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Phenotypic Profiling of *Candida glabrata* in Liquid Media

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[Abstract] Here, we describe a method for a large-scale liquid screening approach in *C. glabrata*. This liquid media method offers several distinct advantages over solid media approaches. This includes growth measurement on a plate reader instead of comparing growth by eye-sight. Furthermore, the liquid method requires lower amounts of antifungals and offers a higher sensitivity. While this method has been optimized for *C. glabrata* it might be used for other *Candida* species and yeast-like fungi as well.
Keywords: *Candida glabrata*, Liquid media screening, Antifungals, Large-scale screening, Antifungal tolerance, Antifungal resistance

Materials and Reagents

1. Filtered pipet tips 1, 250 µl XL (Starlab, TipOne®, catalog number: S1122-1830)
2. Pin pad, 96 RePad (Singer Instruments, catalog number: RP-MP-2L)
3. Pipet tips 300 µl (Starlab, TipOne®, catalog number: S1110-8810)
4. Filtered pipet tips 200 µl (Starlab, TipOne®, catalog number: S1120-8810)
5. 96-well plates (Starlab, CytoOne®, catalog number: CC7682-7596) (200 µl/well)
6. Aluminum foil, self-adhesive (Starlab, catalog number: E2796-9792)
7. Breathable foil (Sigma-Aldrich, catalog number: Z380059)
8. Petri dishes (92 mm) (SARSTEDT)
9. Gas-permeable material
10. Culture tube (for at least 5 ml of culture)
11. Strains to be screened
    Examples: Deletion mutants of *C. glabrata* (Schwarzmüller *et al.*, 2014) or clinical isolates of *C. glabrata*
12. Glycerol
13. Compound(s) used for the screening
    Example: Fluconazole (Discovery Fine Chemicals, catalog number: 86386-73-4)
14. Control strains (see the ‘Notes’ section for further details)
15. DMSO
16. (Optional) Ethanol

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17. Sterile water (double distilled)
18. Bacto™ peptone (BD, Bacto™, catalog number: 211820)
19. Bacto™ yeast extract (BD, Bacto™, catalog number: 212720)
20. Bacto™ agar (BD, Bacto™, catalog number: 214030)
21. Glucose (Merck Millipore, catalog number: 108337)
22. YPD media (yeast extract peptone dextrose) (see Recipes)
23. Solid YPD media (see Recipes)

Equipment

1. Yeast replica robot (Singer Instruments, model: RoToR HDA)
2. Plate reader (Victor^{3}V) (PerkinElmer, model: Victor^{3}V Multilabel Plate Reader)
3. Electronic pipette 1,200 µl (Gilson, model: Pipetman Concept, catalog number: F31015)
4. Electronic pipette 5,000 µl (Gilson, model: Pipetman Concept, catalog number: F31016)
5. Electronic pipette 12 x 300 µl (Gilson, model: Pipetman Concept, catalog number: F31044)
6. Plate mixer (Eppendorf, model: MixMate®, catalog number: 5353000014)
7. Rotary shaker for culture flasks (Eppendorf, New Brunswick™, model: Innova® 44)
8. Incubator (Heraeus Instruments, catalog number: B6120)
9. (Optional) 48-well replica stamp (V&P Scientific, catalog number: VP 408H)
10. (Optional) 96-well replica stamp (V&P Scientific, catalog number: VP 407)
11. (Optional) Library copier (guide for stamps) (V&P Scientific, catalog number: VP 381)

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Procedure

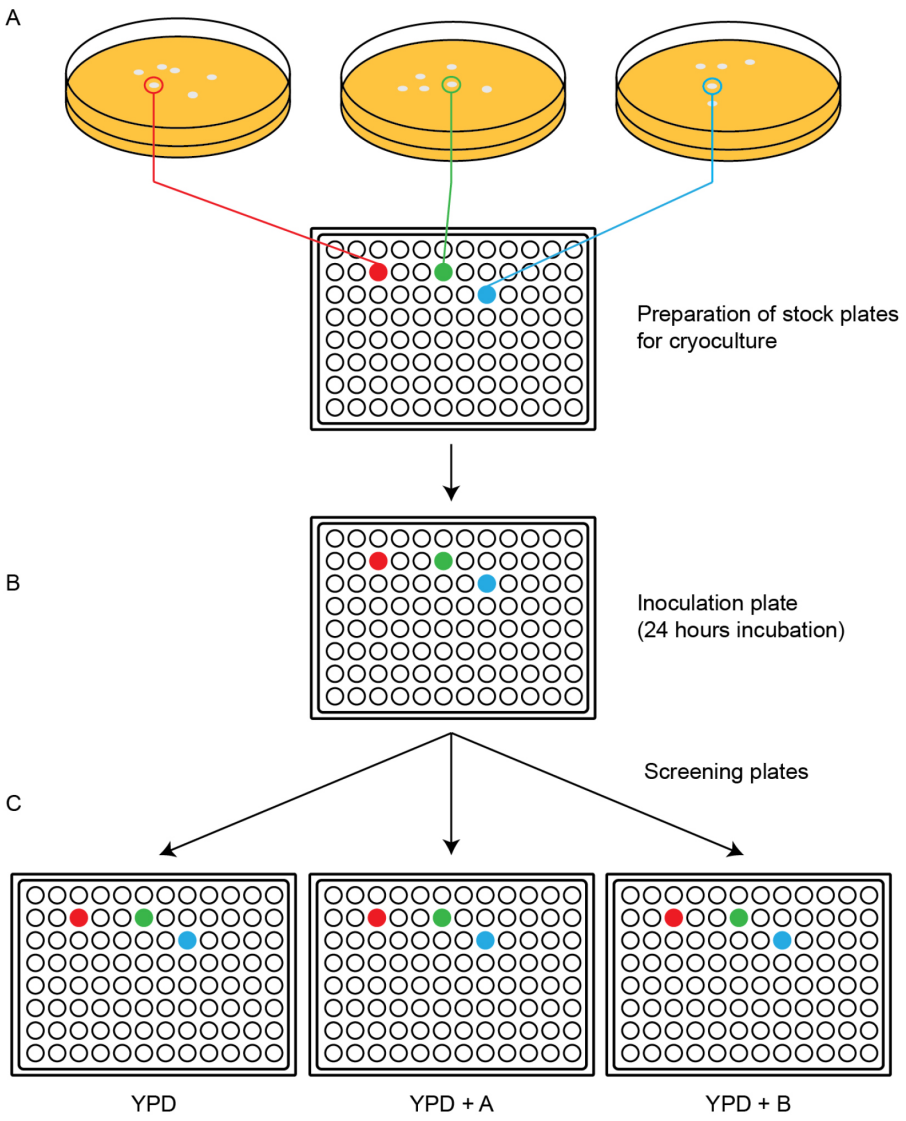

Figure 1. Overview of the plates used in the screening process. A. Single colonies are picked with sterile pipette tips and used to inoculate the wells on a 96-well plate filled with liquid YPD medium. After incubation overnight, this plate is used to inoculate several 96-well plates that are later used as stock plates and frozen at -80 °C. B. A stock plate can be thawed and used to inoculate a plate with fresh media. After 24 h of incubation this plate holds the culture that is used to inoculate the plates for the (C) actual screening.

Note: All of the working steps shown in this section should be performed under sterile conditions unless stated otherwise.

A. Arraying of the stock plates and preparation for storage

1. Array the strains in a 96-well format and include the appropriate control strains on each plate (at least two control strains or two replicates of the same control strain are recommended per plate), which is shown in part A of Figure 1. The plates should be filled with 200 µl YPD medium

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per well. Each well can be inoculated by transferring cells from a solid media plate using a sterile pipette tip. For further tips regarding control strains see the 'Notes' section.

2. Prepare a 96-well plate with 200 µl of YPD medium in each well and inoculate it using a Singer RoToR HDA with a long-pin pin pad. The recommended settings for the Singer RoToR are shown in the 'Notes' section.
3. Incubate the plates at 30 °C overnight without agitation.
4. Centrifuge the plates for 5 min at 47 *x g* and remove the supernatant by pipetting.
5. Resuspend the cell pellets in 200 µl of sterile 15% glycerol by careful pipetting. Avoid quick aspirations that might cause cross contaminations.
6. Seal each 96-well stock plate with an aluminum foil and store the plates immediately at -80 °C.

B. Preparation of the inoculation culture

1. Prepare at least one 96-well plate with 200 µl of YPD medium in each well for each stock plate to be screened.
2. Remove one stock plate at a time from the -80 °C freezer and wait until the contents of all wells have thawed. Frozen or partly frozen wells might result in malfunctions of the Singer RoToR system.
3. Immediately inoculate the prepared plates using the liquid to liquid option of the Singer RoToR HDA. The settings are shown in Table 3 of the 'Notes' section.
4. Reseal the stock plate with an aluminum foil and immediately store the plate at -80 °C in a freezer again.
5. Incubate the inoculated plates at 30 °C and 120 rpm for 24 h. Seal the plates with breathable foils or cover the sides of the plates with a gas-permeable material to avoid excessive evaporation. Else the exposure to the warm air flow of the incubator might cause excessive evaporation and render some wells unusable. This step is shown in Figure 1 part B.

C. Large-scale liquid screening

1. Prepare at least one 96-well plate with 200 µl of YPD medium only in each well for each inoculated plate. These plates will serve as control plates and the growth of the tested strains on all supplemented plates will be compared to the growth on these plates. These plates are presented in Figure 1 part C, as well as in Figure 2.

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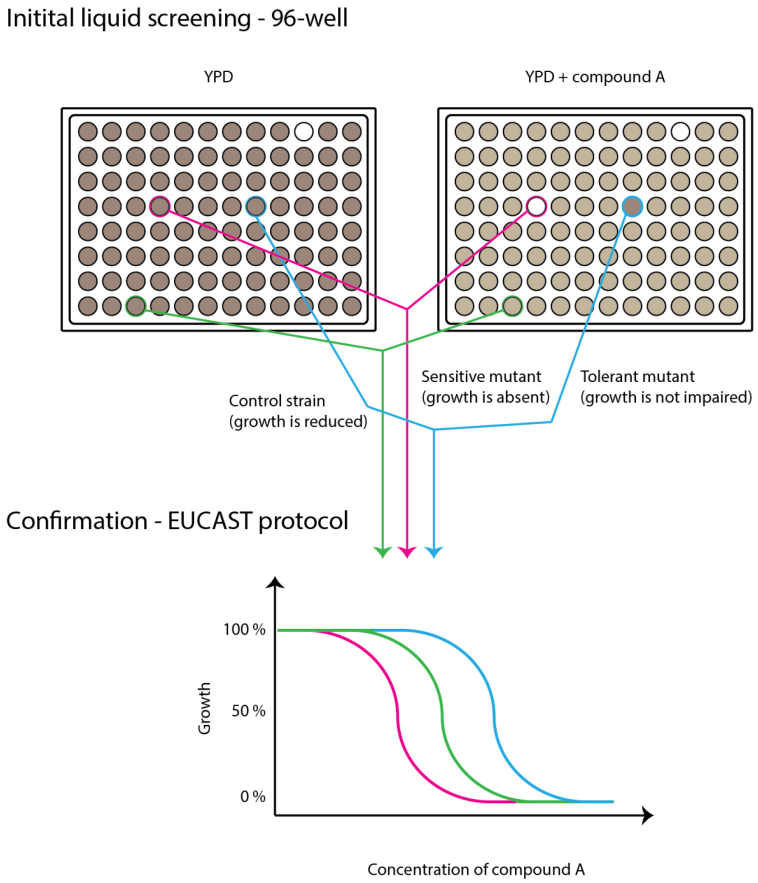

Figure 2. Schematics of the liquid screening approach. During the initial screening the growth of strains on two plates is monitored by measuring the OD_{600} on a plate reader. The growth on the plate supplemented with compound A (see 'Notes' section for further information regarding the screening concentrations) is compared to a plate filled with YPD media only. In case of an antifungal the wildtype (green) is likely to exhibit a decreased growth on the plate supplemented with compound A compared to the control plate. For a sensitive strain (pink) the growth will be lower or even completely absent on the plate supplemented with compound A. A tolerant strain (blue) will show an increased growth on the plate supplemented with compound A compared to the wildtype. The putative hits from the initial screening are confirmed using a broth microdilution based on the EUCAST protocol.

2. Prepare at least one 96-well plate with 200 µl of YPD supplemented with the compound to be tested at the appropriate concentration. The determination of the screening concentrations is further explained in the 'Notes' section.
3. Despite the shaking during the incubation many cells of the inoculation plates may have settled on the bottoms of the wells. Resuspend the cells by shaking the plate at 700 rpm on a MixMate for 15 sec. Carefully remove the plates from the MixMate to avoid cross-contamination.
4. Place one of the inoculum plates (in case several plates were prepared for each stock plate) in the Singer RoToR HDA and use the liquid to liquid transfer. The settings are shown in the 'Notes' section.

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5. Incubate all the inoculated screening and control plates at 30 °C and 120 rpm for 24 h. In case some compounds cause a heavily reduced growth or some strains have a very slow growth this can be extended to 48 or 72 h. In any case seal the plates with breathable foils or cover the sides of the plates with a gas-permeable material to avoid excessive evaporation.
6. Immediately before the measurements, shake the plates at 900 rpm for 15 sec and check if any wells have lost more than 25% of their content due to evaporation. This step and the subsequent measurement on a plate reader do not require sterile conditions.
7. Measure the OD_{600} right after the shaking on a Victor^3 plate reader using the settings shown in the 'Notes' section. Remove the breathable foil from each plate prior to measurement.

D. Analysis and interpretation of the screening results
1. Use the OD_{600} value of the YPD medium-only control well as blank value and subtract it from all other wells of the same plate.
2. Calculate the relative growth of the strains grown on the compound-supplemented plates in relation to the growth on the YPD medium plate.
3. Sensitive strains should demonstrate a significantly reduced growth compared to the control strain. Similarly, tolerant or resistant strains should show a significantly increased growth compared to the wildtype controls.
4. Known sensitive and tolerant or resistant strains should be added to each plate layout to help with the determination of proper cut-off values.

E. Verification by a microdilution assay

The method described for the microdilution assay has been adapted from the protocol of the European Committee on Antimicrobial Susceptibility Testing (EUCAST) for antifungal susceptibility testing (AST) (Arendrup *et al.*, 2012). Among other differences, this AST method uses YPD medium instead of the MOPS-buffered RPMI-1640 medium described in the EUCAST guideline. Alternatively, the Clinical and Laboratory Standards Institute (CLSI) offers a standard AST protocol designated M27-A3 that might be adopted as well.

Plate preparation:

The steps in this protocol show the preparation of plates for Fluconazole susceptibility testing. However, it can easily be adapted for other antifungals and testing compounds. The dilution scheme is summarized in Table 1.

1. Prepare a stock solution of 12.8 mg ml^{-1} Fluconazole in DMSO. To allow for pipetting, the necessary volume of the stock solution should be at least 500 µl.
2. Add 200 µl of the stock solution to a 50 ml tube labeled '1'.
3. Add 100 µl of the stock solution to a 50 ml tube labeled '2'.
4. Add 50 µl of the stock solution to a 50 ml tube labeled '3'.
5. Add 50 µl of the stock solution to a 50 ml tube labeled '4'.
6. Add 100, 150 and 350 µl of DMSO to the tubes 2, 3 and 4, respectively.

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7. Thoroughly mix the contents of tube 4 on a vortex shaker for at least 15 sec.
8. Centrifuge tube 4 quickly to avoid any droplets on the lid of the tube.
9. Transfer 100 µl of the solution in tube 4 to a 50 ml tube labeled ‘5’.
10. Transfer 50 µl of the solution in tube 4 to a 50 ml tube labeled ‘6’.
11. Transfer 50 µl of the solution in tube 4 to a 50 ml tube labeled ‘7’.
12. Add 100, 150 and 350 µl of DMSO to the tubes 5, 6 and 7, respectively.
13. Thoroughly mix the contents of tube 7 on a vortex shaker for at least 15 sec.
14. Centrifuge tube 7 quickly to avoid any droplets on the lid of the tube.
15. Transfer 100 µl of the solution in tube 7 to a 50 ml tube labeled ‘8’.
16. Transfer 50 µl of the solution in tube 7 to a 50 ml tube labeled ‘9’.
17. Transfer 25 µl of the solution in tube 7 to a 50 ml tube labeled ‘10’.
18. Add 100, 150 and 175 µl of DMSO to the tubes 8, 9 and 10, respectively.
19. Remove and discard further 25 µl from tube 7. Every tube should hold 200 µl of Fluconazole dilution at this step.
20. Add 19.8 ml of YPD medium to each tube and thoroughly mix each tube on a vortex mixer.
21. Aspirate 800 µl from tube 1 and dispense 100 µl into each well in column 1 of a 96 well plate. Repeat this step for tube 2 to 10 and pipette 100 µl into each well in columns 2 to 10. Columns 11 and 12 remain empty and will serve as positive and negative control wells.
22. Seal each plate with a self-adhesive aluminum foil and place the lid on top. Mark each plate with the preparation date.
23. Immediately freeze the plates at -80 °C.
24. Use the frozen plates within 6 months of preparation.

Table 1. Dilution scheme for Fluconazole

Stepv	Conc. [mg ml^{-1}]	Source [µl]	Vol. FCZ [µl]	Vol. DMSO [µg ml^{-1}]	200x dilution [µg ml^{-1}]	2x dilution [µg ml^{-1}]
1	12.8	Stock	200	0	12800	128
2	12.8	Stock	100	100	6400	64
3	12.8	Stock	50	150	3200	32
4	12.8	Stock	50	350	1600	16
5	1.6	Step 4	100	100	800	8
6	1.6	Step 4	50	150	400	4
7	1.6	Step 4	50	350	200	2
8	0.2	Step 7	100	100	100	1
9	0.2	Step 7	50	150	50	0.5
10	0.2	Step 7	25	175	25	0.25

a. Preparation of the inoculum:
   i. Pick a single colony from a fresh culture on a solid YPD medium plate (no more than 3 days old).
   ii. Resuspend the colony in 1,000 µl of sterile ddH_2O and vortex mix for at least 5 sec.

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iii. Measure the OD_{600} and adjust it to a value of 0.01 by diluting in YPD medium (corresponds to about 5 x 10^5 cell ml^{-1} for *C. glabrata*). Prepare at least 1.2 ml of cell suspension per row for each strain.
iv. Vortex mix the cell suspensions immediately before dispensing them into the wells.

b. Inoculation of the plates:
i. Add 100 µl of YPD medium to each well in column 11.
ii. Add 200 µl of YPD medium to each well in column 12.
iii. Add 100 µl of a cell suspension to each well in a row. Start with well 11 and then move to the higher concentrations. Thereby, an electronic pipette might be used to dispense 100 µl 11 times without aspirating again. Column 12 contains only YPD medium and serves as blank and negative growth control.
iv. After pipetting close the plates and incubate them at 35 °C. This temperature is recommended by the EUCAST guideline, however it might be changed to suit experimental needs.

c. Readout and analysis of the results:
Note: The steps of this part do not require sterile conditions.
i. Shake each plate on a plate mixer at 700 rpm for 15 sec.
ii. Measure the OD_{600} on a Victor^3 plate reader.
iii. Export the results to an Excel file.

Notes

1. Plate layout for the large-scale screenings
Each plate should contain at least two control strains, shared by all other plates. In addition, each plate should have one empty well for the negative growth control and as a blank value. This empty well can also be used to identify the plate as shown in Figure 3.

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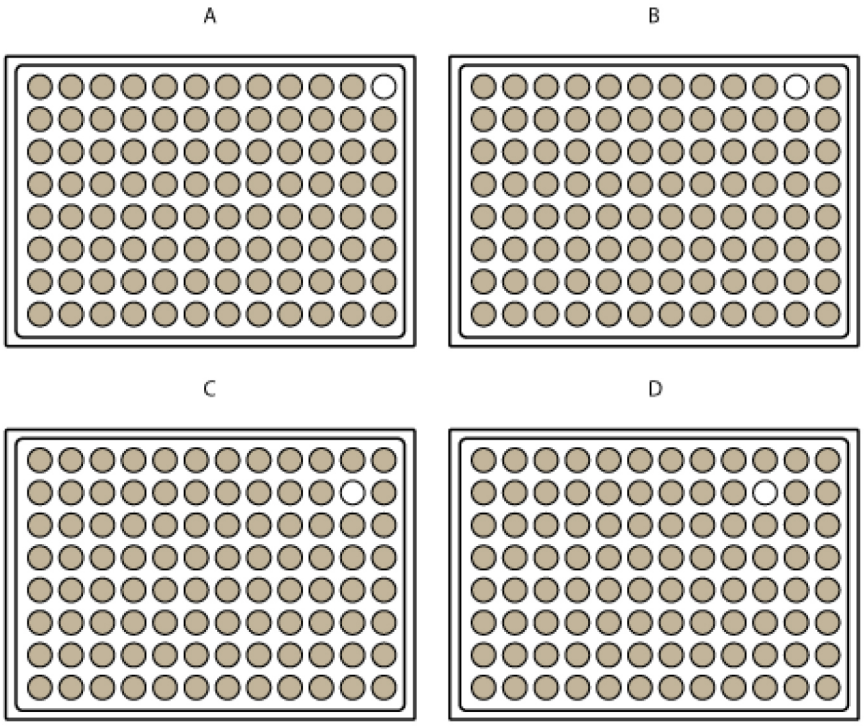

Figure 3. Plate identification by a moving empty well. The empty well is placed in the upper right quadrant of each plate. However, it moves to a different location on each plate. This gives each plate a unique attribute for identification and provides an easy way to control the correct orientation of a plate.

2. Selection of control strains

   As mentioned earlier, each plate should hold at least two control strains. Both strains should be well characterized and the response to a certain stress condition should be known. Therefore, it is possible to immediately detect an antifungal agent that is not working as expected. In case of deletion mutants, at least one of the control strains should be the background strain (the parental strain of the deletion mutants). When screening *Candida* clinical isolates it is recommended to use well-characterized strains such as *C. krusei* ATCC6258 and *C. parapsilosis* ATCC22019 (Arendrup *et al.*, 2012).
3. Backups of the stock plates

   In case anything happens to a stock plate during the inoculation of the source plate, at least one backup stock plate should be frozen at all times.
4. Settings for the Viktor^3 plate reader

   Table 2 summarizes the setting recommended for the Victor^3 plate reader. While it is possible to add a shaking step in the readout program, the shaking on a plate mixer seems more effective and less prone to cross-contaminations.

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Table 2. Settings for the Victor^3 plate reader

Setting	Value/Preset
Read time	0.1 sec
CW lamp filter	600 nm
Shaking	none

5. Settings for the Singer RoToR HDA

   Table 3 summarizes the settings for the Singer RoToR HDA robot. Due to contamination risks the 'Recycle' function should be turned off. For similar reasons the 'wet mix' options should also be deactivated.

Table 3. Settings for the Singer RoToR HDA

Setting	Value/Preset
Program	replicate many
Recycle	none
Quantity of targets	1-4
Source wet mix	off
Target wet mix	off

6. Videos of the Singer RoToR HDA

   The manufacturer of the RoToR HDA system has provided several videos that showcase the possibilities of this system.

   Use of liquid media: https://www.youtube.com/watch?v=1JabqneqXPE

   Other videos: https://www.youtube.com/watch?v=VvLQVWErZmA

   https://www.youtube.com/watch?v=AjbdJkmjZmw

7. Determination of the screening concentrations

   Prior to the large-scale screening the screening concentration for each compound was determined. To do so, the MICs for the background strain and representative strains were determined using the broth microdilution method described earlier. For the large-scale screening a concentration was chosen that inhibited the growth of the tested strains by about 20-30%.

8. Practice removing plates from the MixMate with dummy plates. If it is not done carefully it is likely to result in extensive cross-contaminations.

9. Analysis of synergism and/or antagonism of two compounds in different strains

   While this protocol was primarily used to screen drug sensitivities of a collection of gene deletion mutants, it might also be adapted to analyze synergistic and antagonistic effects.

   If two antifungal compounds do not interact with each other, the growth in the presence of both compounds ($Growth_{AB}$) should be as follows:

$$Growth_{AB} = Growth_A \times Growth_B$$

   $Growth_A$: Growth in the presence of compound A

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$Growth_B$: Growth in the presence of compound B

As an example, if a strain reaches 90% of the growth of the control condition in the presence of compound A and 70% in the presence of compound B, the growth in the presence of A and B should be 63%:

$$0.63 = 0.9 \times 0.7$$

However, two compounds might be synergistic or antagonistic and therefore the growth in the presence of A and B might be different to the calculated growth based on the results for A and B separately. If compounds A and B are antagonistic the growth in the presence of A and B will be higher than the calculated value:

$$Growth_{AB} > Growth_{AB\ calculated} = Growth_A \times Growth_B$$

If the two compounds are synergistic, the growth in the presence of both should be lower than the calculated value:

$$Growth_{AB} < Growth_{AB\ calculated} = Growth_A \times Growth_B$$

To analyze the synergistic or antagonistic effect on a large number of strains, four screening plates are required for each stock plate. One plate holds only YPD medium as control, while the other plates hold medium supplemented with compound A and compound B and both compounds respectively. For analysis **the growth is calculated first for each strain** in relation to the growth on the control plate with YPD medium only. Then the measured growth in the presence of both compounds ($Growth_{AB\ measured}$) is compared to the calculated growth in the presence of both compounds ($Growth_{AB\ calculated}$). If compounds A and B do not interact, the measured growth $Growth_{AB\ measured}$ is equal to the calculated growth $Growth_{AB\ calculated}$:

$$Growth_{AB\ measured} = Growth_{AB\ calculated} = Growth_{A\ measured} \times Growth_{B\ measured}$$

In case there are synergistic or antagonistic effects, the measured growth $Growth_{AB\ measured}$ will significantly differ from the expected growth based on the plates with compound A and B separately:

$$Growth_{AB\ measured} \neq Growth_{AB\ calculated} = Growth_{A\ measured} \times Growth_{Bmeasured}$$

The **difference between the measured growth $Growth_{AB\ measured}$ and the calculated growth $Growth_{AB\ calculated}$** is calculated for all strains. This difference ($\Delta Growth_{AB}$) will be positive in the case of synergism and negative if compounds A and B are antagonistic.

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$$Growth_{AB\ calculated} - Growth_{AB\ measured} = \Delta Growth_{AB}$$

The **difference $\Delta Growth_{AB}$ is then compared** between the control strain and the tested strains.

10. YPD medium concentration for the serial dilution

    Instead of 1x YPD medium it is possible to use 2x YPD medium, similar to the 2x RPMI-1640 medium described in the EUCAST guideline. In case 2x YPD medium is used, the inoculum should be prepared in distilled and sterile H_2O.

11. Storage time of microdilution plates

    The EUCAST guideline recommends no more than 6 months of storage at -80 °C. For testing purposes results after 6 and 12 months of storage were compared but no significant differences were found. Therefore, it seems possible to use plates even after more than 6 months of storage at -80 °C. However, at least one control strain should be included on each plate to monitor possible changes.

12. Preparation of the inoculum for the microdilution assay

    Alternatively, to pick a colony from an agar medium plate, it is possible to use cells from an overnight culture in liquid YPD medium. To do so, inoculate 5 to 10 ml of YPD medium in a culture tube and incubate overnight at 220 rpm and 30 °C.

    Dilute the cell suspension the following day to OD_{600} = 0.3 in fresh YPD medium and regrow to OD_{600} = 1.0.

13. Manual screening without a robot

    As an alternative to a RoToR HDA, the screening plates may be inoculated with replicating stamps. However, the replicating stamps have to be sterilized after each plate. Otherwise traces of a compound might be pulled into the source plate. Therefore, the pins should be submerged in Ethanol and subsequently pulled through an open flame. Before using the stamp again it should be left to cool to room temperature.

14. Computational analysis of the microdilution data

    An example of the analysis is shown in Figure 4. First, calculate the mean of all the blank wells in column 12 for each plate. The mean value is shown in a red box in Figure 4. Deduct this mean blank value from all other wells of the same plate. Following this background subtraction, calculate the growth in each well relative to the growth in well 11 of the same row. The drug concentration that inhibits the growth by more than 50% is considered the minimal inhibitory concentration (MIC). Therefore, the growth in this well should be less than 50% of the growth in well 11. Accordingly, those wells are marked with bold red letters in Figure 4.

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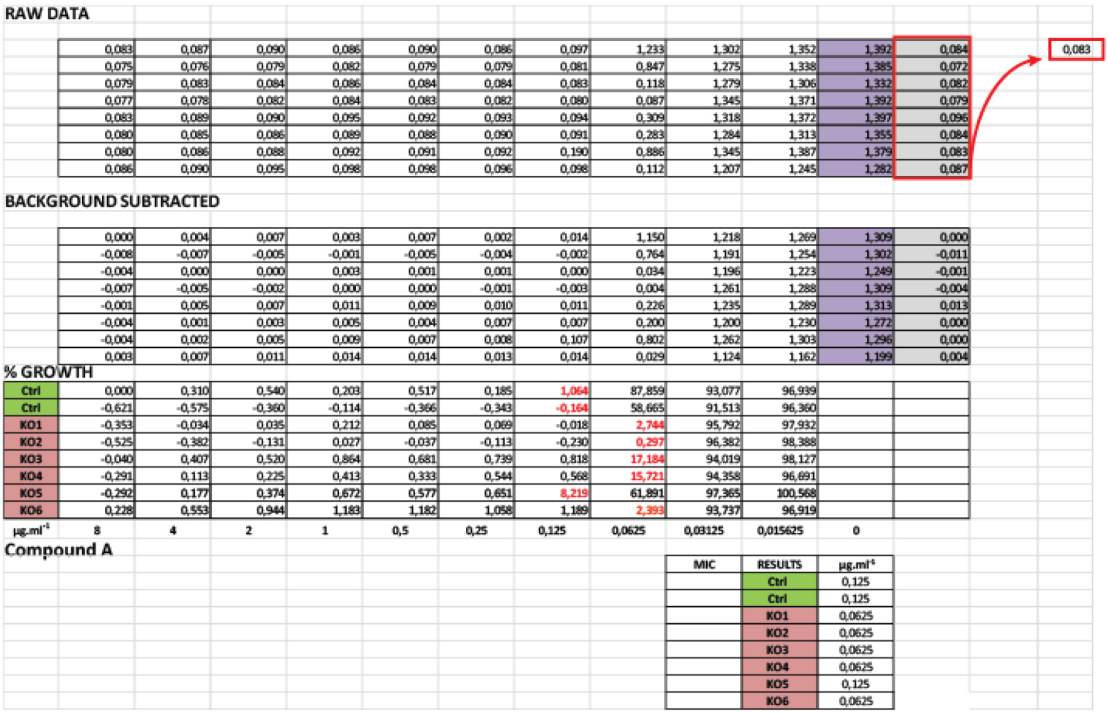

RAW DATA

	0,083	0,087	0,090	0,086	0,090	0,086	0,097	1,233	1,302	1,352	1,392	0,084
	0,075	0,076	0,079	0,082	0,079	0,079	0,081	0,847	1,275	1,338	1,385	0,072
	0,079	0,083	0,084	0,086	0,084	0,084	0,083	0,118	1,279	1,306	1,332	0,082
	0,077	0,078	0,082	0,084	0,083	0,082	0,080	0,087	1,345	1,371	1,392	0,079
	0,083	0,089	0,090	0,095	0,092	0,093	0,094	0,309	1,318	1,372	1,397	0,096
	0,080	0,085	0,086	0,089	0,088	0,090	0,091	0,283	1,284	1,313	1,355	0,084
	0,080	0,086	0,088	0,092	0,091	0,092	0,190	0,886	1,345	1,387	1,379	0,083
	0,086	0,090	0,095	0,098	0,098	0,096	0,098	0,112	1,207	1,245	1,282	0,087

0,083

BACKGROUND SUBTRACTED

	0,000	0,004	0,007	0,003	0,007	0,002	0,014	1,150	1,218	1,269	1,309	0,000
	-0,008	-0,007	-0,005	-0,001	-0,005	-0,004	-0,002	0,764	1,191	1,254	1,302	-0,011
	-0,004	0,000	0,000	0,003	0,001	0,001	0,000	0,034	1,196	1,223	1,249	-0,001
	-0,007	-0,005	-0,002	0,000	0,000	-0,001	-0,003	0,004	1,261	1,288	1,309	-0,004
	-0,001	0,005	0,007	0,011	0,009	0,010	0,011	0,226	1,235	1,289	1,313	0,013
	-0,004	0,001	0,003	0,005	0,004	0,007	0,007	0,200	1,200	1,230	1,272	0,000
	-0,004	0,002	0,005	0,009	0,007	0,008	0,107	0,802	1,262	1,303	1,296	0,000
	0,003	0,007	0,011	0,014	0,014	0,013	0,014	0,029	1,124	1,162	1,199	0,004

% GROWTH

Ctrl	0,000	0,310	0,540	0,203	0,517	0,185	1,064	87,859	93,077	96,939		
Ctrl	-0,621	-0,575	-0,360	-0,114	-0,366	-0,343	-0,164	58,665	91,513	96,360		
KO1	-0,353	-0,034	0,035	0,212	0,085	0,069	-0,018	2,744	95,792	97,932		
KO2	-0,525	-0,382	-0,131	0,027	-0,037	-0,113	-0,230	0,297	96,382	98,388		
KO3	-0,040	0,407	0,520	0,864	0,681	0,739	0,818	17,184	94,019	98,127		
KO4	-0,291	0,113	0,225	0,413	0,333	0,544	0,568	15,721	94,358	96,691		
KO5	-0,292	0,177	0,374	0,672	0,577	0,651	8,219	61,891	97,365	100,568		
KO6	0,228	0,553	0,944	1,183	1,182	1,058	1,189	2,393	93,737	96,919		
µg.ml^{-1}	8	4	2	1	0,5	0,25	0,125	0,0625	0,03125	0,015625	0	

Compound A

MIC	RESULTS	µg.ml^{-1}
	Ctrl	0,125
	Ctrl	0,125
	KO1	0,0625
	KO2	0,0625
	KO3	0,0625
	KO4	0,0625
	KO5	0,125
	KO6	0,0625

Figure 4. Analysis of the plate reader results

Recipes

1. YPD media (yeast extract peptone dextrose)
   25 g/L Bacto™ peptone
   12.5 g/L Bacto™ yeast extract
   2% glucose
2. Solid YPD media
   25 g/L Bacto™ peptone
   12.5 g/L Bacto™ yeast extract
   2% glucose
   2% agarose

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