CRISPR/Cas9 in vitro assembled gene deletion protocol for Coccidioides posadasii

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

[1]Northern Arizona University; [2]Arizona State University

Bridget M. Barker: Lab PI;

Bridget M. Barker

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ABSTRACT

This protocol was developed to use CRISPR/Cas9 technology to delete genes in Coccidioides posadasii. This methodology is useful for creating targeted deletions in any strain of Coccidioides, and has been successful in our lab. The method will allow for users to design and implement targets of their preference, and specific details regarding resistance markers and targets was removed. The user will need to define these specific parameters. This protocol will not work if a gene is essential, and certain genomic regions may be recalcitrant to modification.

MATERIALS

1. MOPS buffer containing sorbitol (MS)
   A.10 mM MOPS, 1 M sorbitol (pH 6.5)

Keywords: CRISPR, Cas9, Coccidioides

B.500 ml/transformation experiment

2. MSC buffer (MSC)
   A.MS with20 mM $CaCl_2$
   B.100 ml/transformation experiment

3. 0.9% phosphate buffered saline (PBS)

4. DNA repair template (*hph* or other selective marker as appropriate)

5. Cas9:crRNA:tracrRNA ribonucleoprotein (RNP) complex (See Table 1)

6. 60% (w/v) polyethylene glycol (PEG)
   A.Made in MSC
   B.PEG quality varies depending on the source. The precise function of PEG is not known, although it may contribute to the efficiency of DNA construct uptake by protoplasts (2). Long term storage of PEG solution will decrease transformation efficiency. Do not use PEG solution >3 months old.

7. GYES soft agar
   A.1% glucose, 0.5% yeast extract, 1 M sucrose, 0.7% Bacto agar
   B.25 ml/transformation experiment

8. GYES agar plates
   A.1% glucose, 0.5% yeast extract, 1 M sucrose, 2% Bacto agar

9. 1X GYE soft agar with appropriate concentration of a selective agent
   A.1% glucose, 0.5% yeast extract, 0.7% Bacto agar
   B.Potential selective agents
      i.e. 375 µl hygromycin B (Hyg) (Sigma)/L media

10. 1X GYE agar plates with appropriate concentration of a selective agent
   A.1% glucose, 0.5% yeast extract, 2% Bacto agar
   B.Potential selective agents
      i.e. 375 µl hygromycin B (Hyg) (Sigma)/L media
   C.May be made in untreated 6-well culture plates or small petri plates for initial selection step. Standard size petri plates (150 mm) are best for subsequent purification steps.

Table 1. CRISPR-Cas9 Protocol: Buffers and Reagents

Buffer	Composition	Recipe	Preparation and Notes
Cas9 enzyme working buffer	20 mM HEPES; 150 mM KCl; pH 7.5	200 µl HEPES (1 M, pH 7.5) 750 µl KCl (2 M) 9 ml Nuclease-Free Water	**Preparation** - Mix components. - Verify pH 7.5. - Store at RT. **Notes:** - HEPES and KCl are nuclease free buffers.
Nuclease-Free Duplex Buffer	Nuclease-Free Duplex Buffer should be stored at 4°C for short storage or at -20°C for long storage.		
Ordering Alt-R CRISPR-Cas9 crRNAs	Items to Order: Alt-R™ CRISPR-Cas9 crRNA*, 2 nmol 20 nt length, custom Alt-R™ CRISPR-Cas9 tracrRNA*, 100 nmol Cat#: 1072534 Alt-R™ S.p. Cas9 Nuclease 3NLS, 500 µg Cat#: 1074182 Nuclease Free Duplex Buffer (included with tracrRNA order) **The crRNA you will custom design for each PAM site you want to target. The tracrRNA is universal.* 1. We order our CRISPR-Cas9 components from IDT: https://www.idtdna.com/site/order/oligoentry/index/crispr		
	2. Design/choose your protospacer sequence(s). We typically choose 20 nt protospacer(s) that are adjacent to CGG, AGG, or TGG PAM sites. All selected protospacer(s) are used to BLAST search the *C. posadasii* genome prior to ordering. The protospacer(s) must not have greater than 15 nt shared with off-target sites throughout the genome. 3. To order the crRNA, just enter the 20 nt protospacer directly into the CRISPR-Cas9 Oligo Entry web page (above link). IDT completes the full crRNA generation for you.		
Resuspend crRNA and tracrRNA	1. Resuspend the crRNA and tracrRNA to 100 µM stock concentrations in Nuclease-Free Duplex Buffer (or Nuclease-Free IDTE Buffer). Use the following volumes: **Normalized amount delivered (nmol)** / **Duplex buffer resuspension volume (µl)** 2 / 20 5 / 50 10 / 100 20 / 200 100 / 1000 2. Store resuspended RNA oligos at -20°C.		

Assembly of guide RNA (gRNA) duplex

3. For complete gene deletion, we currently use two gRNAs, one that targets a 5' PAM site and one that targets a 3' PAM site. Targeting a single PAM site for complete deletion using microhomology repair templates (<50 bp regions of homology) causes reduced gene targeting efficiency. **For each gRNA, prepare 33 µM RNA duplex solution by mixing crRNA and tracrRNA oligos in equimolar concentrations in a sterile microcentrifuge tube.** For example, create a final duplex concentration of 33 µM using the following table:

Component	Amount (µl)
100 µM Alt-R CRISPR-Cas9 crRNA	5
100 µM Alt-R CRISPR-Cas9 tracrRNA	5
Nuclease-Free Duplex Buffer	5
Total volume	15

4. Heat at 95°C for 5 min.

5. Remove from heat and allow to cool to room temperature (20 - 25°C) on bench top.

Note: The crRNA:tracrRNA guide complex can be used for up to 3 months with no loss in activity when stored at -20°C at a concentration of at least 30 µM.
Note: Each gRNA duplex should only contain either a 5' PAM site or 3' PAM site. Do not mix before assembling the RNP complex.

Preparation of Cas9 working solution

6. Before use, thoroughly mix the stock Alt-R S.p. Cas9 Nuclease 3NLS by inverting the tube several times, and briefly centrifuge the tube. **Dilute Cas9 1:10 to a final concentration of 1 µg/µl using Cas9 working buffer**. Store at -20°C.

Assemble the Cas9:crRNA:tracrRNA ribonucleoprotein (RNP) complex

7. Combine the following:

Component	Volume per well (µL)
33 µM RNA duplex solution 1 (gRNA 1)	1.5
33 µM RNA duplex solution 2 (gRNA 2)	1.5
Cas9 nuclease [1 µg/µl]	1.5
Cas9 Working buffer	22
Total volume	26.5

8. Incubate at room temperature for 5 min to allow the assembly of the RNP complexes.

***Repair templates are produced by a single PCR reaction using primers that are designed to amplify a selection cassette (hygR, phleoR, ptrA, or pyrG) while simultaneously incorporating microhomology arms (5' and 3' of the double-strand breaks introduced by Cas9) that are only 35 - 50 bp in length. Therefore, each primer used to amplify the repair template is ~60 - 70 bp in length.*

SAFETY WARNINGS

1. The selective media used for isolating *Coccidioides* spp. transformants includes an acutely toxic compound: hygromycin B. No specific risk mitigation is required for this compound due to the low amount and dispersed nature of this compound in the semi-solid/solid waste generated in this SOP. However, concentrated hygromycin B stocks used to prepare media should be handled in a fume hood and all appropriate PPE should be worn (i.e., lab coat, gloves, eye protection). If a spill involving this compound occurs, spill mitigation should include removing any potentially contaminated PPE and a thorough hand washing.
Some of the buffer reagents (e.g., MOPS) contain compounds that are incompatible with bleach. No specific risk mitigation is required for these reagents due to the diluted nature of these reagents in the waste generated in this SOP

BEFORE START INSTRUCTIONS

Read materials page and order and make needed reagents prior to start. The creation of protoplasts should be complete before starting this procedure

1 Prepare all needed reagents, prepare RNP complex, prepare protoplasts on ice, warm GYES agar plates to 30C, warm 60% PEG to room temperature: see materials list for recipes and ordering information. 2d

2 Place 1.5 ml microcentrifuge tube(s) containing protoplasts and other supplies needed in BSC. 3m
A.**Keep protoplasts on cold beads/ice.**
B.If the protoplasts were stored at -80°C, they will need to thaw on cold beads.

3 Adjust protoplast volume in each 1.5 ml microcentrifuge tube to be processed to ~100 µl. Use additional 1.5 microcentrifuge tubes as necessary. 5m
A.One 1.5 ml microcentrifuge tube containing ~100 µl protoplasts is needed per transformation.
B. Avoid multiple freeze thaws of protoplasts

4 Add desired DNA repair template (1 - 3 µg) to each 1.5 ml microcentrifuge tube containing ~100 µl protoplasts and gently stir with pipette tip to mix; do not pipette or vortex. 1m
A.DNA repair template volume should be ~10 µl and no more than 20 µl.
B.DNA repair template concentration must be at least 1 µg.

5 Add 26.5 µl RNP complex to each protoplast:DNA mixture in a 1.5 ml microcentrifuge tube.Do not mix. 1m

A.Cas9 endonuclease concentration should be maximized.

6 Add 25 µl 60% PEG to each protoplast:DNA:RNP complex mixture in a 1.5 ml microcentrifuge tube and mix by slowly pipetting twice 2m

7 Incubate 1.5 ml microcentrifuge tubes on cold beads for 50 min. 50m

8 Add 900 µl additional 60% PEG to each 1.5 ml microcentrifuge tube and mix gently by inversion until 60% PEG is fully incorporated. 2m

9 Lay 1.5 ml microcentrifuge tubes horizontally on a paper towel on the BSC work surface and incubate at room temperature for 30 min 30m

10 Using a p1000 with large pore tip, overlay each suspension onto a pre-warmed GYES agar plate and gently rotate or use soft loop to spread suspension evenly 5m

11 Place GYES plates agar side down in incubator at 30°C for 48 hr to allow cell wall regeneration and colony growth. 2d

Note: At this point, the protoplasts are delicate due to a lack of cell walls; the cell walls should regenerate in 12 - 24 h

Observe for growth, should see faint growth by 48 hours. Occasionally up to 72 hours is needed.

12 **When growth is observed:** 30m

Prepare 1X GYE soft agar with appropriate selective agent

i. Melt 1X GYE soft agar by microwaving.

ii. Transfer needed amount 1X GYE soft agar to 50 ml conical centrifuge tube and incubate at 46°C.

iii. Add appropriate amount selective agent to melted 1X GYE soft agar.

NOTE: Many selective agents are temperature sensitive. Ensure 1X GYE soft agar has cooled to

46°C (or appropriate temperature for specific selective agent) before adding selective agent

13 Add 2.5 mL 1xGYE soft agar with selective agent to each GYES plate with emerging fungal growth and incubate agar side down at 30C for 4-7 days. Avoid light is selective agent is light sensitive. 1w

14 When colonies are observed (small, star shaped) growing through the selective media, pick colonies and transfer to fresh 1xGYE agar in culture plates (12-well or 6-well depending on preference) and incubate at 30C for 7-14 days, until colonies reach ~2cm in diameter. 2w

15 Re-plate surviving colonies in the same manner as step 14, and incubate at 30C for 7 days, until colonies reach ~2cm in diameter 1w

16 Select a well grown, isolated colony and mark on the back of the plate. Sample the outer edge of the selected colony using an inoculating loop and use to streak for isolated colonies on a fresh selective agar plate. Repeat steps for desired number of transformants (we usually select 12). Incubate selective agar plates at 30°C for 3-5 days 1w

17 Pick single colonies and transfer to fresh selective media plates. Passage 1-2 times to ensure homokaryons. From final plate, scrape a small amount of mycelia to a DNA lysis buffer and extract DNA using appropriate method. 2w

18 Screen extracted DNA for homokaryotic transformants using PCR, design PCR primers to target both selective marker and DNA segment that should be removed in transformant 1d

19 Southern blot analysis with a gene-specific probe on PCR selected transformants can be used to confirm the formation and purification of desired homokaryotic mutants