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Phenol Chloroform DNA Extraction Optimized for HMW gDNA from *Coccidioides* spp.

mitchellbryant [1], Heather Mead[1], Bridget M. Barker[1], Austin Blackmon[1]

[1]Pathogen and Microbiome Institute

Northern Arizona Center for Valley Fever Research

mitchellbryant

Coccidioides spp. are fungal pathogens present in the arid soil of the American Southwest.This group of pathogenic fungi are responsible for coccidioidomycosis, a disease commonly known as Valley fever.Although *Coccidioides* infects thousands of people each year, the organism is rather poorly characterized, and little is known about mechanisms of infection.Currently, there is no effective vaccine or treatment against coccidioidomycosis.Understanding the function of an organism's genes is essential to developing vaccines and treatments because each gene may reveal a vulnerability in the organism which can be targeted with new treatments.Molecular biology is one of the primary tools used to gain an enhanced understanding of how gene function relates to pathogenesis and virulence.Many molecular techniques including DNA sequencing and Southern blotting, require input of large quantities of in-tact genomic DNA.However, *Coccidioides spp.* cells are notoriously hard to disrupt without vigorous mechanical force which is likely to fragment genomic DNA.This protocol details a phenol chloroform DNA extraction optimized to obtain high yields of unfragmented genomic DNA from *Coccidioides spp.* using liquid nitrogen grinding to disrupt the cell wall without damaging the DNA inside. Although conventional bead beating and homogenization methods offer a high level of workflow convenience, they are incapable of extracting a large amount of unsheared genomic DNA.The protocol outlined here will provide a detailed workflow for extraction of unfragmented genomic DNA from *Coccidioides spp.* which is essential for downstream molecular genetics applications such as DNA sequencing and Southern blotting.

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Fungal Lysis Buffer

1 Lysis Buffer (prepare in sterile 50 ml falcon tube while in hood)
• 30 ml Mol. Biol grade water
• 5 ml 3M NaOAc at pH 6.0
• 2.5 ml 10% SDS
• 10 ml 0.5 M EDTA at pH 8.0
• 2.5 ml 1M Tris HCl at pH 7.5
• Prepare stock in small batches with mol. bio. grade water or purchase premade stock solns
TOTAL Volume: 50 ml
Before hand
Check lysis buffer to see if needs to be heated (precipitate has formed) and turn on water bath to 65C. You should also add DTT or β-mercaptoethanol to lysis buffer just before use. I use 10uL of β-ME per 10mL of buffer and make it in small batches. Prepare buffer ahead of time (in DNA free zone) and place 1 ml of lysis buffer in each bead tube (e.g. sterilized 1mm BioSpec glass beads and sterile screw cap 2 ml tubes) needed for extraction (if lysis is still not working well, MP Biomedicals has a few other prepared tubes, lysis matrix D seems to work well for RNA too).

2 Move all supplies into hood to begin extraction. Before scraping plates, take 25:24:1 phenol:chloro:isoamyl tubes out of the –20 degree freezer (or 4C) in order to defrost. Chloroform and alcohol can be left in freezer- works better if cold.

3 If using N2:
1 day before the extraction, scrape plates with a #10 scalpel and place material in a conical. 1 plate is good for 2 or 3 bead tubes. When all plates are scraped and placed in conical, flash freeze mycelia by putting in the -80C freezer overnight.
Alternatively, you can drop the conical into liquid nitrogen for 5-10 minutes before grinding to flash freeze immediately before extraction

4 If bead beating:
Add mycelia directly to bead tube containing lysis buffer on the day of the extraction.

Bead beat for a total of 60-120 seconds using a speed of 6m/s. Rest periods should be used when bead beating for extended periods to prevent the sample from overheating. Usually, 2 minutes of rest per 30 second cycle is sufficient.

Extraction

5 A. Scrape plate with a sterile scalpel (#10 blade/ or any similar shape), making sure to avoid as much media as possible. Change scalpel between plates/strains. This is good for PCR.

6 Place fungal sample into bead tube with 1 ml of lysis buffer.

7 Heat kill @80C for 30 min. This should denature DNAse. This is optional if the rest of the extraction will be occurring in a vented BSC.

8 Homogenize at high speed for 30 seconds at 6m/s. Only do 1 cycle for a total of 30 seconds of bead beating. Skip this if DNA shearing is a concern.

9 Add 50uL of RNAse A to each sample, incubate @37C for

10 minutes. Be sure to mix well via inversion.

11 Spin down in centrifuge for two minutes at 10,000g's.

12 Pipet off approximately 650 µl of supernatant and place into a new 1.5 ml tube.

13 While working in ducted cabinet, add equal volume of phenol:chloroform:isoamyl (25:24:1) pH 8 into sample (If extracted 650 µl previously, add 650 µl).

14 Mix well by inversion for a full ten minutes (DO NOT VORTEX). Use a nutator or slow rotation.

15 Centrifuge samples for ten minutes at full speed (14,000 g's)

16 Pipet off the top layer (approximately 600 µl) and transfer into a new 1.5 ml tube. BE VERY CAREFUL AT THIS STEP TO AVOID WHITE, LOOSE PHASE LAYER! (Hint: take tube out of centrifuge in same direction as pellet in order to avoid disturbing layer. It is better to sacrifice some of the top layer than to risk taking any of the white layer). Place waste into phenol waste bottle inside the fume hood or ducted BSC. This is where you can also consider using phase-lock tubes, if you have issues with getting phenol contamination in your final sample.

17 If sample seems dirty (i.e. accidentally getting some of the phase layer into sample), do another phenol:choloroform:isoamyl wash (again, adding equal volume, mixing by inversion for ten minutes, and centrifuging for ten minutes at full speed). This will greatly reduce final yield of DNA.

18 Add equal volume of choloroform:isoamyl (24:1) to sample and mix by inversion for two-ten minutes (If extracted 600 µl previously, add 600 µl).

19 Centrifuge in 4 degree Celsius fridge for ten minutes at maximum speed.

20 Pipet off top layer (500 µl), making sure to avoid phase layer. Transfer this into a new 1.5 ml tube.

Ethanol Precipitation

21 Add 2/3rds volume of sample (333 µl) of COLD isopropanol and mix by inversion. Incubate tube on ice for approximately 10-20 minutes. (IF NECESSARY, THESE SAMPLES MAY BE KEPT OVERNIGHT AT 4C AT THIS STAGE IN THE EXTRACTION. Be careful not to leave samples too long as salt precipitate will form after about 12 hours).

22 Centrifuge in 4 degree Celsius fridge for ten minutes at maximum speed.

23 Decant isopropanol into waste container. Add approximately 500 µl of COLD 70% ethanol. Try to loosen pellet by flicking the tube.

24 Centrifuge in 4 degree Celsius centrifuge for five minutes at maximum speed.

25 Decant ethanol into waste container (Be careful to avoid losing pellet because it is potentially loose). Add approximately 300 µl of COLD 95% ethanol.

26 Centrifuge in 4 degree Celsius fridge for five minutes at maximum speed.

27 Pour off ethanol and invert tubes on paper towel in order to allow excess ethanol to evaporate off (pellet will be very loose). Should only take ten to twenty minutes to dry.

28 Depending on size of pellet, add ddd nuclease free water (or TE, etc) to sample. If small pellet, only add 50 µl; large pellet, add 200 µl. (Just add 50uL)

29 Let sit in 4C fridge overnight

Quantification

30 The next morning, make sure to flick tubes to mix and spin down for 2s. Check concentration: 2 µl of DNA, 8 µl water, 2 µl loading dye on 1% gel. Run at 70 V for approximately 2 hours.

31 Solid band = good to go. RNA = treat with Rnase. Smear = sheared DNA (may be OK)

32 Will need to dilute sample, depending on amount of DNA present. (can do series dilution for basic PCR, 1:10, 1:100 and 1:1000, to see what works best.)

33 OR: Quantify DNA for other applications. 1-2 ug of DNA will be best for WGS.

Cross contamination notes:

34 The main consideration when prepping DNA for PCR is the problem of contamination.We are analyzing small variations among isolates, so we must be sure to not cross-contaminate.If possible, do not prep your DNA extractions of different isolates at the same time.Rather, it

would be useful to prep multiple DNA preps (say 4 to 6 reps for each patient strain) so you have large quantities of DNA to use and share.Frequently wipe down benches and equipment with light bleach solution and 70% EtOH. Change gloves frequently.