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Quantitation of Cellular Components in *Cryptococcus neoformans* for System Biology Analysis

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Abstract

Methods and procedures in molecular biology used to study fungal pathogenesis have significantly improved during the last decade. In this chapter, we provide step-by-step procedures for performing genetics and biochemical studies in the human pathogenic fungal microorganism *Cryptococcus neoformans* (*Cn*). These methods are employed for studying the pathobiology of *Cn* and for experimental validation of theoretical models of fungal pathogenicity.

Keywords

Cryptococcus neoformans; Fungal infection; Genetic; Molecular biology; Biochemistry; Sphingolipid; DNA; RNA; Protein; Lipids

1. Introduction

Cryptococcus neoformans (*Cn*) is the causative agent of cryptococcosis, a fungal disease acquired by inhalation of infectious particles from the environment. Cryptococcosis is a relatively frequent disease in immunocompromised subjects and in certain regions of the world such as sub-Saharan Africa in which the estimated number of deaths associated with cryptococcal disease, at half a million per year, is comparable with the number attributed to tuberculosis (1, 2). In the USA, the prevalence of cryptococcosis in HIV positive patients is 5–10%, which is approximately the same as that for meningococcal meningitis (3). Emerging groups at risk include patients suffering from chronic lymphatic leukemia, Hodgkin's disease, chronic myelogenous leukemia, and multiple myeloma (4). The median overall survival of patients with lymphoproliferative disorders affected by cryptococcosis is 2 months, which is significantly shorter than the 9-month median survival of an AIDS patient with cryptococcosis (5). Cryptococcosis is also associated with organ transplantation (6, 7), and was documented in 2.8% of organ transplant recipients with an overall death rate of 42% (8). Some cases of cryptococcosis occur in patients with apparently normal immune function (9–12).

One area of investigation that has significantly improved in the last 2 decades is the molecular biology of this microorganism. The development of molecular epidemiology and phylogeny and molecular technology for clinical diagnosis have significantly helped the clinicians to better manage this life-threatening disease. However, it was the advent of genetics and biochemistry of this microorganism that allowed basic and clinical investigators

to address mechanistic questions and study the pathophysiology of cryptococcosis. This was (and still is) an essential step to define fungal features and characteristics necessary for the organism to cause disease (13). These fungal factors can then be exploited for the understanding of fungal pathogenicity and fungal interaction with the host cells and, ultimately, and for the development of new therapeutic strategies. With the rise of its importance as a human pathogen, there has been a concurrent rise in the ability to molecularly study its physiopathology.

In Chapter 9, we provide a mathematical model of the regulation of melanin production by the sphingolipid pathway. In particular, we show that a specific enzyme of the sphingolipid pathway, inositol phosphoryl ceramide synthase 1 (Ipc1), regulates melanin formation in *Cn* through the production of diacylglycerol (DAG) and the consequent activation of protein kinase C 1 (Pkc1). Thus, the downregulation or/and deletion of *IPC1* or/and *PKC1* genes by homologous recombination should produce mutant strains that make less or no melanin. We would expect IPC and DAG lipid measurements to be decreased in the mutant in which Ipc1 is downregulated. Also in this mutant, Pkc1 enzymatic activity should be decreased. This experimental approach is necessary to validate the changes in the network behavior simulated by the mathematical model. Therefore, the deletion of the gene of interest by homologous recombination and confirmation by Southern or/and Northern blot of the isolated genomic DNA or total RNA, respectively, and the analysis of protein and lipid levels regulated by those genes are useful methods to refine and validate the mathematical model.

In this chapter, we provide basic molecular methods for performing genetics and biochemistry studies in *Cn*. These methods can be employed to validate hypotheses and theoretical models of *Cn* pathogenicity or simply to study the pathobiology of this important human pathogen.

2. Materials

2.1. DNA Isolation from Cryptococcus neoformans

1 Yeast Peptone Dextrose (YPD) agar plates and YPD broth (see Note 1).

2 Sterile PBS 1×.

3 1 M Tris–HCl pH 7.5.

4 0.5 M, EDTA pH 8.0.

5 5 M NaCl.

6 100% Triton X-100.

7 20% SDS.

8 TENTS: 10 mM Tris–HCl, pH 7.5, 1 mM EDTA, pH 8.0, 100 mM NaCl, 2% Triton X-100, 1% SDS.

[1]1. All solutions should be prepared in water which has a resistivity of 18.3 MΩ-cm and total organic content of less than 5 ppb. This water is referred to double distilled water in this text.

9 Acid washed 0.425–600 μm glass beads (SIGMA).

10 Phenol:chloroform:isoamyl alchohol =25:24:1 (SIGMA).

11 3 M Sodium acetate (NaOAC).

12 TE buffer, pH 8.0, sterile.

2.2. Biolistic Delivery in Cryptococcus neoformans

1 YPD agar + 1 M Sorbitol plates.

2 YPD agar + Nourseothricin/Hygromycin (100 μg/ml) plates.

3 0.6 μm Gold beads (BIORAD).

4 MacroCarriers (BIORAD).

5 Rupture Disks, 1,350 psi, (BIORAD).

6 Stopping Screens (BIORAD).

7 2.5 M $CaCl_2$ sterile.

8 1.0 M spermidine (filter sterilize) (SIGMA), can be stored at −20°C.

9 100% Ethanol.

10 Isopropanol.

11 This instruction assumes the use of PDS-1000/He Biolistic Particle Delivery System from BIORAD.

2.3. Southern Hybridization of DNA Extracted from Cryptococcus neoformans

1 Denaturing solution: 1.5 M NaCl, 0.5 M NaOH.

2 Neutralizing Solution: 1 M Tris–HCl pH 8.0, 1.5 M NaCl.

3 Nytran SPC (0.45 μm Nylon Transfer Membrane) (Whatman).

4 Whatman 3MM Blotting Paper.

5 Paper towels, preferably single fold.

6 20 × SSC: 175.3 g of NaCl, 88.2 g of sodium citrate in 800 ml of double distilled water. Adjust pH with NaOH pellets and adjust the total volume to 1 L. Autoclave. Can be stored at room temperature.

7 20 × SSPE : 175.3 g of NaCl, 27.6 g of $NaH_2PO_4 \cdot H_2O$, 7.4 g EDTA in 800 ml of double distilled water. Adjust the pH with NaOH pellets to 7.4. Final volume made up to 1 L. Autoclave. Can be stored at room temperature.

8 20% SDS.

9 Nonfat dry milk.

10 Prehybridizing solution: 10 ml 20× SSPE, 10 ml 20% SDS, 2 ml 10% nonfat dry milk in a total volume of 40 ml. Can be stored at 4°C with 0.02% sodium azide for 2–3 days.

11 Random Primers DNA labeling system kit (Invitrogen).

12 ^{32}P dCTP (Perkin Elmer).

13 Microspin G-25 column (Amersham Biosciences).

14 Sterile TE buffer, pH 8.0.

2.4. Highly Pure Total RNA Isolation from Cryptococcus neoformans (e.g., for Microarray Studies)

1 YPD agar plate and YPD broth.

2 Phosphate Buffered Saline (PBS) 1× sterile.

3 Tri Reagent (Molecular Research Centre).

4 BAN as a phase separation reagent, molecular biology grade (Molecular Research Centre).

5 RNeasy Mini Kit and RNeasy MinElute Cleanup Kit (Qiagen).

6 RNase Zap for removing RNase contamination from external surface (Ambion).

7 RNase/DNase free plastic wares.

2.5. Protein Extraction from Cryptococcus neoformans

1 YPD media and agar plates (made from YPD 50 g/L and agar 20 g/L).

2 Buffer for *Cn* cell lysis: 1 ml 1 M Tris–HCl pH 8, 9 ml H_2O, 1.5 ml glycerol (13% v/v), 10 µl CLAP: chymostatin, leupeptin, antipain, and pepstatin A (each at 10 mg/ml in DMSO and stored at −20°C), and 20 µl 100 mM solution phenylmethylsulfonyl fluoride (PMSF) in isopropanol.

3 Glass beads, acid washed, 425 µm (30–40 US sieve) (Sigma).

2.6. Lipid Extraction

1 Mandala lipid extraction buffer: 150 ml ethanol, 150 ml distilled water, 50 ml diethyl ether, 10 ml pyridine, and 180 µl 14.2 N ammonium hydroxide.

2 Use glass tubes for all extraction steps (VWR) fit best in the ThermoSavant SPD2010 SpeedVac system we use).

3 Waters Sep-Pak Classic Silica cartridges (WAT 051900, 690 mg) for analytical scale or WAT036930 200 cc, 5 g cartridges for semipreparative scale lipid isolation and purification.

4 10″ × 10″ glass tank for thin layer chromatography (TLC).

5 3MM Whatman chromatography paper (Fisher).

6 TLC chromatography plates (Fisher M5628-5 or 05-713-329 depending on analytical or semipreparative purposes).

7 Soy glucosylceramide standard (Avanti Polar Lipids) made up to 3.5 mM (2.5 μg/μl) in chloroform/methanol (2:1).

8 Prepare 70% H_2SO_4 by adding 14 ml H_2SO_4 slowly to 6 ml water on ice, with mixing. Add 40 mg resorcinol to 20 ml 70% H_2SO_4. Stir well at room temperature with a magnetic stirrer bar. Pour solution into a glass TLC sprayer.

2.7. In Vitro Enzyme Activity Assay

1 NBD-C6-ceramide (Avanti Polar Lipids).

2 Lysis buffer: 25 mM Tris–HCl pH 7.4, 5 mM EDTA, 1 mM PMSF, and CLAP: chymostatin, leupeptin, antipain, and pepstatin A (each at 10 mg/ml in DMSO and stored at −20°C).

3 Silica gel 60 TLC plates (EM Sciences, Fisher).

2.8. Mass Spectrometry of Lipids

1 Commercially available synthetic lipid standards.

3. Methods

3.1. DNA Isolation (14, 15)

1 Inoculate a 10–15 ml YPD broth with a single colony from a fresh YPD agar plate and grow them for 20–24 h at 30°C with constant shaking. Pellet cells from this culture at 1,200 × *g* 4°C for 10 min.

2 Wash with sterile PBS 1× twice and resuspend in 1 ml of sterile double distilled water and transfer to a 2-ml screw cap tube (see Note 2).

3 Pellet cells in a Microcentrifuge for 30 s at 1,200 × *g* at room temperature.

4 Pour off the water; add 0.5 ml of TENTS and vortex at 7–8 speed for three times, 45 s each. This step assumes the use of Vortex Genie 2 from Scientific Industries.

5 Add two cups (1 cup =400–500 μl) of acid washed 0.45 μm glass beads (see Note 3) and 0.5 ml phenol–chloroform–Isoamyl alcohol (see Note 4).

6 This step assumes the uses of Bead Beater 16 from Scientific Industries. Tubes were vortexed/homogenized in a Bead Beater three times, 45 s each, with a gap of 45 s on ice, in between each cycle (see Note 5).

[2]Each tube should contain ~100 μl of cell pellet.
[3]A cup was made by cutting out from the bottom till 0.5 ml marking of a 1.5-Eppendorf tube. Drive 23 gauge BD needle into the cup through the upper part to make a makeshift handle.
[4]Tubes should be capped properly and the mouth should be wiped with kimwipes ensuring that tubes seal properly before vortexing.
[5]The Vortex should have the single unit assembly during vortexing.

7 After homogenizing (or lysing) of the cells, centrifuge the cells for 10 min at 8,000 × g at room temperature to separate the cell debris and unbroken cells.

8 Remove the upper aqueous phase which now contains the DNA, to a fresh 1.5-ml Eppendorf and add 1 ml of ethanol 100% and keep at −20°C overnight (see Note 6).

9 Centrifuge the tube at 8,000 × g for 30 min at 4°C. Remove the supernatant, dissolve the pellet in 200 μl of TE containing RNase A at a concentration of 100 μg/ml and then incubate at 37°C for 20 min.

10 After incubation, add equal volume of phenol–chloroform–isoamyl alcohol and mix gently by inverting 4–6 times. Centrifuge at 8,000 × g, 10 min, 4°C. Remove the aqueous phase and repeat the step with the aqueous phase.

11 Add 20 μl of 3M NaOAC and 400 μl of ethanol (100%) to the final aqueous phase and incubate at −20°C for 30–60 min for complete precipitation.

12 After precipitation, centrifuge the tube at 8,000 × g at 4°C for 5 min.

13 Wash the DNA pellet twice with 200 μl of ice-cold 70% ethanol and air dry the pellet (see Note 7).

14 Dissolve the pellet in 30–50 μl of sterile TE gently and store at −20°C.

3.2. Biolistic Delivery in Cryptococcus neoformans (14, 16)

1 Spin down 19–20 h grown culture (15 ml) of the recipient strain and throw off 12 ml of the supernatant (see Note 8).

2 Plate 200–250 μl of this cell suspension on prewarmed YPD agar + 1 M sorbitol plates and let them dry for 4–5 h at 30°C (see Note 9). This should include a “non-shot” control plate.

3 During this time of incubation, prepare the shot. For preparing a stock of Gold Beads – 60 mg/ml, 30 mg was weighed out and dissolved in 100% ethanol, vortexed vigorously for 3 min, incubated at room temperature for 15 min and spun for 1 min. Discard the supernatant and suspend the gold beads in 1 ml of sterile water. Incubate or allow the particles to settle down, pellet and discard the supernatant. Add 500 μl of 50% Glycerol to make a final concentration of 60 mg/ml. This stock can be stored in 4°C.

4 Each Shot should be prepared as follows in the same sequence:

10 μl of 60 mg/ml of gold beads

[6]The volume of ethanol should be 2–2.5 times the volume of the aqueous phase. Incubating at −20°C at 2 h can also be done, however, the yield may be less.
[7]The tube containing the DNA pellet can be covered by Para film, punctured and kept at 4°C to let the ethanol dry off. However, it should not be too much dried.
[8]200–250 μl was to be used from this cell suspension, so this would suffice for 15–12 plates for biolistic delivery. If more number of shots is desired, the culture volume should increase proportionately as during shooting the recipient cell density should be high.
[9]The cells should be spread in a monolayer over the plate. To do this, spread the cell suspension with a sterile glass hockey stick in a single direction.

1 μl of ≥1 μg/μl of DNA

10 μl of 2.5 M $CaCl_2$

2 μl of 1.0 M Spermidine

Vortex the mix for 3–5 min and let it settle for 5 min at room temperature. Spin for 20 s and take off the supernatant. Wash the Bead-DNA mix once with 500 μl of 100% ethanol by vortexing and spin down the Bead-DNA. Throw off the supernatant. Finally, resuspend the Bead-DNA in 25 μl of 100% ethanol (see Note 10).

5 The Macrocarriers should be prepared inside a Laminar Hood to prevent contamination. Dip the macrocarriers (one for each shot) in 100% ethanol. Blot off the excess liquid on a sterile wiper and keep in a sterile Petri dish until completely dry.

6 Vortex the Bead-DNA well so that the beads are uniformly coated with the DNA (see Note 11). Spread 10 μl of this mix, first onto the center of the macrocarrier then working outward, within 5mmto the edge in a slow circular motion. Let it dry. If there is any extra Bead-DNA, it can be added to each macrocarrier in the same fashion (see Note 12).

7 The machine (PDS-1000/He Biolistic Particle Delivery System) should be sterilized with 70% ethanol and dried before shooting. The chamber should be kept closed as much as possible. Open the Helium tank pressure valve and set the pressure regulator at 1,800–2,100 psi.

8 Soak the rupture disk, 1,350 psi in isopropanol, place in the retaining cap and screw the unit onto the gas acceleration tube of the machine with the retaining cap torque wrench (see Note 13).

9 Unscrew the macrocarrier cover lid and place a stopping screen on the stopping screen support. Place the macrocarrier on top (Bead-DNA side up) of the macrocarrier holder, invert and place on the fixed nest. The dried microcarriers should face toward the stopping screen. Screw the macrocarrier cover lid to the assembly until tightened and place this in the top slot inside the chamber.

10 Place the target shelf on the second to bottom shelf (see Note 14). Place the YPD agar + 1Msorbitol Petri dishes with cells, on this shelf without the lid on.

11 Close the chamber and set the vacuum switch at “VAC” position till the desired vacuum of 28.5–29″ is reached. Hold the vacuum chamber at this level of

[10]10–15 μl extra ethanol was added to compensate for evaporation. It was always wise to include at least two extra shot when preparing for the Bead-DNA.
[11]Spread Bead-DNA mix immediately as they have a tendency to settle down. Best is to spread from a continuously vortexed mix.
[12]The macrocarrier should be used for shooting within 1–2 h of its preparation.
[13]The rupture disk should not be kept for more than 30–60 s in the isopropanol and excess liquid should be blotted off as this may cause delamination. The rupture disk should also be wet while being loaded as the liquid reduces failure rate of the rupture disk. The retaining cap should be clean for any residual rupture disk part from previous shooting as this may cause rupture of the disk at a wrong pressure and thereby no delivery of the DNA into the Cryptococcal cells.
[14]This distance is the best for delivering DNA into Cryptococcal cells.

vacuum by quickly pressing the switch to “HOLD” position and press the “FIRE” switch to bombard the sample into the plate until the rupture disk pops. Vent the chamber and immediately cover the Petri dish with lid and remove it from the chamber.

12 Repeat the shooting until all the macrocarriers coated with Bead-DNA were utilized. All the parts should be cleaned and surface sterilized with 70% ethanol between two different DNA samples.

13 Incubate the “shot” along with a “non-shot control” plates for 2 h at 30°C (see Note 15).

14 Label Falcon 2054 tubes, one for each of the shot and non-shot plates.

15 Aliquot 1 ml of prewarmed YPD broth onto each plate. Rub the liquid broth across the whole surface of the plate with sterile hockey stick and scrape off the cells. Tilt the plate and pipette the liquid into the labeled Falcon tubes.

16 Plate 200–250 μl of the scraped liquid and spread uniformly onto prewarmed YPD Nourseothricin/Hygromycin plates. Incubate the plates at 30°C for several days.

3.3. Southern Hybridization (17)

1 After taking picture of the Gel (see Note 16), denature in the Denaturing Solution (use fresh) for 1 h at room temperature with constant shaking.

2 Neutralize the gel in Neutralizing Solution for 1–2 h.

3 Wash the gel with double distilled water.

4 Wet the membrane and the 3MM Whatman paper in 2× SSC until complete wet and assemble the transfer. Transfer overnight at room temperature or at least for 16–18 h.

5 Before removing the gel, mark with pencil the wells on the membrane (see Note 17). Keep the membrane on a filter paper presoaked with 6× SSC at room temperature and semidry. Auto cross-link for 1 min at 1,200 (μJ × 100; this instruction assumes the use of UV Stratalinker 1800 from Stratagene). The membrane, if not set for hybridization can be stored at 4°C for 2–3 days in a sealed bag.

6 Prehybridize the membrane in prehybridizing solution for 1–2 h at 65°C.

7 Labeling of probes: Spun down the contents of the Random Primer Labeling kit for 30 s in microcentrifuge after thawing. Boil 9 μl of DNA (for probe) for 5 min and cool it down on ice for 1 min. Add to the DNA 1 μl each of dATP,

[15]If not transforming with any selectable marker like Nourseothricin/Hygromycin, these plates, after shooting can be incubated at 30°C directly, for several days.
[16]The amount of DNA before restriction enzyme digestion is quantified by agarose gel electrophoresis and the DNA should be completely digested.
[17]The total well should be marked with a pencil.

dGTP, dTTP, 2 μl of Random Primers, 5 μl of ^{32}P dCTP, and lastly 1 μl of Klenow. Incubate the mix for 30 min at 37°C. After incubation, add 2 μl of Stop buffer and 20 μl of TE. Snap the tip of a microspin G 25 column and put in a 1.5-ml Eppendorf and spin for 30 s in a centrifuge inside the Laminar Hood and then run the probe through the column (see Note 18). Boil the probe for 5 min and cool it on ice for 1 min. Add 1 ml of 5× SSPE with a syringe into the probe and transfer it to the hybridizing chamber carefully. Hybridize overnight and wash sequentially with 50 ml of 0.1% SDS in 2× SSC for 20–30 min at 65°C, 50 ml of 0.5% SDS in 0.1× SSC thrice, each for 20–30 min at 65°C.

8 Dry the membrane over a filter paper and saran wrap and tape it on a cassette. Inside the darkroom put the film on top of the membrane and expose the film at −80°C overnight or at the least 4–5 h before developing.

3.4. Isolation of Total RNA (18)

1 Harvest cells (20–24 h) grown in the required media by pelleting down at 1,200 × g at 4°C for 10 min (see Note 19).

2 Wash the pelleted cells with sterile PBS twice and spin down at 1,200 × g, 4°C for 5 min. Drain out the PBS on a sterile wipe and flash freeze in a dry ice – ethanol bath and set for lyophilization (see Note 20).

3 Aliquot ~100 μl (about 50–75 mg) of lyophilized cells in a 2-ml screw cap tube and grind or smash the cells to powder form with the help of the spatula used to scoop out the lyophilized cells (see Note 21). Add 1–1.25 ml of Tri reagent. Cap the tubes properly and homogenize in Bead Beater 16 with pulses as follows 45 s thrice, 30 s once with a gap of 45 s between each cycle on ice.

4 Incubate the tubes for 10 min at room temperature. Centrifuge for 10 min at 4°C at 8,000 × g to pellet the cell debris and unbroken cells.

5 Transfer the supernatant to a fresh tube and add 50–60 μl of BAN (50 μl of BAN/ml of Tri reagent added) and shake vigorously for 20–30 s. Incubate for 5 min at room temperature and centrifuge at 8,000 × g at 4°C for 10 min.

6 Transfer the aqueous phase to a new tube and add equal volume of 70% Ethanol and mix gently and properly (see Note 22).

7 Load the aqueous phase (700 μl at a time) onto an RNeasy isolation column and spin for 30 s in a centrifuge at 8,000 × g at room temperature. If the

[18]The amount of the DNA used as probe should be at least 100 ng. The angle of the Eppendorf with the G25 column after loading of the Probe should be the same as before in the microcentrifuge.
[19]Be extremely cautious about RNase contamination. Wipe with RNase ZAP the whole external surface of the working area, pipettes, etc., before starting and change gloves frequently. If Minimal Media (YNB or DMEM) is to be used, it can be supplemented with 50 mMHepes, 1Msorbitol, and 10% FCS if required.
[20]Lyophilization for a 75–100 ml culture should be at least for 24 h but not more than 48 h.
[21]The lyophilized cells in powder form give better yield.
[22]Do not let the tip touch into the interphase while transferring the aqueous phase.

volume exceeds 700 μl, the same column can be reloaded until the whole aqueous phase had passed through it.

8. Discard the flow-through and wash the column with RW1 buffer provided with the kit. Discard the flow-through and wash with 500 μl of RPE twice, spin for 30 s at 8,000 × g and discard the flow-through. Transfer the RNA isolation column to a new 2-ml collection tube and spin for 2 min at 8,000 × g at room temperature.

9. Elute the RNA in 50 μl of RNase free water in a fresh 1.5-ml Eppendorf. Re-elute the residual RNA in another aliquot of 50 μl of RNase free water in the same tube.

10. Concentrate the RNA with the column from RNeasy MinElute Cleanup kit following instructions of the manufacturer. Elute in a final volume of 20 μl of DNase–RNase free water.

3.5. Protein Extraction from *Cn*

1. These instructions assume the use of a Bead Beater 8.

2. Streak out *Cn* strains of interest (e.g., wt or mutant) onto YPD agar plate and incubate at 30°C for 48 h.

3. Pick a single colony into a 50-ml Corning Centrifuge tube containing 10 ml YPD media and allow to grow at 30°C with shaking for 24 h.

4. Centrifuge the culture 10 min at 1,200 × g at ambient temperature (20°C), wash once with doubly distilled water and then resuspend into 7 ml doubly distilled water.

5. Aliquot 1 ml each into 1.5-ml conical tubes with screw caps and centrifuge at 3,500 × g for 10 min at 25°C.

6. Meanwhile prepare the lysis buffer.

7. Following centrifugation, discard the supernatant and resuspend each pellet into 200 μl lysis buffer.

8. Add one “cupful” glass beads (see Note 3), then vortex and place on ice.

9. Place each tube into the beadbeater in a 4°C coldroom and beadbeat for 40 s, followed by 1 min on ice. Repeat this four times (see Note 23).

10. Centrifuge each tube at 3,500 × g for 12 min at 4°C.

11. Collect the supernatant and carry out Bio-Rad protein assay to determine the amount of protein.

[23]It is important to go through four cycles on the beadbeater when lysing the *Cn* cells otherwise insufficient protein will be extracted.

3.6. Lipid Extraction

1. Under sterile conditions, fill 50-ml tube with 9 ml yeast-peptone (YP) and 1 ml 20% glucose. Add a single colony of the strain of interest (in this case *Cn* Gcs1^{REC}) and incubate 48 h at 30°C, 250 rpm.
2. Centrifuge at 1,200 × g for 10 min at 4°C. Wash pellet twice with water then resuspend in 9 ml sterile water.
3. Count the cells after appropriate serial dilution and aliquot 5 × 10^8 cells per tube. Centrifuge 10 min 1,200 × g at 4°C. Suction out water carefully (see Note 24).

3.6.1. Mandala Extraction (for Extraction of Inositol-Containing Phospholipids and Phosphatidylcholine) (see Note 25)

4. Add 1.5 ml Mandala extraction buffer (19) to each tube. Vortex and sonicate 20 s each.
5. Incubate at 60°C in a water bath for 15 min, vortex and sonicate for 20 s each then reincubate at 60°C for 15 min.
6. Sonicate 20 s then centrifuge 10 min at 1,200 × g at 4°C. Using a glass Pasteur pipette, combine supernatant from two tubes together into a clean tube.
7. Evaporate the solvent in the Speedvac (see Note 25).

3.6.2. Bligh and Dyer Lipid Extraction (for Determination of Neutral Lipids)

8. Following evaporation, add 2 ml methanol and vortex. Sonicate if necessary.
9. Add 1 ml chloroform and vortex. Ensure there is one phase, even if turbid (see Note 26).
10. Incubate the samples at 37°C for 1 h. During this period, vortex each sample twice for 30 s.
11. Centrifuge at 1,200 × g for 5 min at room temperature, then transfer the lower phase to a clean tube with a glass Pasteur pipette. Add 1 ml Chloroform and 1 ml water and vortex twice for 30 s each. Recentrifuge samples at 1,200 × g for 5 min at room temperature.
12. Once again, using a glass Pasteur pipette transfer lower phase to a clean tube. Up to three tubes can be combined into one to lessen the amount of tubes being handled.
13. Evaporate the solvent in the Speedvac (see Note 25).

[24]At this stage, the cell pellet can be frozen at −80°C until ready for extraction.
[25]To see the original references on how this protocol was established, see reference by Barbara Hanson (24).
[26]The tubes can be left at 4°C overnight if there are time constraints.

3.6.3. Additional Purification Steps (e.g., Isolation of Glucosylceramide Using a Silica Column)

3.6.3.1. Silica Column Purification 1

14 Resuspend the lipids in 1 ml chloroform/acetic acid (99:1).

15 Wash the SepPak cartridges with 15 ml chloroform (see Note 27). Apply sample (in 1 ml) and rinse with 1.5 ml chloroform/acetic acid (99:1). Collect flow-through after 0.5 ml has been allowed to collect into waste.

16 Add 15 ml chloroform/acetic acid (99:1) and collect 5 ml per tube.

17 Add 15 ml acetone and collect 5 ml per tube. Evaporate acetone from these tubes in the SpeedVac then resuspend in minimum amount acetone to combine into one tube. Reevaporate (see Note 28).

3.6.4. Base Hydrolysis

18 Add 0.5 ml chloroform, followed by 0.5 ml 0.6 M KOH in methanol to each sample. Vortex well and leave at room temperature for 1 h.

19 Add 0.325 ml 1 M HCl followed by 0.125 ml distilled water. Vortex well then centrifuge at 1,200 × g for 10 min at room temperature. Transfer lower organic phase to a clean tube.

20 Evaporate solvent in the SpeedVac. You should have a small dark brown pellet at this stage (see Note 25).

3.6.5. Silica Column Purification 2

21 Resuspend the pellet in 1 ml chloroform/acetic acid (99:1). Repeat steps 16 and 17.

22 Change eluting solvent to chloroform/methanol (95:5); add 10 ml and collect in two tubes.

23 Change eluting solvent to chloroform/methanol (90:10); add 15 ml and collect into 3 tubes. *These are the tubes that will contain glucosylceramide, the lipid of interest for this example*. Evaporate solvent using a SpeedVac. Do not combine the tubes (see Note 29).

24 Wash the column with 15 ml methanol and collect in case needed.

3.6.6. Thin Layer chromatography

25 Prepare a 10′ × 10′ glass TLC tank by adding chloroform/methanol/water (97.5:37.5:6) to a *clean, dry* tank lined with white chromatography paper. Apply a thin layer of vacuum grease around the top lip of the tank to ensure a

[27]For analytical scale, use WAT051900; 15 ml is equivalent to a 5 bed volume wash.
[28]Dry down other tubes as well in case needed later, then store at −20°C.
[29]Try to get as much compound down as possible by rinsing the walls of the glass tube with 9:1 chloroform:methanol.

good seal (see Note 30). Leave until paper is well saturated, usually at least 5 h to overnight.

26 Spot the soy standard onto a TLC plate 1.5 cm from the bottom using a 10 μl pipette, using 1, 2, and 3 μl in three separate lanes (equivalent to 2.5, 5, and 7.5 μg, respectively).

27 Resuspend the dried lipid from step 24 in 30 μl chloroform/methanol (2:1), and spot either 30 μl (analytical) or 5 μl (semipreparative scale) onto the TLC plate into a fourth lane. Allow solvent to evaporate in fume hood (~1–2 min) before placing the TLC plate in the tank.

28 Make sure the TLC tank is tightly closed. Allow the solvent front to migrate up to 1 cm from the top of the plate, before removing the plate from the tank.

29 Dry the TLC plate in the hood at room temperature prior to placing it in another tank containing only iodine crystals to allow visualization of the lipids. Alternatively, the plate can be sprayed with resorcinol in 70% H_2SO_4, and then placed in an oven for 10 min to allow a dark purple color to develop wherever sugar moieties are located on the lipids.

3.7. In Vitro Enzyme Activity Assay

1 This protocol describes the in vitro activity assay of Ipc1 (20) but could be adapted for assaying any enzyme from *Cn*. Ipc1 activity is measured by using the fluorescent ceramide analog NBD-C6-ceramide as substrate and monitoring the formation of NBD-C6-IPC, as described by Fischl et al. (21) with some modifications.

2 Grow wt and mutant *Cn* strains in YPD media in a shaker incubator for 24 h at 30°C. Harvest the cells by centrifugation and wash with sterile distilled water (see Note 24).

3 Resuspend the pellets in lysis buffer, add acid-washed glass beads for a volume equal to ¾ of the cell suspension and homogenize three times for 45 s, followed by 1 min on ice each time, using the Bead Beater 8.

4 Centrifuge at 2,500 × g for 10 min at 4°C, then transfer the supernatant (~100 μl) to a sterile 1.5-ml microcentrifuge tube for protein quantification.

5 Following protein determination, incubate 100 μg protein from the cell lysates for 30 min at 30°C in 50 mM bis-Tris–HCl buffer (pH 6.5) containing 1 mM phosphatidyl inositol, 5 mM Triton X-100, 1 mM $MnCl_2$, 5 mM $MgCl_2$, and 20 μM NBD-C6-ceramide in a final reaction volume of 100 μl.

6 Terminate the reaction by addition of 0.5 ml 0.1 N HCl in methanol.

7 Add 1 ml chloroform and 1.5 ml 1 M $MgCl_2$, mix well and centrifuge at 1,000 × g for 10 min to separate the phases.

[30] You can add two weights on top to ensure the cover seals well. The weights can be 2 × 250 ml glass bottles filled with water.

8 Analyze the chloroform-soluble product, NBD-IPC, by TLC on silica gel 60 plates (EM Science) as described above using chloroform/methanol/water (65:25:4).

9 Identify and quantify NBD-IPC by direct fluorescence using a Molecular Dynamics 840 Storm unit.

3.8. Mass Spectrometry of Lipids (22, 23)

1 This protocol describes MS and MS/MS of Cn glucosylcersamide but is applicable to any *Cn* lipid molecule.

2 Following Bligh and Dyer extraction described above under lipid extraction, MS and MS/MS scans of glucosylceramide were carried out on a Thermo Finnigan TSQ7000 triple quadrupole mass spectrometer equipped with electrospray ionization as described in ref. 6.

3 A 31 min method was used with A; water/0.2% formic acid/2 mM ammonium formate and B: methanol/0.2% formic acid/1 mM ammonium formate, on a 150 × 3 mm Spectra 3 μm C8SR column (Peeke Scientific) using gradient elution and addition of internal standards.

4 Include multiple reaction monitoring (MRM) for the characteristic production *m/z* 276.2.

5 Quantify Cn glucosylceramide using soy glycosylceramide (Avanti Polar lipids) for standard curve generation.

6 Normalize mass spectral data to inorganic phosphate determination.

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