SOP code	ST_EXTR
File name & version	SOP_007_v02
Full SOP title	Stool DNA extraction for multiplex qPCR and resistance studies
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, 7th December 2021
LUMC (Lisette van Lieshout)	Lisette van Lieshout, 25th November 2021

Contents

1. Introduction

Faeces represents one of the most complex biological materials for DNA extraction as it also contains remains of human DNA, bacterial DNA, food DNA and many inhibitors preventing subsequent PCR amplification. DNA extraction from faeces represents one of the essential steps in obtaining good quality DNA and ensuring accurate identification of parasites and relative quantification. It is therefore critical to follow the procedures to obtain high quality DNA samples for molecular diagnostics. In case samples have been stored in ethanol, the ethanol should be washed away before starting the DNA extraction. Recent studies have confirmed that a bead-beating procedure during the DNA extraction process increases the DNA yield in human faecal samples, in particular that of *T. trichiura*. The ideal extraction system efficiently combines an inhibitor removal technology with chemical and mechanical lysis by bead beating.

2. Scope and application

This Standard Operating Procedure (SOP) describes the method for extracting genomic DNA from either ethanol preserved or unpreserved stool samples. The SOP includes two different steps. The first step describes how to remove ethanol from the preserved samples. The second step describes the process of DNA extraction using the QIAamp DNA Mini Kit, including a bead beating phase which is recommended to facilitate the DNA extraction by mechanical disruption of the helminth's eggs.

3. Responsibilities

Head of Site Laboratory: must ensure that all technicians and fieldworkers 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

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

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

5. Materials

Equipment

1. Magnetic stirrer
2. Microcentrifuge (for eppendorf tubes)
3. Pipettes (10, 200, 1000 µL)
4. Heat-block (preferably two) or oven to be used at 55°C, 70°C and 100°C
5. Bead beater (recommended TissueLyser LT or PowerLyser Qiagen)
6. Vortex

Consumables

1. Pipette tips with filters (10, 200, 1000 µL)
2. Eppendorf tube 1.5 mL (e.g. Merck cat. Z606340)

3. Screw cap Micro tubes 2 mL (e.g. Sarstedt, cat. 72.694)
4. PowerBead tubes, Ceramic 1.4 mm pack of 50 (e.g. Qiagen, cat. 13113-50). Storage at room temperature (15-30°C)

Samples and reagents

1. Faecal samples ethanol preserved or unpreserved
2. QIAamp DNA Mini Kit (e.g. Qiagen, cat. 51306). Storage: solutions AL, ATL, AW1, AW2, AE and columns at room temperature (15-30°C), Proteinase K solution at 2-8°C
3. Extra AL buffer (e.g. Qiagen, cat. 19075) - 264 mL
4. Absolute ethanol (e.g. Merck, cat. 32205-M), storage at 5-30 °C.
5. Polyvinylpolypyrrolidone (PVPP), Sigma (e.g. cat. N. 77627), storage at 5-30°C.
6. Phosphate Buffered Saline (PBS) pH 7.4 ready to use (e.g. Sigma cat. D8537-1L) storage at room temperature (15-30 °C)
7. Phocine herpesvirus 1 (PhHV), (EVAg cat.N. 011V-00884) storage at -80°C.

Other SOPs

- SOP_001 (ST_COLLECT: Stool collection, labelling and transportation to the laboratory)
- SOP_002 (ST_FLOW: Stool sample flowchart)
- SOP_004 (Reception, aliquoting and storage of stool samples)
- SOP_008 (qPCR: Multiplex real-time PCR for the detection of STH)
- SOP_014 (Reception, aliquoting and storage of Phocine Herpes Virus 1 (PhHV) stock solution)

6. Procedure

6.1 Organization of samples

Samples will be processed in batches of 24. Each batch should contain one sample of PBS as a negative control for the DNA extraction (position 1 on the extraction worksheet appendix A). The negative extraction control is treated as a sample and the technician must track the location of it every batch of samples. Before starting, order the samples in a rack, label the PowerBead tubes with the samples identification code and fill the DNA extraction worksheet with all the information required (see sample collection form_SOP_001 in **Appendix A**). Store the form in the appropriate folder. DNA extraction is performed in the "DNA extraction laboratory". This laboratory is exclusively used for this purpose and is free of DNA amplicons.

For instructions on the sample identification codes to be used please refer to SOP_001 on Stool collection, labelling and transportation to the laboratory.

6.2 Preparation of buffers and reagents

- If a precipitate has formed in Buffer ATL or Buffer AL, warm the bottles of buffers at 55°C until the precipitate has fully dissolved.
- 2% PVPP solution (w/v), weight 1 gr of PVPP in a screw cap glass bottle containing 50 mL of PBS. Mix until a homogenous suspension is obtained, label as 2% PVPP and add the date of preparation. Store the solution at 2-8°C for up to one year from preparation. Remark: PVPP does not dissolve, so you have to stir it regularly while using it.
- AL solution spiked with PhHV working solution: add 50 µL of PhHV working solution to the AL solution (33 mL). Mix thoroughly and keep at room temperature. In the qPCR the PhHV should have an average outcome of approximately Ct=29 (± 3 Ct).

- PhHV working solution: dilute the stock solution with PBS and process according to protocol (see SOP_014), make aliquots of 50 µL and store at -80°C.

6.3 Sample preparation

For samples preserved in ethanol

1. Put the bottle with 2% PVPP on a stir plate
2. Put 250 µL of the ethanol-faeces suspension in the previously labelled PowerBead tubes using a 1000 µL tip (make sure you use a very slow aspiration and dispensing speed with a tip to which the end was cut off) and centrifuge at 14000 x g for 1 minute.
3. Remove the ethanol with a micropipette and wash the pellet by adding 1000 µL of PBS, mix and centrifuge at 14000 x g for 1 minute.
4. Remove the PBS with a micropipette and add 200 µL of 2% PVPP to the pellet. **PVPP does not dissolve so stir it regularly while using it.**
5. Proceed with section 6.4.

For concentrated eggs

1. Put the bottle with 2% PVPP on a stir plate
2. Let the concentrated egg sample thaw and resuspend it using 600 µL of PBS and transfer the suspension into the previously labelled PowerBead tubes using a 1000 µL tip (make sure you use a very slow aspiration and dispensing speed with a tip to which the end was cut off) and centrifuge at 14000 x g for 1 minute.
3. Remove the PBS with a micropipette and add 200 µL of 2% PVPP to the pellet. **PVPP does not dissolve so stir it regularly while using it.**
4. Proceed with section 6.4.

For unpreserved samples

1. Put the bottle with 2% PVPP on a stir plate.
2. Aliquot 200 µL of 2% PVPP into a PowerBead tube. **PVPP does not dissolve so stir it regularly while using it.**
3. Transfer approximately 0.1 gr of sample to the tube containing 2% PVPP, using a wooden stick, carefully opening the container with unpreserved stool over a tissue to avoid contamination.
4. Proceed with section 6.4.

6.4 Mechanical disruption

1. Firmly tighten the caps (to prevent leaking) and place the tubes inside the homogenizer.
2. Operate the bead beater at high speed (50 Hz) for 10 minutes
3. Freeze the samples for 30 minutes at -80°C or overnight at -20°C
4. Proceed with section 6.5

6.5 DNA extraction with QIAamp DNA Mini kit[3]

1. Turn on the heat-blocks in advance and adjust one of them at 55°C and the other one at 100°C.
2. Take the samples from the freezer and, once thawed, mix them and heat them for 10 minutes in the heat-block at 100°C.
3. Briefly centrifuge to remove drops from the inside of the lid.

4. Add 180 µL of Buffer ATL and 20 µL Proteinase K, mix by vortexing and incubate at 55°C for 2 hours or overnight
5. Briefly centrifuge to remove drops from the inside of the lid.
6. Add 400 µL of AL solution spiked with PhHV (see section 6.2 for instructions). Mix thoroughly by vortexing for 15 s. For resistance testing, use a "clean" non-spiked AL buffer.
7. Incubate at 70°C for 10 minutes
8. Centrifuge for 30 seconds at full speed and transfer the supernatant to an Eppendorf containing 400 µL of ethanol (95-100%) and mix thoroughly
9. Briefly centrifuge to remove drops from the inside of the lid
10. Pipet 600 µl of the mixture into the QIAamp Mini spin column (in a 2 mL collection tube). Centrifuge at 14000 x g for 1 min. Discard the flow through and the collection tube
11. Place the QIAamp Mini spin column in a new 2 mL collection tube and add the remaining mixture onto the column. Centrifuge at 14000 x g for 1 min. Discard the flow through and the collection tube
12. Place the QIAamp Mini spin column in a new 2 mL collection tube and add 500 µL Buffer AW1. Centrifuge at 14000 x g for 1 min. Discard the flow-through
13. Add 500 µL: Buffer AW2. Centrifuge at full speed 20000 x g for 3 min. Discard the flow-through and collection tube
14. Place the QIAamp Mini spin column in a new 1.5 mL microcentrifuge tube (not provided) labelled with the sample identification number, add 200 µL of Buffer AE and incubate at room temperature for 1 min. If extracting DNA for resistance testing elute the DNA in 50 µL of Buffer AE. Centrifuge at 6000 x g for 1 min to elute the DNA
15. **Preferably store the DNA at 2-8°C**. Alternatively store at -20°C, but be aware that repeated freezing/thawing can shear DNA.

7. General remarks

Waste management

Dispose of potentially contaminated material without contaminating the local environment.

Precautions

Ensure that local SOPs on screening and sample delivery are available to staff.

8. References

1. Kaisar M, Brienen E, Djuardi Y, Sartono E, Yazdanbakhsh M, Verweij J, Supali T and Van Lieshout L: Improved diagnosis of Trichuris trichiura by using a bead-beating procedure on ethanol preserved stool samples prior to DNA extraction and the performance of multiplex real-time PCR for intestinal parasites. Parasitology 2017, 144:965-974.
2. Ayana M, Cools P, Mekonnen Z, Biruksew A, Dana D, Rashwan N, Prichard R, Vlaminck J, Verweij J, Levecke B. Comparison of four DNA extraction and three preservation protocols for the molecular detection and quantification of soil-transmitted helminths in stool. PLoS Negl Trop Dis. 2019, 13(10): e0007778
3. www.qiagen.com (QIAamp DNA Mini Kit Handbook)

Appendix A: DNA extraction worksheet

SOP_007

Appendix A: DNA extraction worksheet

DNA extraction date _____**Operator**_____**Site**_____

	Sample code	Date of collection	Elution volume	Comments
1	Negative control (PBS)			
2				
3				
4				
5				
6				
7				
8				
9				
10				
11				
12				
13				
14				
15				
16				
17				
18				
19				
20				
21				
22				
23				
24				

Quality control:

Check storage temperature of each reagent ☐

Check the expiry date of each reagent before use ☐

DNA extraction kit lot:_____Proteinase K lot:_____