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Protoplast Formation and Purification for Coccidioides

Bridget M. Barker[1], Ashley Itogawa[1], Heather Mead[2], mitchellbryant[1], Austin Blackmon[1], Seth Charley[1]

[1]Northern Arizona University; [2]TGen-North

Bridget M. Barker: Lab PI;

Bridget M. Barker

ABSTRACT

This protocol will allow the researcher to create protoplast of Coccidioides spp.

MATERIALS

1.Polypropylene 250 ml baffled Erlenmeyer flasks with 0.2 µm filter caps
2.*Coccidioides* spp. arthroconidia
3.Serological pipettes (10 ml, 25 ml, 50 ml)
4.Disposable hemocytometers (iNCYTO C-Chip NI)
5.Filtered pipette tips (10 µl, 200 µl, 1000 µl)
6.Liquid waste container (bottle with screw cap)
7.50 ml polycarbonate, screw cap, conical centrifuge tubes
8.Empty tip box (for use as 50 ml tube holder during horizontal incubation with shaking)
9.15 ml polypropylene, screw cap, conical centrifuge tubes
10.Cold beads in small ice bucket (-20°C)
11.Inoculating loops (1 µl, 10 µl)
12.1.5 ml microcentrifuge tubes

Reagents

Note: Prepare all media and buffers using ultrapure water (resistivity of 18 M Ω cm at 25°C). Use only molecular biology grade reagents. Store all reagents at 4°C unless otherwise indicated.

1. 1X GYE broth
    A.1% glucose, 0.5% yeast extract
    B.Two 100 ml bottles/transformation experiment
2. Dry enzyme powder for cell wall digestion enzyme mixture prepared in a 50 ml (polypropylene) conical centrifuge tube
    A.75 mg Driselase from *Basidiomycetes* (Sigma), 40 mg lysing enzymes from *Trichoderma harzianum* (Sigma), 10 units chitinase (optional) from *Streptomyces griseus* (Sigma)
        i.The use of chitinase is optional, but it appears to enhance the transformation

Keywords: protoplast, transformation, Coccidioides

competency of the protoplasts.

3. Osmotic buffer A (OB)
   A.50 mM potassium citrate, 0.6 M KCl (pH 5.8)
   B.200 ml/transformation experiment
4. Osmotic buffer B (OM)
   A.10 mM sodium phosphate, 1.2 M $MgSO_4$(pH 5.8)
   B.100 ml/transformation experiment
5. Trapping buffer (TB)
   A.100 mM MOPS, 0.6 M sorbitol (pH 7.5)
   B.100 ml/transformation experiment
6. MOPS buffer containing sorbitol (MS)
   A.10 mM MOPS, 1 M sorbitol (pH 6.5)
   B.500 ml/transformation experiment
7. MSC buffer (MSC)
   A.MS with20 mM $CaCl_2$
   B.100 ml/transformation experiment

SAFETY WARNINGS

Working with Coccidiodies required access to a BSL3 laboratory and IBC approvals

BEFORE START INSTRUCTIONS

Make fresh reagents

1 Place quantified *Coccidioides* spp. arthroconidia and other supplies needed in BSC. Inoculate 100 ml 1XGYE broth in a 250 ml filter-capped, baffled Erlenmeyer flask with up to 5×10^8 *Coccidioides* spp. arthroconidia and incubate at 30°C with shaking (200RPM). 10m

2 Monitor the germination process using microscopy or culture observation. 16h

A. Germ tube formation typically occurs between 14 - 17 hrs of incubation. Synchronization of germ tube formation results in higher quality protoplast production. Initiate protoplast isolation procedure before the germ tube form branches (typically occurs after ~14 hrs growth).

B. Microscopy monitoring of germination:

i. Swirl 250 ml Erlenmeyer flask to mix culture and evenly suspend germlings

ii. Remove 10 µl culture and load onto one side of a disposable hemocytometer.

iii. View cells in disposable hemocytometer at appropriate magnification on microscope and look for germling formation specifically look for germ tube formation without branch formation.

C. Alternatively, germling formation can be approximated based on culture settling.

i. Tip germling culture flask 45° and let settle.

ii. The germlings will settle after about 10 - 15 min and the media will start to clear. A

yellowish-cream color will begin to concentrate at the bottom of the flask. Conidia will tend to stay in solution so it will remain cloudy with no evidence of settling.

3 Once germlings look ready, Cool centrifuge to 10°C. Then, prepare cell wall digestion mixture. Must be prepared fresh, immediately before use. Add 10 ml OB to prepared dry enzyme powder. **Osmotic buffer A (OB) is 50 mM potassium citrate, 0.6 M KCl (pH 5.8).** Dry enzyme powder for cell wall digestion enzyme mixture is 75 mg Driselase from *Basidiomycetes* (Sigma), 40 mg lysing enzymes from *Trichoderma harzianum* (Sigma), and 10 units chitinase (optional) from *Streptomyces griseus* (Sigma). The use of chitinase is optional, but it appears to enhance the transformation competency of the protoplasts. Make 200mL of OB per transformation. Incubate cell wall digestion mixture at 39°C for 20 min with shaking (~180-200 rpm). This may need to be adjusted for lab specific equipment. Ensure that material shakes enough to stay in suspension. 20m

4 Return to germlings flasks. Remove some culture supernatant and discard as liquid waste to concentrate germlings. Do not remove >15 ml supernatant. 5m

5 Transfer even volumes of germ tube culture to each of an appropriate number of 50 ml polycarbonate conical centrifuge tubes and load into aerosol-resistant fixed angle rotor for 50 ml tubes. 10m

6 Centrifuge 50 ml polycarbonate conical centrifuge tubes at 2800×*g* at 10°C for 10 min using aerosol-resistant fixed angle rotor for 50 ml tubes. Bring rotor into BSC. Remove supernatant from each 50 ml polycarbonate conical centrifuge tube and discard as liquid waste. 10m

7 Add 10 ml cell wall digestion enzyme mixture to first pellet and resuspend by pipetting. Transfer resuspended pellet to next 50 ml polycarbonate conical centrifuge tube and resuspend that pellet by pipetting. Repeat until all pellets have been resuspended in a single 50 ml polycarbonate conical centrifuge tube. **Pellets must be completely re-suspended without clumps; pipetting 15 - 20 times for each pellet is usually sufficient. Do not vortex.** 15m

8 Incubate 50 ml polycarbonate conical centrifuge tube at 30°C for 30 - 60 min with gentle shaking (~120 rpm). Incubation time can be adjusted based upon the rate of protoplast formation but should not exceed 1 hr. Monitor for protoplast formation using microscopy. Remove 10 µl culture and load one side of a disposable hemocytometer. View cells in disposable hemocytometer at appropriate magnification on microscope and look for cell wall digestion progress. Microscopy checks are recommended at 30 and 45 min timepoints. **Do not allow cell walls to be over digested.** 1h

9 Once digestion is complete, centrifuge at 900 ×*g* at 10°C for 10 min using aerosol-resistant fixed angle rotor for 50 ml tubes. Remove supernatant from centrifuge tube, being careful not to disturb the pellet, and discard as liquid waste. Add 10 ml OM to 50 ml polycarbonate conical centrifuge 10m

tube and resuspend protoplast pellet by gently pipetting until there are no visible clumps. **Osmotic buffer B (OM) is 10 mM sodium phosphate, 1.2 M $MgSO_4$ (pH 5.8), need 100 mL per transformation.**

10 Add 10 ml Trapping Buffer (TB) as an overlay over the OM in the 50 ml polycarbonate conical centrifuge tube by holding tube at 45°and **very slowly** dispensing the TB along the centrifuge tube wall a few mm above the surface of the OM to form two layers, slowly moving up as the volume is added to the tube. The lower OM layer is denser than the TB and *should not be disturbed*. **This step is critical to get proper separation of the protoplasts. Handle the 50 ml polycarbonate conical centrifuge tube VERY gently from this point on.** TB is 100 mM MOPS, 0.6 M sorbitol (pH 7.5). Make 100 ml/transformation. Can be made ahead and stored for up to 6 months. 10m

11 Gently place 50 ml polycarbonate conical centrifuge tube in aerosol-resistant **swing bucket** compatible with 50 ml tubes to avoid disturbing the layers. Centrifuge at max rpm at 10°C for 20 min (no more than 4000 RPM). 20m

12 Place 50 ml polycarbonate conical centrifuge tube in a 50 ml tube rack in such a way as to provide good visibility of the buffer layers. Cell debris should be present as a pellet at the bottom of the centrifuge tube and a **white layer of protoplasts should be present at the interphase** of the OM and TB. 10m

13 Pre-warm 60% PEG to room temperature if you will be proceeding directly to CRISPR/Cas9 procedure.

14 Carefully remove the protoplast layer from the 50 ml polycarbonate conical centrifuge tube using a P1000 pipette with wide bore tip (or cut small amount of tip off) and transfer even volumes to each of two to four 15 ml conical centrifuge tubes stored on cold beads or ice. Do not transfer >2 ml per 15 ml conical centrifuge tube. Discard 50 ml polycarbonate conical centrifuge tube containing remaining OM:TB layers as waste. Approximately 4 - 5 ml of protoplast suspension should be recovered in total. Avoid excess cellular debris when pipetting the protoplast layer. **Keep protoplasts cold.** 5m

15 Add 12 ml MS to each 15 ml conical centrifuge tube and gently mix by pipetting. Handle suspensions gently to avoid rupturing the protoplasts. Centrifuge 15 ml conical centrifuge tubes at 6000 rpm at 10°C for 8 min using aerosol-resistant fixed angle rotor for 15 ml tubes. MOPS buffer containing sorbitol (MS) is 10 mM MOPS, 1 M sorbitol (pH 6.5). Use 500 ml/transformation experiment. Can be made ahead and stored for 6 months. 10m

16 Remove supernatant from each 15 ml conical centrifuge tube and discard as liquid waste. Use a 10 µl inoculating loop to remove any brown cell debris from each white protoplast pellet, if 5m

observed. Add 240 µl MSC to each 15 ml conical centrifuge tube and gently resuspend white protoplast pellet by pipetting with large bore pipet tip (p1000).

17 Transfer even volumes (~120 µl) protoplasts into each of two 1.5 ml microcentrifuge tubes. Centrifuge 1.5 ml microcentrifuge tubes at 13,000 rpm for 30 sec. Remove supernatant from each 1.5 ml microcentrifuge tube and discard as waste. 1m

18 If needed, perform quality control check on purified protoplasts using microscopy and/or culturing. 20m

Microscopy check of purified protoplasts:

i.Add 20 µl protoplasts to 180 µl PBS in a fresh 1.5 ml microcentrifuge tube using a P200 pipette and mix by gently pipetting.

ii.Remove 10 µl PBS suspended protoplasts and load onto one side of a disposable hemocytometer.

iii.View protoplasts in disposable hemocytometer at appropriate magnification on microscope and look for clean protoplasts. Look for low levels of cellular debris and protoplast formation (i.e., clear “circles” that are not over digested or otherwise damaged).

Viability check of protoplasts:

Remove 20 µl protoplasts and plate on 1X GYE agar to verify viability. Incubate plates at 30°C for 4 - 7 days and inspect for growth.

19 Continue with CRISPR Cas9 Editing step 1 or store 1.5 ml microcentrifuge tubes at 4°C for brief storage or at -80°C for up to 2 weeks until ready to proceed.