A Novel Method for Testing Antifungal Interactions: Assessing Synergy and Antagonism in *Candida albicans* Clinical Isolates.

Hanna Lundvik[1], Ramona Santini[2], Tugce Unalan Altintop[3], Volkan Özenci[2], Dan I. Andersson[1*] and Nikos Fatsis-Kavalopoulos[1*]

* Corresponding authors

1 Department of Medical Biochemistry and Microbiology, Uppsala University

2 Department of Clinical Microbiology, Karolinska Institutet

3 Department of Medical Microbiology, Hacettepe University

Abstract

The rise in antifungal resistance has limited treatment options for serious fungal infections, emphasizing the need for effective combination therapies. However, low-cost and rapid systems to evaluate synergy and antagonism in antifungal combinations are lacking. Here, we introduce a novel in vitro testing method for assessing antifungal interactions in C. *albicans*, enabling the simultaneous testing of three antifungal agents in a single agar plate with overnight results. This method, validated against the checkerboard assay, provides consistent fractional inhibitory concentration (FICi) measurements with reduced variability and workload. We applied this method in a comprehensive screen of 92 clinical C. *albicans* isolates for three antifungals—amphotericin B, fluconazole, and anidulafungin—yielding assessments of a total of 276 distinct combinations of antifungals and isolates. Results revealed isolate-specific interaction patterns, with amphotericin B and fluconazole showing synergy in 1% of isolates, anidulafungin and fluconazole in 19.5%, and amphotericin B and anidulafungin in 23.9%. These findings underscore the need for isolate-specific testing in clinical settings, which this scalable, high-throughput assay can facilitate effectively.

Introduction

Fungal infections pose a significant health risk, causing life-threatening diseases such as meningitis, pneumonia, and fungaemia, as well as chronic and recurrent conditions like oral and vaginal candidiasis, and asthma (1). The growing threat from fungal pathogens is exacerbated by the increasing prevalence of antifungal resistance, which limits both treatment options and efficacy (2). During the last decades, several resistant fungal species have emerged, such as C. *glabrata*, *C. parapsilosis*, and *C. auris*, that have very few therapeutic options due to the limited available antifungal arsenal (3–6). Currently, only three major classes of antifungals are routinely used to treat invasive fungal infections, namely; echinocandins, azoles, and polyenes, of the latter only one drug, amphotericin B, is approved for systemic use.

With a limited array of antifungals and the continued rise of resistance, clinicians are increasingly turning to combination therapies to enhance treatment outcomes (7). Combination therapy, used either consecutively or sequentially (3,8,9) is particularly

important in treating immunocompromised patients with underlying comorbidities, where monotherapy may not be sufficient for eradication (10).

One antifungal combination, amphotericin B and 5-flucytosine, is already regularly recommended for the treatment of candidiasis (11) and several other combinations are also frequently employed to treat infections (3,8,9). Combination therapy however is not a simple addition of antimicrobial effects. Administering multiple antifungal drugs can lead to synergistic interactions, where their combined action is greater than the sum of their individual effects, or conversely, antagonistic interactions may occur, reducing the overall effectiveness of treatment (11).

In cases of synergistic interactions, the required doses of each drug could be lowered, potentially reducing toxic side effects (12,13). This is especially beneficial given that several antifungal agents are associated with toxicity or contraindications in high-risk patient populations (14). Moreover, the emergence of antifungal resistance could be slowed, preserving the efficacy of available drugs (15) particularly with newly introduced antifungals like ibrexafungerp, where prolonging their clinical lifespan is essential (16).

The potential benefits of identifying synergistic combinations and avoiding antagonistic ones have led to numerous studies aimed at evaluating antifungal interactions (7,17–19). However, the field lacks consensus, with some combinations reported to show either synergistic or antagonistic effects depending on the isolate suggesting that the efficacy of antifungal combinations may be isolate specific (19,20).

In recent years, several high-throughput methods have been developed to predict the combinatorial outcome of antifungal combinations, mainly by utilizing chemogenomic screenings (21–23). However, in vitro methods for studying isolate-specific antifungal interactions have limitations. The checkerboard assay is the most widely used, but it lacks standardization, resulting in significant interlaboratory variability in both performance and data collection (24). Moreover, it is labour-intensive and requires skilled personnel, which may limit its application in resource-constrained settings. Other methods, such as disc diffusion and time-kill assays, are also used but face similar challenges with standardization (25). In contrast, recent advances in antibiotic combination studies have introduced innovative approaches that could be adapted for antifungal combinations (26–29).

In this study, we aimed to develop a reliable and user-friendly method for studying antifungal interactions, with the goal of creating a straightforward pipeline that could lead to much-needed standardization in the field.

Our method builds on an in vitro assay previously used for testing antibiotic interactions in bacteria (26). The CombiANT interaction method, uses a custom culture plate designed to create defined diffusion gradients of three antimicrobials simultaneously. Each antimicrobial is loaded into separate reservoirs surrounding a triangular interaction area. After overnight incubation, distinct zones of growth inhibition appear, allowing for the rapid quantification of drug interactions. Automated image analysis calculates Fractional Inhibitory Concentration indices (FICis) with high accuracy, enabling precise identification of synergistic, additive, or antagonistic effects between antimicrobial pairs.

Here, we optimized and adapted this method into a novel combination plate for testing antifungal interactions, using C. *albicans* as a model organism. This plate allows for the simultaneous testing of three antifungal agents in one agar plate, effectively replacing the need for three individual checkerboard assays. We validated the performance of the combination plate against the commonly used checkerboard assay and applied it to screen interclass antifungal combinations (amphotericin B, anidulafungin, and fluconazole) across 92 clinical C. *albicans* isolates. Through this method validation and its application in the largest screening of clinical C. *albicans* isolates to date, we aim to provide a valuable tool for antifungal combination testing, generate large-scale data on Candida interactions, and facilitate the development of patient-specific combination therapies.

Results

The primary aim of this study was to develop a method for rapid and straightforward identification of interactions between antifungal agents. To achieve this, we adapted a previously established technique used for detecting interactions between antibiotics targeting bacteria (26,30,31).

Our pipeline is based on a combination plate assay that allows testing interactions between three antifungals simultaneously (Fig. 1). The assay uses a standard petri dish format, incorporating three antifungal-loaded reservoirs (A, B, and C) surrounding a triangular interaction area (Fig. 1I). To prepare the assay, agar infused with antifungal agents is added to the reservoirs and left to solidify. At this stage, the plate remains inert and can be stored refrigerated, limited only by the shelf life of the antifungals. This design allows for the preparation of large batches of plates in advance, which can be used when needed or in large-scale experiments, ensuring consistent handling of reagents.

The assay is activated by adding a 25 mL layer of agar to the combination plate, enabling the antifungals to diffuse from the reservoirs (Fig. 1II). After that layer solidifies, fungal isolates are overlaid by inoculating low-temperature gelling agarose, which is spread across the previously solidified agar layer (Fig. 1III). This step is critical for achieving results after an overnight incubation, as the inoculum must contain enough cells to produce readable outcomes despite the longer generation times of fungi compared to bacteria.

Following overnight incubation at 30°C, antifungal interaction patterns specific to each isolate are visible on the plates, with zones of inhibition and growth clearly distinguishable (Fig. 1IV).

On the combination plate, specific points of interest are annotated for analysis. These points include the edges of inhibition zones where individual antifungals act alone (termed inhibitory concentration points, or ICs indicated with asterisks in the pink marked regions of Fig.1 right panel) and the corners of growth zones where combinations of two antifungals produce their combined effects (termed combination inhibitory points, or CPs indicated with asterisks in the green zones of Fig.1 right panel). The ICs are located along the outer boundary of the interaction area, opposite the corresponding antifungal reservoir, marking the point where the antifungal concentration alone is sufficient to inhibit growth. The CPs are located within

120 the interaction area, at the corners of triangular growth zones, representing the combined
121 inhibitory effect of two antifungals.

122 Using the previously described analysis algorithm(26) , these annotated points are aligned
123 with a predefined diffusion model that maps the concentration gradients of the antifungals
124 across the agar. The algorithm calculates the concentrations of the antifungals at both the IC
125 and CP points. Fractional inhibitory concentration index (FICi) values for the three pairwise
126 combinations of antifungals are then calculated using the following formulae and the
127 extrapolated concentrations of the CP and IC points (32):

128

129 $$FICi_{ab} = \frac{MIC_a \text{ in combination}}{MIC_a \text{ alone}} + \frac{MIC_b \text{ in combination}}{MIC_b \text{ alone}}$$

130 $$FICi_{ac} = \frac{MIC_a \text{ in combination}}{MIC_a \text{ alone}} + \frac{MIC_c \text{ in combination}}{MIC_c \text{ alone}}$$

131 $$FICi_{bc} = \frac{MIC_b \text{ in combination}}{MIC_b \text{ alone}} + \frac{MIC_c \text{ in combination}}{MIC_c \text{ alone}}$$

132

133 After establishing the protocol, we conducted a large-scale screen for antifungal interactions
134 using a collection of 92 clinical C. *albicans* isolates (Fig. 2). The screening provided FICi values
135 for combinations of fluconazole (FLC), anidulafungin (ANI), and amphotericin B (AMB)—
136 antifungals chosen as representatives of the three main classes used to treat systemic fungal
137 infections. A FICi value of 1 was used as the threshold between synergistic and antagonistic
138 interactions (33,34). Isolates with a mean FICi below 1 were categorized as having synergistic
139 interactions, while those with a mean FICi above 1 were classified as antagonistic (35–38).
140 Our results showed that the AMB-FLC combination exhibited a synergistic interaction in 1%
141 (1/92) of the strains (Fig. 2). The ANI-FLC combination showed synergy in 19.5% (18/92) of
142 the strains (Fig. 2), and the AMB-ANI combination had a synergistic interaction in 23.9%
143 (22/92) of the strains (Fig. 2).

144 To verify the accuracy of our method, we compared its results with those obtained using
145 checkerboard interaction assays for the same three combinations (AMB-FLC, ANI-FLC, and
146 AMB-ANI). Checkerboards of AMB-FLC, ANI-FLC, and AMB-ANI were performed for ATCC
147 90028 C. *albicans* reference strain. FICi values were calculated (32) at 95% growth inhibition
148 for the combinations of AMB-ANI and AMB-FLC and 75% for of ANI-FLC in concordance with
149 the Broth micro dilution (BMD) thresholds for determining the inhibitory effect of each
150 antifungal (39) (supplementary protocol 1). FICi values from the combination plate and
151 checkerboard assays were similar (Fig. 3 left), with a paired nonparametric t-test showing no
152 significant differences between the methods.

153 To test the discrepancy between the two methods when applied on clinical strains, we
154 proceeded to checkerboard test the most synergistic and antagonistic clinical strains
155 identified in our screen (Fig. 3 right). Strains DA81057 and DA81040 were most synergistic

156 and antagonistic, respectively, for the combination of AMB-FLC; DA81050 and DA81109 were
157 most synergistic and antagonistic, respectively, for the combination of ANI-FLC; and DA81027
158 and DA81024 were most synergistic and antagonistic, respectively, for the combination of
159 AMB-ANI. FICi values for the clinical isolates with both methods were plotted against each
160 other and fitted to a linear regression model (Fig. 3 right). The slope of the regression ranged
161 from 1.066 to 1.317 (95% confidence interval, p = 0.001), indicating a strong agreement
162 between the methods, though the combination plate tended to slightly overestimate FICi
163 values compared to the checkerboard assay. Additionally, Bland-Altman analysis showed a
164 low intrinsic bias (0.2) between the two methods, with 95% limits of agreement ranging from
165 -0.67 to 1.09, further confirming the high level of concordance between results, despite the
166 slight tendency of the combination plate to yield higher FICi values.

167

168 Discussion

169 In this study, we present a new method for studying antifungal interactions. The combination
170 plate allows testing of three antifungal combinations simultaneously and has been developed
171 to provide overnight results. By replacing three checkerboard assays with a single, less labour-
172 intensive, agar-based test, the combination plate provides an easier and faster approach to
173 conducting antifungal interaction screenings, without compromising accuracy. For example,
174 hands-on preparation time for one combination plate is approximately 5-10 minutes whilst
175 one checkerboard may require upwards of60. Additionally, the method can easily be adjusted
176 for the screening of isolates with varying susceptibility levels by simply altering the antifungal
177 concentration in the loading chambers as needed.

178 Applying the combination plate to determine interactions in susceptible, tolerant or resistant
179 fungal species could be clinically useful. This approach could help identify synergistic
180 combinations against invasive infections, and development of new resistances could
181 potentially be delayed. Perchance most worryingly is the increase in resistance towards
182 echinocandins, found in both C. *glabrata* and C. *auris*. Combining empirical echinocandin
183 treatment with other synergistic antifungals could prove to be an effective measure to
184 widespread resistance emerging.

185 One of the primary goals with using this method was to obtain interaction results within an
186 overnight incubation. Two key steps contributed to achieving this goal. First, we used YPD
187 medium and agar throughout the process instead of RPMI. Even though RPMI medium is most
188 commonly used in regular antifungal susceptibility testing, it has been argued that nutrient?
189 deprivation from RPMI medium may lead to artificially low MIC results, as inhibited growth
190 can be misinterpreted as susceptibility (40). For this assay, RPMI failed to support sufficient
191 fungal growth for clear interaction patterns after overnight incubation, so YPD medium was
192 used instead. While further standardization and optimization of media formulations for
193 antifungal interaction testing is needed, results from the combination plates on YPD medium
194 were similar to those obtained with RPMI in checkerboard assays, indicating that media
195 choice has low impact on interaction results for this technology. The second step to reaching
196 assay readability quickly was the use of an inoculated agar overlay. Using a suspension of cells
197 in agar instead of streaking a liquid culture onto an agar plate enhanced fungal growth and

198 produced a smooth lawn with distinct inhibition zones, aiding in the analysis of the
199 combination plates.

200 In vitro studies of antifungal interactions have increased in recent years, as combination
201 therapy is increasingly used to treat resistant pathogens. Traditionally, these studies have
202 relied on checkerboard assays and typically involved smaller isolate collections (20,41–46).
203 Our study was performed on a substantially larger isolate collection, and quantified 3 distinct
204 combinations at once and on the same clinical collection. We observed striking differences
205 between the interaction pattern of echinocandins compared to that of amphotericin and
206 fluconazole.

207 Specifically, combinations involving an echinocandin displayed a variety of interaction profiles
208 in our study, with close to 20% of strains exhibiting a positive interaction and 80% a negative
209 one. This pattern, studies comprised of fewer isolates may not be able to capture. Indeed,
210 varying responses to the same combinations are evident between studies (7,17), which has
211 been posed to arise both due to the non-standardization of methodology and limited isolate
212 collections (7). The isolate-specific variability in interaction pattern we observed here, makes
213 a compelling argument for case-to-case testing of antifungal interaction in hospital clinics.
214 Such routine clinical use of interaction testing of antifungals requires an easy to use and
215 affordable test, which we think this combination plate can provide.

216 The combination of amphotericin B and fluconazole studied here showed, in contrast to the
217 echinocandin combinations, limited variability and was overwhelmingly antagonistic, with all
218 but one strain exhibiting antagonism. This combination is known to show discrepancies
219 between in vivo an in vitro studies. Thus, the combination plate and other in vitro
220 methodologies consistently report this combination as antagonistic (44,45,47), despite the
221 favourable effects seen in vivo (48). A recent study showed that the difference seen may be
222 due to concentration dependant interactions based on the free-drug levels in plasma (49).
223 Deciphering these various physiological factors which may affect drug availability is necessary
224 to help bridge the knowledge between in vitro and in vivo, aiding the tailoring of antifungal
225 combination therapy regimens.

226 To categorize the interactions quantified in this study, we adhered to the original definition
227 of the FIC index (33,34). Interactions with an index above 1 were classified as antagonistic,
228 and those below 1 as synergistic. Some studies use alternative thresholds, such as an upper
229 limit of 0.5 for synergy and a lower limit of 4 for antagonism, to reduce misclassification from
230 the intrinsic variability in checkerboard assays (see below), though these thresholds lack
231 clinical validation. Therefore, we chose to present our findings using a straightforward
232 classification. We hope that large-scale studies enabled by the here proposed and similar
233 technologies will ultimately help define clinically relevant thresholds for synergy and
234 antagonism, though this goal remains a future step in method development.

235 Checkerboard assays, while common, have inherent difficulties in readability. They rely on
236 serial dilutions, which have a margin of error of one well, leading to potential variability of up
237 to four-fold in the FICi readout due to this inherent ambiguity. In contrast, the combination

238 plate employs a continuous gradient of antifungals rather than serial dilutions, reducing
239 variability as shown in the consistent FICi values from our screening.

240 Advances in the field of antifungal interaction testing have been made with the invention of
241 several predictive chemogenomic methods(21–23). These methods hold great strength and
242 can be valuable when exploring new combinations, developing new antifungals or evaluating
243 interactions with other drugs. However, they may miss out on isolate-specific interactions
244 which need to be tested on a case-to-case basis. Here the combination plate can serve as a
245 complement to the in-silico methods to quickly evaluate if predicted synergies are present in
246 clinical isolates, and if interaction profiles vary.

247 Although the combination plate is less labour-intensive and faster to perform, it maintains
248 accuracy. Our cross-validation of the most synergistic and antagonistic drug-pathogen
249 combinations with checkerboard assays demonstrates that results from this assay are directly
250 comparable to those obtained from checkerboards, which are widely used but significantly
251 more time-consuming to perform and interpret.

252 One limitation of the combination plate is that it primarily samples the concentration space
253 near the MICs of the individual antifungal agents. Interactions between compounds, however,
254 may vary at different concentrations (50,51), which a checkerboard assay could detect. We
255 argue that the reduced inherent variability of the combination plate compensates for this
256 limitation, especially as clinically relevant interactions are likely to occur around inhibitory
257 concentrations, which is precisely where the combination plate operates.

258 In conclusion, the combination plate method presented here offers reproducible, overnight
259 results when testing antifungal interactions in vitro. It is easily scalable, allowing larger
260 screens as the one presented here which showed isolate-specific interaction profiles to the
261 combinations tested. The current results highlight the need for individual testing prior to
262 implementation of combination treatment, which could both improve the therapeutic
263 outcome and slow down resistance emergence. Due to its simplicity the combination plate
264 could readily be implemented in both clinical and research labs, to further advance the field
265 of antifungal interactions.

266

267 Materials and Methods

268 **Fungal isolates and growth conditions**

269 For technical validation and method calibration the *Candida albicans* American Type Culture
270 Collection (ATCC) strain 90028, obtained from the Department of Clinical Microbiology at
271 Karolinska Institutet, was used. The fungus was grown overnight at 30°C, in liquid cultures
272 using Yeast Peptone Dextrose (YPD) broth (Sigma-Aldrich, Ref. Y1375) in an orbital shaking
273 incubator set at 190 rpm. All clinical isolates (n=92) were obtained from the Department of
274 Clinical Microbiology of Karolinska Institutet, Huddinge. The isolates tested in this study
275 were sampled from *C. albicans* bloodstream and vulvovaginal infections collected between
276 2021-2024 at Karolinska University Hospital, Stockholm, Sweden. Isolates were stored at -
277 80°C in freezing medium (-Nutrient broth (Oxoid CM0067) 21,25 g/L (2,13%)-Glycerol 85%
278 (VWR 24384) 184,5 g/L (=150 mL/L) (12,75%)) until use and subsequently tested. Overnight

279 cultures were prepared by transferring the yeast from frozen vials into 2 ml of YPD broth
280 and incubated at 190 rpm orbital shaking.

281 **Antifungals**

282 Antifungals were suspended in DMSO or water, per European Committee on Antimicrobial
283 Susceptibility Testing (EUCAST) recommendation (52) to a final concentration of 10 mg/ml,
284 aliquoted in 20 μl, and stored at -20°C until use. Antifungals used in this study include
285 micafungin (Sigma-Aldrich, Ref. SML2268), anidulafungin (Sigma-Aldrich, Ref. SML2288),
286 amphotericin B (ThermoFisher Scientific, Ref. J61491.03), fluconazole (Sigma-Aldrich, Ref.
287 PHR1160) and voriconazole (ThermoFisher Scientific, Ref. 458670010).

288 **Broth microdilutions**

289 Minimum inhibitory concentration (MIC) was determined for all antifungals for C. *albicans*
290 ATCC strain 90028 using broth microdilution according to EUCAST guidelines (52) with minor
291 modifications. RPMI medium with HEPES (Gibco, Ref. 130118031) was used instead of MOPS,
292 plates were incubated at 30°C, and results were read by eye. Measurements were conducted
293 with one biological replicate. Two-fold serial dilutions of the antifungals were prepared in 96-
294 well round bottom microtiter plates, leading to a 100 μl suspension of the antifungals at 2X
295 the final concentration. Wells were inoculated with 100 μl of yeast suspension prepared to
296 0.5 McFarland cells in RPMI medium. The MIC was defined as the concentration where 50%
297 growth inhibition was seen (MIC50), except for amphotericin B where the 90% growth
298 inhibition was deemed as the MIC (MIC90), in accordance to EUCAST guidelines.

299 **Optimization of fungal growth conditions**

300 The six laboratory medium tested were:

301 1. BD BACTO™ Brain Heart Infusion (BHI) broth (Becton Dickinson, Ref. 237500)
302 2. BD DIFCO™ Mueller-Hinton (MH) broth (Becton Dickinson, Ref. 275730)
303 3. MH-II broth (Becton Dickinson, Ref. 212322)
304 4. Lysogeny Broth (LB) (Sigma-Aldrich, Ref. L3522)
305 5. Yeast Peptone Dextrose (YPD) broth (Sigma-Aldrich, Ref. Y1375)
306 6. RPMI medium (Gibco, Ref. 130118031)

307 2% D-glucose (w/v) was supplemented to all media, except YPD, and plates were prepared in-
308 house with 1.5% agarose (w/v) (Sigma-Aldrich, Ref. A9639), except for YPD agar which was
309 bought (Sigma-Aldrich, Ref. Y1500).

310 Three different plating methods were evaluated, namely:

311 1. Glass beads. 100 μl of undiluted fungal overnight culture was added directly to plates
312 and spread using the beads.
313 2. Cotton swabbing, performed according to EUCAST guidelines but using undiluted
314 overnight culture (53).
315 3. Inoculating an overlay using low-temperature gelling agarose. Overlay was prepared
316 by mixing equal volumes of 3% (w/v) low-temperature gelling agarose (Sigma-Aldrich,

317 Ref. A4018) with undiluted overnight culture, yielding a 1:2 dilution of the overnight.
318 The low temperature gelling agarose was homogenized in a water bath set at 55°C.
319

320 **Combination plate calibration**

321 Prior to the screening of clinical isolates, the concentrations of the antifungals to be used on
322 the assay were calibrated using the ATCC type strain. MIC values obtained from the BMDs lay
323 the basis for the calibration which was done for every antifungal. Optimal assay concentration
324 was defined as the concentration where clear inhibition zones with defined edges formed as
325 this aids the annotation of the key points during data analysis. During calibration, the three
326 antifungal-loading reservoirs in a plate contained the same antifungal, but at different
327 concentrations. Concentrations up to 600 times the MIC of each antifungal was tested, using
328 an increment of either 10 (between 10-100X the MIC) or 100 (between 100-600X the MIC).

329 **Protocol validation and ATCC strain**

330 After optimal growth conditions and antifungal concentrations were established for the assay,
331 the developed protocol (supplementary protocol 2) was evaluated using the ATCC type strain.
332 The strain was tested for the pairwise combination of ANI, AMB, and FLC in technical
333 triplicates.

334 The antifungal-loading chambers were loaded with YPD agar containing one of the three
335 antifungals. After the agar solidified, the assays were kept at 4°C for a minimum of 45 min,
336 after which an underlay of 25 ml YPD agar was added. The agar was left the rest and solidify
337 at room temperature for at least 2h before plating. Plating was done by using an inoculated
338 agar overlay which was prepared by mixing equal volumes of a 3% low-temperature gelling
339 agarose with a dense fungal overnight culture diluted 1:50 in fresh YPD medium, resulting in
340 a final 1:100 dilution of the overnight culture. Methylene blue was added to the 3% agarose
341 solution at a concentration of 1 µg/mL for a final concentration of 0.5 µg/ml

342 **Screening of clinical isolates**

343 All clinical isolates (n=92) were screened for the pairwise combination of ANI, AMB, and FLC.
344 Isolates were tested in technical triplicates of biological duplicates. Isolates were grown
345 overnight as described and subsequently screened using the developed protocol
346 (Supplementary protocol 2).

347 **Checkerboard technique**

348 Checkerboard assays were performed for the ATCC 90028 C . *albicans* reference strain and
349 the most synergistic and antagonistic clinical strains identified in the screen to verify the
350 results. The checkerboard protocol used here was modified from the protocol published by
351 Vitale *et.al* (54) and a step-by-step protocol is available in Supplementary protocol 1.
352 Checkerboards were performed using 96-well round bottom microtiter plates in biological
353 triplicates. The antifungal stocks prepared previously were suspended in RPMI medium at 4X
354 the highest concentration to be used. The suspensions was 2-fold serially diluted and 50 µl of
355 each antifungal, at 4X the final concentration, was added to their corresponding well. 100 µl

356 of fungal suspension prepared at 0.5 McFarland was added to all experimental wells. RPMI
357 medium served as a sterility control. After an overnight static incubation at 30°C the wells
358 were resuspended and the optical density (OD) was measured at 450 nm using a Thermo
359 Fisher Scientific Multiscan FC Type 357. For analyses, the OD values of the sterility control
360 were subtracted from all experimental wells and the growth inhibition was calculated as a
361 percentage by dividing the experimental wells with the growth control. To obtain the MIC
362 values for the antifungals when used in combinations, points of theoretical additivity were
363 picked. Based on the EUCAST method of MIC determination of yeasts in BMDs, the theoretical
364 additivity is at 95% growth inhibition for the combinations of AMB-ANI and AMB-FLC whilst
365 for the combination of ANI-FLC it is at 75% growth inhibition.

366 **Statistical analysis**

367 Statistical analyses were performed using Graphpad Prism version 9, significance levels are
368 noted at p=0.05 probability unless otherwise stated.

369 Author contributions

370 Conceptualization: NFK, DA, VÖ, TUA

371 Formal analysis: NFK, HL

372 Funding acquisition: DA, VÖ

373 Investigation: NFK, HL, RS

374 Methodology: NFK, HL

375 Writing – original draft: NFK, HL

376 Writing – review and editing: all authors

377 Funding

378 Funding was provided by grants awarded to VÖ and DIA …..Funders had no input in the
379 investigation, analysis or decision to publish.

Figures

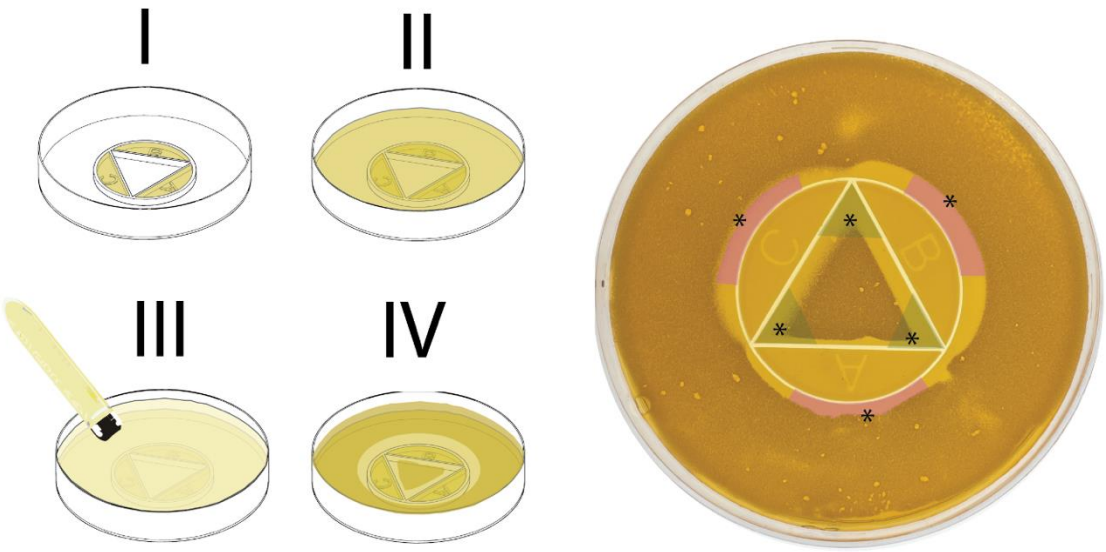
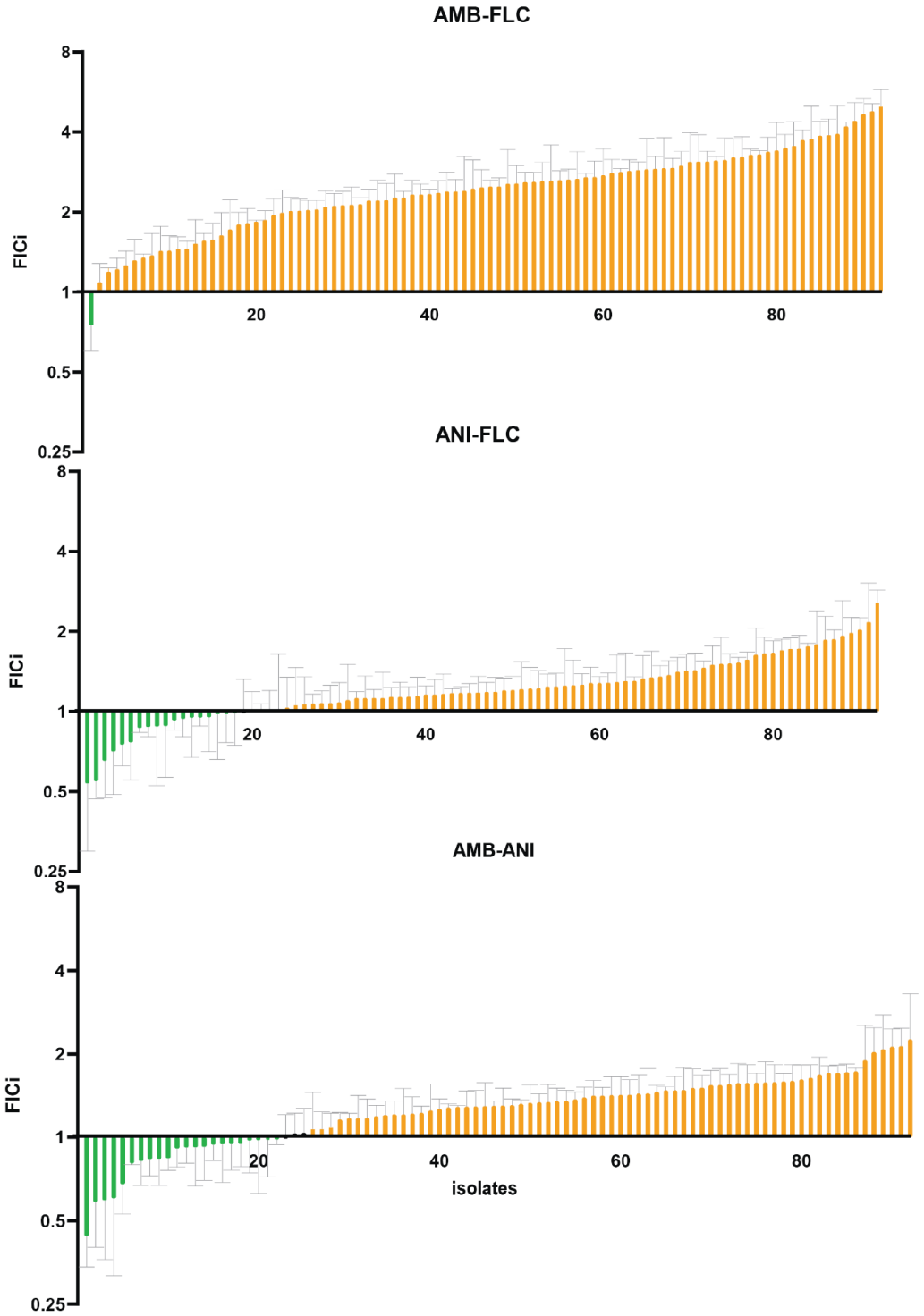


Figure 1. **Schematic and workflow of the antifungal combination plate assay for interaction testing.**
The combination plate assay, designed for testing interactions between three antifungal agents, uses a standard petri dish format with antifungal-loaded reservoirs (A, B, and C) surrounding a triangular interaction area (I). Agar infused with antifungal agents is added to the reservoirs, solidified, and refrigerated until use. The assay is activated by adding a 25 mL agar underlay to allow antifungal diffusion (II), followed by inoculating the plate with a low-temperature gelling agarose overlay containing a fungal inoculum (III). After overnight incubation at 30°C, inhibition zones and growth patterns indicating antifungal interactions are visible (IV). Areas of inhibition for individual antifungals appear (marked in pink), while growth inhibition zones due to antifungal interactions are marked in green. Six key points of interest, marked by asterisks, represent the inhibition edges for each antifungal and the internal growth zone edges near each corner of the interaction triangle, used for calculating Fractional

395 Inhibitory Concentration index (FICi) values for each antifungal pair.



396

397 Figure 2. **Screening results of antifungal interactions across clinical *Candida albicans***
398 **isolates.**
399 Results from a large-scale screen of 92 *C. albicans* isolates tested with the combination plate
400 assay for three antifungal pairs,: amphotericin B (AMB) with fluconazole (FLC) , anidulafungin
401 (ANI) with FLC and AMB with ANI, shown in ascending FICi order for every combination . FICi

values were used to categorize interactions as synergistic (mean FICi < 1,marked in green) or antagonistic (mean FICi > 1, marked in orange). Grey bars mark SEM for n=3.

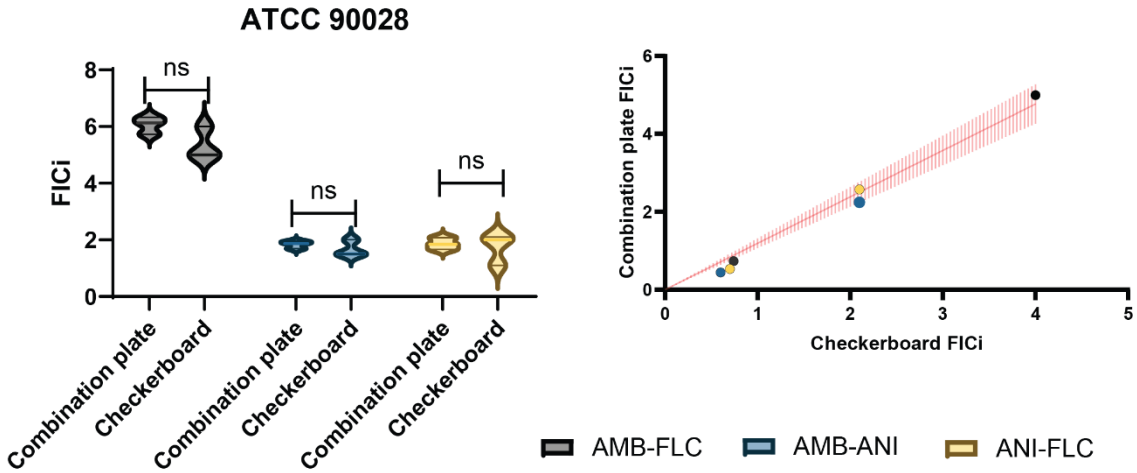


Figure 3 **Validation of combination plate results against checkerboard assay for antifungal interaction testing.** Left: FICi values of the ATCC 90028 *C. albicans* reference strain with the Combination plate and Checkerboard assay, non-parametric t test shows no significant difference between the two methods. Right: Regression analysis of FICi values obtained from both methods for selected clinical isolates (those with the most synergistic and antagonistic FICi values), regression slope shown in read between 1.066 and 1.317 (95% CI, p = 0.001).

References

1. Perlin DS, Rautemaa-Richardson R, Alastruey-Izquierdo A. The global problem of antifungal resistance: prevalence, mechanisms, and management. Lancet Infect Dis. 2017 Dec 1;17(12):e383–92.

2. Hendrickson JA, Hu C, Aitken SL, Beyda N. Antifungal Resistance: a Concerning Trend for the Present and Future. Curr Infect Dis Rep. 2019 Nov 16;21(12):47.

3. Pfaller M, Neofytos D, Diekema D, Azie N, Meier-Kriesche HU, Quan SP, et al. Epidemiology and outcomes of candidemia in 3648 patients: data from the Prospective Antifungal Therapy (PATH Alliance®) registry, 2004–2008. Diagn Microbiol Infect Dis. 2012 Dec 1;74(4):323–31.

4. Chakrabarti A, Sood P. On the emergence, spread and resistance of Candida auris: host, pathogen and environmental tipping points. J Med Microbiol. 2021 Feb 18;70(3):001318.

5. Daneshnia F, Júnior JN de A, Ilkit M, Lombardi L, Perry AM, Gao M, et al. Worldwide emergence of fluconazole-resistant Candida parapsilosis: current framework and future research roadmap. Lancet Microbe. 2023 Jun 1;4(6):e470–80.

6. Kaur R, Dhakad MS, Goyal R, Kumar R. Emergence of non-*albicans Candida* species and antifungal resistance in intensive care unit patients. Asian Pac J Trop Biomed. 2016 May 1;6(5):455–60.

7. Fioriti S, Brescini L, Pallotta F, Canovari B, Morroni G, Barchiesi F. Antifungal Combinations against Candida Species: From Bench to Bedside. J Fungi. 2022 Oct 13;8(10):1077.

430 8. Qiu K yin, Liao X yu, Fang J pei, Xu H gui, Li Y, Huang K, et al. Combination antifungal treatment for
431 invasive fungal disease after hematopoietic stem cell transplantation in children with
432 hematological disorders. Transpl Infect Dis. 2019;21(3):e13066.

433 9. Haider S, Rotstein C, Horn D, Laverdiere M, Azie N. The Prospective Antifungal Therapy Alliance®
434 registry: A two-centre Canadian experience. Can J Infect Dis Med Microbiol. 2014 Spring;25(1):17.

435 10. Campitelli M, Zeineddine N, Samaha G, Maslak S. Combination Antifungal Therapy: A
436 Review of Current Data. J Clin Med Res. 2017 Apr 26;9(6):451.

437 11. Pappas PG, Kauffman CA, Andes DR, Clancy CJ, Marr KA, Ostrosky-Zeichner L, et al.
438 Clinical Practice Guideline for the Management of Candidiasis: 2016 Update by the Infectious
439 Diseases Society of America. Clin Infect Dis. 2016 Feb 15;62(4):e1–50.

440 12. Johnson MD, Perfect JR. Use of Antifungal Combination Therapy: Agents, Order, and
441 Timing. Curr Fungal Infect Rep. 2010 May 1;4(2):87.

442 13. Shrestha SK, Fosso MY, Garneau-Tsodikova S. A combination approach to treating
443 fungal infections. Sci Rep. 2015 Nov 23;5(1):17070.

444 14. McKeny PT, Nessel TA, Zito PM. Antifungal Antibiotics. In: StatPearls [Internet].
445 Treasure Island (FL): StatPearls Publishing; 2024 [cited 2024 Oct 23]. Available from:
446 http://www.ncbi.nlm.nih.gov/books/NBK538168/

447 15. Fuentefria AM, Pippi B, Dalla Lana DF, Donato KK, de Andrade SF. Antifungals
448 discovery: an insight into new strategies to combat antifungal resistance. Lett Appl Microbiol.
449 2018;66(1):2–13.

450 16. El Ayoubi LW, Allaw F, Moussa E, Kanj SS. Ibrexafungerp: A narrative overview. Curr
451 Res Microb Sci. 2024 Jan 1;6:100245.

452 17. Wake RM, Allebone-Salt PE, John LLH, Caswall BA, Govender NP, Ben-Ami R, et al.
453 Optimizing the Treatment of Invasive Candidiasis—A Case for Combination Therapy. Open Forum
454 Infect Dis. 2024 Jun 1;11(6):ofae072.

455 18. O'Brien B, Liang J, Chaturvedi S, Jacobs JL, Chaturvedi V. Pan-resistant Candida auris:
456 New York subcluster susceptible to antifungal combinations. Lancet Microbe. 2020 Sep
457 1;1(5):e193–4.

458 19. Jacobs SE, Jacobs JL, Dennis EK, Taimur S, Rana M, Patel D, et al. Candida auris Pan-
459 Drug-Resistant to Four Classes of Antifungal Agents. Antimicrob Agents Chemother. 2022 Jun
460 30;66(7):e00053-22.

461 20. O'Brien B, Chaturvedi S, Chaturvedi V. In Vitro Evaluation of Antifungal Drug
462 Combinations against Multidrug-Resistant Candida auris Isolates from New York Outbreak.
463 Antimicrob Agents Chemother. 2020 Mar 24;64(4):10.1128/aac.02195-19.

464 21. Gaikani H, Smith AM, Lee AY, Giaever G, Nislow C. Systematic Prediction of Antifungal
465 Drug Synergy by Chemogenomic Screening in Saccharomyces cerevisiae. Front Fungal Biol
466 [Internet]. 2021 Jul 2 [cited 2024 Oct 15];2. Available from:
467 https://www.frontiersin.org/journals/fungal-biology/articles/10.3389/ffunb.2021.683414/full

468 22. Jansen G, Lee AY, Epp E, Fredette A, Surprenant J, Harcus D, et al. Chemogenomic
469 profiling predicts antifungal synergies. Mol Syst Biol. 2009 Jan;5(1):338.

470 23. Wambaugh MA, Denham ST, Ayala M, Brammer B, Stonhill MA, Brown JC. Synergistic
471 and antagonistic drug interactions in the treatment of systemic fungal infections. eLife. 2020 May
472 5;9:e54160.

473 24. Chaturvedi V, Ramani R, Andes D, Diekema DJ, Pfaller MA, Ghannoum MA, et al.
474 Multilaboratory Testing of Two-Drug Combinations of Antifungals against Candida albicans,
475 Candida glabrata, and Candida parapsilosis. Antimicrob Agents Chemother. 2011 Jan
476 31;55(4):1543.

477 25. Bidaud AL, Schwarz P, Herbreteau G, Dannaoui E. Techniques for the Assessment of In
478 Vitro and In Vivo Antifungal Combinations. J Fungi. 2021 Feb 4;7(2):113.

479 26. Fatsis-Kavalopoulos N, Roemhild R, Tang PC, Kreuger J, Andersson DI. CombiANT:
480 Antibiotic interaction testing made easy. PLOS Biol. 2020 Sep 17;18(9):e3000856.

481 27. Lee WB, Chien CC, You HL, Kuo FC, Lee MS, Lee GB. An integrated microfluidic system
482 for antimicrobial susceptibility testing with antibiotic combination. Lab Chip. 2019 Aug
483 6;19(16):2699–708.

484 28. Shao F, Li H, Hsieh K, Zhang P, Li S, Wang TH. Automated and miniaturized screening
485 of antibiotic combinations *via* robotic-printed combinatorial droplet platform. Acta Pharm Sin B.
486 2024 Apr 1;14(4):1801–13.

487 29. Cacace E, Kim V, Varik V, Knopp M, Tietgen M, Brauer-Nikonow A, et al. Systematic
488 analysis of drug combinations against Gram-positive bacteria. Nat Microbiol. 2023;1–17.

489 30. Fatsis-Kavalopoulos N, Roelofs L, Andersson DI. Potential risks of treating bacterial
490 infections with a combination of β-lactam and aminoglycoside antibiotics: A systematic
491 quantification of antibiotic interactions in E. coli blood stream infection isolates. eBioMedicine.
492 2022 Apr 1;78:103979.

493 31. Tang PC, Sánchez-Hevia DL, Westhoff S, Fatsis-Kavalopoulos N, Andersson DI. Within-
494 species variability of antibiotic interactions in Gram-negative bacteria. mBio. 2024 Mar
495 13;15(3):e0019624.

496 32. Fatsis-Kavalopoulos N, Sánchez-Hevia DL, Andersson DI. Beyond the FIC index: the
497 extended information from fractional inhibitory concentrations (FICs). J Antimicrob Chemother.
498 2024;79(9):2394–6.

499 33. Hall MJ, Middleton RF, Westmacott D. The fractional inhibitory concentration (FIC)
500 index as a measure of synergy. J Antimicrob Chemother. 1983 May 1;11(5):427–33.

501 34. LOEWE S. The problem of synergism and antagonism of combined drugs.
502 Arzneimittelforschung. 1953 Jun;3(6):285–90.

503 35. Meletiadis J, Pournaras S, Roilides E, Walsh TJ. Defining fractional inhibitory
504 concentration index cutoffs for additive interactions based on self-drug additive combinations,
505 Monte Carlo simulation analysis, and in vitro-in vivo correlation data for antifungal drug
506 combinations against Aspergillus fumigatus. Antimicrob Agents Chemother. 2010 Feb;54(2):602–
507 9.

508 36. Te Dorsthorst DTA, Verweij PE, Meletiadis J, Bergervoet M, Punt NC, Meis JFGM, et al.
509 In vitro interaction of flucytosine combined with amphotericin B or fluconazole against thirty-five
510 yeast isolates determined by both the fractional inhibitory concentration index and the response
511 surface approach. Antimicrob Agents Chemother. 2002 Sep;46(9):2982–9.

512 37. Deng S, Lei W, de Hoog GS, Yang L, Vitale RG, Rafati H, et al. Combination of
513 Amphotericin B and Terbinafine against Melanized Fungi Associated with Chromoblastomycosis.
514 Antimicrob Agents Chemother. 2018 Jun;62(6):e00270-18.

515 38. Marchetti O, Moreillon P, Glauser MP, Bille J, Sanglard D. Potent synergism of the
516 combination of fluconazole and cyclosporine in Candida albicans. Antimicrob Agents Chemother.
517 2000 Sep;44(9):2373–81.

518 39. J Guinea1#, J. Meletiadis2,3#, , S. Arikan-Akdagli4, , C. Giske5, , K. Muehlethaler6, , M.
519 C. Arendrup7,8 and the, Subcommittee on Antifungal Susceptibility Testing (AFST) of the ESCMID
520 European Committee for, et al. Method for the determination of broth dilution minimum
521 inhibitory concentrations of antifungal agents for yeasts [Internet]. EUCAST; 2023. Available from:
522 https://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/AFST/Files/EUCAST_E.Def_7.4_
523 Yeast_definitive_revised_2023.pdf

524 40. Vahedi-Shahandashti R, Stubenböck MM, Lass-Flörl C. The Influence of Medium
525 Composition on EUCAST and Etest Antifungal Susceptibility Testing. J Fungi. 2023 Oct;9(10):973.

526 41. Tobudic S, Kratzer C, Lassnigg A, Graninger W, Presterl E. In vitro activity of antifungal
527 combinations against Candida albicans biofilms. J Antimicrob Chemother. 2010 Feb;65(2):271–4.

528 42. Nagy F, Tóth Z, Nyikos F, Forgács L, Jakab Á, Borman AM, et al. In vitro and in vivo
529 interaction of caspofungin with isavuconazole against Candida auris planktonic cells and biofilms.
530 Med Mycol. 2021 May 22;59(10):1015.

531 43. Bidaud AL, Botterel F, Chowdhary A, Dannaoui E. In Vitro Antifungal Combination of
532 Flucytosine with Amphotericin B, Voriconazole, or Micafungin against Candida auris Shows No
533 Antagonism. Antimicrob Agents Chemother. 2019 Nov 21;63(12):e01393.

534 44. Katragkou A, McCarthy M, Meletiadis J, Hussain K, Moradi PW, Strauss GE, et al. In
535 vitro combination therapy with isavuconazole against Candida spp. Med Mycol. 2017 Nov
536 1;55(8):859–68.

537 45. Vazquez JA, Arganoza MT, Vaishampayan JK, Akins RA. In vitro interaction between
538 amphotericin B and azoles in Candida albicans. Antimicrob Agents Chemother. 1996
539 Nov;40(11):2511.

540 46. Yang Q, Liu Z, Wang Y, Xie J, Zhang K, Dong Y, et al. In vitro synergistic antifungal
541 activities of caspofungin in combination with fluconazole or voriconazole against Candida species
542 determined by the Etest method. Int J Infect Dis. 2022 Sep 1;122:982–90.

543 47. Lewis RE, Lund BC, Klepser ME, Ernst EJ, Pfaller MA. Assessment of Antifungal
544 Activities of Fluconazole and Amphotericin B Administered Alone and in Combination
545 againstCandida albicans by Using a Dynamic In Vitro Mycotic Infection Model. Antimicrob Agents
546 Chemother. 1998 Jun;42(6):1382–6.

547 48. Rex JH, Pappas PG, Karchmer AW, Sobel J, Edwards JE, Hadley S, et al. A randomized
548 and blinded multicenter trial of high-dose fluconazole plus placebo versus fluconazole plus

549 amphotericin B as therapy for candidemia and its consequences in nonneutropenic subjects. Clin
550 Infect Dis Off Publ Infect Dis Soc Am. 2003 May 15;36(10):1221–8.

551 49. Meletiadis J, Beredaki MI, Elefanti A, Pournaras S, Muller A. In Vitro–In Vivo
552 Correlation of Posaconazole–Amphotericin B Combination against Candida albicans: In Vitro
553 Interacting Concentrations Are Associated with In Vivo Free Drug Levels. J Fungi. 2023 Apr
554 1;9(4):434.

555 50. Drusano GL. Antimicrobial pharmacodynamics: critical interactions of "bug and drug."
556 Nat Rev Microbiol. 2004 Apr 1;2(4):289–300.

557 51. Bollenbach T. Antimicrobial interactions: mechanisms and implications for drug
558 discovery and resistance evolution. Curr Opin Microbiol. 2015;27:1–9.

559 52. Arendrup MC, Cuenca-Estrella M, Lass-Flörl C, Hope W. EUCAST technical note on the
560 EUCAST definitive document EDef 7.2: method for the determination of broth dilution minimum
561 inhibitory concentrations of antifungal agents for yeasts EDef 7.2 (EUCAST-AFST)*. Clin Microbiol
562 Infect. 2012 Jul 1;18(7):E246–7.

563 53. Matuschek E, Brown DFJ, Kahlmeter G. Development of the EUCAST disk diffusion
564 antimicrobial susceptibility testing method and its implementation in routine microbiology
565 laboratories. Clin Microbiol Infect. 2014 Apr 1;20(4):O255–66.

566 54. Vitale RG, Afeltra J, Dannaoui E. Antifungal Combinations. In: Ernst EJ, Rogers PD,
567 editors. Antifungal Agents: Methods and Protocols [Internet]. Totowa, NJ: Humana Press; 2005
568 [cited 2024 Oct 9]. p. 143–52. Available from: https://doi.org/10.1385/1-59259-943-5:143

569

570 Supplementary information

571 **Supplementary protocol 1: Checkerboard protocol for antifungal combinations**

572 Protocol has been adapted from E. Dannouli et al and EUCAST protocol for MIC
573 determination of yeasts by broth microdilution. The RPMI medium used throughout the
574 protocol is supplemented with 2% glucose

575 1. Grow the fungal isolates of interest on YPD plates overnight at 30°C.
576 2. Prepare antifungal stocks in RPMI medium at 4X the highest concentration to be
577 used in the assay.
578 3. Perform a 2-fold serial dilution in RPMI medium of each antifungal stock prepared,
579 yielding a 4X dilution of all antifungal concentrations to be used.
580 4. Drug A is added to rows B-H. Add 50 ul of the prepared dilutions to each well with
581 the highest concentration in row B and lowest concentration in row H to a 96-well
582 round bottom microtiter plate.
583 5. Drug B is added to columns 2-11. Add 50 ul of the prepared dilutions to each well
584 with the highest concentration in column 2 and lowest concentration in column 11.
585 a. Based on step 3-4, wells B-H in column 1 contains only drug A and wells 2-11
586 in row A only drug B. Add 50 ul of RPMI medium to these wells for a final
587 volume of 100 ul.

6. Prepare the fungal inoculum by suspending colonies in RPMI medium until a turbidity of 0.5 McFarland.
7. Add 100 ul of the fungal inoculum to all wells in column 1-11.
8. Well A1 serves as the growth control. Add 100 ul of RPMI medium to the 100 ul of inoculum
9. Well A12 serves as the sterility control. Add 200 ul of RPMI medium.
10. Incubate plate at 30°C overnight.
    a. To reduce evaporation, plates can be placed in plastic bags prior to incubation.
11. Analyse plates by measuring the OD at 450 nm
    a. If needed, resuspend wells prior to the OD measurement
12. Ensure sterility by comparing the sterility control with the empty wells in column 12.

Supplementary protocol 2: Protocol for antifungal combination testing using combination plates

Day 1:

1. Grow chosen strains in 2 ml of Yeast-Peptone-Dextrose (YPD) medium at 30°C in an orbital shaker overnight (ON).
    a. Places tubes diagonally in rack.
2. Prepare YPD agar according to manufacturer's instruction, keep at 55°C.
3. Prepare a 1 mg/mL of methylene blue in sterile dH_2O. Shield from direct light and store at 4°C until use.

Day 2:

1. Dilute antifungal stock to the desired input concentration in YPD agar. Keep antifungal-agar in a water bath set at 55°C until use.
2. Load 0.5 mL of the antifungal agar to the assigned chamber of the CombiANT assay
    a. Ensure entire chamber is filled and remove any air bubbles.
3. Let agar solidify at room temperature, then keep at 4°C for at least 45 minutes until further use.
4. To activate the assay, remove plates from 4°C and add a 25 mL underlay of YPD agar to each plate.
    a. Add agar centrally of the assay, ensure all chambers are submerged and pipette away any bubbles.
    b. Do not move the plates until agar has solidified as it may disrupt the diffusion pattern
5. Let the agar set for at least 2 hours at room temperature after the last plate is cast

6. Prior to plating of the strains, prepare n + 1 mL (n = number of plates) of low-gelling temperature agarose at 3% (w/v) in sterile dH_2O. Melt and keep agar at 55°C in a water bath.
    a. Add 1 μL of the methylene blue solution per mL of low-gelling temperature agarose solution prepared.
7. Dilute the ON cultures of the chosen strains 1:50 in YPD medium.
8. Per plate, mix 1 mL of the diluted ON cultures with 1 mL of the low-gelling temperature agarose with methylene blue. Mix by pipetting and pour immediately onto the plate.
    a. Ensure entire plate is covered by the overlay.
9. Let overlay solidify at room temperature for 10 min, then incubate ON at 30°C.

Day 3.

1. Take pictures of the plates. Orient plates so chamber A is furthest down for easier analysis.
2. Identify the points of interest using the CombiANT Imager software (Rx Dynamics AB).
3. Input the data into the analysis algorithm.