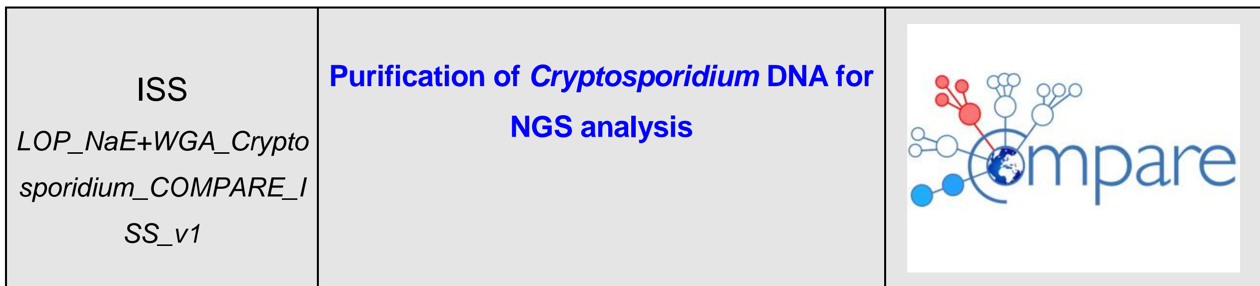

ISS *LOP_NaE+WGA_Cryptosporidium_COMPARE_ISS_v1*	**Purification of *Cryptosporidium* DNA for NGS analysis**	

Laboratory Operating Procedure

Purification of *Cryptosporidium* oocysts and extraction of DNA for NGS analyses

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Introduction

The only source of genomic DNA of *Cryptosporidium* spp. is the oocyst, a small and very robust stage. Oocysts are found in human and animal feces, as well as in environmental samples (water) or as a contaminant of food. The organism cannot replicate outside the host, and in vitro cultivation methods are only use as research tools and not for an actual amplification of *Cryptosporidium* isolates. This means that oocysts need to be concentrated and purified from other components of the matrix. This is accomplished by a combination of flotation and immune-magnetic separation (IMS) steps. The purified oocysts are enumerated and checked for integrity by a direct immuno-fluorescence assay. DNA is extracted from IMS-purified oocysts after several cycles of freezing and thawing to induce breaks in the oocyst wall. Both mechanic (bead-beater or FastPrep) and enzymatic (Qiagen) methods can be used. If the amount of genomic DNA is too small, it may be necessary to use Whole Genome Amplification to generate sufficient material for subsequent NGS applications.

1. Concentration of stools

Description of the procedure

Feces can contain large amounts of gross particles, undigested fibers and fat. This procedure is intended to enrich the concentration of the target organism, and can be applied to both fresh and preserved fecal samples. Concentrated feces are used for immunofluorescence detection and for the immuno-magnetic separation of *Cryptosporidium* oocysts. When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, consult the appropriate material safety data sheets (MSDSs), available from the product supplier.

Equipment and Material

- Bench centrifuge
- 50 ml Falcon tubes
- Chemical hood
- Vortex
- Sievers
- Pipettes
- Disposable gloves

Reagents to be supplied by the user

- Diethyl ether
- Phosphate Buffered Saline (PBS)

Procedure

1. Under a chemical hood, draw the proper amount of feces (3 to 5 ml, or 3 to 4 gram) into a 50ml Falcon tube.
2. Add 40 ml of PBS and vortex well.
3. Sift the suspension into a clean 50 ml Falcon tube using a sieve (funnel white). Adjust the volume to 40 ml with PBS.
4. Centrifuge the sample at 4,200 rpm for 10 min at room temperature.
5. Discard the supernatant by aspiration.
6. Suspend the pellet in 21 ml PBS, and add 9 ml diethyl ether. Emulsify thoroughly by vortexing for a few seconds. Open the tube to release gases, and close again the tube.
7. Centrifuge at 1600 rpm for 5 min at room temperature.
8. After the centrifugation, 3 phases should be visible. From above: 1) ether, 2) a lipid interface, and 3) an aqueous phase. A sediment is present at the bottom of the tube. Remove the three liquid phases by aspiration and leave the sediment undisturbed.
9. Wash the sediment with 40 ml of PBS to remove ether residues. Centrifugation at 4,600 rpm for 5 min at room temperature. Repeat three times.
10. Suspend the sediment in an appropriate volume of PBS (typically 1-2 ml). Make sure that the fecal suspension is not too dense.

2. Flotation

Description of the procedure

Oocysts have a low specific gravity (about 1.05 g/cc) and can be purified from fecal material by flotation. To flotate oocysts by buoyant density gradient separation, different solutions can be used, such as saturated sodium chloride and sucrose. The procedure described herein is based on saturated sodium chloride. When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, consult the appropriate material safety data sheets (MSDSs), available from the product supplier.

Equipment and Material

- Preparative centrifuge
- 50 ml Falcon tubes
- Pipettes
- Disposable gloves

Reagents to be supplied by the user

- Sodium chloride (NaCl)
- Reverse osmosis water (e.g., MilliQ)

Procedure

1. Prepare a solution of saturated NaCl (*d*=1.18 g/ml).
2. Pipette 10 ml of saturated NaCl into the bottom of a 50 ml Falcon tube.
3. Gently overlay 1 ml of concentrated fecal suspension.
4. Add 1 ml of cold water to prevent oocysts damage due to exposure to hypertonic NaCl solution.
5. Centrifuge at 2300 g for 10 min at 4°C.
   Note: do not use the break.
6. After the centrifugation, recover the oocysts that accumulate at the basis of the water phase.
7. Bring the volume to 40 ml with water.
8. Centrifuge at 2300 g for 10 min at 4°C.
9. Discard the supernatant by aspiration, leaving the pellet undisturbed.
10. Repeat steps 6-8 two times more.
11. Suspend the pellet containing the oocysts in water.

3. Detection of *Cryptosporidium oocysts* by ImmunoFluorescence Assay (IFA)

Description of the procedure

Purified *Cryptosporidium* oocysts should be enumerated and their integrity (i.e., presence of nuclei) evaluated prior to DNA extraction. To this end, a direct immunofluorescent assay (IFA) using fluorescein isothiocyanate (FITC) labeled monoclonal antibodies directed against cell wall antigens of *Cryptosporidium* is used. The procedure herein described is based on the commercially available MERIFLUOR *Cryptosporidium/Giardia* (MERIFLUOR C/G), but other kits are also available on the market. When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, consult the appropriate material safety data sheets (MSDSs), available from the product supplier.

Equipment and Material

- Fluorescent microscope equipped with filter system for FITC
- Treated microscope slides
- Humid chamber
- Aerosol-free tips
- Pipettes
- Disposable gloves
- Wash bottle
- Microscope coverslips
- Application sticks

Reagents to be supplied by the user

- 4'6 diamidino-2-phenyl indole (DAPI).

Procedure

1. Transfer approximately 50 µl of sample, after flotation or IMS, to a treated slide well. Spread the specimen over the entire well. Do not scratch the treated surface of the slide.
2. Place a drop (~50 µl) of Positive Control to a treated slide well. Spread the Positive Control over the entire well. Do not scratch the treated surface of the slide.
3. Place a drop (~50 µl) of Negative Control to a treated slide well. Do not scratch the treated surface of the slide.
4. Allow the slides to dry completely at room temperature (about 30 minutes).
5. Place one drop of Detection Reagent in each well.
6. Place one drop of Counterstain in each well.
7. Mix the reagents with an applicator stick and spread over the entire well. Do not scratch the treated surface of the slide.
8. Incubate the slides in a humidified chamber for 30 minutes at room temperature.
   Note: Protect from light.
9. Use a wash bottle to rinse the slides with a gentle stream of 1X Wash Buffer until excess Detection Reagent and Counterstain is removed.
   Note: Do not submerge the slides during rinsing**.** Avoid disturbing the specimen or causing cross contamination of the specimens.
10. Remove excess buffer by tapping the long edge of the slide on a clean paper towel.
    Note: Do not allow slide to dry.
11. Add one drop of Mounting Medium containing DAPI (0.4 µg DAPI/mL in PBS) to each well and apply a coverslip.
12. Scan each well thoroughly at 100 (or 400 X) magnification using a fluorescent microscope.
    Note: *Cryptosporidium* oocysts are round to slightly oval in shape, 2-6 µm in diameter. The oocyst wall will stain bright apple green. A suture line may also be visible on the oocyst wall. Each sporozoite contains one nucleus that can be stained by DAPI. Since one oocyst contains four sporozoites, up to four blue spots can be observed within each oocyst.

4. Immuno-magnetic separation of oocysts

Description of the procedure

This procedure further purifies oocysts from bacteria, fungi, host cells and residual fecal debris. It is based on the use of magnetisable beads coupled with anti-*Cryptosporidium* monoclonal antibodies. There are several commercial kits available; the procedure describe herein refers to the Dynabeads™ anti-*Cryptosporidium* kit (Thermo Fisher). When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, consult the appropriate material safety data sheets (MSDSs), available from the product supplier.

Equipment and Material

- Leighton 10 (L-10) tubes
- Magnets (MPC™-1 or MPC™-6)
- Rotating sample mixer
- Vortex
- Micropipettes
- Aerosol-free tips
- Disposable gloves

Reagents to be supplied by the user

- Reverse osmosis water (e.g., MilliQ)
- Hydrogen chloride (HCl)
- Sodium hydroxide (NaOH)
- Sodium hypochlorite

Procedure

1. Sample must be suspended in final volume of 10 ml of water.
2. Allow the sample to equilibrate to room temperature.
3. Dilute 1 ml of 10X SL™–buffer A with 9 ml of demineralized water. Retain this solution for a later use.
4. To a flat-sided L-10 tube, add 1 mL of 10X SL™-Buffer A, and 1 mL of 10X SL™-Buffer B.
5. Immediately transfer the sample to the L-10 tube containing the SL™-Buffer. Label the tube with a sample identifier code.
6. Vortex the Dynabeads™ anti-Cryptosporidium vial for 10 sec.
7. Suspend the beads completely by inverting the vial. Add 100 µL of Dynabeads™ anti-*Cryptosporidium* to the L-10 tube.
8. Affix the L-10 tube to a rotating mixer (e.g. MX1) and rotate at 15–20 rpm for 1 hour at room temperature.
9. Place in the MPC™-1 or MPC™-6 with the flat side of tube facing towards the magnet.
10. Without removing the tube from the magnet, place the magnet side of the MPC™-1 downwards (tube is horizontal and above the magnet).
11. Gently rock the tube end to end through approximately 90°, tilting the cap-end and base-end of the tube up and down in turn. Tilt for 2 min with approximately one tilt per sec.

12. Return the magnet to the upright position, tube vertical, with the cap at the top. Remove the cap and pour off all the supernatant.
13. Remove the tube from the magnet and suspend the sample in 1 mL 1X SL™-Buffer A. Mix gently to suspend all material in the tube.
14. Transfer all the liquid and beads from the L-10 tube to a labelled 1.5-mL microcentrifuge tube.
15. Place the microcentrifuge tube into the MPC™-S, with magnetic strip in place in the vertical position.
16. Without removing the microcentrifuge tube from the MPC™-S, gently tilt the MPC™-S back and forth 90°. Continue for 1 min with approximately one 90°-tilt per sec.
17. Immediately aspirate the supernatant from the tube and cap held in the MPC™-S. If more than one sample is being processed, conduct three 90° back-and-forth motions before removing the supernatant from each tube.
18. Remove magnetic strip from the MPC™-S.
19. Add 50 μL of 0.1 N HCl to the microcentrifuge tube and vortex for 10 sec.
20. Place the tube in MPC™-S without magnetic strip in place and allow to stand in a vertical position for at least 10 min at room temperature.
21. Vortex for 10 sec.
22. Ensure that the sample is at the base of the tube. Place the microcentrifuge tube in MPC™-S.
23. Insert the magnetic strip in the MPC™-S in the tilted position and allow the tube to stand undisturbed for about 10 sec.
24. Transfer all fluid onto a clean microcentrifuge tube containing 5 μL of 1 N NaOH. Mix immediately.
25. Adjust the volume to 1 ml with reverse osmosis water.
26. Add an equal volume of 0.6 % active chlorine (as sodium hypochlorite).
27. Wash three times with nuclease free water. Centrifuge at 1,100 X g for 5 min, using a swing rotor and soft acceleration-deceleration profile to minimize oocyst damage.
28. Suspend the pellet in 215 μL of nuclease-free water. Use 15 μL for IFA and 200 μL for DNA extraction.

5. DNA extraction from purified oocysts

Description of the procedure

DNA is extracted from purified oocysts using either silica-based commercial kits (e.g., Qiagen or similar) or a mechanic-based procedure (e.g., FastPrep). Considering that a single oocyst contain about 40 femtograms of DNA, the procedure is to be applied only to samples containing from 10^5 to 10^6 purified oocysts in order to extract some nanograms of DNA. To maximise yield, the purified oocysts are first submitted to cycles of freezing and thawing. The DNA extraction procedure here described uses a commercial kit (QIAamp DNA Mini Kit). When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, consult the appropriate material safety data sheets (MSDSs), available from the product supplier.

Equipment and Material

- Micro centrifuge
- Thermomixer or heating block
- Vortex
- Micropipettes and aerosol-free tips

- Disposable gloves

Reagents to be supplied by the user

- Liquid nitrogen
- Ethanol 96-100%

<u>Preparation of reagents</u>

Buffer AL:

- store at room temperature, 15–25°C
- stable for 1 year when stored closed at room temperature
- shake before use

Note: Do not add QIAGEN Protease or proteinase K directly to Buffer AL.

Buffer AW1:

- before using for the first time, add the appropriate amount of ethanol (96–100%) to the buffer concentrate, as indicated on the bottle
- stable for 1 year when stored closed at room temperature
- shake before use

Buffer AW2:

- before using for the first time, add the appropriate amount of ethanol (96–100%) to the buffer concentrate, as indicated on the bottle
- stable for 1 year when stored closed at room temperature
- shake before use

Procedure

1. Transfer the IMS-purified oocysts (200 µl) in a 1.5 ml Eppendorf tube.
2. Freeze the tube in liquid nitrogen for 1 min and then transfer to a heating block (set at 90°C) until completely thawed (~3 min).
3. Repeat step 2 four more times.
4. Pipet 20 µl QIAGEN Proteinase K into the bottom of a 1.5 ml microcentrifuge tube.
5. Add the solution containing oocysts to the tube containing Proteinase K.
6. Add 200 µl Buffer AL to the sample. Mix by pulse-vortexing for 15 s.
7. Incubate at 56°C for 10 min.
8. Briefly centrifuge the 1.5 ml microcentrifuge tube to remove drops from the inside of the lid.
9. Add 200 µl ethanol (96–100%) to the sample, and mix again by pulse-vortexing for 15 s. After mixing, briefly centrifuge the 1.5 ml microcentrifuge tube to remove drops from the inside of the lid.
10. Carefully apply the mixture from step 9 to the QIAamp Mini spin column (in a 2 ml collection tube) without wetting the rim. Close the cap, and centrifuge at 6000 x g (8000 rpm) for 1 min. Place the QIAamp Mini spin column in a clean 2 ml collection tube, and discard the tube containing the filtrate.
11. Carefully open the QIAamp Mini spin column and add 500 µl Buffer AW1 without wetting the rim. Close the cap and centrifuge at 6000 x g (8000 rpm) for 1 min. Place the QIAamp Mini spin column in a clean 2 ml collection tube, and discard the collection tube containing the filtrate.
12. Carefully open the QIAamp Mini spin column and add 500 µl Buffer AW2 without wetting the rim. Close the cap and centrifuge at full speed (14,000 rpm) for 3 min.

Note: to eliminate the chance of possible Buffer AW2 carryover, place the QIAamp Mini spin column in a new 2 ml collection tube and discard the old collection tube with the filtrate. Centrifuge at full speed for 1 min.

13. Place the QIAamp Mini spin column in a clean 1.5 ml microcentrifuge tube, and discard the collection tube containing the filtrate. Carefully open the QIAamp Mini spin column and add 100 µl Buffer AE or distilled water.
    Note: If Whole Genome Amplification is to be performed, elute the DNA in TE buffer.
14. Incubate at room temperature (15–25°C) for 1 min, and then centrifuge at 6000 x g (8000 rpm) for 1 min.
15. DNA extracts are stored either at +4°C (for immediate use) or at -20°C (for prolonged storage).

6. Whole Genome amplification (WGA)

Description of the procedure

The Whole Genome Amplification method is based on MDA technology, which carries out isothermal genome amplification utilizing a DNA polymerase capable of replicating up to 100 kb without dissociating from the genomic DNA template. The DNA polymerase has a 3'–5' exonuclease proofreading activity to maintain high fidelity during replication and is used in the presence of exonuclease resistant primers to achieve high yields of DNA product. The procedure described herein uses a commercial kit (Repli-g Midi kit, Qiagen).
When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, consult the appropriate material safety data sheets (MSDSs), available from the product supplier.

Equipment and Material

- Microcentrifuge
- Thermomixer or heating block
- Micropipettes and aerosol-free tips
- Vortex
- Disposable gloves

Reagents to be supplied by the user

- Ice
- Nuclease-free water

Preparation of reagents

Prepare Buffer DLB by adding 500 µl of nuclease-free water to the tube. Mix thoroughly and centrifuge briefly.
Note: Reconstituted Buffer DLB can be stored for 6 months at –20°C. Buffer DLB is pH-labile. Avoid neutralization with CO_2.

Procedure

1. Thaw REPLI-g Mini DNA Polymerase on ice. Thaw all other components at room temperature, vortex, then centrifuge briefly.
2. Prepare sufficient Buffer D1 (denaturation buffer) and Buffer N1 (neutralization buffer) for the total number of whole genome amplification reactions. For buffer D1, mix 9 µl of reconstituted buffer DLB with 32 µl of nuclease free water (sufficient for 7 samples); for buffer N1 mix 12 µl of Stop solution with 68 µl of nuclease free water (sufficient for 7 samples).
3. Place 5 µl template DNA into a 1.5 microcentrifuge tube.
4. Add 5 µl Buffer D1 to the DNA. Mix by vortexing and centrifuge briefly.
5. Incubate the samples at room temperature for 3 min.
6. Add 10 µl Buffer N1 to the samples. Mix by vortexing and centrifuge briefly.
7. Prepare a master mix on ice. For each reaction, first add 29 µl of REPLI-g Midi Reaction Buffer and then 1 µl of REPLI-g Midi DNA Polymerase.
8. Incubate at 30°C for 16 h.
9. Inactivate REPLI-g Midi DNA Polymerase by heating the sample for 3 min at 65°C.
10. Store amplified DNA at 4°C for short-term storage or –20°C for long-term storage.
11. Check the quality of the amplified genomic DNA on a 0.7% agarose gel and measure the concentration using a Qubit or Nanodrop.

Literature

-Kir S. et al. Quantitative comparison of different purification and detection methods for *Cryptosporidium parvum* oocysts. Veterinary Parasitology 2011, 177: 360-370. (Flotation)

- Dynabeads™ anti-*Cryptosporidium* (IMS)

--MerIFluor® *Cryptosporidium*/*Giardia* handbook (IFA)

-QIAamp® DNA Mini and Blood Mini Handbook, Qiagen (DNA extraction)

-REPLI-g® Mini/Midi Handbook, Qiagen (WGA)