SYTOX Orange DNA Stain Protocol for *Cryptococcus neoformans*

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Materials and Reagent Solutions

Materials:

- SYTOX Orange - Cat. No. S11368; Thermo Fisher Scientific
- RNase A – 10mg/mL; Cat. No. 19101; Qiagen
- 2-mercaptoethanol – Cat. No. 1610710; Bio-Rad

1µM SYTOX Orange Stock Solution

- Prepare a 1 µM SYTOX stock solution, store in the -20ºC freezer.

1X SYTOX Orange Staining Solution

- 500 µL 1µM SYTOX Orange Stock Solution in 50 mM Tris-HCl Buffer pH 7.5
- 2.5 µL RNAse A (final concentration of 0.5 mg/mL)
- 1 µL 2-mercaptoethanol

Note: Adjust volume amounts proportionally to the number of samples that will be stained.

Protocol

Fixation with 70% Ethanol

1. Remove 1 mL of *C. neoformans* culture and place into a conical tube.
2. Spin down cells at 3000 x g for 4 minutes.
3. Remove media supernatant, ensure the pellet remains at the bottom of the tube.
4. Wash the cells with 1.5 mL diH2O, vortex to resuspend, then spin the cells down again.
5. Remove the wash and proceed to ethanol fix.
6. Add 0.5 mL of diH2O to the tube, and vortex to resuspend.
7. Add 1.3 mL of 100% Ethanol for a final fixation of cells with 70% (w/v) Ethanol.
8. Fix cells at room temperature for at least one hour, then place in 4ºC overnight or longer.

Staining with SYTOX Orange

9. Next day, aliquot cells from the tube of fixed cells into a new 1.5 mL eppendorf tube to have a total number of cells ranging from $5x10^5$ – $1x10^7$ cells.
    a. Note: we recommend counting the cultures using a hemocytometer for reproducibility in SYTOX fluorescence across experiments.
10. Spin down the cells in the new aliquoted tubes using a tabletop centrifuge at max rpm for 2 minutes.

11. Remove the ethanol while ensuring not to disturb the pellet. Wash the cells with 300 µL of diH2O. Vortex to resuspend, then spin the cells down.
12. Remove the diH2O, ensuring not to disturb the pellet.
13. Prepare the SYTOX Orange Staining Solution with volumes proportional to the number of samples that will be stained.
    a. This solution should be prepared immediately before adding to the cells.
14. Add 500 µL of SYTOX Orange Staining Solution to each new set of eppendorf tubes.
    a. It is best to bulk prepare SYTOX Orange Staining Solution per the number of samples and controls.
15. Vortex to resuspend the cells.
16. Two incubation conditions you can use depending on time availability:
    a. Place the samples in 4ºC in the dark overnight. The next day, 1 hour before running flow cytometry, place the stained cells at room temperature in the dark. Run flow cytometry.
    b. Place the samples in the dark at room temperature for at least 1 hour. Run flow cytometry.

    Note: if using stress growth conditions, we recommend staining with haploid and diploid controls to ensure artifactual fluorescence shifting is not occurring with the shorter incubation time.

Running Flow Cytometry

17. Before running samples, sonicate them (using a low amplitude ~10% for 5-10 seconds) to disperse cell aggregates.
18. Determine fluorescent channels and instrument settings using the log controls before running samples.
19. Flow cytometry settings used for the Attune NxT Flow Cytometer (Thermo Fisher Scientific):
    a. Forward scatter (FSC) and the YL1 fluorescence channel were used for analysis. Instrument settings were as follows: FSC voltage 100, YL1 voltage 280, FSC threshold 5,000, and YL1 threshold 2,000. The acquisition volume was set to 40 µL with a flow rate of 25µL/min, and data collection was stopped after 10,000 events.

Controls

- We recommend using log-phase *C. neoformans* wild-type haploid and diploid strains that have been validated for ploidy.
    - Grow the haploid and diploid cells to mid-log phase by growing in 2% YEPD for 4-5 hours (window after the lag phase). To ensure log-phase, count the budding percentage of the population using a hemocytometer. Mid-log populations should have a budding percent around 50%.
    - Fix and stain as described above with your samples.
    - The haploid strain will provide fluorescent peaks for 1C and 2C lines. The diploid strain will provide fluorescent peaks for 2C and 4C lines.