i) Cell viability assays for *Candida auris*

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ii) Abstract

Cell viability assays are useful for assessing the efficacy of antifungal therapeutics and disinfection strategies *in vitro*. In recent years these assays have been fundamental for the testing of conventional and novel therapies against the nosocomial fungal pathogen *Candida auris*. Here we provide detailed descriptions of methods for assessing cellular viability of *Candida auris in vitro*, such as metabolic assays (XTT and resazurin), colony forming unit counting, live/dead quantitative PCR, and fluorescent staining for microscopic analyses.

iii) ***Key words:*** *Candida auris*, cell viability, XTT, Resazurin, colony forming units, live/dead qPCR, fluorescent staining

1) Introduction

Cell viability assays are largely simple, yet extremely effective techniques utilised by most microbiology research laboratories. These methodologies can involve several different assays which allow for assessment of cell viability of planktonic or sessile cells of bacterial and fungal species. These include, but are not limited to the following;

a) Colorimetric microtiter plate assays using salts (e.g., tetrazolium, XTT) or dyes (e.g., resazurin) are platforms for assessing metabolic activity of microbial biofilms [1-5]. These assays allow for high-throughput screening of various antimicrobial compounds with their “low input, high output” applications.
b) Colony forming units (CFU) using the Miles and Misra counting technique, and/or colony forming equivalents (CFE) using quantitative polymerase chain reaction (qPCR) in microbial samples. qPCR can be utilized in combination with photoreactive DNA-binding dyes such as propidium monoazide (PMA) to allow for detection of viable vs. total microorganisms [6, 7].

c) Fluorescent staining with live/dead viability dyes such as SYTO™ 9 and propidium iodide to allow for microscopical discrimination between viable and dead cells [8]. Fungal-specific dyes have been reported, such as calcofluor-white and FUN-1™, with the latter allowing for accurate assessment of viability in yeasts [9].

In the context of medical mycology, these above methods have previously been used to study the response of *Candida albicans* to antifungal therapeutics and disinfection strategies with great success. However, in recent years similar techniques have also been employed for *Candida auris*, a nosocomial pathogen first identified in Japan in 2009 [10]. Since its emergence, *C. auris* has rapidly gained worldwide attention due to its genetic heterogeneity and ability to survive and persist in environments for prolonged periods of time [11]. Furthermore, resistance profiles from *C. auris* clinical isolates vary considerably to the three main classes of antifungals, with a significant number of isolates being multi-drug resistant [12]. At this juncture, a decade of research into *C. auris* has prioritised widespread antifungal susceptibility testing, with more recent investigations into the efficacy of a range of novel therapeutics against the species [13-16]. All such publications have utilised some, if not all, of the techniques highlighted above to assess effectiveness of a given treatment. Herein, we will document in detail, the methodologies available for assessing cell viability of *C. auris in vitro*.

2) Materials

For all media and working solutions, it is recommended to purchase readily available microbiological powders or tablets to ensure the most accurate batch-to-batch

preparations and follow manufacturers' instructions in preparing such solutions. It not possible, ingredients for each media and buffers are described below to allow for preparation in-house. However, concentrations included here for each component may differ slightly from commercially available preparations, which may further vary from company to company.

2.1) *Candida auris* storage, revival and cell standardization

1) Microbank™ beads.
2) Sabouraud's dextrose (SAB) agar (ingredients; 40.0 g/L glucose, 10.0 g/L peptone and 15.0 g/L agar dissolved in 1000 mL of double distilled H_2O (ddH_2O), pH 5.6 ± 0.2 at 25ºC). The agar should be sterilized via autoclaving at 121ºC.
3) Disposable 10 µL inoculation loops.
4) Yeast extract peptone dextrose (YPD) medium (ingredients; 10.0 g/L yeast extract, 20.0 g/L peptone and 20.0 g/L dextrose dissolved in 1000 mL of ddH_2O, pH 6.5 ± 0.2 at 25ºC). The medium should be sterilized via autoclaving at 121ºC.
5) Phosphate-buffered saline (PBS) (ingredients; 8.0 g/L NaCl, 0.2 g/L KCl, 1.44 g/L Na_2HPO_4, and 0.24 g/L KH_2PO_4 dissolved in 800 mL of ddH_2O. pH can be adjusted to 7.4 with concentrated HCl, before adding an additional 200 mL of ddH_2O). PBS should be sterilized via autoclaving at 121ºC.
6) Roswell Park Memorial Institute (RPMI) 1640 medium (main ingredients; 2000 mg/L dextrose, 5.0 mg/L phenol red, 5000 mg/L phenol red [pH indicator], 100 mg/L $Ca(NO_3)_2$. $4H_2O$, 48.84 mg/L anhydrous $MgSO_4$, 400.0 mg/L KCl, 2000 mg/L $NaHCO_3$, 6000 mg/L NaCl and 800.0 mg/L anhydrous Na_2HPO_4 in 1000 mL of ddH_2O. The remaining components include amino acids and vitamins which are listed elsewhere (https://www.thermofisher.com/uk/en/home/technical-

resources/media-formulation.114.html). The resulting solution should be pH 8.2 ± 0.3 at 25ºC. Due to the complexity of the media composition, it is highly recommended to purchase RPMI-1640 medium from an appropriate supplier. RPMI media should be sterilized via autoclaving at 121ºC.

7) Dey-Engley Neutralizing Broth (ingredients; 5.0 g/L casein enzymatic hydrolysate, 2.5 g/L yeast extract, 10.0 g/L dextrose, 6.0 g/L sodium thiosulfate, 1.0 g/L sodium thioglycollate, 2.5 g/L sodium bisulfite, 7.0 g/L lecithin, 5.0 g/L polysorbate 80 and 0.02 g/L bromocresol purple in 1000 mL of ddH_2O. pH 7.6 ± 0.2 at 25ºC). Dey-Engley Neutralizing Broth should be sterilized via autoclaving at 121ºC.
8) 6-well, 12-well, 24-well, 48-well and/or 96-well flat bottom microtiter plates for biofilm growth (round-bottom 96-well microtiter plates are necessary for planktonic cell preparation).
9) Haemocytometer (0.1 mm depth, 0.0025 mm^2 diameter).

2.2) Metabolic assays and biomass assessment

1) XTT [2,3-bis(2-methoxy-4-nitro-5-sulfo-phenyl)-2H-tetrazolium-5-carboxanilide] assay working solution. To prepare the working solution, 0.25 mg/L XTT is added to sterile distilled water and fully dissolved by gentle agitation, then filter sterilized through a 0.22-µm-pore-size filter, aliquoted, and stored at -80°C. All aliquots should be single use, and not be freeze-thawed. Upon thawing, XTT solution should be pre-warmed to room temperature prior to addition of menadione which should be prepared to a concentration of 10 mM in 100% acetone in a glass beaker, and fully dissolved by gentle agitation. A final concentration of 1 µM should be added to the working solution of XTT (higher concentrations may be required).

2) Resazurin sodium salt (or commercial equivalent e.g., AlamarBlue™ or PrestoBlue™) working solution. For the resazurin assay, a stock solution of 1% is prepared in sterile PBS by vigorous vortexing to completely dissolve the powder. Once dissolved, the solution is filter sterilized through a 0.22-µm-pore-size filter, aliquoted, and stored at 4-5°C for up to 3 months.
3) Crystal violet (CV) powder working solution. To prepare this, CV powder should be dissolved in ddH_2O to give a 1% solution. This is then further diluted in ddH_2O to a working concentration of 0.5%. There is no requirement to filter sterilize or autoclave the CV. The stock solution can be stored at room temperature for up to 3 months.

2.3) Viable cell quantification

1) Nunc® Thermanox™ 13mm coverslips (different sizes available for larger microtiter plates) or alternative substrates for biofilm growth such as 3.14 cm^2 circular stainless-steel coupons or cellulose matrix cut to an appropriate size e.g., 1.25 cm^2.
2) 23-gauge needle (or equivalent)
3) Tweezers
4) 5 mL bijoux tubes
5) Ultrasonic bath for biofilm sonication (40 cm handle, 16 mm blade, sterile cell scrapers can also be used for removing biofilm biomass)
6) 1.5 mL Eppendorf tubes
7) Propidium monoazide (PMA) is used for qPCR quantification of viable *C. auris* cells. The PMA is supplied as a 1 mg lyophilized solid that requires reconstitution in 200 µL of RNase free H_2O, dissolved by gentle pipetting – the

resulting working solution should be a rich red colour. Following dissolution, single-use PMA aliquots should be prepared and stored at -20ºC.

8) 650 W halogen light
9) QIAamp DNA mini kit
10) Screw-cap 2 mL Beadbug™ O-Ring tube
11) Glass beads, acid-washed (425-600 µm diameter)
12) BeadBug™ microtube homogenizer or equivalent.
13) Quantitative PCR machine and reagents of choice e.g., SYBR™ Green PCR Master Mix or TaqMan™ Universal PCR Master Mix with appropriate primers (and probes if applicable).

2.4) Microscopy imaging

1) FUN-1™ cell stain working solution of 20 µM (the 10 mM stock solution is diluted 1:500 in sterile ddH2O to get the desired working concentration). Stock solutions of FUN-1™ should be stored at -20ºC in single use aliquots to limit the amount of freeze-thaw cycles.
2) Glass coverslips (different sizes available)
3) Microscope slides and cover glass
4) VECTASHIELD® Vibrance™ Antifade Mounting Medium
5) Clear nail varnish
6) Superglue

3) Methods

3.1) Cell Standardization

The assays described herein can be utilised for planktonic or sessile forms of *C. auris*. To generate such preparations, the following steps can be used for all clinical isolates of *C. auris*. It is noteworthy that all steps should be performed under aseptic conditions wearing appropriate personal protective equipment.

1) Isolates of *C. auris* are stored in vials containing Microbank™ beads at -80ºC, by which stocks are prepared according to manufacturer's instructions **(note 1).**
2) For revival, vials can be thawed at room temperature, and one bead containing the organism transferred to a Sabouraud's dextrose agar using a disposable 10 µL inoculation loop. The bead is streaked across the agar then plates are transferred to a 30ºC incubator and incubated for 24 to 48 hrs (**Figure 1A-i**).
3) *C. auris* is propagated in liquid media by taking 1-2 colonies using a disposable 10 µL inoculation loop then transferred to 10 mL of yeast peptone dextrose (YPD). This suspension is then cultured at 30ºC, shaking at 150-200 rpm for 16-18 hrs (**Figure 1A-ii**).
4) Following growth in liquid media, *C. auris* is pelleted by centrifugation (3,000 x g), before pellets are washed via resuspension twice in 10 mL of sterile phosphate buffered saline (PBS; pH 7.2-7.6) (**Figure 1A-iii**). Following the final wash step, pellets are then resuspended in 10 mL of PBS prior to counting.
5) *C. auris* cells are standardised via counting on a haemocytometer (0.1 mm depth, 0.0025 mm^2 diameter) using the following formula (cell count × dilution factor × volume of square = colony forming unit [CFU per mL]) (**note 2) (Figure 1A-iv).** Following standardization, *C. auris* cells can then be utilised for planktonic or biofilm growth in Roswell Park Memorial Institute 1640 (RPMI) media in microtiter plates and/or on appropriate substrates **(Figure 1A-v)**.

3.2) Planktonic and Biofilm Preparation

1) The standardised suspensions of cells are then diluted to appropriate concentrations for experimental procedures. It should be noted that for all antifungal susceptibility testing, appropriate guidelines should be followed using standardized methods such as those of the Clinical and Laboratory Standards Institute (CLSI) and European Committee on Antimicrobial Susceptibility Testing (EUCAST) [2, 17, 18]. Briefly, to prepare plates for planktonic susceptibility testing, all drugs or other therapeutics and cells should be prepared to double the required concentration. Treatments of choice are firstly diluted to working concentrations in RPMI media with 200 µL drug added to the wells (usually the first column) on a round-bottom 96-well microtiter plate using a multi-channel pipette.
2) Drugs are then serially diluted 2-fold in RPMI media across the plate. *C. auris* cells can then be added to the desired wells which will then return the contents of all wells to the desired concentration. Media only and untreated wells should be included for negative and positive controls, respectively. Planktonic minimum inhibitory concentrations (PMICs) are defined as the drug concentration that inhibits cell growth. For PMIC testing, the usual cell concentration initially used is 2×10^4 CFU per well (100 µL of 2×10^5 CFU/mL), thereby when diluted 1:1 with the drug will give a final concentration of 1×10^4 CFU per well. PMIC testing is usually conducted for 24-48 hours, with plates incubated at 37°C (**Figure 1B-i**).
3) For biofilm growth, cells should be diluted to appropriate concentrations in RPMI media. Although these concentrations may vary from laboratory to laboratory, we would recommend using a final cell density of 2×10^5 per well (usually in 200 µL e.g., 200 µL of 1×10^6 CFU/mL suspension) of a flat-bottom 96-well microtiter plate

to initiate biofilm formation (concentrations and volumes should be adjusted accordingly for larger microtiter plates).

4) Biofilms can be formed on the bottom of flat-bottom microtiter plates or on appropriate substrates aseptically placed in microtiter plates using sterilized tweezers. Alternative substrates are described in the Materials section and documented elsewhere [19]. From our experience, *C. auris* can be left to form biofilms for a varied amount of time ranging from 4 hours to 14 days at different temperatures (20ºC e.g., room temperature, to 37ºC) depending on the experimental hypothesis (**Figure 1B-ii**). However, usually, biofilms are formed between 4-48 hours at 37ºC.

3.3) Metabolic activity assessment using microtiter plate assays

To assess the metabolic activity of *C. auris*, two main assays can be employed; XTT and resazurin colorimetric assays. These methods can be used for both planktonic and sessile forms of *C. auris*, although predominantly utilised for the latter. For the methods described below, biofilms are formed in flat-bottom 96-well microtiter plates (larger microtiter plates can be used for biofilm formation, with concentrations and volumes adjusted accordingly). For treatment studies, biofilms can be tested with therapeutics for appropriate times prior to assessment of metabolic activity. Both methodologies are useful techniques for assessing efficacy of treatments *in vitro* e.g., a reduction in metabolic activity likely equates to an enhanced antifungal activity, and thereby less of a colorimetric change with the associated assay (**note 3**). However, it is important to note that careful consideration of assay measurements should be made when comparing the efficacy of antifungals against different isolates of *C. auris* (**note 4**). Other limitations attached to such assays are discussed elsewhere in detail [20]. It

is also recommended that studies using the metabolic assays described below should consider parallel assessment of biofilm biomass using the crystal violet assay. An optional step is described in 3.3.2, part 4 for this additional assay.

3.3.1) XTT assay

1) Biofilms are formed by addition of 200 µL of 1 x 10^6 CFU/mL *C. auris* in RPMI media to flat-bottom 96-well microtiter plates (final concentration of 2 x 10^5 CFU/well). Biofilms are left to develop for an appropriate amount of time as discussed above (**section 3.2, step 4**).
2) Following growth (and treatment if necessary), media is aspirated, and biofilms are gently washed three times with 200 µL of sterile PBS to remove non-adherent cells. For the wash steps, media is aspirated and replaced with PBS carefully using a multichannel pipette, with tips placed into the wells at a 45º angle at the same point within the plate (bottom left of the well) to limit unnecessary disruption of the biofilm (as seen in **Figure 1B-ii**). After the final wash step, XTT (**section 3.3.1, steps 2-6**) or resazurin (**section 3.3.2**) can be used to assess metabolic activity of the *C. auris* biofilms.
3) For the XTT assay, a total of 100 µL of XTT working solution (0.25 mg/L XTT with 1 µM of menadione (**note 5**) is added to every well of the 96-well plate containing the pre-washed biofilms using a multichannel pipette. Wells with no biofilms are used as negative controls. For treatment studies, positive control biofilms minus the drug or therapeutic of choice should be included on every plate.
4) During the reaction the positively charged “oxidised” XTT compound will be broken apart in the presence of NADH and other reducing (electron donating) agents

produced by metabolically active cells (**Figure 2A**). This reaction is accelerated by use of an intermediate electron acceptor such as menadione.

5) XTT plates are incubated in the dark for 2 hours at 37ºC, with the colorimetric change measured in a microtiter plate reader at absorbance of 492 nm (**note 6).** We recommend transferring 75 µL of the XTT reagent following incubation to a new flat-bottom 96-well microtiter plate before reading as the biofilms are likely to interfere with the absorbance reading.
6) Within the assay, a colorimetric change occurs in the presence of metabolically active cells. A colourless solution of XTT changes to a bright orange coloured soluble compound called formazan which can then be quantified at OD_{492nm} (**Figure 2B-i**). Absorbance readings should be blank corrected with negative control wells (e.g., media only) to compensate results for background XTT conversion. In treatment studies, results are generally presented with treatment type on x-axis and absorbance values at OD_{492nm} along y-axis (**Figure 2B-ii**).

3.3.2) Resazurin assay

1) Biofilms are formed as described above (**section 3.3.1, steps 1 and 2**). For the resazurin assay, the stock solution of resazurin (1% in PBS) is diluted to 0.001% in RPMI media (**note 7**). A total of 100 µL is added to all wells containing biofilm and to negative control wells minus biofilms using a multichannel pipette.
2) In this assay, NADH and other reducing (electron donating) agents produced by metabolically active cells (**Figure 3A**) will convert the weakly fluorescent purple phenoxazine dye reduced to form resofurin, a strongly fluorescent pink compound (**Figure 3B-i**).

3) Resazurin plates are incubated for up to 3 hours, at 37ºC, with the fluorescence change measured at 530ex/590em on a fluorescence microplate reader with data presented as arbitrary units (AU) of fluorescence (**Figure 3B-ii**) (**note 6**). Alternatively, if you do not have access to a fluorescence plate reader, the colour change can also be measured by reading the absorbance at 570 and 600 nm, respectively. The extent of resazurin reduction can be calculated compared to the negative control wells using the following equation following the manufacturer's instructions:

$$\%\, reduction = \frac{(\varepsilon_{ox}\lambda 2)(A\lambda 1) - (\varepsilon_{ox}\lambda 1)(A\lambda 2)}{(\varepsilon_{RED}\lambda 1)(A\lambda' 2) - (\varepsilon_{RED}\lambda 2)(A\lambda' 1)} \text{ x } 100$$

In the equation above, ελ1 and ελ2 are constants that represent the molar extinction coefficient of resazurin (AlamarBlue™) at 570 and 600 nm, in their oxidised ($_{ox}$) and reduced ($_{RED}$) forms, respectively. Again, we recommend using blank corrected values in the equation in order to account for background conversion. In the equation, the operands are defined as follows:

$\varepsilon_{ox}\lambda 1$ = 80,586

$\varepsilon_{ox}\lambda 2$ = 117,216

$\varepsilon_{RED}\lambda 1$ = 155,677

$\varepsilon_{RED}\lambda 2$ = 14,652

Aλ1 = Observed absorbance measurement of test well at 570 nm

Aλ2 = Observed absorbance measurement of test well at 600 nm

Aλ'1 = Observed absorbance measurement of control well at 570 nm

Aλ'2 = Observed absorbance measurement of control well at 600 nm

4) **[Optional step]** After quantifying metabolic activity using one of the above assays, biofilm biomass can also be quantified to reinforce metabolic activity measurements. This can be achieved using the easily obtainable crystal violet (CV) dye. Detailed protocols of CV staining procedures can be found elsewhere [21, 22]. In brief, biofilms are washed, air-dried then stained with 0.5% CV for 20 minutes at room temperature, washed thoroughly to remove excess stain and finally de-stained with 95-100% ethanol. Absorbance of the solubilised dye can be read spectroscopically at 570 nm.

3.4) Cell counting and live/dead quantitative PCR

Colony forming unit (CFU) cell counting is still an effective and common methodology used in microbiology research laboratories worldwide. This method employs the Miles and Misra technique [23] for quantifying the number of viable cells in a microbial suspension (e.g., in this case, a sonicated *C. auris* biofilm) (**Figure 4**). A second methodology, known as live/dead quantitative PCR, has become a useful tool for assessing the viability of *C. albicans* in planktonic or sessile mono- and mixed species models following treatment [7, 24, 25]. However, the same methodology can be applied to *C. auris* cultures as well (**Figure 5**). The following sections provide detailed descriptions for biofilm formation, sonication and subsequent CFU counting and/or total and viable CFE quantification via qPCR. In the following example, *C. auris* biofilms will be formed on Nunc® Thermanox™ 13 mm coverslips placed at the bottom of 24-well microtiter plates, prior to CFU counting and live/dead assessment (**note 8**). As discussed above, treatments can be tested on these biofilms for an appropriate time prior to the steps described below (**note 3**).

3.4.1) Biofilm preparation and sonication for viable counts

1) Biofilms are formed by addition of 500 µL of 1 x 10^6 CFU/mL *C. auris* in RPMI media to flat bottom 24-well microtiter plates (final concentration of 5 x 10^5 CFU/well) containing Nunc® Thermanox™ 13mm coverslips. Biofilms can be left to develop for an appropriate amount of time as discussed above (**section 3.2, step 4**).
2) After growth, media is aspirated, and biofilms are gently washed three times with 500 µL of sterile PBS to remove non-adherent cells. As above, these steps were carried out carefully and pipette tips were placed to the edge of the coverslips to remove media and wash biofilms to ensure limited mechanical disruption of biofilms by pipetting (see **Figure 1B-ii**).
3) After washing, coverslips containing biofilms are removed from microtiter plates using a 23-gauge needle and tweezers, then transferred to a bijoux tube containing 1 mL of sterile PBS (**note 9**) (see **Figure 1B-ii**).
4) Biofilms are then sonicated at 35 kHz for 10 minutes in an ultrasonic water bath followed by vortexing for 30 seconds (**Figure 4-i**). Alternative methods to sonication exist such as manual removal of the biofilm using cell scrapers. For this, 1 mL of PBS should be pipetted onto the biofilm and biomass mechanically disrupted using the cell scraper. The biofilm cells can then be transferred to a bijoux tube via a pipette in 1 mL of PBS. For this, ensure that no biomass is remaining on the cell scraper following mechanical disruption.
5) The biofilm sonicate can then be used for two purposes: Miles and Misra technique for CFU counting (**section 3.4.2**), and the live/dead qPCR method for CFE quantification (**section 3.4.3**). To prepare for these methods, 20 µL of the 1 mL of

sonicate is taken for CFU counting, with the remaining 980 µL split equally into 2 x 1.5 mL Eppendorf tubes for live/dead qPCR (**note 10**).

3.4.2) Cell counting using the Miles and Misra technique

1) For CFU counting, 180 µL of sterile PBS is added to every well of a round bottom 96-well microtiter plate using a multichannel pipette (**Figure 4-ii**). Depending on the number of samples, each column should represent a different sample (multiple microtiter plates are required if >8 samples).
2) To row A, add 20 µL of the *C. auris* biofilm sonicate to 180 µL of PBS; this will represent the 10^{-1} dilution. Once all samples are added, the suspensions should be serially diluted 1 in 10 by adding 20 µL of samples from row A to 180 µL of PBS in row B, then 20 µL of diluted sample from row B to 180 µL of PBS in row C, and so on down to row H. At each row, the 20 µL of suspension should be mixed well by pipetting with 180 µL of PBS. It is recommended to change pipette tips between each dilution step. Once complete, the dilution factors for each row should range from 10^{-1} in row A down to 10^{-8} in row H (**Figure 4-iii**).
3) One Sabouraud's dextrose agar plate per sample should be split into 6 or 8 equal sectors. Starting from the lowest dilution factor (e.g., 10^{-8}), transfer 3 x 10 µL drops from the microtiter plate in the appropriate sector. This step is repeated for all dilutions, until all sectors of the agar plate are covered (**Figure 4-iv**). As above, it is suggested that the pipette tip is changed between each 10 µL drop and/or dilution factor.
4) Plates should be left to completely dry before inverting for incubation. For this, plate lids can be left slightly askew so that they can air-dry (**note 11**). Once dry, the

plates can be incubated overnight at 37ºC for up to 48 hours, with monitoring of growth after 24 hours.

5) After incubation, visible colonies will appear on the plates at lower dilutions, whilst higher dilutions will appear as heavily concentrated lawns of growth without discernible colonies. The sector with the dilution containing between 10-100 colonies should be selected for cell counting. The colonies should be counted in the 3 x spot dilutions per sector. From these triplicate values, take an average, then multiply by the dilution factor (e.g. 10^4) and also by 100 to account for the scaling factor (e.g., 10 µL to 1 mL). This will now give you the number of colony forming units in each mL of the original sample (CFU/mL). For example, if an average of 25 colonies were counted in the 10^{-4} sector, the concentration of cells in the original sample would be 2.5 x 10^7 CFU/mL (e.g., 25 x 10^4 x 100).

3.4.3) Viable and total cell count quantification using live/dead qPCR

1) Live/dead quantification by qPCR is achieved using a photoreactive DNA-binding dye such as propidium monoazide (PMA) (**note 12**) prior to qPCR analyses. In its mode of action, the compound will penetrate dead fungal cells or those with compromised membranes e.g., following antifungal treatment, and preferentially bind double stranded DNA (dsDNA) within the cell (**Figure 5A-i**). The 2 x 1.5 mL Eppendorfs containing the microbial samples (approx. 490 µL in each) are used for live/dead qPCR. One sample is treated with 2.5 µL of PMA working solution. The other sample remains untreated (no PMA added) and will act as the control for total fungal cells in the initial sample.
2) Both Eppendorf tubes should then be incubated in the dark for 10 minutes to allow uptake of the dye. All samples are then transferred to ice.

3) Samples are then exposed to a 650 W halogen light for 5 minutes for photoactivation of the PMA compound; exposure to the bright visible light will induce cross-linking of the PMA with dsDNA. Cross-linking between the compound and DNA structure following exposure to intense visible light will render it unsuitable for qPCR. Thereby, after DNA extraction, only DNA from viable cells will amplify in the qPCR, whilst untreated samples will allow for distinguishing total fungal cells. This will give rise to lower Ct values for untreated (total) cells and higher Ct values for PMA-treated (viable) cells (**Figure 5A-ii**).
4) The Eppendorf tubes should be evenly laid on a 'bed of ice', with the halogen light positioned approx. 20 cm away (as seen in **Figure 5A-i**). The 'bed of ice' should be approx. 5-10 cm thick and placed in a polystyrene box lid or equivalent heat-resistant container. The purpose of the ice is to prevent the Eppendorf tubes from melting due to the intense heat of the halogen light. The halogen light should be positioned so that all of the samples are exposed to the same degree of light. After 2 mins and 30 seconds, samples should be inverted and placed back on the ice for the remaining duration to ensure light exposure to all the sample. It is essential to take extra caution when handling the Eppendorf tubes following this step as they may be hot.
5) Following photoactivation, samples are centrifuged at 13,000 x g, for 10 minutes, before PBS is carefully aspirated and discarded. Here, pelleted cells treated with PMA may appear red in colour.
6) DNA is then extracted from the pellets using the QIAamp® DNA mini kit, as per manufacturer's instructions. A minor modification during the extraction process should be made, so that samples can be mechanically disrupted using glass beads to ensure high DNA yield. This additional step should be done following the initial

lysis step using the QIAamp® DNA mini kit. For this, firstly, an equivalent of 100 µL of sterile acid-washed glass beads of 0.5 mm diameter should be added to a screw-cap 2 mL Beadbug™ O-Ring tube.

7) Samples are transferred to the 2 mL O-Ring tubes, then mechanically disrupted using a bead beating machine (BeadBug™ microtube homogenizer or alternative), for a total of 90 seconds (3 x 30 second time intervals, with intermittent cooling of samples on ice).
8) Following mechanical disruption of the samples, the homogenised mixture is then used for DNA extraction with the above kit, with the manufacturer's instructions followed accordingly.
9) Following extraction, fungal DNA can be used for quantitative PCR using specific primers for *C. auris* (e.g., 18S or ITS). [**optional**] At this stage, if necessary, the quality and quantity of the extracted DNA can be assessed using a UV spectrophotometer (e.g., Nanodrop or equivalent).
10) To generate a standard curve for quantification, serial dilutions of pure planktonic *C. auris* cultures (e.g., from 10^8 to 10^3 CFU/mL) should be extracted using the same kit and method as described above (**note 13**). Unknown concentrations of viable and total *C. auris* can then be determined using the standard curves for each isolate. For example, highly concentrated samples of *C. auris* (e.g., 10^8) will give rise to lower Ct values, whilst higher Ct values are characteristic of lower concentrations of the organism (e.g., 10^3) (**Figure 5B-i**). Unknown live and total CFE/mL values can be extrapolated from the Ct values gathered for the pure planktonic *C. auris* cultures of known concentrations (**Figure 5B-ii**).

3.5) Live/dead fluorescent imaging

Fluorescent staining and imaging are also useful tools for assessing viability in planktonic or sessile *C. auris*. A combination of different dyes has been reported in the literature for *C. auris* isolates, such as nucleic acid dyes (SYTO™ 9, propidium iodide, and DAPI), cell membrane/wall stains (calcofluor white (CFW), fluorescently conjugated-Concanavalin A), and the two-colour fluorescent viability probe FUN-1™ [26-30]. In the below protocol, we will describe the fluorescent staining of *C. auris* biofilms using the viability probe FUN-1™ (**Figure 6-i**). In the following example, *C. auris* biofilms will be formed on glass coverslips placed at the bottom of 24-well microtiter plates, prior to fluorescent staining with FUN-1™. As above, it is likely that this methodology will be used to assess efficacy of antifungal drugs or therapies against *C. auris*, therefore these treatments can be tested during or following biofilm formation prior to fluorescent staining and microscopy (**note 3**).

1) Biofilms are formed by adding 500 µL of 1 x 10^6 CFU/mL *C. auris* in RPMI media to flat bottom 24-well microtiter plates (final concentration of 5 x 10^5 CFU/well) containing glass coverslips for an appropriate amount of time (see **section 3.2, step 4**). Other substrates may be used for such studies (**note 14**).
2) Following biofilm maturation, media is aspirated, and biofilms washed three times with 500 µL of sterile PBS to remove non-adherent cells.
3) After the wash steps, biofilms are then stained with 500 µL working solution of FUN-1™ at a final concentration of 20 µM (**note 15**). The dye is gently added to the wells as to not disrupt the adhered biofilms, and plates incubated for 30 minutes in the dark at 37°C.
4) Following incubation, biofilms are washed three times with PBS to remove excess dye. **[optional step]** At this stage, biofilms can be fixed using an appropriate aldehyde or alcohol fixative such as 4% paraformaldehyde (PFA) or ice-cold 100%

methanol. It is recommended that 4% PFA should be used for 10 minutes at room temperature, whilst alcohol fixation should be used ice-cold (-20°C) for 10-20 minutes. Fixation will increase longevity of fluorescently labelled samples (**note 16**). Following fixation, biofilms should be gently washed three times in PBS to remove excess fixative.

5) Glass coverslips can now be removed using a pair of tweezers with a needle (see **Figure 1B-ii**) and transferred to a microscope slide (**note 9**). The coverslips are placed in the centre of the microscope slide, and 10 µL mounting media added to the biofilm. A microscope cover glass is gently placed over the biofilm ensuring no bubbles form. The sides of the cover glass are then kept in place using small amounts of clear nail varnish and allowing it to dry (**note 17**).
6) Biofilms are then ready to be imaged on a fluorescent microscope or confocal laser scanning microscope. To image biofilms using a conventional fluorescent microscope, the substrate must allow for light penetration. If this is not possible (i.e. biofilms are grown on steel coupons or other non-transparent substrates), it is recommended that images should be captured using a confocal laser scanning microscopy.
7) For the FUN-1™ dye, it is recommended that a Fluorescein isothiocyanate (FITC) filter set with excitation ~488 nm and emission ≥ 530 nm is used.
8) Visually, in viable cells with intact membranes, FUN-1™ will be internalized, and the dye then transported to a cytoplasmic vacuole in metabolically active cells. FUN-1™ will subsequently be compacted into distinctive fluorescent red cylindrical intravacuolar structures. Dead cells fluoresce bright yellow-green, with no discernible red structures [31] (**Figure 6-ii**). If used in conjunction with CFW (**note 15**), CFW will bind chitin in the cell wall of fungal cells, staining these structures a

fluorescent blue colour which can be visualised at ~365 nm excitation and ~435 nm emission (DAPI filter set).

Concluding remarks

We have documented here a detailed description of the methodologies available to assess the viability of *C. auris* grown in planktonic or sessile forms. With the recent emergence of this nosocomial pathogen, and with no sign of the organisms' global health threat subsiding any time soon, more and more research laboratories are starting to investigate conventional and alternative treatments to combat this fungus. Thus, *in vitro* research into treatment modalities against *C. auris* is essential moving forward, and we hope one or more of these methodologies described here provide useful assistance for other laboratories.

4) Notes

1) Most isolates of *C. auris* can be obtained from the American Type Culture Collection (ATCC), Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), National Collection of Type Cultures (NCTC) and other microbial culture collections.
2) When using the haemocytometer, fungal cell suspensions should be diluted in PBS by at least 1:100 to enable accurate cell counting. This dilution factor should then be taken into account when calculating the total cell count, as per the following equation (cell count × dilution factor × volume of square = colony forming unit [CFU per mL]).

3) The methodologies described here document viability assays for untreated *C. auris* biofilms. However, it is likely that such assays will be utilised during treatment studies testing the efficacy of conventional and/or novel therapeutics. It is recommended for such studies, that following treatment, biofilms are washed again three times with sterile PBS to remove any residual treatment. This is essential as the residues of certain drugs or antiseptics (e.g., chlorohexidine) may interfere with activity of compounds such XTT and resazurin. In some cases, treatments may be neutralised with Dey-Engley Neutralizing Broth for 15 minutes at 37ºC, prior to wash steps and assessment of viability using metabolic assays.
4) With metabolic assays such as XTT, there still remains a need for standardized testing of fungal biofilms [32]. Therefore, such assays must be used with a certain level of caution in regard to data analysis and interpretation. Here, it is important to note that different isolates of *C. auris* have low, intermediate and high biofilm-forming capabilities (which can be assessed by a CV assay) each with possible variations in metabolic rates. Therefore, it is not wise to use the XTT or resazurin assay measurements as an absolute assessment of metabolic output.
5) Higher concentrations of menadione can be used to further accelerate the colorimetric change in the XTT assay. The concentration should be optimised for the cellular density of the tested biofilm e.g., an increased concentration of metabolically active cells will lead to a faster colorimetric change, and therefore may not require higher concentrations of menadione, and vice versa.
6) Regular monitoring of the XTT and resazurin plates is recommended, especially if using new isolates with unknown biofilm-forming capabilities or metabolic profiles. If possible, absorbance/fluorescence of the biofilms should be measured on the plate reader at regular intervals, to prevent saturation of colour. For treatment

studies, untreated controls should act as “positive controls”, which will further allow for determining the optimal incubation period for the metabolic assay.

7) It should be noted that resazurin-containing assays are available commercially as AlamarBlue™ or Presto Blue™ cell viability reagents. As described on the Thermo-Fisher Scientific website, these reagents contain resazurin in a proprietary stabilizing formulation that allows for a convenient “mix, incubate, and read” protocol. Manufacturer’s instructions should be followed when using these alternative reagents, although the principle of resazurin conversion and colorimetric change is the same as described in this methodology.
8) For the CFU counting and live/dead qPCR methods, biofilms were formed on Nunc® Thermanox™ 13 mm coverslips. However, biofilms can be formed directly on the bottom of flat-bottom 24-, 12- or 6- well microtiter plates for this assay, with the resulting biomass mechanically disrupted in 1 mL of PBS using a cell scraper (and transferred to the bijoux tube for sonication or vortexing, where appropriate). It is recommended not to use smaller microtiter plates (e.g., 48- or 96-well plates) as there would be insufficient biomass recovered for DNA extraction. Other substrates can also be used as described previously for growth of *C. auris* on clinically relevant substrates [19]. These alternative substrates are listed in the materials section. It should be noted that volumes of inoculum added may need to be adjusted accordingly to ensure appropriate coverage of the substrate tested.
9) To improve ease of which coverslips can be removed from microtiter plates, the end of the 23-gauge needle should be formed into a hook. This can be achieved by twisting the end of the needle with tweezers. The hook can then be positioned underneath the coverslips to slowly prise it away from the bottom of the plate (as seen in **Figure 1B-ii**). The coverslip can then be grasped by the tweezers and

transferred to the bijoux tube for live/dead qPCR, or to the microscope slide for fluorescent imaging. For imaging, ensure that the tweezers touch the edges of the coverslips only, so that the biofilm is not disrupted.

10) It is highly recommended to utilise both methodologies of cell counting and quantification (e.g., Miles and Misra, and live/dead qPCR) for studies into treatment efficacy against *C. auris* biofilms. This is to overcome the limitations attached to these techniques, such as underestimation of cell counts in CFU counting due to microbial aggregation (particularly problematic if working with aggregative forms of *C. auris*) compared to overestimation of number of cells associated with qPCR, through detection of extracellular DNA. Combining results from the two methodologies should serve to circumvent these limitations. It is also noteworthy that advantages and other limitations for these techniques are described elsewhere [20].

11) Where possible, for Miles and Misra counting, a category II microbiological hood should be used for preparing dilution plates. This will allow for numerous plates to be prepared at the same time, whilst also minimizing the chances of airborne contamination when leaving plates to dry with lids askew. If not possible, spot dilutions should be done aseptically directly adjacent to a Bunsen burner to reduce chances of contamination.

12) Other photoreactive dyes such as ethidium monoazide (EMA) are available for quantification of viable and dead microorganisms. However, it has been reported that PMA has a higher charge than EMA and is therefore less likely to penetrate membranes of viable cells [33]. Recently, Biotium have released a PMAxx dye, which has been designed to be a superior alternative to PMA. However, the

live/dead qPCR methodology is still the same for PMAxx as described here for PMA.

13) For extractions from pure planktonic *C. auris* cultures, DNA should be extracted from each isolate used to ensure that accurate standard curves are generated. Furthermore, it is suggested that DNA should be extracted from serially diluted cultures of each *C. auris* isolate, ranging from 10^8 to 10^3 CFU/mL to determine the lowest limit of detection. We find that 10^3-10^4 CFU/mL is the lower limit for which the QIAamp® DNA mini kit can extract from *C. auris* isolates efficiently; however, this may vary from lab-to-lab and may be different for other extraction kits. Finally, live/dead qPCR can be utilised with SYBR™ Green and Taqman™ qPCR technologies.

14) Alternative growth substrates suitable for confocal microscopy are multi-well chamber slides (typically ranging from 2-16 wells) and steel coupons. Steel coupons can be placed in 12-well plates with the same protocols for biofilm growth on Nunc® Thermanox™ 13mm coverslips or glass coverslips can be followed as described in **section 3.5** (concentrations and volumes may need to be adjusted accordingly for thicker substrates to ensure complete coverage of the surface). Alternatively, Nunc® Lab-Tek® 8-well chamber slides (0.8 cm^2/well) or ibidi µ-Slide 8-well chamber slides are specifically manufactured with cell imaging in mind. We recommend those supplied by ibidi GmbH, which work best for clear biofilm imaging. These are specifically made for confocal imaging and can be ordered coated or uncoated, with the latter requiring manual coating with poly-D-lysin which can be dissolved in ddH_2O. Coating with poly-D-lysin aids in fungal cell attachment and biofilm formation. Chamber slides should be incubated with 200 µL of 0.1 mg/mL poly-D-lysine for 30 min at 37ºC. Coated wells are then washed once with

200 µL PBS. *C. auris* cells can now be grown and exposed to desired treatments before staining, imaging and fixing as described above. Volumes given are for use in 8-well chamber slides and should be adjusted accordingly if using slides with a different number of wells.

15) If using FUN-1™ in conjunction with CFW, it is recommended as per manufacturer's instructions that concentrations of 10 µM and 25 µM are used, respectively. Both dyes can be added simultaneously and CFW visualized at ~365 nm excitation/~435 nm emission. It is recommended for all new fluorescent stains to be serially diluted to optimize dye concentration, given that cellular density of *C. auris* cells may impact staining efficiency. This could be achieved by using standardized suspensions of planktonic cells of *C. auris* diluted in different concentrations of fluorescent dye. Thermo-Fisher supply a useful manual called "probes for yeast viability" (https://www.thermofisher.com/document-connect/document-connect.html?url=https%3A%2F%2Fassets.thermofisher.com%2FTFS-Assets%2FLSG%2Fmanuals%2Fmp07009.pdf&title=UHJvYmVzIGZvciBZZWFzdCBWaWFiaWxpdHk=) for optimization of dye concentration. It should be noted that other viability stains are available for confocal microscopy and can be utilised for such experiments. SYTO™ 9 green fluorescent nucleic acid stain and propidium iodide can be used in conjunction to distinguish between live and dead fungal cells.

16) Any fixation method used should be optimized by each laboratory. The suggestions provided are only examples, and fixation may also vary depending on dye used.

17) For larger and/or thicker substrates, e.g., the steel coupons, it is recommended not to use the additional microscope cover glass to cover the biofilm. For these

samples, they should be fixed as described above, then the underside of the substrate superglued into place on a microscope slide. From experience, it is difficult to keep the additional microscope slide in place on these larger substrates which can lead to complications during imaging.

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Figure Legends

Figure 1

***Candida auris* cell preparation for planktonic and biofilm growth.** Isolates stored in vials are revived by cross-streaking a bead containing the organism on Sabouraud's dextrose agar (SAB). Plates are incubated for 24-48 hours at 30°C to permit growth (**A-i**). Following growth on solid media, 1-2 colonies are propagated into 10 mL of yeast extract peptone dextrose (YPD) and cultured for 16-18 hours at 37°C, gently shaking at 150-200 rpm (**A-ii**). Following growth in liquid media, suspensions at centrifuged at 3,000 x g for 5 minutes (**A-iii**). Pelleted cells are washed and resuspended in sterile phosphate buffered saline (PBS). An aliquot of the pure planktonic *C. auris* culture is diluted 1 in 100 in PBS, then counted using a haemocytometer. The number of colonies counted, the dilution factor, and volume of the square is used to calculate the total CFU/mL (**A-iv**). The standardized cell cultures can then utilized for planktonic or

biofilm growth in Roswell Park Memorial Institute 1640 (RPMI) medium in microtiter plates and/or on appropriate substrates (**A-v**). Following standardization, cells can be used for planktonic minimum inhibitory concentration (PMIC) testing. For this, cells are diluted to appropriate volume (usually 2 x 10^5 CFU/mL) in RPMI, and 100 µL transferred to wells on a round-bottom 96-well microtiter plate containing 100 µL of serially diluted concentrations of drug using a multi-channel pipette. Appropriate positive controls (*C. auris* suspensions minus drug) and negative controls (media only) are recommended for all plates (**B-i**). Biofilms can be formed directly in flat-bottom microtiter plates, or on appropriate substrates (e.g., coverslips, steel discs, or cellulose matrix) placed into the microtiter plates with sterile tweezers. For media changes, or washing of biofilms following drug treatment, it is recommended to tilt the pipette at a 45º angle to minimize disruption of the biofilm biomass. A 23-gauge needle with the end formed into a hook, and tweezers can be used to remove biofilms grown on substrates from microtiter plates. *C. auris* biofilms can be grown for 4 hours to 14 days, at temperatures ranging from 20°C to 37°C (**B-ii**). Figure compiled using Biorender.com.

Figure 2

XTT metabolic assay. The positively charged “oxidised” XTT tetrazolium salt can be used to assess the metabolic activity of *Candida auris*. In the presence of NADH and other reducing (electron donating) agents produced by metabolically active cells, the XTT tetrazole ring structure will be broken down into the water-soluble formazan. An intermediate electron acceptor such as menadione can also be used to accelerate the rate of reaction (**A**). In the reaction, a colorimetric change from a colourless solution

(XTT) to a bright orange solution (formazan) is characteristic of increasing levels of XTT-conversion, and by thereby, increased level of metabolic activity within the biofilm (**B-i**). The colorimetric change can be quantified at OD_{492nm} (**B-ii**). Figure assembled using Biorender.com.

Figure 3

Resazurin metabolic assay. The phenoxazine dye, resazurin can be used to assess the metabolic activity of *Candida auris*. In the presence of NADH and other reducing (electron donating) agents produced by metabolically active cells the positively charged resazurin will be converted into resofurin (**A**). In the reaction, the weakly fluorescent resazurin will be converted into the fluorescent resofurin, visually seen by a change from purple to pink in increasing levels of metabolic activity within the biofilm (**B-i**). The colorimetric change can be quantified on a fluorescence microtiter plate reader at 530 excitation/590 emission, with data presented as arbitrary units (AU) of fluorescence (**B-ii**), or alternatively, at OD_{570nm} and OD_{600nm}, with extent of conversion calculated relative to a negative control. Figure created using Biorender.com.

Figure 4

Cell counting using the Miles and Misra technique. *Candida auris* biofilms (following treatment) are sonicated at 35 kHz for 10 minutes in an ultrasonic water bath **(i).** In the meantime, a total of 180 µL of sterile PBS is added to every well of a round bottom 96-well microtiter plate using a multichannel pipette **(ii),** prior to addition of 20 µL of biofilm sonicate into well A1. Each column can be used for individual biofilm sonicates. Suspensions are then serially diluted 1 in 10 down the microtiter plate from

row A (10^{-1}) to row H (10^{-8}) (**iii**). Following serial dilutions, starting from the lowest dilution factor (e.g., 10^{-8}), 3 x 10 µL drops from each dilution is transferred to the appropriate sector on a Sabouraud's dextrose agar plate. Once dry, plates are then incubated at 37ºC for up to 48 hours until visible growth. Cells can then be counted from sectors containing between 10-100 colonies (**iv**). Figure generated using Biorender.com.

Figure 5

Live/dead quantitative PCR. The viability of *Candida auris* cells can be assessed using quantitative PCR (qPCR) with the photoreactive DNA-binding dye, propidium monoazide (PMA). In the reaction, the dye can only penetrate cells with compromised membranes (e.g., following antifungal treatment) and not viable cells with an intact membrane structure. Once inside the cell, PMA will intercalate the double stranded DNA. Samples are incubated with PMA in the dark for 10 minutes to allow uptake of the dye, then the samples undergo photo inactivation for 5 minutes (samples should be laid on a bed of ice approx. 20 cm away from a 650 W halogen light) (**A-i**). Following DNA extraction, the proportion of total and viable cells within a sample can then be determined using qPCR, whereby, lower Ct values are observable for untreated (total) cells and higher Ct values for PMA-treated (viable) cells (**A-ii**). To calculate the colony forming equivalent (CFE)/mL for unknown samples treated with and without PMA, a standard curve of known CFE/mL of *C. auris* is required. For this, DNA is extracted from pure planktonic *C. auris* cultures ranging from 10^8 to 10^3 and used in the qPCR (**B-i**). Unknown viable and total CFE/mL is then extrapolated from the standard curve compiled from the Ct values vs. known CFE/mL for the pure planktonic *C. auris*

cultures. In the example provided, the total CFE/mL would be 3 x 10^6, whilst the viable CFE/mL would be 4 x 10^4 (**B-ii**). Figure compiled using Biorender.com.

Figure 6

Fluorescent imaging for assessment of viability. *Candida auris* cells can be stained with the two-colour fluorescent viability probe FUN-1™. For such studies, it is likely that cells will be stained following antifungal treatment (**i**). In the mechanism of action, the dye will passively diffuse though all cell membranes staining the cytoplasm green. In viable cells, intracellular processing of the dye in compact, distinctive vacuole structures gives rise to a red fluorescence. This allows for distinguishing between dead and viable cells following confocal imaging (**ii**). In the example provided in (**ii**), images on the computer screen are visual representations of findings presented in Short et al (2019). In the study, the aggregative phenotype of *C. auris* strain 8978 was found to be more tolerant to antiseptic treatment then the non-aggregative strain 8973, 14-days post treatment. Image created using Biorender.com.

Fig 1

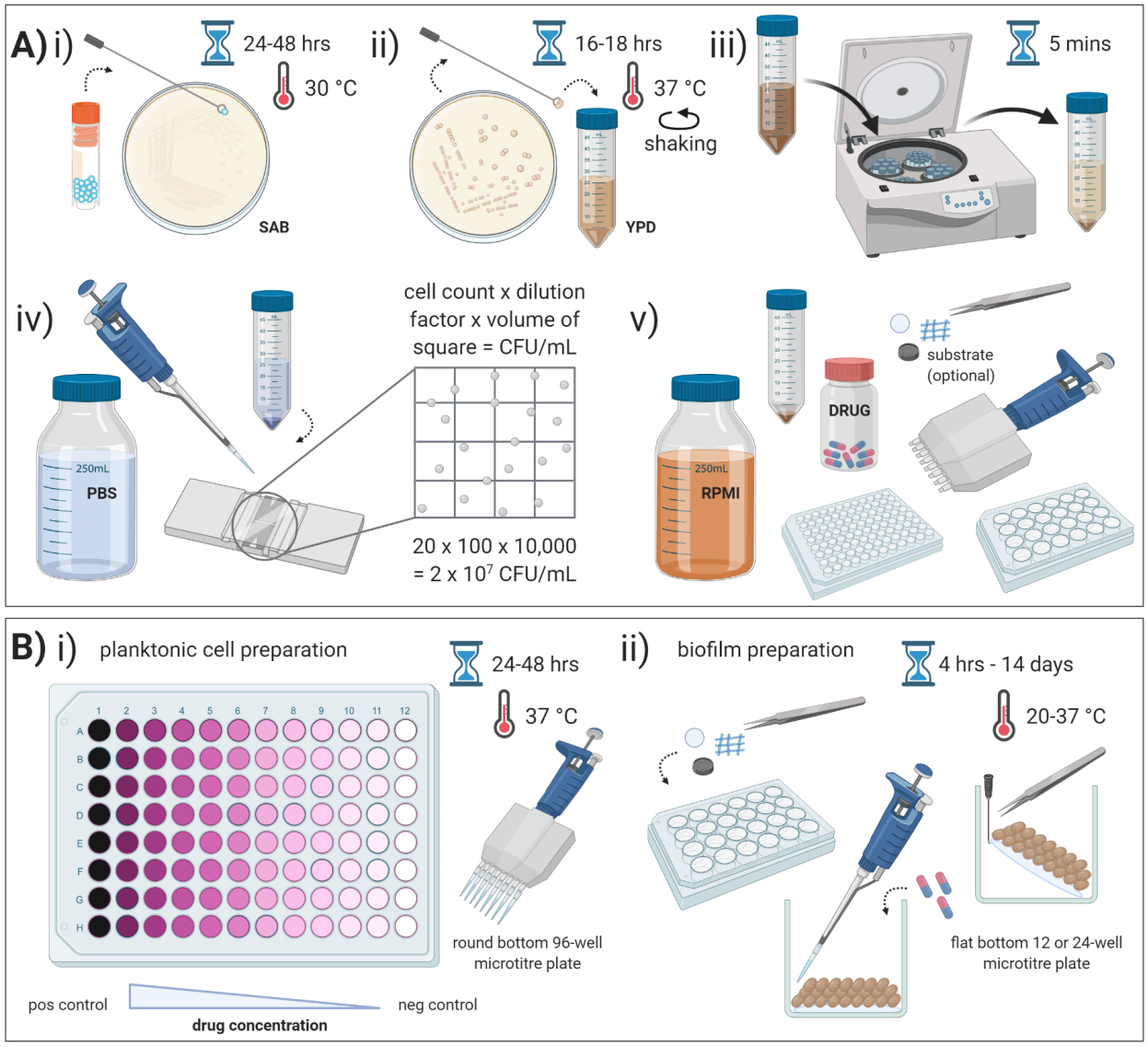

A) i)
24-48 hrs
30 °C
SAB
ii)
16-18 hrs
37 °C
shaking
YPD
iii)
5 mins
iv)
250mL
PBS
cell count x dilution factor x volume of square = CFU/mL
20 x 100 x 10,000
= 2 x 10^7 CFU/mL
v)
250mL
RPMI
DRUG
substrate (optional)
B) i)
planktonic cell preparation
24-48 hrs
37 °C
round bottom 96-well microtitre plate
pos control
neg control
drug concentration
ii)
biofilm preparation
4 hrs - 14 days
20-37 °C
flat bottom 12 or 24-well microtitre plate

Fig 2

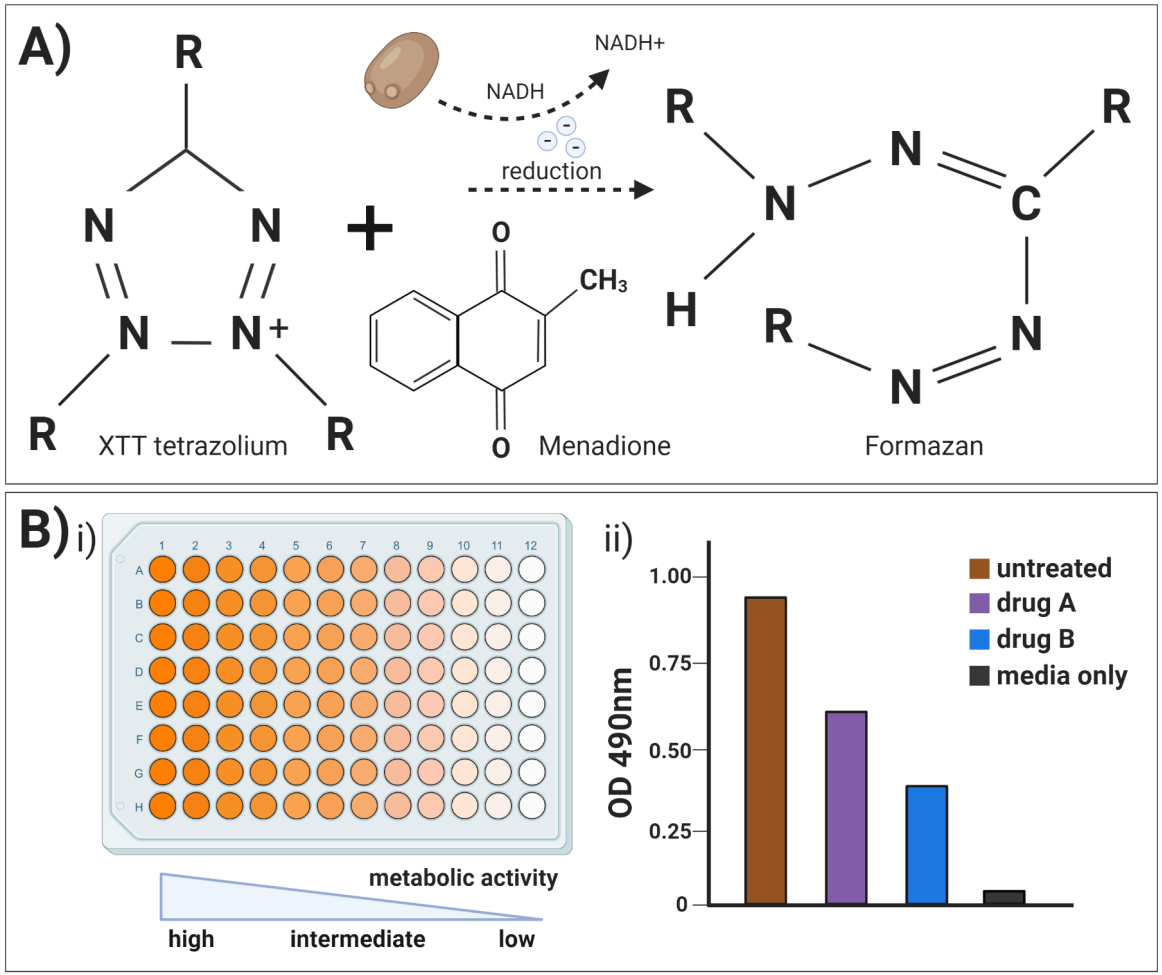

A)
R
N
N
N
N+
R
XTT tetrazolium
NADH
NADH+
reduction
O
CH3
O
Menadione
R
N
N
C
R
H
R
N
N
Formazan
B) i)
1 2 3 4 5 6 7 8 9 10 11 12
A
B
C
D
E
F
G
H
metabolic activity
high
intermediate
low
ii)
1.00
0.75
0.50
0.25
0
OD 490nm
untreated
drug A
drug B
media only

Fig 3

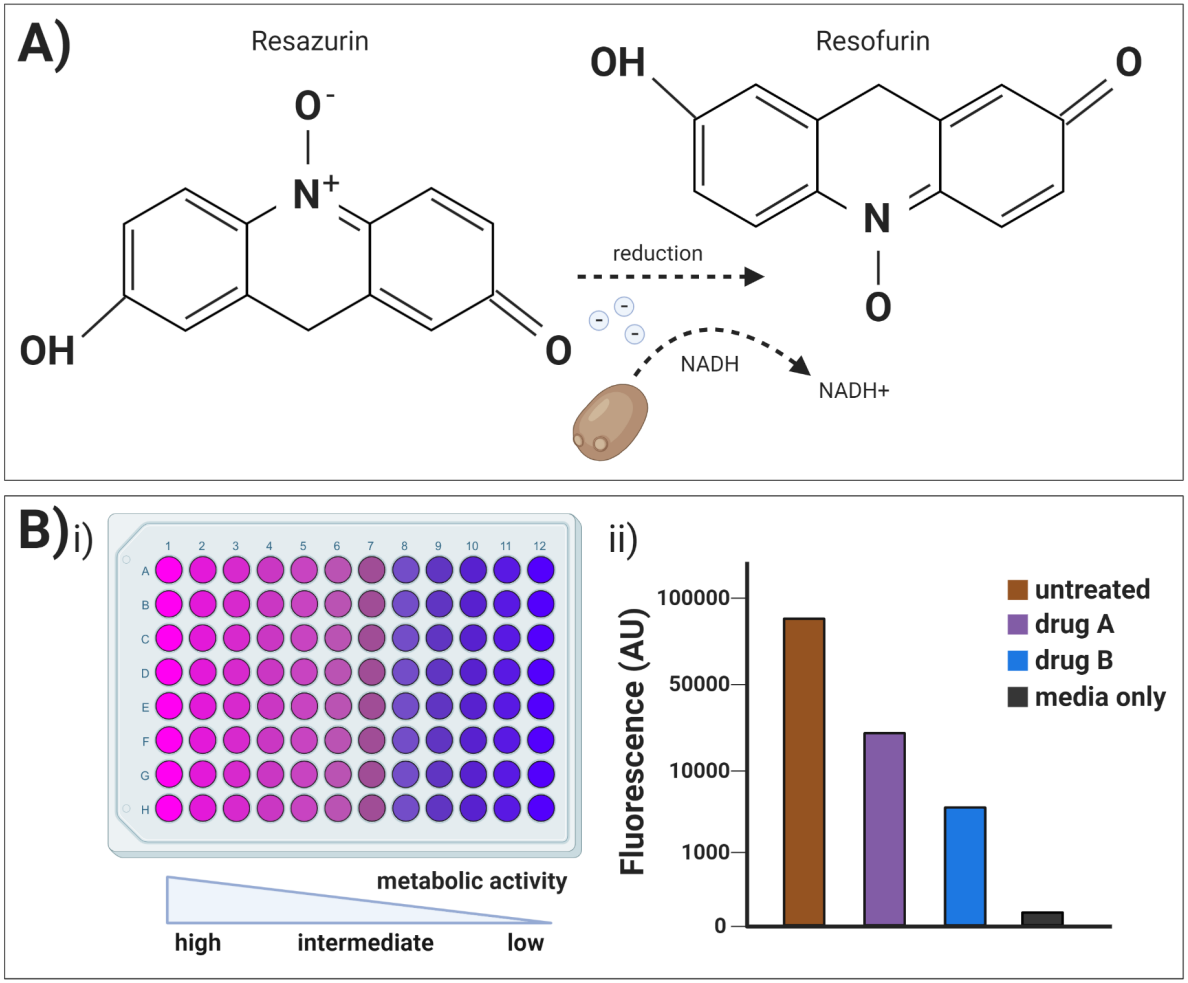

A)
Resazurin
Resofurin
OH
O
N+
O-
N
reduction
NADH
NADH+
B) i)
1 2 3 4 5 6 7 8 9 10 11 12
A
B
C
D
E
F
G
H
metabolic activity
high
intermediate
low
ii)
Fluorescence (AU)
100000
50000
10000
1000
0
untreated
drug A
drug B
media only

Fig 4

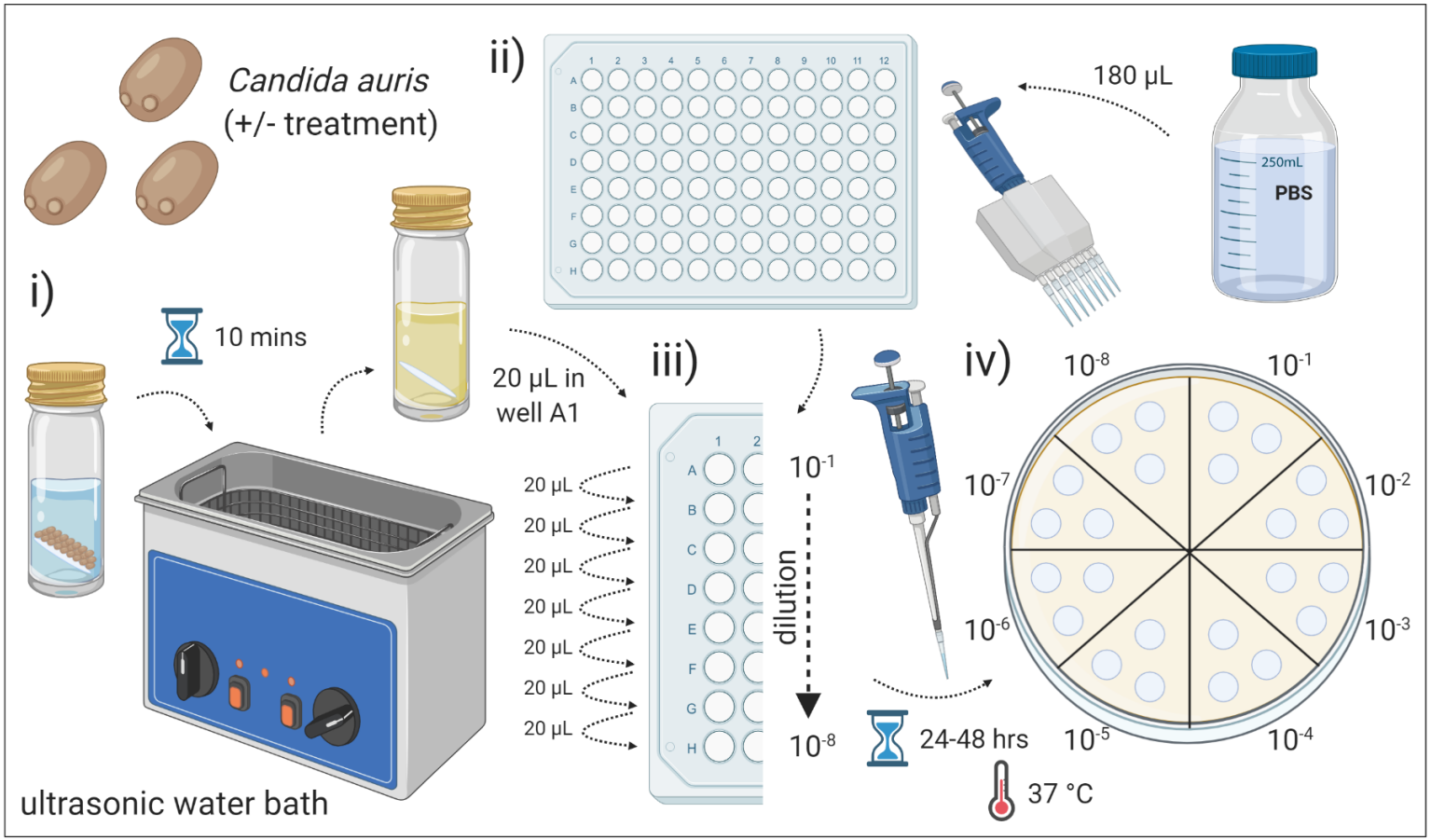

Candida auris
(+/- treatment)
i)
10 mins
ultrasonic water bath
ii)
180 µL
250mL
PBS
20 µL in
well A1
iii)
20 µL
20 µL
20 µL
20 µL
20 µL
20 µL
20 µL
10-1
dilution
10-8
24-48 hrs
37 °C
iv)
10-8
10-1
10-7
10-2
10-6
10-3
10-5
10-4

Fig 5

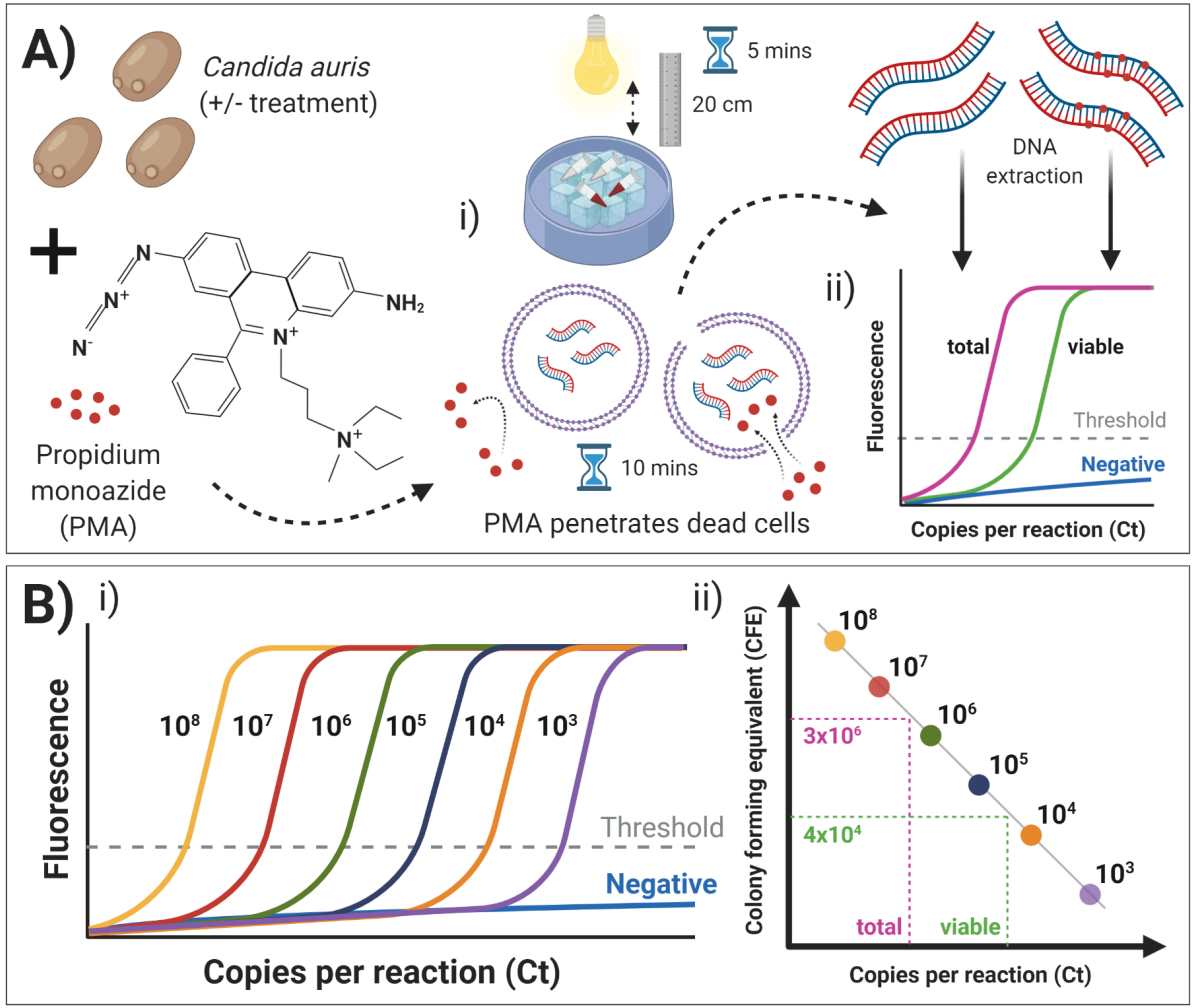
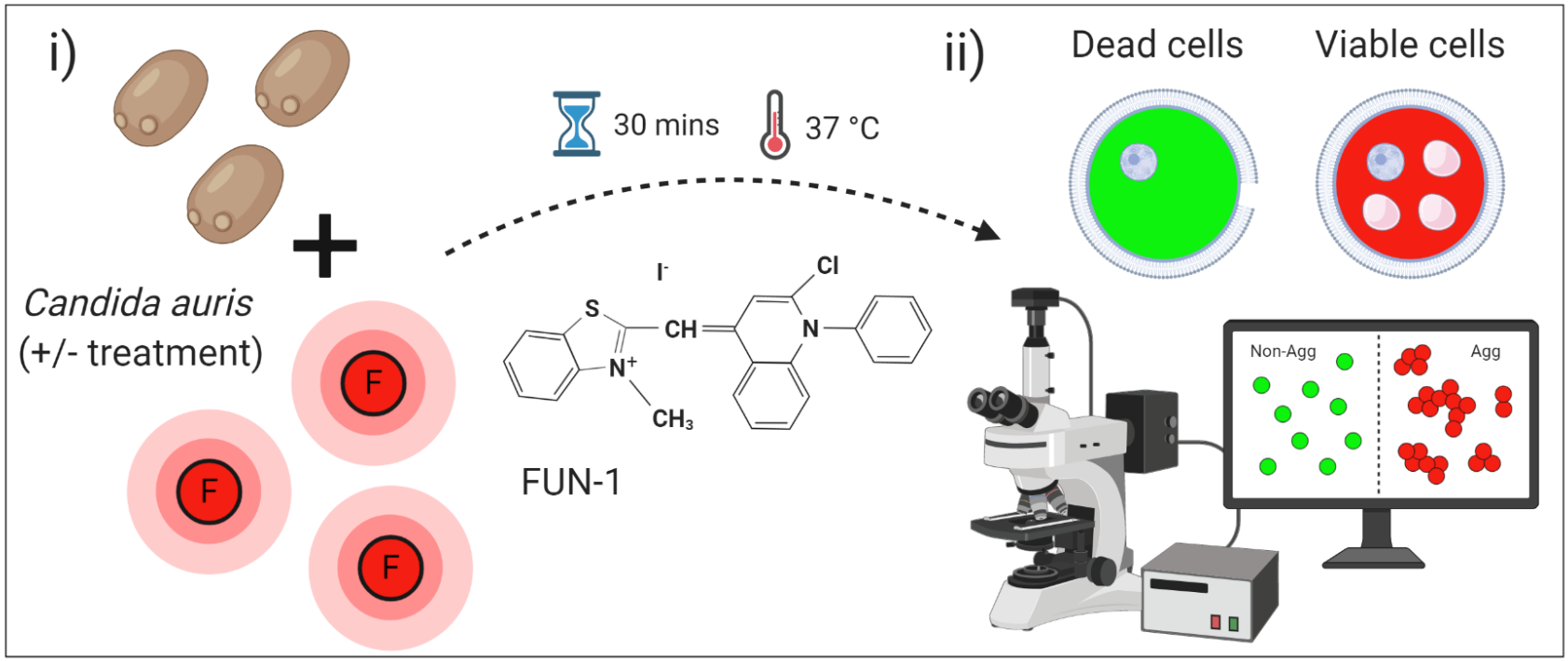

A)
Candida auris
(+/- treatment)
+
Propidium
monoazide
(PMA)
i)
5 mins
20 cm
10 mins
PMA penetrates dead cells
DNA
extraction
ii)
Fluorescence
total
viable
Threshold
Negative
Copies per reaction (Ct)
B)
i)
Fluorescence
10⁸ 10⁷ 10⁶ 10⁵ 10⁴ 10³
Threshold
Negative
Copies per reaction (Ct)
ii)
Colony forming equivalent (CFE)
10⁸
10⁷
10⁶
10⁵
10⁴
10³
3x10⁶
4x10⁴
total
viable
Copies per reaction (Ct)

Fig 6


i)
Candida auris
(+/- treatment)
F
F
F
30 mins
37 °C
I-
Cl
S
CH
N
N+
CH3
FUN-1
ii)
Dead cells
Viable cells
Non-Agg
Agg