Chapter 8

Genetic transformation of *Candida auris* via homology-directed repair using a standard lithium-acetate protocol

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Abstract Reverse genetics is a particularly powerful tool in non-model organisms with known whole-genome sequences enabling the characterization of gene and, thus, protein function via a mutant phenotype. Reverse genetic approaches require genetic manipulation techniques which often need to be specifically developed for non-model organisms, this can be fraught with difficulties. Here, we describe a genetic transformation protocol for the recently emerged human pathogen *Candida auris* to target the integration of DNA constructs into genomic locations via homology-directed repair using long flanking homologous sequences (> 1kb). We detail the generation of DNA constructs for gene deletion with dominant drug-resistance markers via fusion-PCR, the transformation of these constructs into chemically competent *C. auris* cells, and the confirmation of correct integration by PCR. This strategy can be adapted to deliver DNA constructs other than deletion cassettes, including promoter exchanges and protein tags.

Key words: *Candida auris*, gene deletion, gene targeting, genetic manipulation, genetic transformation, dominant drug-resistance markers.

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1. Introduction

Candida auris emerged about a decade ago as a multidrug-resistant fungal pathogen of humans. Since its first identification, this yeast has been found across all continents, causing clonal outbreaks in intensive care settings [1, 2]. Our ability to explain the life cycle and unique phenotypic characteristics of *C. auris* is currently impeded by a lack of information about its basic biology [3]. Development of molecular tools for *C. auris* to make it genetically tractable and traceable will be key for elucidating which mechanisms underpin its (multi)drug resistance, virulence, pathogenicity, and other relevant traits.

To date, genetic manipulation of *C. auris* has been successfully performed by several laboratories delivering genome alteration cassettes by various methods [4–13]. Also, a CRISPR (clustered regularly interspaced short palindromic repeats)-Cas9 system was developed for non-*albicans* *Candida* species [4] and proved successful in genetically manipulating *C. auris* [8–10]. However, this system relies on the delivery of the genome-editing tool as a ribonucleotide protein. Additionally, we and others have started to generate deletion mutants by introducing dominant drug resistance markers flanked by sequence homologies to the target locus [5, 11–13]. The targeting method via homology-directed repair can be used more widely for applications, such as gene deletion, placing genes under the control of heterologous promoters, protein engineering, or introducing point mutations. Currently, there are three dominant drug-resistance marker (DDRMs) cassettes available

for *Candida auris*, these cassettes confer resistance to the antibiotics nourseothricin (NAT or clonNAT), hygromycin B, and mycophenolic acid [6]. Other (model) yeasts such as *Saccharomyces cerevisiae* and *Candida albicans* only need short flanking regions (30-40 bp) to carry out homologous recombination [14, 15]. Although short flanking regions have been used successfully to delete *HOG1* in *C. auris* [5], our experience indicates that *C. auris* is less efficient at homology-directed targeting and requires homologous flanking regions in excess of 1 kb for correct integration of a DNA construct at a reasonable frequency [11].

Here, we detail all the steps necessary for homology-directed targeting of a DDRM cassette to a desired genomic region of *C. auris*. The protocol described here uses chemically competent cells subjected to heat shock to deliver a DDRM cassette generated by a fusion-PCR method [16] to delete the target region. However, this protocol can easily be adapted to deliver other genetic manipulation constructs (promoter exchange, protein tags, etc).

2. Materials

Prepare all solutions using ultrapure H_2O (prepared by purifying deionized H_2O, to attain a sensitivity of 18 MΩ-cm at 25 °C) and analytical grade reagents. Autoclaving is performed at 121 °C for 15 min. Prepare and store all reagents at room temperature (unless indicated otherwise). Diligently follow local regulations for disposal of waste materials.

2.1 General materials

1. Thermocycler.
2. Heat block (or water bath with temperature regulation).
3. Static and shaking incubators.
4. Standard and Nanodrop spectrophotometers.
5. Agarose gel electrophoresis system: gel tray, electrophoresis chamber, and power source.
6. Loading dye.
7. Ethidium bromide.
8. DNA size marker.
9. UV light lamp.
10. 0.22-μm pore size filter system for sterilizing solutions
11. YPD (yeast-peptone-dextrose): dissolve 10 g of yeast extract, 20 g of mycological peptone and 20 g of glucose in 700 mL of H_2O. When dissolved, add H_2O to a final volume of 1 L and autoclave. For solid YPD medium add 20 g of agar before autoclaving.
12. 0.5 M EDTA disodium salt stock solution, pH 8.0: dissolve 93.05 g of EDTA disodium salt in 400 mL of H_2O and adjust the pH with sodium hydroxide (NaOH) pellets. EDTA will not dissolve completely until the pH is about 8.0. Add H_2O to a final volume of 500 mL and autoclave.
13. 1× TAE buffer: 50 mM EDTA disodium salt, 2 M Tris base and 1 M acetic acid. Preparation of a concentrated 50× stock solution is recommended: dissolve 242 g of Tris base in 750 mL of H_2O, add 57.1 mL of acetic acid and 100 mL of 0.5 M EDTA (pH 8.0), and finally, adjust the volume to 1 L by adding H_2O. The final pH of the 50× TAE buffer should be ~8.5. Prepare the 1× TAE working buffer by adding 49 parts of H_2O to 1 part of 50× TAE buffer.
14. 1 M Tris-HCl buffer, pH 8.0: dissolve 121.14 g of Tris base in 700 mL of H_2O, adjust the pH using concentrated HCl, add H_2O to a final volume of 1 L and autoclave.

2.2 Fusion PCR

1. Specific oligonucleotide primers designed for the target locus.
2. High-fidelity DNA polymerase kit (see **Note 1**).
3. DNA clean-up kit and DNA gel extraction kit.
4. 1× TE buffer (10 mM Tris-HCl and 1 mM EDTA disodium salt). Preparation of a concentrated 10× stock solution is recommended: add 10 ml of 1M Tris-HCl buffer (pH 8.0) and 2 mL of 0.5 mM EDTA (pH 8.0) to 88 mL of H_2O for a final volume of 100 mL and autoclave. Prepare the 1× TE working buffer by adding 9 parts of H_2O to 1 part of 10× TE buffer.

2.3 Transformation

1. 1 M lithium acetate: dissolve 6.6 g of lithium acetate in 70 mL of H_2O, then add H_2O to 100 mL, sterilize the solution using a 0.22-μm pore size filter system.
2. 50% polyethylene glycol MW 3,350 (PEG-3,350): mix 50 g of PEG-3,350 and 50 mL H_2O, then autoclave.
3. Transformation Mix: Combine 240 μL 50 % PEG-3,350, 36 μL 1 M lithium acetate, and 10 μL of 10 mg/mL denatured herring sperm DNA (denature the herring sperm DNA by incubating at 95 °C on a heat block for 10 min followed by cooling on ice for 5 min).
4. Solid YPD medium (YPD plates) with selection drug at appropriate concentration.

2.4 Checking transformants

1. Taq DNA polymerase or high-fidelity DNA polymerase (see **Note 1**).
2. 0.02 M NaOH dilution: dissolve 0.08 g of NaOH in 70 mL H_2O, then add H_2O to 100 mL.
3. 50 % Glycerol: mix 50 mL glycerol with 50 mL H_2O, then autoclave.

2.5 Genomic DNA extraction

1. Acid-washed glass beads.
2. Multi-tube mixer or sample homogenizer.
3. DNA extraction buffer: prepare a bottle with around 100 mL of H_2O, then add 20 mL of 1 M NaCl, 4 mL of Triton X-100, 2 mL of 1 M Tris-HCl buffer at pH 8.0, 400 μL of 0.5 M EDTA and 2 g of sodium dodecyl sulfate (SDS). After mixing add H_2O up to 200 mL and autoclave.
4. Phenol:chloroform:isoamyl alcohol (25:24:1).
5. 3 M sodium acetate pH 5.2: dissolve 12.3 g of sodium acetate in 30 mL H_2O, then add H_2O up to 50 mL and autoclave.
6. 100 % ethanol.
7. 70 % ethanol: mix 70 mL 100 % ethanol with 30 mL H_2O.
8. 1 mg/mL RNase stock solution: dissolve 10 mg of RNase A in 10 mL 10 mM Tris-HCl (pH 7.5), 15 mM NaCl, incubate at 100 °C for 5 min to inactivate contaminating DNases, and cool slowly to room temperature. Finally dispense into aliquots and store at - 20 °C.

3. Methods

All procedures are carried out at room temperature unless otherwise specified. The protocol described here is for one transformation and one control.

3.1 Obtaining the transformation cassette by fusion-PCR

The fusion-PCR method allows joining of DNA fragments, which share homology with the target region, with a DDRM [16]. Using this method, a cassette consisting of a DDRM gene [17] flanked by 1-2 kb of the up- and downstream sequence of the target DNA region is constructed (Fig. 1a). Afterwards, this cassette is directly used for transformation.

Initially, three DNA fragments are obtained by standard PCR (Fig. 1a): PCR1 and PCR2 correspond to the upstream and downstream sequences of the target DNA region including overhang regions (20-30 bp) at the 3' and 5' end, respectively (Fig. 1a). The overhang regions are homologous to the promotor and terminator sequences flanking the DDRM gene. PCR3 amplifies the complete DDRM gene including promoter and terminator region (Fig. 1a). These three fragments are joined by fusion-PCR, followed by a final standard PCR4 to amplify the final transformation cassette using nested primers (Fig. 1a).

All these PCRs are performed using a high-fidelity DNA polymerase, the manufacturer's instructions need to be followed for primer design, concentration of reaction components, and PCR programme. For PCR mixtures combine reagents in small PCR tubes as summarised in Table 1. PCR reagents should be kept on ice while handling. Transfer the PCR tubes containing the reagent mixtures to a thermocycler, and amplify following the conditions detailed in Table 2.

Table 1. Representative PCR reagents concentrations (see **Note 32**). Add items in the indicated order from top to bottom.

Reagent	Final concentration
H_2O	add to final volume
Buffer	1 ×
$MgCl_2$	1.5 mM
dNTPs	200 µM (each)
Forward primer (10 pmol $µL^{-1}$)[a]	0.5 µM[b]
Reverse primer (10 pmol $µL^{-1}$)[a]	0.5 µM[b]
Template DNA	0.01-50 ng[c]
DMSO (optional)[d]	5%
Polymerase	1 U

[a]Primer must be diluted to a working concentration of 10 pmol $µL^{-1}$. Primer dilutions can be made in H_2O but TE buffer is recommended.
[b]Final primer concentrations can vary in a range of 0.2 - 1.0 µM.
[c]For low-complexity DNA (e.g. plasmid), it is recommended to use 0.01 - 2 ng. For high-complexity genomic DNA, use 10 - 50 ng.
[d]Recommended for GC-rich amplicons, such as the *CaNAT1* and *CaSAT1* cassettes.

Table 2. Representative cycling conditions (see **Note 33**).

Cycle step	Temperature	Time	Cycles
Initial denaturation	95-98 °C	30-60 s	1
Denaturation	95-98 °C	10-30 s	
Annealing	T_m[a]	30-60 s	25-35
Extension	72 °C	30-60 s/kb	
Final extension	72 °C	5-10 min	1

[a]A T_m calculator can be found at www.thermofisher.com/tmcalculator.

1. Oligonucleotide primer design: Eight primers will be necessary (Fig. 1a) (see **Note 2**), two primers (p1 and p2) for the upstream flanking region (PCR1), where the reverse primer (p2) includes a homologous overlapping "tail" sequence corresponding to the DDRM promoter region. Two primers (p3 and p4) for the downstream flanking region (PCR2), on this occasion the homologous "tail" sequence corresponds to the DDRM terminator region in the forward primer (p3) (Fig. 1a). Note that "tail" sequences always need to be placed at the 5'-end of an oligonucleotide during primer design, as the 3'-end targets it to the desired genomic region. Furthermore, two primers (p5 and p6) are needed to amplify the whole DDRM cassette (PCR3), including its promoter and the terminator, as well as two nested primers (p7 and p8) for the amplification (PCR4) of the transformation cassette (Fig. 1a).
2. PCR1 and PCR2 (Fig. 1a): Use genomic DNA (gDNA) (gDNA extraction is described in section 3.4) from the target strain as template and the primers previously designed to obtain the up- (p1 and p2) and downstream (p3 and p4) fragments. A final PCR reaction volume of 10 µL is sufficient (Table 1).
3. PCR3 (Fig. 1a): Use a plasmid (or DNA) containing the DDRM [6] as template and the primers previously designed to obtain the DDRM fragment (p5 and p6). A final volume of 10 µL is sufficient (Table 1).
4. Purify the PCR products using a DNA clean-up kit following the manufacturer's instructions.
5. Check the PCR results by electrophoresing 1 µL of the samples in an agarose gel. A loading dye must be mixed with the sample before loading onto the gel, usually, in a proportion of 1 µL of sample, 7 µL of H_2O and 2 µL of loading dye (check manufacturer's instructions). A DNA ladder marker (5 - 10 µL) must be included for size comparison. Prepare a 0.7 - 1 % agarose gel in 1× TAE buffer and add ethidium bromide to a final concentration of approximately 0.2 - 0.5 µg/mL for DNA

visualization. Pour the agarose into a gel tray with the gel comb in place to create wells for loading the DNA into. Place the gel into an electrophoresis unit and cover it with 1× TAE, load the samples in the wells and run at 100 - 125 constant V for 45-60 min (see **Note 3**). Finally, visualize your DNA fragments using a UV light lamp.

6. Determine DNA concentration of your purified samples using a NanoDrop spectrophotometer (see **Note 4**).

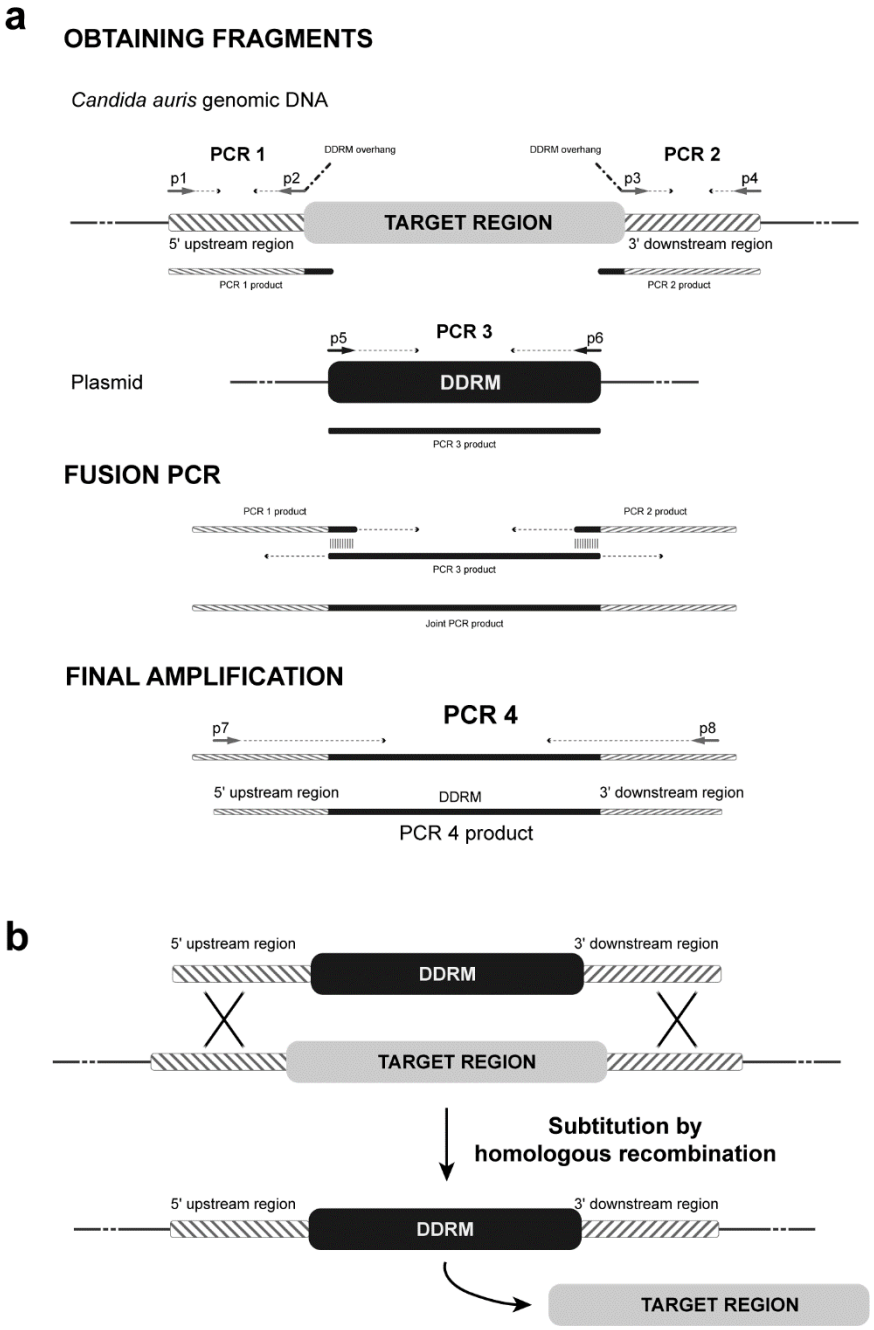

Fig. 1 Schematic representation of the transformation method. (**a**) Marker cassette obtaining by fusion PCR (see main text, section 3.1). (**b**) Target region substitution by homologous recombination within the cell after transformation (see main text, section 3.2).

7. Use the three previously obtained PCR fragments to perform the fusion-PCR (Fig. 1a). Combine the three products in roughly equimolar amounts (~0.15 pmols each, see **Note 5**) with the high-fidelity DNA polymerase, its components and DMSO as indicated by the manufacturer (Table 1) but

without oligonucleotide primers. Subject samples to eight PCR cycles (according to the polymerase manufacturer's instructions, Table 2) (see **Notes 6, 7**). Do not purify this PCR or run it in gel electrophoresis, proceed directly to the next step.

8. Directly use 1 µL of fusion-PCR as template with nested primers p7 and p8 in PCR4 to obtain the final transformation cassette (Fig. 1a) via standard PCR conditions. Before proceeding to the transformation, DNA purification of the transformation cassette is recommended. Check the purified PCR products by electrophoresing 1 µL of the samples in a DNA agarose gel (see section 3.1, step 5) and determine DNA concentration (see section 3.1, step 6) (see **Note 8**).

3.2 *C. auris* Transformation

The transformation protocol described here is adapted from a *C. albicans* protocol [18] using chemically competent cells and heat-shock for the uptake of the transformation cassette. Within the cell, the transformation cassette is integrated at the target DNA region by homologous recombination guided by the up- and downstream regions flanking the DDRM gene (Fig. 1b).

1. Grow *C. auris* cells in 5 mL YPD in a sterile 50-mL flask or 50-mL tube overnight at 30 °C in an incubator shaking at 200 rpm.
2. Dilute overnight culture 1:100 in 50 mL of fresh YPD in a sterile 250-mL Erlenmeyer flask (see **Note 9**) and incubate at 30 °C and shaking at 200 rpm until reaching mid-exponential growth 1 – 2 ×10^7 cells/ml (corresponding to an OD_{600} reading of 0.5 - 0.8 on a spectrophotometer) (see **Notes 10, 11**).
3. Transfer the culture into a centrifugation tube and harvest cells by centrifugation at 1,000 ×g for 2 min. Decant supernatant, resuspend cells gently in 30 ml of sterile H_2O (see **Note 12**). Repeat centrifugation at 1,000 ×g for 2 min. Remove H_2O by decanting as before.
4. Resuspend the cell pellet gently in 1 mL 0.1 M lithium acetate, transfer the cell suspension to a 1.5-mL reaction tube, and centrifuge at 1,000 ×g for 60 sec. Then, decant the supernatant and resuspend in 100 µL 0.1 M lithium acetate. Split the sample into two new reaction tubes, 50 µL each, one for the actual transformation and the other one as negative control (see **Note 13**).
5. Add Transformation Mix.
6. Mix the sample and add around 1 µg of transformation cassette DNA (see **Note 14**) or the same volume of sterile H_2O for the negative control.
7. Mix samples by a short pulse on a vortex mixer, and incubate at 30 °C overnight.
8. After overnight (16 -18 hrs) incubation, briefly mix the samples and heat-shock at 44°C for 15 min in a heat block or water bath.
9. Centrifuge samples at 1,000 ×g for 60 sec. Remove supernatant, resuspend the samples in 1 mL YPD, transfer to a 50-mL centrifugation tube, add 2 mL YPD, and incubate for cell recovery at 30 °C in an incubator shaking at 200 rpm (see **Note 15**).
10. Centrifuge samples at 1,000 ×g for 2 min; remove supernatant and resuspend in 100 µL sterile H_2O. Plate transformation sample (with DNA) onto selective media (YPD + selection drug at appropriate concentration) (see **Notes 16, 17**). For the negative control sample, use a small aliquot to prepare a 1:10,000 dilution in sterile H_2O and plate 100 µL (1-5 ×10^3 cells) on a YPD plate as regeneration control, plate the rest of the neat negative control sample on a YPD plate + selection drug (no colonies should grow).
11. Incubate plates at 30 °C. Colonies should be visible 2 - 4 days after plating (see **Note 18**).
12. When visible, patch colonies one-by-one to a fresh YPD plate + selection drug. Make sure that individual colonies are numbered to keep track of them. Grow overnight at 30 °C.

3.3 Transformant screening

Homology-directed repair does not seem to work very well in *C. auris*, it tends to integrate DNA randomly into the genome. For this reason, it might be necessary to screen a large number of colonies before finding a transformant in which the marker cassette is integrated at the desired locus. We recommend a fast screening of the colonies by colony PCR followed by a second check of

the positive transformants using high-quality gDNA (gDNA extraction is described in section 3.4) as PCR template (see **Note 19**). Colony PCR allows screening hundreds of colonies in one or two days.

1. Add 40 µL of a 0.02 M NaOH solution to a 1.5-mL reaction tube.
2. Collect cells with a sterile tip or toothpick taking a small section of the colony. Avoid touching the agar. Transfer the cells in the NaOH solution and mix until the solution turns slightly cloudy, then discard the tip or toothpick (see **Note 20**).
3. For cell lysis, boil the sample at 100 °C for 10 min using a heat block, then, place samples on ice for 5 min.
4. Centrifuge 2 min at 4,000 ×g. Do not mix or resuspend the pellet after centrifugation.
5. Use 1 µL from the supernatant as template for a standard 10 µl PCR as described in section 3.1 (Tables 1 and 2) (see **Note 21**). At least three PCRs are recommended for each sample (Fig. 2) (see **Note 22**): PCRa and PCRb using a primer located within the DDRM gene and another placed in the up- (p9 and p10) or downstream (p11 and p12) region of the target site, respectively (ensure that these primers are outside of the homologous flanking sequences of the transformation cassette); and PCRc using primers located within the target DNA (p13 and p14) to check the correct elimination of this region. Genomic DNA from the parental strain should be included as control, negative for the transformation cassette integration PCRs and positive for target DNA PCR (see **Notes 23, 24**).

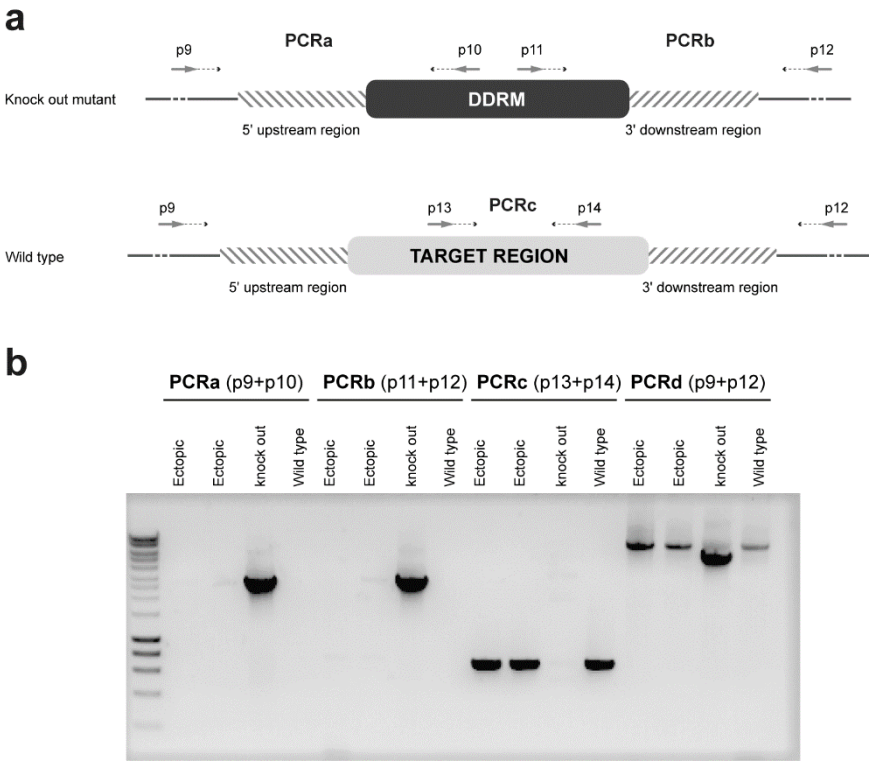

Fig. 2 Transformants screening (see main text, section 3.3). (a) Schematic representation of PCRs necessary for checking transformants. (b) Agarose gel electrophoresis showing a real example of transformants screening including two false positives (ectopic), one positive (knock-out) and the wild-type.

6. Perform gel electrophoresis using the whole volume of the PCRs (as described in section 3.1.5) to check the results. The expected result for correct integration of the cassette and deletion of the gene is: positive for both PCRs using the primers located within the marker and in the up- and downstream region (PCRa and PCRb) and negative for the PCR using primers of the target gene (PCRc) (Fig. 2b)

7. Double-check the positive transformants using high-quality gDNA (gDNA extraction is described in section 3.4). Repeat PCRs as described above (Fig. 2) using 1 µL of a dilution 1:10 of the high-quality gDNA (see **Note 25**).
8. Finally, to store transformants, grow confirmed transformants in 5 mL YPD + selection drug in a 50-mL flask or 50-mL tube overnight at 30 °C shaking at 200 rpm in an incubator. Harvest cells by centrifugation at 1,000 ×g for 2 min and wash with 5 mL of sterile H_2O. Repeat centrifugation at 1,000 ×g for 2 min. Decant the supernatant, resuspend cells in 500 µL of sterile YPD (or H_2O) and mixed with 1 ml of 50 % of sterile glycerol (final concentration 33 %) (see **Note 26**).

3.4 Extraction of high-quality genomic DNA (gDNA)

The gDNA extraction protocol described here is adapted from a method used in budding yeast [19].

1. Grow *C. auris* cells in 5 mL YPD using a 50-mL flask or 50-mL tube overnight at 30 °C in an incubator shaking at 200 rpm.
2. Harvest the cells by centrifugation at 1,000 ×g for 2 min.
3. Decant supernatant, gently mix cells with 500 µL of H_2O and transfer cells to a 2-mL reaction tube.
4. Centrifuge sample for 2 min at 12,000 ×g. Decant the supernatant and resuspend cells in the residual liquid.
5. Add 0.3 g of acid-washed glass beads and 300 µL of DNA extraction buffer. Disrupt cells in a multi-tube mixer or a sample homogenizer for ~3 min.
6. In a fume hood, add 300 µL of phenol:chloroform:isoamyl alcohol (25:24:1) and mix.
7. Centrifuge for 10 min at 12,000 ×g. In a fume hood, transfer the top aqueous layer to a new 1.5-mL reaction tube (see **Note 27**).
8. Determine the volume of the top aqueous layer, either by weighing or using a micropipettor (typically 300 - 400 µL are recovered), add 0.1 volumes of 3 M sodium acetate pH 5.2 and 2 volumes of ice-cold 100 % ethanol. Mix by inversion 4 - 5 times.
9. Incubate at -20°C for at least 20 min to allow DNA precipitation (see **Note 28**).
10. Centrifuge for 10 min at 12,000 ×g.
11. Decant supernatant and wash pellet in 70 % ethanol. Centrifuge for 5 min at 12,000 ×g. Repeat this step (see **Note 29**).
12. Remove ethanol and air-dry the pellet (optional, use a SpeedVac) (see **Note 30**).
13. Dissolve DNA pellet in 50-100 µL of either TE buffer or sterile H_2O (see **Note 31**).
14. Add 2.5-5 µL of 1 mg/mL RNase A stock solution (final concentration of 50 µg/mL) and incubate sample 30 - 60 min at 37 °C.
15. Check gDNA quality by electrophoresing 1 µL of the sample in an agarose gel (as described in 3.1.5). High-quality gDNA size should run slower than commercial DNA markers (>10 kb) and form a tight band without a lower-weight smear which would indicate degradation or shearing of the DNA sample.

4. Notes

1. Commercially available DNA polymerase kits contain all necessary buffers and supplements.
2. Good primer design is fundamental for efficient PCRs. Many primer design guidelines are available online, for example https://www.thermofisher.com/blog/behindthebench/pcr-primer-design-tips/.
3. Voltage and time might need to be adjusted depending on the make of your DNA gel electrophoresis kit, always allow for enough time to separate the DNA size marker bands without running them off the gel.
4. Alternatively, DNA concentration can be determined by the band intensities on a gel in comparison with DNA ladder marker bands similar in size to the PCR product. The DNA amount included in certain bands of the DNA ladder is usually provided by the manufacturer.
5. To calculate the pmols of a double-stranded DNA, use the following equation: pmol = M/(0.66*N), where M is the mass of the DNA in ng, and N is the number of nucleotide pairs; 0.66 accounts for

the average molecular weight of a nucleotide pair. 1 µg (1,000 ng) of a 1,000 base pair DNA fragment thus corresponds to 1.52 pmol. DNA amount conversion calculators can also be found online, e. g. https://www.promega.co.uk/resources/tools/biomath/.

6. A melting temperature (T_m) of 55°C is recommended, but It could be higher to minimize the formation of unspecific DNA fragments.
7. Fusion-PCR is not meant to amplify the product but to join the DNA fragments. Therefore, only a few cycles are needed.
8. Due to the complexity of the process, it is not unusual to obtain unspecific DNA fragments in PCR4. In this case, it is recommended to run the whole PCR reaction on an agarose DNA gel and purify the fragment of correct size from the gel using a DNA gel extraction kit. This extraction process is of lower efficiency than using a DNA purification kit to clean up the PCR reaction itself. Therefore, a large volume of the final PCR product might be required to generate sufficient amounts of the transformation cassette.
9. If several transformations are to be performed with the same strain, or more cells/transformation are required, using a higher volume of YPD is advisable. Please note, the indicated volume on the Erlenmeyer flask must be 5-times bigger than the volume of culture medium used. If in this step a higher volume is used, the volumes in following steps must be adjusted accordingly.
10. Usually, an OD_{600} of 0.5 - 0.8 is reached after 3 - 5 hours. Cells in late-exponential growth or stationary phase can be used, but the transformation efficiency will be considerably reduced.
11. Please note, that OD_{600} readings are influenced by make and model of spectrophotometer and the strain background.
12. Mix cells gently by pipetting up and down, or on a vortex mixer at low-medium velocity, mixing conditions harsher than that could cause cell death and, thus, reduce transformation efficiency.
13. If the culture volume was changed in section 3.2 step 2, ensure that each tube contains between 1 - 5 × 10^8 cells.
14. A higher amount of DNA will result in a higher total number of transformants. However, the frequency of correctly targeted transformants is not affected. Therefore, the use of a higher concentration of DNA will allow the screening of a higher number of transformants, which might be advantageous when targeting efficiency is low.
15. This incubation step gives cells time to express DDRM gene after the transformation in order to become resistant. The incubation time would depend on the DDRM used, but a few hours tend to be sufficient.
16. Samples are best split over 2 - 3 plates to avoid overcrowding of colonies.
17. The antimicrobial drugs shown to work in *C. auris*, at the final concentrations indicated, are: nourseothricin (also known as NAT or clonNAT) at 200 mg/L, hygromycin B at 400 mg/L, and mycophenolic acid at 50 mg/L.
18. If the mutant has a growth defect, colonies may need more than 4 days to appear. However, after prolonged incubation small colonies can appear, likely untransformed background colonies tolerant or spontaneously resistant to the antimicrobial. In such circumstances it can be difficult to separate true transformants from background.
19. The DNA yield and quality in colony PCR tends to be low. Therefore, the colony PCR might not work or give ambiguous results. For this reason, we highly recommended confirming possible positive transformants with a standard PCR using high-quality gDNA as template. Performing the initial screen with good-quality gDNA would be too laborious.
20. A very high amount of cells can negatively affect the PCR efficiency.
21. This PCR can be carried out using a high-fidelity or a Taq polymerase, follow manufacturer's instructions to set up reactions.
22. A PCR multi-well plate can be used when a very large numbers of transformants are analysed.
23. An additional PCR using a pair of primers from a region known to be present in all strains, for example the actin gene (*ACT1*), can be included as a positive control for DNA quality.

24. When a large number of transformants need to be screened, preparing at least three PCRs for each one will be tedious. A faster and cheaper alternative is to perform PCRa first, and continue only with positive candidates. Then, perform the other PCRs to confirm the correct integration at the downstream end (PCRb) and the elimination of the target gene (PCRc).
25. Alternatively, if the target DNA region and the marker sizes are different, a PCRd using primers located in the up- or downstream region flanking the targeted DNA sequence (p9 and p12) can be done (Fig. 2). Using these primers, the fragment would be amplified in both cases, either the cassette has been correctly integrated or not, but the size of the fragment would be different.
26. Any final glycerol concentration between 25 – 50 % works well.
27. After centrifugation three layers are formed. It is important to take only the top layer without touching the middle white layer. Transfer of material from the middle layer would contaminate the gDNA, decreasing its quality and its efficiency as a PCR template.
28. Samples can be kept overnight at -20 °C. However, long incubation times at low temperature will precipitate undesired components such as large amounts of sodium acetate, reducing the quality of the gDNA sample.
29. For a better elimination of salt in the final gDNA, washes with serially diluted ethanol can be performed. Perform 3 - 4 washes using ethanol dilutions of descending concentrations between 95 % and 70 %.
30. Samples are dry when ethanol smell cannot be detected anymore. Do not over-dry the DNA pellet as this makes it difficult to dissolve it again.
31. Heating the sample to 50 °C for a few min after adding H_2O or buffer improves solubility of the sample.
32. Concentrations are indicative since different polymerase kits have particular specifications. Always check manufacturer's instruction for specific reaction conditions.
33. Conditions are indicative since different polymerase kits have particular specifications. Always check manufacturer's instruction for specifics.

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