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## Purification of germination-competent *E. intestinalis* spores

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**We use this protocol and it's working**

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## Abstract

*Encephalitozoon intestinalis* is a human-infecting microsporidian species, which can cause fatal illnesses in immunocompromised patients. As an obligate intracellular parasite, *E. intestinalis* replicates exclusively within host cells, and then is transmitted via a non-replicating spore. Studying spores requires culturing the parasite within host cells, initially yielding a mixture of parasite stages, including both germination-competent and germination-incompetent spores. Starting from previously described protocols<sup>1–4</sup>, here, we describe an optimized method to reproducibly enrich and isolate mature, germination-competent *E. intestinalis* spores from infected Vero cells. We provide characterization of germination rates, infectivity as assessed using a parasite FISH staining protocol<sup>5</sup> and genomic content of germination-competent spores.

## Materials

### Purification and propagation of *Encephalitozoon intestinalis*

- *Encephalitozoon intestinalis* (ATCC: 50506)
- Vero cells (ATCC CCL-81)
- DMEM:HG (Dulbecco's Modified Eagle Medium, high glucose)(Gibco 11995065)
- FBS (Fetal Bovine Serum) (VWR 89510-188)
- Non Essential Amino Acids (Gibco 11140050)
- L-Glutamine, pH 6 to 8 (Gibco 25030081)
- T-75 flask (CELLTREAT - 229341)
- Cell scraper, 30 mm (CELLTREAT - 229315)
- Percoll, pH range 8.5-9.5 (Cytiva 17-0891-02)
- Sterile PBS, without calcium, without magnesium: 137.9 mM Sodium Chloride, 1.47 mM Potassium Phosphate Monobasic, 2.67 mM Potassium Chloride, 8.09 mM Sodium Phosphate Dibasic, pH 7.4±0.2
- Ultracentrifuge tube (Thermo Scientific Cat #06752: 12 mL PET)
- 20% (w/v) SDS solution
- Swinging bucket ultracentrifuge rotor (TH-641, Thermo Scientific Cat # 54295)
- 15 mL centrifuge tube (VWR 89039-664)
- 50 mL centrifuge tube (VWR 89039-656)
- 3 mL BD Luer-Lok Syringe sterile (BD 309657)
- 27G x 1/2 (0.4mm x 13mm) BD PrecisionGlide Needle (BD 305109)
- BIOFlex HC Swinging-Bucket Rotor (Thermo Scientific Cat # 75003000)
- Sorvall X4 Centrifuge (Thermo Scientific Cat # 75016052)

### Germination Assay

- *E. intestinalis* germination buffer stock  
140 mM NaCl + 5 mM KCl + 1 mM CaCl<sub>2</sub> + 1 mM MgCl<sub>2</sub> + 5% (v/v) H<sub>2</sub>O<sub>2</sub> pH 9.5 (Final volume 1.5 mL in distilled water)
- Fisherbrand Square Cover Glasses (Cat#: 12-541-013)
- Eprelia Polysine Microscope Adhesion Slides (Eprelia Cat#: P4981-001)

### Infectivity Assay

- Fisherbrand Cover Glasses: circles (Cat#: 12-541-013)
- Fisherbrand Selectfrost Microscope Slides (Cat#: 12-550-003)
- Humidity chamber (Falcon 150mm x 15mm Not TC-treated Bacteriological Petri Dish (cat#:351058) lined with a damp paper towel)
- Life Technologies prolong Diamond Antifade 1 (Cat#: P36965)
- *E. intestinalis* 16S rRNA-specific FISH probe conjugated to Quasar 570 (LGC Biosearch Technologies).  
Oligonucleotide mix: tagtcccagagaatagcatc, tgtcaaaaacactcctagcc, taggcctacaacttacttg, actcacttctcataaccgcc, gactcctttacactcgaaaa, caacactatatagtcctcgc, cccacatactcaagatttct, atccatttcaaacggccatg, cccaaagagcactgtcaaag, gggacccattcaaaacaact
- Hybridization buffer (FISH):  
180 µl 5M NaCl



20 µl 1M Tris pH 7.5

800 µl H<sub>2</sub>O

0.5 µl of 20% SDS

- Wash buffer (FISH)

180 µl 5M NaCl

20 µl 1M Tris pH 7.5

10 µl 5M EDTA

790 µl dH<sub>2</sub>O

0.5 µl of 20% SDS

- DRAQ5 (Novus Biologicals Cat#: NB016658)
- Calcofluor white (Sigma-Aldrich, Cat#: 18909-100ML-F)
- 20% (w/v) Tween-20 solution

### **Genomic DNA Isolation and qPCR assay**

- Proteinase K-Zymolyase (PKZ) buffer

10 mM Tris (pH 7.5)

1 mM EDTA

25 mM NaCl

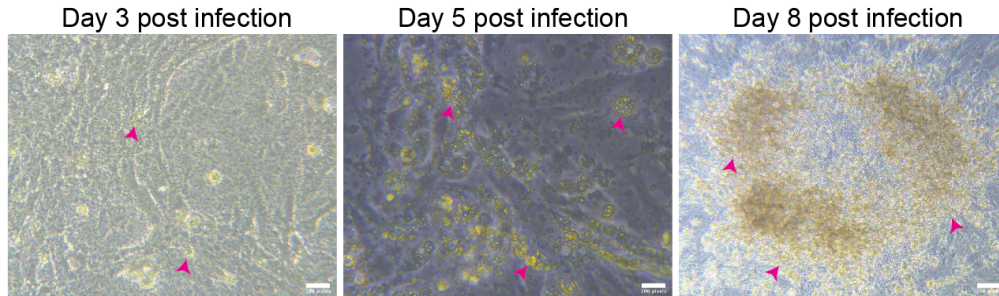
- Proteinase K (Roche 03115879001)
- Zymolyase (Genesee Scientific E1004)
- 2X SYBR Green qPCR Master Mix (APExBIO K1070)
- Nuclease-free water (cat# 221-239-05)
- Primer set (Stock concentration 5 µM): (Primer 1 (GGCGAGGGGTGAAATACGAA)/ Primer 2 (CGCACACTCCACTCCTTGT))

## Section 1: Parasite propagation and host cell lysis

- 1 Seed  $2.1 \times 10^6$  Vero cells in a T-75 tissue culture flask with DMEM:HG supplemented with 10% FBS and 1X Non Essential Amino Acids.
- 2 After 48 hours, change media to DMEM: HG supplemented with 3% FBS and 1X Non Essential Amino Acids. Total volume of media in the flask should be 30 mL.
- 3 Add *E. intestinalis* spores to the Vero cells at a multiplicity of infection (MOI) of 10.
- 4 Replace the media (DMEM: HG supplemented with 3% FBS and 1X Non Essential Amino Acids) every 48 hours over the next 7 days.

### Expected result

As infection proceeds, clusters of infected cells will be visible. At Day 3 post infection, very few clusters will be visible. As infection proceeds, larger infection clusters are visible (Day 5 post infection) until the spores are ready to be harvested (Day 8 post infection).



**Infected Vero cells at 3, 5 and 8 days post infection.** Magenta arrowheads indicate infected cell clusters at 3, 5 and 8 days post infection.

- 5 Using a cell scraper (30 mm), detach infected cells from the base of the flask.
- 6 Transfer the flask contents to a 50 mL conical tube.



- 7 Centrifuge the 50 mL conical tube at 2000 x *g* for 10 min at room temperature (RT).
- 8 Remove and discard the supernatant.
- 9 Resuspend the cell pellet in 12 mL of pre-chilled sterile Phosphate buffered saline (PBS) + 0.1% SDS.

#### Note

Mechanical lysis using a needle and syringe can also be performed, but is only suitable for small culture volumes. We recommend resuspending the cell pellet from step 8 in 12 mL pre-chilled sterile PBS and dividing the suspension equally into two 15 mL conical tubes (6 mL each). Next, using a 3 mL syringe with a 27 gauge needle, draw up and push the sample through the needle to lyse the cells. We recommend passing the sample through the needle 5 times. This should be done for samples in both conical tubes. If mechanical lysis is used, proceed directly to step 15.

- 10 Incubate on ice for 5 minutes.
- 11 Vortex at max speed for 5 seconds.
- 12 Incubate on ice for 5 minutes.
- 13 Vortex at maximum speed for 5 seconds.
- 14 Divide the cell suspension equally into two 15 mL tubes (6 mL per tube).

## Section 2: *Crude spore* purification using Percoll

- 15 Add an equal volume (6 mL) of Percoll to each 15 mL tube containing the cell suspension.



- 16 Mix the contents of the 15 mL tube well by inverting the tube until homogenous, followed by vortexing at maximum speed for 5 seconds.
- 17 Centrifuge at 4000 x *g* for 30 minutes at RT.
- 18 Remove and discard the supernatant.
- 19 Resuspend the spore pellet in 10 mL PBS and transfer to a new 15mL tube. This is wash 1.
- 20 Centrifuge at 4000 x *g* for 10 minutes at RT.
- 21 Remove and discard the supernatant.
- 22 Repeat steps 19-21. This is wash 2.
- 23 Resuspend the pellet in 1 mL of PBS and transfer to a 1.5 mL tube.
- 24 Centrifuge at 4000 x *g* for 5 minutes at RT. This is wash 3.
- 25 Remove and discard the supernatant. Resuspend pellet in 1.5 mL PBS. We refer to this fraction as Crude spores.

### Section 3: Enrichment of germination-competent spores using a discontinuous Percoll gradient

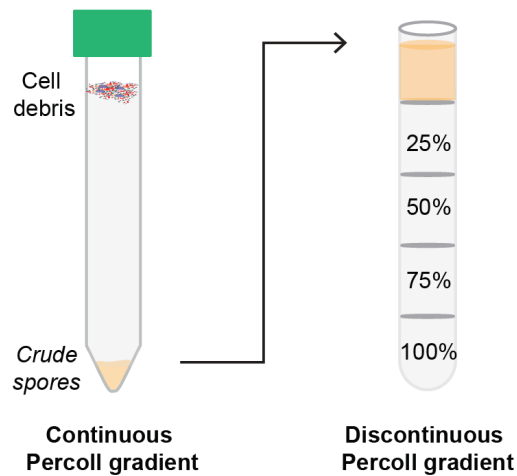
- 26 Prepare Percoll solutions by diluting 100% Percoll in sterile PBS. Prepare the following percentages of Percoll solutions: 100% Percoll; 75% Percoll; 50% Percoll; and 25% Percoll. Mix dilutions by vortexing until homogeneous.
- 27 Prepare a discontinuous Percoll gradient in an ultracentrifuge tube. Pipet 2.5 mL of each solution in the following order into the ultracentrifuge tube: 100% Percoll; 75% Percoll; 50% Percoll; 25% Percoll. Pipet each Percoll solution carefully along the wall of the tube,

taking care not to disturb the layers. After pipetting is complete, all layers should be well defined and clearly visible.

#### Note

We have also carried out steps 27-30 in this protocol in a closed 15 mL conical tube in a floor centrifuge with a Bioflex HC rotor at 7000 x *g* speed, which preserves the sterility of the sample. Individual bands may appear diffuse; to minimize this appearance, reduce the braking speed to 3 Decel.

- 28 Add the resuspended *Crude spore* pellet purified from the continuous Percoll gradient to the top of the discontinuous gradient.

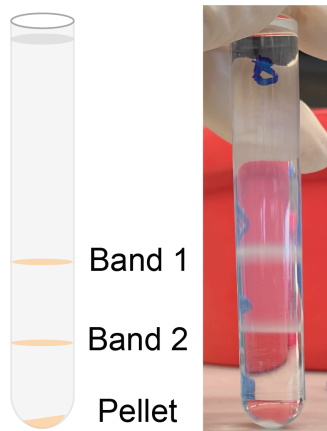


**Schematic of spore purification using a discontinuous Percoll gradient.**

- 29 Place tubes in a swinging bucket rotor.
- 30 Centrifuge at 12,700 x *g* for 30 minutes at RT.
- 31 After centrifugation, the spores will separate into two bands and a pellet. Band 1: Between the 75% and 50% Percoll zones, Band 2: Between the 100% and 75% Percoll zones, Pellet: Bottom of the tube.



### Expected result



**Spore fractions following purification using a discontinuous Percoll gradient.**

- 32 Remove and discard all the contents of the tube, leaving behind ~0.5 mL containing the pellet fraction at the bottom of the tube.

### Note

Spores in Band 1 and Band 2 can also be separated and purified following Step 31. To do this, carefully decant each fraction into a new ultracentrifuge tube containing 9 mL of PBS and follow Steps 34-39.

- 33 Resuspend the pellet in 1 mL PBS and transfer to a new ultracentrifuge tube containing 9 mL PBS and mix by pipetting.
- 34 Centrifuge at 12,700 x g for 10 minutes at RT. This is wash 1.
- 35 Discard the supernatant and resuspend the pellet in 1 mL PBS.
- 36 Transfer the resuspended pellet to a 1.5 mL tube.



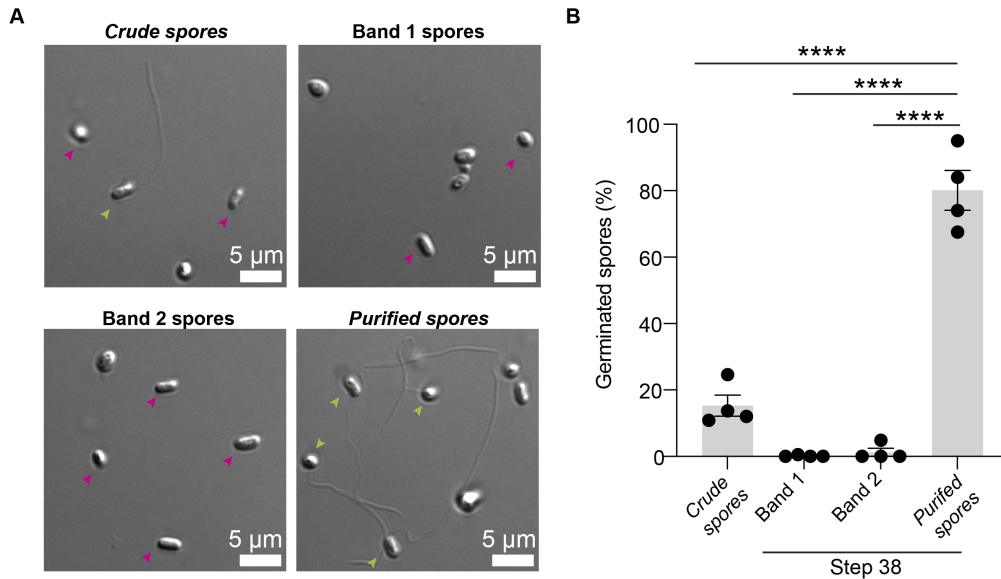
- 37 Centrifuge at 4000 x *g* for 5 minutes. This is wash 2.
- 38 Discard the supernatant and resuspend the pellet in fresh 1 mL PBS. We refer to this fraction as *Purified spores*.
- 39 Store the tube from step 38 at 4 °C until further use. For *E. intestinalis* spore preparations, we typically observe minimal decline in germination rates over the course of several weeks. For other microsporidian species, this may vary, and the optimal storage solution may also vary.

#### Section 4: Measurement of germination rates

- 40 Pipet 2 µl of spores purified in Step 38 (*Purified spores*, Band 1 and Band 2 spores) and *Crude spores* into 1.5 mL tubes.
- 41 Add 10 µl of germination buffer to each tube and mix by pipetting gently.
- 42 Incubate at 37 °C for 15 minutes.
- 43 Apply 4 µl of the germinated spore mixtures to poly-lysine coated slides. Cover with a square coverslip and image samples on a light microscope with a 60X or 63X objective under brightfield illumination. Polar tube germination can be visualized by phase contrast microscopy or DIC microscopy.
- 44 To quantify germination rates, count at least 100 spores across multiple fields of view.

## Expected result

Germination rates for *Purified spores* are significantly higher than germination rates for *Crude spores*, Band 1 and Band 2 spores. Germination rates for *Crude spores* are typically <25%, while germination rates for *Purified spores* can approach 95%. Germination rates for Band 1 and Band 2 spores are negligible, suggesting that these may be immature spores or otherwise defective parasites.



**Germination rates of *E. intestinalis* parasites from different stages of purification.** **A.** Representative images for samples after *Crude spore* purification (Step 25), and major fractions from gradient enrichment of *Purified spores* (Step 38). Magenta arrowheads indicate ungerminated spores, green arrowheads indicate germinated spores. **B.** Quantification of germination rates from micrographs. Mean  $\pm$  SD are from four biological replicates for (B).  $n = 100$  parasites per experiment. \*\*\*\* $p < 0.0001$ , calculated from an unpaired Student's  $t$ -test for (B).

## Note

Germination efficiency was recorded on a Zeiss Axio Observer at 63X magnification with DIC and under brightfield illumination. A minimum of 100 spores were counted per germination condition.

## Section 5: Measurement of infectivity in Vero cells

- 45 Seed  $2 \times 10^5$  Vero cells per well in a 6 well tissue culture plate. Cells should be cultured in DMEM:HG supplemented with 3% FBS and 1X Non Essential Amino Acids. Each well should have five 12 mm coverslips.

### Note

The number of wells being seeded will depend on the number of conditions being tested. In this case we seeded four wells to measure the infectivity of *Crude spores*, *Purified spores*, Band 1 and Band 2 spores.

- 46 To infect cells, add *Crude spores*, *Purified spores*, Band 1 and Band 2 spores at an MOI of 30.
- 47 After 24 hours, gently aspirate media without disturbing the cells, and wash cells with 2 mL PBS.
- 48 Add 2 mL of fixative (4% PFA in PBS-T (0.1% Tween 20)) and incubate at RT for 45 minutes.
- 49 Aspirate fixative and wash cells with 2 mL of PBS-T (0.1% Tween 20).
- 50 Transfer coverslips to a humidity chamber.
- 51 Wash coverslips with 500  $\mu$ L FISH hybridization buffer.
- 52 Add 50  $\mu$ L FISH staining solution (125 nM FISH probe in hybridization buffer).
- 53 Incubate at 37 °C overnight. Protect samples from light by covering the humidity chamber with aluminium foil.
- 54 Aspirate FISH staining solution.



55 Add 500 µl FISH wash buffer to each coverslip and incubate at 37 °C for 30 minutes. Protect samples from light by covering the humidity chamber with aluminium foil. This is wash step 1.

56 Aspirate FISH wash buffer.

57 Add 500 µl FISH wash buffer to each coverslip and incubate at 37 °C for 30 minutes. Protect samples from light by covering the humidity chamber with aluminium foil. This is wash step 2.

#### Note

We have utilized a FISH probe to clearly identify infection at earlier stages of parasite development. However, infectivity can be assessed using a DNA dye (DRAQ5) and chitin dye (Calcofluor white) alone. If a FISH probe is not readily available, skip steps 51-57 and proceed directly to step 58.

58 Add 100 µl of dye staining solution (Calcofluor white (2 µg/mL) and DRAQ5 (1:200) in PBS-T (0.1% Tween-20)).

59 Incubate at RT for 30 minutes. Protect samples from light by covering the humidity chamber with aluminium foil.

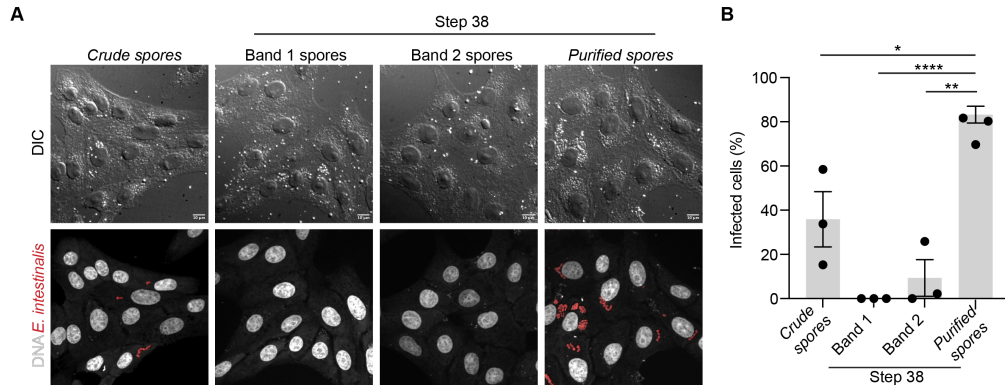
60 Aspirate dye staining solution.

61 Add 500 µl PBS-T (0.1% Tween-20) to each coverslip and incubate for 5 minutes at RT. Aspirate PBS-T solution and repeat step 61 two more times. Protect samples from light by covering the humidity chamber with aluminium foil.

62 Mount coverslips on microscope slides with Prolong Diamond antifade (ThermoFisher Scientific) and image samples on a fluorescence microscope.

## Expected result

We assessed the infectivity of *Crude spores*, *Purified spores*, Band 1 and Band 2 spores in Vero cells. We found that a high percentage of cells exposed to the *Purified spores* were infected (87%), while cells exposed to Band 1 and Band 2 spores remained largely uninfected: 0% for Band 1, and 9% for Band 2.



**Infectivity of *E. intestinalis* parasites collected from different stages of purification.** **A.** Example micrographs showing Vero cells exposed to *Crude spores*, *Purified spores*, Band 1 and Band 2 spores. **B.** Quantification of infected cells resulting from the conditions shown in (A). Mean  $\pm$  SD are from three biological replicates for (B).  $n = 100$  cells per experiment. \*\*\*\* $p < 0.0001$ , \*\* $p < 0.01$ , \* $p < 0.05$ , calculated from an unpaired Student's t-test for (B).

## Section 6.1: Measuring genomic DNA content of *E. intestinalis* parasites: Genomic DNA isolation

- 63 Resuspend 1  $\mu\text{L}$  of Band 1, Band 2 and *Purified spores* in 100  $\mu\text{L}$  of Proteinase K-Zymolyase (PKZ) buffer.

### Note

Spore concentrations from each fraction were normalized. Use  $2.7 \times 10^4$  spores for each reaction.

- 64 Add 2  $\mu\text{L}$  of Proteinase K and incubate at 50  $^{\circ}\text{C}$  for 60 minutes.



- 65 Denature samples by boiling at 95 °C for 5 minutes.
- 66 Add 2 µL of Zymolyase to the samples and incubate at 30 °C for 60 minutes. This will digest the spore wall.
- 67 Denature samples by boiling at 95 °C for 5 minutes.

## Section 6.2: Measuring genomic DNA content of *E. intestinalis* parasites: Quantitative PCR (qPCR) of genomic DNA

- 68 Prepare a qPCR reaction mixture to a total volume of 10 µL, consisting of 5 µL of 2X SYBR Green qPCR Master Mix, 3 µL of nuclease-free water, 1 µL of a specific primer set and 1 µL of the isolated genomic DNA sample.

### Note

Our primer set is designed for *E. intestinalis* 16S rRNA. However, these primers also bind conserved regions in *E. hellem* and *E. cuniculi*.

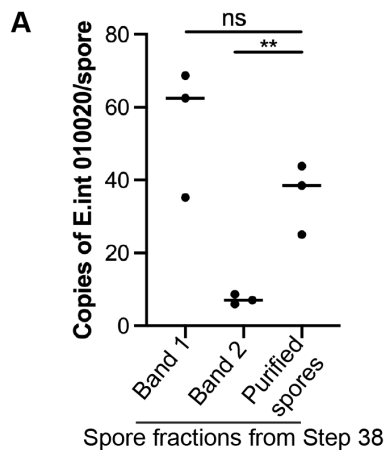
- 69 Initiate the following qPCR: a denaturation step at 95 °C for 10 minutes, followed by 45 cycles of PCR amplification. Each cycle consists of denaturation at 95 °C for 15 seconds, annealing at 60 °C for 10 seconds, and extension at 72 °C for 30 seconds.
- 70 Post-amplification, a melting curve analysis is performed. The PCR product is first re-annealed by heating to 95 °C (at 4.8 °C/s, hold 15 s) then cooling to 60 °C (at 2.5 °C/s, hold 1 s). The melt curve analysis is performed by heating from 60 °C to 95 °C at a rate of 0.06 °C/s, with acquisitions performed at every 0.1 °C. Product identity was confirmed by Sanger sequencing initially, and subsequently by comparison of melting profiles.

### Note

To estimate the concentration of genomic DNA from Cq values, a standard curve from (100 pM to 10 aM) can be prepared from amplifying a larger region (1.2 kb) of the 16S rRNA gene using primers GATTGACGGACGGCTCAGTG / ACTGATCCTGCTGCTGGTTC using a high fidelity polymerase and normalizing by absorbance at 260 nm.

## Expected result

qPCR analysis of the genomic content purified from all three fractions shows that Band 1 and *Purified spores* likely contain the full diploid *E. intestinalis* genome: ~60 and 40 copies of the rRNA gene per cell in band 1 and *Purified spores*, respectively, similar to the expected copy number of 44. However, Band 2 spores appeared to contain ~10 fold less genomic DNA, approximately 5 copies of the rRNA gene per cell. These differences could represent real differences in the genomic content of Band 2 spores or differences in composition of the spore wall in parasites from these different fractions that affects lysis.



**Characterization of genomic DNA content of *E. intestinalis* parasites. A.** Quantification of genomic DNA content in Band 1, Band 2 and *Purified spores* by qPCR. Mean  $\pm$  SD are from three biological replicates for (A) \*\* $p < 0.01$ , ns, not significant ( $p > 0.05$ ) calculated from an unpaired Student's t-test.



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