SOP code	ST_PCR
File name & version	SOP_008_v02
Full SOP title	Multiplex real-time PCR for the detection of soil-transmitted helminths (STH)
Written by	Maria Demontis, Michel Bengtson
Date and Place	15 November, 2021 LUMC, Leiden, Netherlands
Reviewed by	Javier Gandasegui
Date and Place	15 November 2021 ULE, Leon, Spain
Read, reviewed and approved by	**Name and Date**
BDU (Wendemagen Enbiale)	Wendemagegn Enbiale, 1st of December, 2021
FM- CISM (Inácio Mandomando)	Inacio Mandomando, December 20, 2021
KEMRI (Charles Mwandawiro)	Charles Mwandawiro, 2nd December, 2021
LUMC (Lisette van Lieshout)	Lisette van Lieshout, 25th November 2021

Contents

1. Introduction

Soil-transmitted helminths (STH) are a group of parasitic nematodes including *Ascaris lumbricoides*, *Trichuris trichiura*, *Necator americanus*, *Ancylostoma duodenale* and *Strongyloides stercoralis*. Molecular techniques (for soil transmitted helminths, STH), such as the real-time quantitative polymerase chain reaction (real-time PCR or qPCR), are a promising alternative to Kato-Katz (the current gold standard for the diagnosis of STH). Real-time polymerase chain reaction is a laboratory technique based on the Polymerase Chain Reaction (PCR), which is used to amplify and simultaneously detect or quantify a targeted DNA molecule. The procedure follows the general procedure of polymerase chain reaction; its key feature is that the amplified DNA is detected as the reaction progresses in "real time". Real-time PCR has been found to be more sensitive than Kato-Katz, particularly in low prevalence settings. This is of particular importance in the context of elimination, due to the low prevalence and reduced worm burdens expected as the transmission breakpoint is approached. Due to the expected low worm burdens in these settings, Kato-Katz would have lower sensitivity whilst real-time PCR is more likely to detect the very lowest of intensities. Recently, real-time PCR has also been shown to exhibit less measurement error (less variation between readings) compared with Kato-Katz. Molecular techniques have also been shown to have a higher specificity as they are able to distinguish between hookworm species. Thus, real-time PCR is a promising method to evaluate treatment efficacy with anthelmintics in STH infections.

2. Scope and application

The purpose of this standard operating procedure (SOP) is to give a detailed guide on how to perform real-time PCR for STH (*A. lumbricoides*, *T. trichiura*, *N. americanus*, *A. duodenale* and *S. stercoralis*) and *Schistosoma spp* in DNA extracts obtained from stool samples collected in the context of the ALIVE trial.

3. Responsibilities

Head of Site Laboratory: must ensure that all technicians strictly follow this SOP (compliance) and monitor the quality of the results.
Laboratory technicians: must know the contents of this SOP and perform the laboratory activities under the responsibility and guidance of the Head of Site Laboratory.

4. Safety

Samples should be treated as potentially infectious and universal precautions should always be followed, such as:

- ✔ Always wear hand gloves during manipulation of biological materials;
- ✔ Clean the work surface prior and after the work is finished with 70% alcohol and 1 % bleach.

5. Materials

Equipment

Micropipettes (p1000, p200, p20, p10)

Applied Biosystems™ 7500 Real-Time PCR Systems, or Applied Biosystems™ 7500 FAST Real-Time

PCR System or Applied Biosystems™ QuantStudio 5 Real-Time PCR System

96-well PCR plate spinner or a centrifuge with a rotor for 96-well plates

Consumables

Filtered tips (p1000, p200, p20, p10)
Gloves (powderless)
96-well PCR plates (optical permeable)
Optical adhesive films
Eppendorf tubes for master mix
Sealers
Permanent marker
Tube openers
Ice bucket

Samples and Reagents

DNA samples
Primers and probes (See Table 1)
HotStar Taq master mix, 2500 U (e.g. Cat No. 203446; Qiagen)
Rox Passive Reference Dye (e.g. Thermo Scientific, cat. 75768); stock solution 25 µM, working solution dilution 2.5 µM,
Magnesium chloride solution ($MgCl_2$); stock solution 1M, working solution 25mM
Bovine Serum Albumin (BSA); stock solution 20 mg/mL, working solution 5 mg/mL
Tris-EDTA buffer solution; Stock solution 1X TE, working solution 0.1 X TE
Molecular biology grade water (any brand)
Ethanol 70%
Bleach 1%

Table 1. Primers and probes

Species	Primer name	Sequence 5'-3'
A. duodenale	Ad_125_Fwd	GAATGACAGCAAACTCGTTGTTG
	Ad_195_Rev	ATACTAGCCACTGCCGAAACGT
	Ad_Probe	Texas_Red-ATCGTTTACCGACTTTAG-BHQ2
N. americanus	Na_58_Fwd	CTGTTTGTCGAACGGTACTTGC
	Na_158_Rev	ATAACAGCGTGCACATGTTGC
	Na2_Probe	Cy3-CTGTACTACGCATTGTATAC-BHQ2
	Na3_Probe	Cy3-CTGTACTACGCATTGTATGT-BHQ2
A. lumbricoides	Al_96_Fwd	GTAATAGCAGTCGGCGGTTTCTT
	Al_183_Rev	GCCCAACATGCCACCTATTC
	Al_Probe	Yakima_Yellow-TTGGCGGACAATTGCATGCGAT-BHQ1
S. stercoralis	Ss_18S_Fwd	GAATTCCAAGTAAACGTAAGTCATTAGC
	Ss_18S_Rev	TGCCTCTGGATATTGCTCAGTTC
	Ss_Probe	FAM-ACACACCGSCCGTCGCTGC-BHQ1
Schistosoma spp.	Sch_ITS_48_Fwd	GGTCTAGATGACTTGATYGAGATGCT
	Sch_ITS_124_Rev	TCCCGAGCGYGTATAATGTCATTA
	Sch_Probe	FAM-TGGGTTGTGCTCGAGTCGTGGC-BHQ1
T. trichiura	Tt_283_Fwd	TTGAAACGACTTGCTCATCAACTT
	Tt_358_Rev	CTGATTCTCCGTTAACCGTTGTC
	Tt_Probe	Yakima_Yellow-CGATGGTACGCTACGTGCTTACCATGG-BHQ1
Phocine herpes virus-1	PhHV_267_Fwd	GGGCGAATCACAGATTGAATC
	PhHV_337_Rev	GCGGTTCCAAACGTACCAA
	PhHV_Probe	Cy5-TTTTTATGTGTCCGCCACCATCTGGATC-BHQ2

Other SOPs

- SOP_004: Reception, aliquoting and storage of stool samples
- SOP_007: Stool DNA extraction for multiplex real-time PCR and resistance studies
- SOP_014: Reception, aliquoting and storage of Phocine Herpes Virus 1 (PhHV) stock solution

Reagent preparation

Wear gloves and change them FREQUENTLY to avoid contamination of stock reagents; be certain that master mix preparation occurs in the biosafety cabinet of a clean PCR room, using a clean separate set of pipettes and filter tips for all work including stock reagent preparation and that no other work involving nucleic acids: DNA, RNA, Plasmids or any biological material with contaminating genetic material, occurs in this cabinet. Label all the reagents with the name, concentration and date of preparation.

Tris-EDTA buffer solution (TE).
Stock solution 1X: Tris-EDTA buffer solution, Sigma 93583 -100 mL or 500 mL
Working solution: From your 1 X TE stock, take 5 mL (no need of a pipette, add 5 mL to a new 50 mL conical tube) and fill up to 50 mL with molecular biology grade water; this (0.1 X TE) will be used for all primer and probe dilutions.
Tris-EDTA can be also prepared from powder:
Stock solution: For 50 mL of 1X TE, using a spatula put 0.086 gr of the Tris-EDTA powder in a 50 mL conical tube and add up to 50 mL with molecular biology grade water

Rox Passive Reference Dye (Rox).
Stock solution: 25 µM
Working solution: 2.5 µM
1:10 dilution, take 100 µL of stock solution to a new 2 ml or 1.5 mL tube with a screw cap and add 900 µL of molecular biology grade water

Magnesium chloride solution ($MgCl_2$).
Stock solution 25mM
Working solution 25mM (no dilution needed).

Bovine Serum Albumin (BSA).
Stock solution: 20 mg/mL,
Working solution: 5 mg/mL: take 100 µL of stock solution to a new 2 ml or 1.5 mL tube with a screw cap and add 300 µL of molecular biology grade water.

Primer and probe resuspension.
To make a 100 µM concentration stock solution of the primers/probes: spin down briefly the lyophilized primers/probes vials; make sure you see a "cloud" at the bottom of each vial. Take the number of nmoles in the tube, which is indicated by the manufacturer, and multiply that by 10. This will be the number of µL of 0.1 x TE (10mM Tris; 0.1 mM EDTA; pH 8.0)* to add to get a 100 µM solution. For example, if you have 25.6 nmoles of oligo, add 256 µL of 0.1 x TE to make a 100 µM stock solution. Pipette multiple times up and down to make sure that primers/probes are fully resuspended.
To make a 25 µM working solution dilute the 100 µM stock solution in TE buffer (1:4; one part of stock plus three parts of TE). This 25 µM stock is the working stock used for the recipes in the table that follows. For 500 µL of working stock, take 125 µL of the original stock (100 µM) into a new tube and add 375 µL of TE buffer
To make a 10 µM working solution dilute the 100 µM stock solution in TE buffer (1:10; one part of stock plus nine parts of TE). This 10 µM stock is the working stock used for the recipes in the table that follows. For 500 µL of working stock, take 50 µL of the original stock (100 µM) into a new tube and add 450 µL of TE buffer.

REMEMBER:

- Make sure you cover the probe working stock tube in foil and reduce exposure to light as much as possible (the reporter dye contained is super photosensitive!!!)
- You can switch off the cabinet light (but not the cabinet flow) when adding the probes to reduce exposure to light as much as possible
- Disinfect the biosafety cabinet using 70% ethanol.
- Using 1% bleach (make this freshly each day) disinfect your tip boxes, pipettes, ice buckets, pens/markers, racks, bags of tubes, and anything you might be using in the cabinet.
- Use the UV light in the sterile biosafety cabinet with your bleached set of pipettes/tips, racks, markers, adhesive films, etc. for 20 minutes before starting to work.

6. Procedure

The real-time PCR assays targeting the different STHs are designed in two multiplex assays.

- ✔ Multiplex 1 (ANAS) targets *A. lumbricoides*, *N. americanus*, *A. duodenale* and *S. stercoralis*
- ✔ Multiplex 2 (ST) targets Schistosoma spp. (*S. mansoni and* S. *haematobium*) and *T. trichiura*

Mastermix preparation in PCR-1 room

1. Use the UV light in the sterile biosafety cabinet with your set of dedicated pipettes/tips, racks, markers, , etc. for 20 minutes (see the precautions section in this SOP).
2. Outside of the biosafety cabinet (in PCR-1 room), place your HotStar Master Mix aliquot, primer and probe working stocks, aliquot of $MgCl_2$, Rox, BSA and PCR grade/nuclease free water.
3. As soon as your master mix reagents are thawed, flick the tubes to mix and briefly spin them down.
4. In the biosafety cabinet, using micropipettes prepare pooled Master Mix for 100 reactions (see table 2 and table 3 below for recipes per assay per 100 reactions).
5. Ensure that the tube containing the Master Mix is closed properly to prevent contamination when moving to PCR-2 room.
6. Take the tube with the Master Mix to the PCR-2 room.

Sample preparation: adding the template in PCR-2 room

7. Take the DNA samples from the 4°C storage, and place them in a plastic rack. Let the samples thaw if they were stored at -20°C. REMEMBER: Prior to the template addition, you need to flick or vortex and spin down the tubes briefly.
8. Aliquot 20 µL of prepared master mix into each well of the 96-well plate.
9. In every multiplex run, add a negative control (NTC; no template control i.e. reaction mixture with molecular biology grade water instead of DNA sample) and a positive control for each target.
10. Add 5 µL of DNA template to each well. Follow the “unified plate layout” in Appendix A when adding template
11. Cover the plate with an optical adhesive film.
12. Spin down the 96-well plate using a 96-well PCR plate spinner or a centrifuge with a rotor for 96-well plates* for 30 seconds

Amplification in PCR-3 room

13. Perform the amplification, in PCR-3 room, on the ABI 7500, ABI 7500 FAST or QuantStudio5 system (Applied Biosystem) using the following cycling conditions for both multiplex assays: pre-amplification of 15 min at 95°C, followed by 50 cycles of 15 s at 95°C, 30 s at 60°C and 30 s at 72°C (this is the step where fluorescence acquisition has to be performed).

Note 1: if a 96-well PCR plate spinner is not available, tap the plate on your bench when finished loading the template to pop any bubbles seen in the wells.

Note 2: always clean the areas and materials properly after using them to prevent contamination

Table 2: Reaction mixture for multiplex 1 ANAS		
Components	**Concentration**	**Mix 100 rxs**
H_20		65
$MgCl_2$	25 mM	350
BSA	5 mg/ml	50
Rox	1:10	40
Primer Ad - 125F	25 µM	20
Primer Ad - 195R	25 µM	20
Probe Ad	10 µM	25
Primer Na - 58F	25 µM	20
Primer Na - 158R	25 µM	20
Probe Na 2	10 µM	6,25
Probe Na 3	10 µM	6,25
Primer Al - 96F	10 µM	20
Primer Al - 183R	10 µM	20
Probe Al	10 µM	12,5
Primer Ss - 18SF	25 µM	10
Primer Ss - 18SR	25 µM	10
Probe Ss	10 µM	12,5
Primer PHHV - 267F	25 µM	15
Primer PHHV - 337R	25 µM	15
Probe PHHV	10 µM	12,5
HotStar Taq Master Mix		1250
Total		2000
Add 5 µl DNA to the mix		

Table 3: Reaction mixture for multiplex 2 ST		
Components	**Concentration**	**Mix 100 rxs**
H_20		162,5
$MgCl_2$	25 mM	350
BSA	5 mg/ml	50
Rox	1:10	40
Primer Sch - ITS - 48F	25 µM	20
Primer Sch - ITS - 124R	25 µM	20
Probe Sch	10 µM	12,5
Primer Tt - 283F	25 µM	20
Primer Tt - 358R	25 µM	20
Probe Tt	10 µM	12,5
Primer PHHV - 267F	25 µM	15
Primer PHHV - 337R	25 µM	15
Probe PHHV	10 µM	12,5
HotStar Taq Master Mix		1250
Total		2000
Add 5 µl DNA to the mix		

Experimental properties:

Name, username: Plate # - Name of Assay - Samples ###:### - Date (for example: Plate 1 – ANAS-Samples 001:042 – April 6-2021)

Instrument: 7500 (96 wells)

Type of experiment: Quantitation – Comparative Ct

Reagents: Taqman

Ramp speed: Standard (~ 2 hours)

Plate setup

Define targets and samples:

Name your target

Define your reporter/quencher for the probe (for example FAM-None)

Assign targets to wells: match each well with the appropriate sample name defined in the previous step

Run method

Volume of reaction: For all the STH assays, select 25 µL reaction as the reaction volume.

Cycling conditions: For all the STH assays, select 60°C as the annealing and extension temperature for 30 seconds (depending on the real-time PCR machine and software). Per step 11 amplification

procedure: pre-amplification of 15 min at 95°C, followed by 50 cycles of 15 s at 95°C, 30 s at 60°C and 30 s at 72°C (this is the step where fluorescence acquisition has to be performed).

Data collection/analysis
After the run is complete, adjust the threshold of the assay (unclick the “automatic” option and move the mouse on the bar shown on the graph; the threshold needs to be at the beginning of the linear phase of the amplification and above the noise (background)
Click on “Analyze”
Save the run as “previous file name_POST.eds”
Export the data (Results) in the folder of the run

For individual samples, results are reported as either "negative" or "positive". For positives, provide the mean Ct value from the duplicate runs.

Re-test a sample when:

a. there is one positive in one duplicate well (regardless of Ct value) and negative in the other
b. any sample which is positive for both replicates, but gives Ct values > 39 in both replicates. If a sample is positive in both replicates with one Ct value < 39 and the other greater, there is no need for retest.
c. If there is a difference of more than 3.3 Ct values between two replicates
d. Upon re-testing, if a sample is positive in at least 1 re-test well, regardless of Ct value, it is considered positive and this retest Ct value is the result that is reported.
e. Upload the results in box excel sheets.
f. Negative extraction control, NTC - different between plate/run 1 and 2.

7. General remarks

✔ Every real-time PCR includes a NTC (No Template Control). A NTC contains the mastermix and 5 µL molecular grade water instead of the template. This is a standard negative control used to identify set-up contamination and primer-dimer product amplification.
✔ For each PCR plate the negative extraction control is treated as a sample and the technician must track the location of it in every PCR plate.
✔ The passive reference dye is added to the reaction mix to generate a signal for correction of differences in optical sensitivity between distinct sample tubes. It also provides confirmation that an equal volume of reaction mix has been added to each PCR sample.

Waste management

Dispose of potentially contaminated material without contaminating the local environment.

Precautions

Separating pre- and post-amplification areas is key to preventing contamination. You must avoid common types of contamination by following these simple guidelines:

1. Designate and use distinct areas for sample preparation, PCR setup, and post-PCR analysis. To avoid contamination from old amplicons, set up the stations on separate benchtops, two for pre-PCR (PCR-1 and PCR-2) and the other for post-PCR (PCR-3).
    a. **PCR-1** for aliquoting, diluting and storing all the reagents and set up the PCR reaction mix
    b. **PCR-2** for DNA template addition to the plates and storage of DNA samples and controls
    c. **PCR-3** for PCR-amplified DNA purification, gel electrophoresis runs and analysis of PCR products
2. Restrict equipment to these areas. Keep the PCR machine and electrophoresis apparatus in the post-PCR area.
3. Prepare and store reagents for PCR separately and use them solely for their designated purpose. Aliquot reagents in small portions and store them in either location based on their use in pre-PCR or post-PCR applications. Store the aliquots of reagents separately from any DNA samples.
4. Use separate sets of pipettes and pipette tips, lab coats, glove boxes, and waste baskets for the pre-PCR and post-PCR areas. To assist with it coloured stickers can be used to label pipettes (e.g green for pre-PCR work and red for post-PCR work)
5. Use pipettes and pipette tips with aerosol filters dedicated for DNA sample and reaction mixture preparation.
6. Follow the golden rule of PCR: DO NOT bring any reagents, equipment, or pipettes used in a post-PCR area back to the pre-PCR area. This even goes for your lab notebook and pens. Label pre- and post-PCR items, so it is easy to tell where they belong.

8. References

1. Verweij JJ, Brienen EA, Ziem J, Yelifari L, Polderman AM, Van Lieshout L. Simultaneous detection and quantification of Ancylostoma duodenale, Necator americanus, and Oesophagostomum bifurcum in fecal samples using multiplex real-time PCR. The American journal of tropical medicine and hygiene. 2007;77(4):685-90.
2. Verweij JJ, Canales M, Polman K, Ziem J, Brienen EA, Polderman AM, et al. Molecular diagnosis of Strongyloides stercoralis in faecal samples using real-time PCR. Transactions of the Royal Society of Tropical Medicine and Hygiene. 2009;103(4):342-6.
3. Kaisar MMM, Brienen EAT, Djuardi Y, Sartono E, Yazdanbakhsh M, Verweij JJ, et al. Improved diagnosis of Trichuris trichiura by using a bead-beating procedure on ethanol preserved stool samples prior to DNA isolation and the performance of multiplex real-time PCR for intestinal parasites. Parasitology. 2017;144(7):965-74.
4. Cools P, Vlaminck J, Verweij JJ, Levecke B. Quantitative PCR in soil-transmitted helminth epidemiology and control programs: Toward a universal standard. PLoS neglected tropical diseases. 2021;15(3):e0009134.

Appendix A Plate set up: ANAS

	1	2	3	4	5	6	7	8	9	10	11	12
A	1	1	2	2	3	3	4	4	5	5	6	6
B	7	7	8	8	9	9	10	10	11	11	12	12
C	13	13	14	14	15	15	16	16	17	17	18	18
D	19	19	20	20	21	21	22	22	23	23	24	24
E	25	25	26	26	27	27	28	28	29	29	30	30
F	31	31	32	32	33	33	34	34	35	35	36	36
G	37	37	38	38	39	39	40	40	41	41	42	42
H	NTC	NTC	POS Ad	POS Ad	POS Na	POS Na	POS Al	POS Al	POS Ss	POS Ss		

NTC = No Template Control (containing the mastermix and molecular grade water)

POS = Positive control (**Ad**: *A. duodenale;* **Na**: *N. americanus;* **Al**: *A. lumbricoides*; **Ss**: *S. stercoralis*)

Appendix B Plate set up: ST

	1	2	3	4	5	6	7	8	9	10	11	12
A	1	1	2	2	3	3	4	4	5	5	6	6
B	7	7	8	8	9	9	10	10	11	11	12	12
C	13	13	14	14	15	15	16	16	17	17	18	18
D	19	19	20	20	21	21	22	22	23	23	24	24
E	25	25	26	26	27	27	28	28	29	29	30	30
F	31	31	32	32	33	33	34	34	35	35	36	36
G	37	37	38	38	39	39	40	40	41	41	42	42
H	NTC	NTC	POS Schist	POS Schist	POS Tt	POS Tt						

NTC = No Template Control (containing the mastermix and molecular biology grade water)

POS = Positive control (**Schist**: *S. mansoni* or *S. haematobium*; **Tt**: *T. trichiura*)