Laboratory Operating Procedure

Purification of *Giardia* cysts and extraction of DNA for NGS analyses

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Introduction

The main source of genomic DNA of *Giardia* spp. is the cyst, a small and very robust stage. Cysts are found in human and animal feces, in environmental samples (water) or in food. The organism cannot replicate outside the host, and only some strains can be cultivated under defined *in vitro* conditions. Therefore, purification of cysts from other components of the matrix under study is necessary. This is accomplished by a combination of flotation and immune-magnetic separation (IMS) steps. The purified cysts are enumerated and checked for integrity by a direct immuno-fluorescence assay. DNA is extracted from IMS-purified cysts after cycles of freezing and thawing to induce breaks in the cyst wall. Different methods can be used, including those based on mechanical or enzymatic lysis, and on DNA capture by silica or paramagnetic beads. If the amount of extracted 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 immune-magnetic separation of *Giardia* cysts. 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

Cysts have a low specific gravity (about 1.06 g/cc) and can be purified from fecal material by flotation. To flotate cysts by buoyant density gradient separation, different solutions can be used, such as magnesium sulphate and sucrose. The procedure described herein is based on magnesium sulphate. 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
- 15 ml Falcon tubes
- Pipettes
- Disposable gloves
- Hydrometer (available with ranges of 1.000 –1.400)

Reagents to be supplied by the user

- Magnesium Sulfate
- Reverse osmosis water (e.g., MilliQ)

Procedure

1. Dissolve 450 g of MgSO4 into 750 ml of water using a magnetic stirrer. Bring the volume to 1 liter.
2. Measure the specific gravity of the solution with a hydrometer (it should be 1.20).
3. Homogenize the fecal sample using a spatula, and transfer 2 to 5 g of feces into a clean container.
4. Add 10 ml of the MgSO4 flotation solution to the fecal sample, stir vigorously by hand for 1-2 minutes, and then vortex until the consistency is uniform.
5. Sift the suspension into a 15 ml centrifuge tube. Adjust the volume to 12ml with flotation solution.
6. Centrifuge the sample at 280 g for 5 minutes in a bench centrifuge.
7. Remove the tube and let it stand for 10 minutes.
8. Three phases should be visible, a namely white thin layer, a flotation solution phase, and a sediment at the bottom of the tube.
9. Cysts are collected by aspirating the white tin layer in which they concentrate.
10. After collection, wash cysts three times with PBS by centrifugation at 2.000 rpm for 5 minutes.
11. Suspend the pellet containing the cysts in water.

3. Detection of *Giardia cysts* by ImmunoFluorescence Assay (IFA)

Description of the procedure

Purified *Giardia* cysts should be enumerated and their integrity (i.e., presence of intact 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 *Giardia* is used. The procedure herein described is based on the commercially available MERIFLUOR *Cryptosporidium/Giardia* kit (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: *Giardia* cysts are elliptically shaped and range in size from 6 to 10 microns. The cyst wall will stain bright apple green. Each cysts contains eight nuclei that can be stained by DAPI.

4. Immuno-magnetic separation of cysts

Description of the procedure

This procedure further purifies cysts from bacteria, fungi, host cells and residual fecal debris. It is based on the use of magnetisable beads coupled with anti-*Giardia* monoclonal antibodies. There are several commercial kits available; the procedure describe herein refers to the Dynabeads™ anti-*Giardia* 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-*Giardia* vial for 10 sec.
7. Suspend the beads completely by inverting the vial. Add 100 µL of Dynabeads™ anti-*Giardia* 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 cyst 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 cysts

Description of the procedure

DNA can be extracted from purified cysts by silica-based commercial kits (e.g., Qiagen or similar), mechanic-based procedures (e.g., FastPrep) or paramagnetic resin. Considering that a single cyst contain about 120 femtograms of DNA, the procedure is to be applied only to samples containing from 10^5 to 10^6 purified cysts in order to extract nanograms of DNA. To maximise yield, the purified cysts may be first submitted to cycles of freezing and thawing. The DNA extraction procedure here described uses a commercial kit (DNA extraction IQ™ System Promega). 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

- Sterile Eppendorf tubes
- Disposable gloves

Preparation of reagents

Proteinase K Solution:

- Add 5.5 ml of Incubation Buffer to the bottle containing the lyophilized Proteinase K, and gently swirl to dissolve. The final concentration of Proteinase K will be18 mg/ml.
- Dispense into smaller aliquots and store at -20°C.
- stable for 1 year when frozen; can be thawed up to 5 times

DTT Solution:

- Dissolve 5g of DTT in nuclease-free water to a final volume of 32.4 ml. The final concentration of DTT will be 1M.
- Dispense into smaller aliquots and store at -20°C.

Lysis Solution:

- To 138.6 µl of Lysis Buffer, add 1.4 µl of 1M DTT. Mix well.
- Prepare just before use in the necessary amount

Note: the reagents described above are components of the Promega Tissue and Hair Extraction Kit.

Procedure

1. Pellet the cysts obtained after IMS by centrifugation (5 min at 5000 rpm, 4°C, in a micro centrifuge).
2. To the pellet, add 16 µl of Incubation Buffer, 2 µl of Proteinase K, and 2 µl of DTT
3. Incubate for 30 min at 55°C in a thermomixer with agitation (max speed)
4. Add to each tube 40 µl of Lysis Solution and 4 µl or 8 µl of Resin
5. Incubate for 10 min at 25°C in a thermomixer with agitation (max speed)
6. Transfer the tube in a magnetic stand and carefully discard solution without disturbing the resin
7. Wash the resin with 100 µl of Lysis Solution, place the tube in the magnetic stand and discard the washing solution without disturbing resin
8. Wash the resin three times as above using 100 µl of 2x Wash Buffer
9. Place the tube in the magnetic stand with the lid open, and air-dry the resin for 15 min
10. Add 40-60 µl of Elution Buffer and incubate at 65°C for 5 min
11. Vortex and place the tube in the magnetic stand. Recover the eluted DNA in a clean tube
    Note: If Whole Genome Amplification is to be performed, elute the DNA in TE buffer.
12. 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 CO2.

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

- Dynabeads™ anti-*Giardia* handbook (IMS)

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

-DNA extraction IQ™ System, Promega (DNA extraction)

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