Chapter 13

Multi-Copy Genetic Screen in *Aspergillus nidulans*

Hee Soo Park and Jae-Hyuk Yu

Abstract

With the completion of genomes of various *Aspergillus* species, large-scale genome-wide expression studies can be carried out. Genomics, however, is more powerful and efficient when combined with genetics. A multi-copy-based gain-of-function screen is a complementary method to loss-of-function genetic screen and can identify novel genes that may not be easily identifiable through loss-of-function-type screens. Particularly, gain-of-function genetic screens would identify novel activators or repressors of fungal development and secondary metabolism. Here, we describe a working protocol for the identification of novel regulators in *Aspergillus nidulans*.

Key words: Genetics, *Aspergillus*, Multi-copy screening, Sporulation, Secondary metabolism

1. Introduction

Loss-of-function genetic screen is the traditional approach to identify genes of interest based on inactivation of gene function (1). Chemical mutagenesis can be used to introduce random mutation in certain gene. 4-nitroquinoline 1-oxide (NQO) is an aromatic hydrocarbon that can intercalate between DNA base pair causing various loss-of-function mutations (2). However, loss-of-function approaches often miss genes if the inactivation does not result in clear and detectable phenotypes. Gain-of-function mutagenesis is a complementary method to loss-of-function approach and can identify novel genes. Multi-copy genetic screen is one of high-throughput screening method (3). Recently, several studies found novel regulators of fungal development and/or secondary metabolism employing multi-copy genetic screen (4–6). Generally in *Aspergillus nidulans*, *pyrG*-deficient strains and a wild-type genomic library constructed in the vector pRG3-AMAI, which

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contains the *pyr4*+ allele of *Neurospora crassa* and an autonomous replicating sequence called AMA1, are being used (7, 8). Here, we describe a detailed protocol for multi-copy genetic screen in *A. nidulans* using pRG3-AMA1 wild-type library.

2. Materials

2.1. Aspergillus Transformation

1. KOH solution: 1.1 M KOH solution. Dissolve 6.17 g KOH in 70 mL. Adjust volume to 100 mL with additional dH_2O (see Note 1).
2. KCl_Citric acid solution: 1.1 M KCl and 0.1 M citric acid monohydrate. Dissolve 8.2 g KCl and 2.1 g citric acid monohydrate in 40 mL dH_2O. Adjust pH to 5.8 with 1.1 M KOH solution. After adjusting the pH, transfer the solution into a 100 mL graduated cylinder and make the volume up to 100 mL with dH_2O. Sterilize by autoclaving and store at room temperature (see Note 2).
3. 2× Protoplasting solution: KCl_Citric acid solution with 12.5% (w/v) VinoTeste Pro. Dissolve the 1.25 g of VinoTaste Pro (Novozyme) in 10 mL of KCl_Citric acid solution. Sterilize the solution by filtering this solution through a sterile syringe-driven filter (0.22 μm pore size) (see Note 3).
4. Sucrose cushion solution: 1.2 M sucrose. Dissolve 41.08 g sucrose in 80 mL of dH_2O. Adjust volume to 100 mL with additional dH_2O. Sterilize by autoclaving and store at 4°C.
5. KCl solution: 0.6 M KCl. Dissolve 4.47 g KCl in 80 mL of dH_2O. Adjust volume to 100 mL with additional dH_2O. Sterilize by autoclaving and store at 4°C.
6. KCl_$CaCl_2$ solution: 0.6 M KCl and 50 mM $CaCl_2$. Dissolve 4.47 g KCl and 0.74 g $CaCl_2 \cdot 2H_2O$ in 80 mL of dH_2O. Adjust volume to 100 mL with additional dH_2O. Sterilize by autoclaving and store at 4°C.
7. STC buffer solution: 10 mM Tris–HCl (pH 7.5), 1.2 M sorbitol, 10 mM $CaCl_2$. Dissolve 10 mL 1 M Tris–HCl, 218.6 g sorbitol, and 0.47 g $CaCl_2 \cdot 2H_2O$ in 800 mL of dH_2O. Adjust volume to 1 L with additional dH_2O. Sterilize by autoclaving and store at 4°C.
8. 1 M Tris–HCl solution: Dissolve 15.76 g Tris–HCl per 100 mL dH_2O. Store at room temperature.
9. 1 M Tris base solution: Dissolve 12.11 g Tris base per 100 mL dH_2O. Store at room temperature.
10. PEG solution: 20 mM Tris–HCl (pH 7.5), 25% (w/v) PEG, 0.6 M KCl, and 50 mM $CaCl_2$. Dissolve the following in

20 mL dH_2O: 4.47 g KCl, 0.74 $CaCl_2 \cdot 2H_2O$, 0.802 mL 1 M Tris–HCl, 0.196 mL 1 M Tris base, and 25 g PEG (average molecular weight 3,350). Adjust volume to 100 mL with additional dH_2O. Sterilize by autoclaving. Store at room temperature (see Note 4).

11. Minimal medium (MM) with 0.5% yeast extract: 5 g yeast extract, 10 g D-glucose, 50 mL 20× nitrate salt solution, and 1.0 mL trace element solution per 1 L of dH_2O. Adjust pH to 6.5 with NaOH. For a *pyrG*$^-$ strain, add 1 g uridine (0.1% w/v, Acros Organic) and 1 g uracil (0.1% w/v, USB Products) prior to autoclaving. Sterilize by autoclaving. Store at room temperature.
12. 20× Nitrate salt solution: Dissolve the following in 800 mL dH_2O, 120 g $NaNO_3$, 10.4 KCl, 10.4 g $MgSO_4 \cdot 7H_2O$, and 30.4 g KH_2PO_4. Adjust volume to 1 L with additional dH_2O.
13. Trace element solution: Dissolve the following in 800 mL dH_2O, 22 g $ZnSO_4 \cdot 7H_2O$, 11 g H_3BO_3, 5 g $MnCl_2 \cdot 4H_2O$, 5 g $FeSO_4 \cdot 7H_2O$, 1.6 g $CoCl_2 \cdot 6H_2O$, 1.6 g $CuSO_4 \cdot 5H_2O$, 1.1 g $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, and 50 g $Na_2EDTA \cdot 2H_2O$. Adjust pH to 6.0 with NaOH after each component dissolves and add dH_2O to a final volume of 1 L. Store at room temperature (see Note 5).
14. The selection medium: 10 g D-glucose, 50 mL 20× nitrate salt solution, 1.0 mL trace element solution, 218.6 g sorbitol (1.2 M) per 1 L of dH_2O. Add 15 g agar (1.5%, v/w) prior to autoclaving. Adjust pH to 6.5 with NaOH.
15. Top agar: 10 g D-glucose, 50 mL 20× nitrate salt solution, 1.0 mL trace element solution, 218.6 g sorbitol (1.2 M) per 1 L of dH_2O. Add 7 g agar (0.7%) prior to autoclaving. Adjust pH to 6.5 with NaOH.
16. Sterile Miracloth (EMD).
17. Transfer pipettes.
18. 0.22 μm Syringe filter.
19. The recipient strain should be a *pyrG*-deficient strain (*pyrG*$^-$ mutant) that is auxotrophic for uridine and uracil.
20. The pRG3-AMA1-based wild-type genomic library is available from the Fungal Genetics Stock Center, Kansas City, MO.

2.2. Genomic DNA Isolation

1. Minimal medium with 0.5% yeast extract: Add and dissolve 5 g yeast extract, 10 g D-glucose, 50 mL 20× nitrate salt solution, and 1.0 mL trace element solution per 1 L of dH_2O. Adjust pH to 6.5 with NaOH. Sterilize by autoclaving. Store at room temperature.
2. Sterile test tubes (8–10 mL).

3. Breaking buffer: 50 mM Tris–HCl, pH 8.0, 50 mM EDTA (pH 8.0), 5% Triton X-100 (v/v), 8% sucrose (w/v). Dissolve the following in 50 mL dH_2O, 5 mL 1 M Tris–HCl (pH 8.0), 10 mL 0.5 M EDTA, 5 mL Triton X-100, and 8 g sucrose and add dH_2O to a final volume of 100 mL. Sterilize by autoclaving. Store at 4°C.
4. Phenol:Chloroform:Isoamyl alcohol (25:24:1).
5. Zirconia/silica bead (0.5 mm, Research Products International Corp).
6. 2 mL Screw cap microcentrifuge tubes with O-ring cap.
7. TE buffer with RNase A: 10 mM Tris–HCl (pH 7.6) and 1 mM EDTA sterilized by autoclaving. Add 2 mg Ribonuclease A per 100 mL TE buffer. Store at 4°C.
8. Mini-Beadbeater (Bio Spec Products Inc.).

2.3. Plasmid Rescue and Insert Sequencing

1. *Escherichia coli* MAX Efficiency® DH10B™ Competent Cells (Invitrogen).
2. Gene Pulser/Micropulser cuvettes (0.1 cm gap, Bio-Rad).
3. Gene Pulser X cell Microbial system (Bio-Rad).
4. LB medium: Dissolve 25 g LB broth into 1 L dH_2O. To make LB plate add 15 g agar. Sterilize by autoclaving. Allow the medium to cool to 65°C before adding 2 mL of 50 mg/mL ampicillin to a final concentration of 100 μg/mL.
5. S.O.C Medium: Dissolve the following in 70 mL dH_2O, 2 g Bacto® Tryptone, 0.5 g Bacto® Yeast extract, 1 mL 1 M NaCl, 0.25 mL 1 M KCl, 1 mL $MgCl_2$, 1 mL 1 M $MgSO_4$, and 2 mL 1 M Glucose, and add dH_2O to a final volume of 100 mL. The pH should be 7.0. Sterilize by autoclaving.
6. Ampicillin: 50 mg/mL ampicillin. Dissolve 500 mg ampicillin into 10 mL of dH_2O.
7. QIAquick PCR Purification Kit.

3. Methods

3.1. Transformation with Multi-Copy Genomic Libraries

1. Prepare fresh spore suspension of a recipient strain carrying a *pyrG*$^-$ allele.
2. Inoculate 1×10^8 spores into 20 mL MM with 0.5% yeast extract and incubate with shaking at 150 rpm on a shaker for 12–14 h at 30°C.
3. Harvest the hyphae by filtering through the sterile Miracloth and wash the hyphae with about 20 mL MM with 0.5% yeast extract.

4. Scrape hyphae from the Miracloth with a sterile spatula, transfer the hyphae into a sterile 50 mL centrifuge tube, and resuspend the hyphae in 10 mL MM with 0.5% yeast extract.
5. Add 10 mL 2× protoplasting solution.
6. Incubate with 100 rpm in a shaker at 30°C for 2 h.
7. Remove any undigested hyphal material by filtration through sterilized Miracloth.
8. Overlay the protoplasting mixture very gently with 10 mL of sucrose cushion solution.
9. Centrifuge for 10 min at 1,800 × *g* at 4°C using HS-4 rotor (Soval).
10. Using a sterile transfer pipette take the protoplasts at the top of the sucrose cushion.
11. Place the collected protoplasts in a sterile 50 mL centrifuge tube, gently mix with an equal volume of KCl solution, and centrifuge for 10 min at 1,800 × *g* at 4°C using HS-4 rotor.
12. Carefully decant the supernatant and resuspend the pellet in 1 mL of KCl solution and transfer the suspension to 1.5 mL microcentrifuge tubes.
13. Centrifuge at 2,400 × *g* for 3 min at room temperature.
14. Decant the supernatant carefully and resuspend the pellet in 1.0 mL KCl_$CaCl_2$ solution.
15. Centrifuge at 2,400 × *g* for 3 min at room temperature.
16. Decant the supernatant carefully and resuspend the pellet in 0.5 mL KCl_$CaCl_2$ solution.
17. Centrifuge at 2,400 × *g* for 3 min at room temperature.
18. Decant the supernatant. Resuspend the protoplast pellet in a volume of 100 μL of KCl_$CaCl_2$ solution at a concentration of 10^8–10^9 protoplasts per 1 mL. Store on ice.
19. To 100 μL protoplast suspension add ~5 μg of the pRG3-AMA1 wild-type library (the plasmids in library contain *pyr4*$^+$) in less than 15 μL TE buffer.
20. Add 50 μL of PEG solution, and mix gently by turning the tube on its side and rotating it. Incubate for 25 min on ice.
21. Add 1 mL of PEG solution and incubate at room temperature for 25 min.
22. Add 10 mL of STC buffer and mix.
23. Spread about 0.5 mL mixture and plate on the selection medium.
24. Add 5 mL top agar (0.7% agar) and mix (see Note 6).
25. Incubate the plates overnight at 30°C.

26. Flip the plates upside down and incubate at 37°C for an additional 2–3 days.
27. Screen for the transformants of interest, e.g., reduced sporulation, accumulation of unusual pigments, restricted growth, etc. Transfer the transformants of interest into regular solid MM and check phenotypes, including secondary metabolism, further.

3.2. Genomic DNA Isolation

1. Inoculate a loopful of fungal spores (or hyphae) in 2 mL liquid MM with 0.5% yeast extract and supplements in an 8 mL test tube and incubate at 37°C slanted for 14–16 h (stationary culture).
2. Collect the mycelial mat, squeeze it with paper towel to remove the medium as much as possible, and transfer the squeezed sample into a 2-mL O-ring screw cap microcentrifuge tube.
3. Add 500 μL of breaking buffer, 500 μL of phenol: chloroform:isoamyl alcohol (25:24:1), and 300 μL of zirconia/silica beads into tubes.
4. Tightly close the cap and insert tubes in a Mini-Beadbeater in a cold room and homogenize at the highest speed for 2 min.
5. Take out the tubes and centrifuge for 10 min at 16,000 × *g* at 4°C or room temperature.
6. Transfer 300 μL of the aqueous (upper) phase into a new 1.5 mL microcentrifuge tube.
7. Add two volumes of ice-cold absolute ethanol (kept at –20°C) and gently mix.
8. Centrifuge the precipitated DNA for 10 min at maximum velocity and decant the supernatant.
9. Add 1 mL of 70% ethanol to the tubes with the DNA pellets.
10. Centrifuge at 16,000 × *g* for 5 min. Decant the supernatant.
11. Centrifuge at 16,000 × *g* for 1 min and carefully remove the supernatant as much as possible.
12. Let the pellet air-dry for 5–10 min.
13. Resuspend the pellet in 50 μL of TE buffer with RNaseA.
14. Incubate for 1 h in the 37°C incubator.
15. Measure DNA concentration spectroscopically and store DNA samples at –20°C until use.

3.3. Plasmid Rescue and Insert Sequencing

1. Thaw 20 μL of *E. coli* MAX Efficiency® DH10B™ Competent Cells on ice.
2. Add 1 μL of isolated *A. nidulans* genomic DNA to one tube containing 20 μL competent cells (see Note 7).
3. Label 0.1 mm electroporation cuvette for each sample and place on ice.

4. Carefully pipette and transfer the mixture of cells and DNA to a chilled cuvette. Tap the suspension to the bottom (see Note 8).
5. Place the cuvette in the electroporator shock-pod and close the shock-pod.
6. Press the "pulse" button (see Note 9).
7. Add 1 mL of S.O.C. medium and transfer the solution to a 15 mL snap-cap tube (e.g., Falcon tube).
8. Shake at 225 rpm at 37°C for 1 h.
9. Spread 100 μL of the dilution on prewarmed LB plates containing 100 μg/mL ampicillin.
10. Incubate plates overnight at 37°C.
11. Pick 2–5 colonies and inoculate 5 mL LB broth containing 100 μg/mL ampicillin overnight at 37°C.
12. Purify the plasmid DNA using QIAquick PCR Purification Kit.
13. Sequence the plasmid DNA with the primer set OMN33 and OMN35 (see Note 10).
14. Search the full genome sequence (the Broad Institute, http://www.genome.wi.mit.edu/annotation/fungi/aspergillus/index.html) with the insert sequences.
15. Reintroduce the plasmids into the recipient strain for confirmation of the resulting phenotypes.
16. If more than one gene resides within the insert, functional validation of each gene is necessary. This can be done by introducing multiple copies of each gene to the recipient strain.

4. Notes

1. Eye protection and care should be used to prevent contact of the solution with eye and skin. The KOH solution should be made fresh before to prevent reaction with CO_2.
2. KCl–Citric acid solution can be stored for short periods or sealed carefully (e.g., with Parafilm) to prevent evaporation.
3. 2× Protoplasting solution should be made less than 1 h before use.
4. If PEG is precipitated then it must be warmed and mixed thoroughly before use to allow the PEG to dissolve. PEG precipitate will destroy protoplasts and reduce transformation frequencies.
5. After autoclaving, the solution will be green but the color of the solution will change to purple with time.

6. After autoclaving, allow top agar to cool to ~50°C.
7. Solutions containing the DNA to be transformed must contain a minimum of salts.
8. Avoid formation of bubbles.
9. Electroporate using the following electroporator conditions: 1.8 kV, 200 Ω, and 25 μF.
10. Primers to sequence the insert in pRG3-AMA1:
    (a) OMN33 5′-AAATAAGCTTGCATGCGC-3′.
    (b) OMN35 5′-GCCAGTGAATTCGAGCTC-3′.

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