected parasitic disease prevalent in Africa infecting both humans and animals. An estimated 700 million people, living in poor, rural communities without access to basic amenities, such as potable water and adequate sanitation, are at risk of various forms of schistosomiasis (Aula et al., 2021). The disease is transmitted by intermediate snail vector hosts that shed *Schistosoma* infective stages in freshwater bodies. Humans and animals contract the disease from water contact with infested water bodies where the infective stages of the parasite penetrate the human or animal hosts. Three *Schistosoma* species (*S. mansoni, S. japonicum*, and *S. haematobium*) are the widely recognized and studied causative agents of human schistosomiasis. *S. japonicum* and *S. mansoni* are responsible for intestinal schistosomiasis, while *S. haematobium* is responsible for urogenital schistosomiasis (WHO, 2025b). In schistosomiasis infections, the adult stages of the parasites lodge in the host's blood vessels within the intestinal or urogenital systems, where the mature female produces thousands of eggs, which are deposited in various tissues and organs, most notably the liver, bladder, small and large intestine, cervix, and vagina. Most of the disease pathology arises from inflammatory reactions and immune responses due to trapped eggs in the various organs

and can result in complications, such as hepatosplenomegaly, periportal fibrosis, bladder cancer, and female genital schistosomiasis (Colley et al., 2014; McManus et al., 2018). Schistosomiasis infection is also linked to stunting and decreased cognitive performance in children, increased risk of HIV infection in women, ectopic pregnancies, and other long-term irreversible consequences such as infertility (Aula et al., 2021).

Formerly, some schistosome species were thought to be host-specific; however, advanced applications of molecular techniques have provided evidence that human and animal schistosomes can pair to produce zoonotic hybrids capable of infecting both humans and animals. In West Africa, hybrids of *S. haematobium* and livestock schistosomes, *S. bovis* and/or *S. curassoni* has been reported as evidence of possible zoonotic transmission of the parasite (Rey et al., 2021). Recent research on epidemiology of schistosomiasis is being focused on single egg genotyping and hybrid detection. The addition of new genetic markers and a field-deployable sequencing platform should prove instrumental in clarifying the degree to which intra-specific (within a species) and inter-specific (between species) hybridization is occurring, which has important implications in host range expansion, disease, and transmission of this highly prevalent trematode.

Critical Parameters

The initial starting material contains ~2 ng/µl of DNA; therefore, samples should be handled carefully to avoid DNA loss. All PCRs and library preparations should be performed in a biosafety cabinet to minimize environmental contamination. When conducting PCR, negative controls must be included. For library preparation, both negative and positive controls may be included. The positive control can be sourced as genomic DNA from BEI Resources or as adult worms from the Schistosomiasis Collection at the Natural History Museum (SCAN), UK.

The DNA extraction kits and PCR amplification reagents described above successfully isolated *Schistosoma* DNA and produced robust PCR amplifications. Other kits should not be used without proper evaluation. Other high-fidelity Taq reagents may be assessed for performance. If inhibitors are suspected in the PCR reaction, bovine serum albumin (BSA) can be added.

During gel electrophoresis, double bands were observed at the recommended annealing temperature of 58°C. However, the presence of double bands does not affect the sequencing reaction, if *Schistosoma* DNA is confirmed at the correct band size for the targeted markers.

The sequencing should be run depending on the desired read depth. It takes ~6 hr to obtain a read depth of 100× for at least 90% of a 96-barcodes run.

Troubleshooting

These are straightforward protocols with minimal issues. The commonly encountered problems and possible solutions are shown in Table 9.

Understanding Results

Gel electrophoresis images are useful for confirming amplification of target loci and pooling prior to sequencing (Fig. 2). The ONT sequencing platform outputs demultiplexed FASTQ files for each sample based on the number of barcodes. The data analysis follows the recommended NGS data pipeline for variant calling analysis. A curated reference genome for *S. haematobium* can be found in the supplementary file (see Supporting Information). Depending on the analysis, reference genome can be curated for other *Schistosoma* species. Software, e.g., NanoFilt, Minimap2, Samtools, and BCFtools, recommended for analysing ONT data, should be used for read filtering, mapping, and generating SNP data. BAM files can be visualized in IGV. SNP data can be further analysed and visualized by generating consensus sequence files in FASTA format. These can be imported into Geneious for multiple sequence alignments. Phylogenetic data analysis can be conducted in MEGA X using the maximum likelihood method with 1000 bootstraps. Potential outputs include allelic plot, phylogenetic trees, and haplotype networks.

Time Considerations

The PCR depends on the number of samples being processed at once. Each round of PCR takes 1.5 hr. Pooling and library preparation usually takes ~6 hr.

Acknowledgements

This research was supported (in part) by the Intramural Research Program of the National Institutes of Health (NIH). The contributions of the NIH author(s) are considered Works of the United States Government. The findings and conclusions presented in this paper are those of the author(s) and do not necessarily reflect the views of the NIH or the U.S. Department of Health and Human Services.

Table 9 Troubleshooting Guide for PCR, Library Prep, and Sequencing

Problem	Possible cause	Solution
No amplification	No or very low concentration of DNA	Use Qubit fluorometer to detect DNA concentration
	No parasite sample loaded in FTA card	Review extraction kit and protocol
	Primers degraded	Prepare fresh dilutions of primers from stock solutions
Faint gel bands	Very low concentration of DNA	Determine concentration using Qubit fluorometer and calculate pooling volume as recommended
Low number of reads	Poor starting material	Quantify samples pre- and post-pooling
	Short sequencing run	Sequence for longer period

This document has been produced with the financial assistance of the Bill and Melinda Gates Foundation Investment ID INV-007175 (formerly OPP1191735) through the African Postdoctoral Training Initiative (APTI) programme. APTI is implemented by the African Academy of Sciences with support from the Bill and Melinda Gates Foundation and in Partnership with the US National Institutes of Health, where the grantees receive mentorship during their first and second years of the fellowship. The contents of this document are the sole responsibility of the author(s) and can under no circumstances be regarded as reflecting the position of the Bill and Melinda Gates Foundation, the African Academy of Sciences, and the US National Institutes of Health.

Author Contributions

Oluwaremilekun G. Ajakaye: Conceptualization; investigation; writing—original draft; writing—review and editing. **Michael E. Grigg**: Funding acquisition; project administration; supervision; writing—review and editing.

Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

All information required to run the protocol is included in the manuscript.

Supporting Information

cpz170333-sup-0001-SuppMat.xlsx

Excel file containing curated reference genome for S. haematobium.

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Internet Resources

https://www.promega.com/resources/tools/biomath/

The online calculation tool can be used to calculate DNA mass and concentration and to convert between different units of measurement.