XTT drug susceptibility assay for *C. albicans* biofilms formed in 96-well plates (flat-bottom wells; Costar 3590/3361) [updated 4-3-2019 by Hiram Sanchez]

-Culture on warmed YPD (yeast-peptone-dextrose) plates (3-phase streak) from -80°C glycerol stocks-incubate at **30°C for 1-2 days** (after this keep plates in 4°C for 1-2 weeks)

Day 1:

1. From a single colony grow your samples overnight in 15mL conical tubes containing 5 ml of YPD, **incubate at 30°C with gently shaking 50 to 200 rpm**. Include reference strain (i.e. Wild Type-SN250).
   - 4 samples fit per plate, 1 reference strain and 3 tested strains

Day 2:

1. Remove cultures and pulse vortex for proper mixing.
2. To count cells with hemocytometer see addendum for details.
3. Using a 96 well flat-bottom plate (Costar 3590/3361) label and inoculate with the plate with **1 X10^6 cell/ml** as described (see below figure)

   - Place **200µL of 1x PBS** in Rows A and H and column 12 to prevent evaporation of samples
   - Place **100µL of each strain** to be tested into their corresponding quadrant
   - Place **100µL of RPMI** in column 1 as a negative control

Plate Setup (Fluconazole)

<table>
<tr><th></th><th>1</th><th>2</th><th>3</th><th>4</th><th>5</th><th>6</th><th>7</th><th>8</th><th>9</th><th>10</th><th>11</th><th>12</th></tr>
<tr><td>A</td><td colspan="11">PBS 200µL</td></tr>
<tr><td>B</td><td rowspan="6">RPMI 100µL</td><td colspan="5">Wild type 100µL</td><td colspan="4">Sample B 100µL</td><td rowspan="6">PBS 200µL</td></tr>
<tr><td>C</td></tr>
<tr><td>D</td></tr>
<tr><td>E</td><td colspan="5">Sample A 100µL</td><td colspan="4">Sample C 100µL</td></tr>
<tr><td>F</td></tr>
<tr><td>G</td></tr>
<tr><td>H</td><td colspan="11">PBS 200µL</td></tr>
</table>

4. Seal plate with parafilm. Label with your initials, time/date, and testing drug.
5. Place plate in **37°C incubator**, without shaking for **6h**.
6. After **6hrs** of incubation remove plate from incubator, slowly remove media and dose with fluconazole-try not to disrupt biofilm. **(DOSE 1)**
7. Seal plate with parafilm and **incubate at 37°C for 24hrs.**

Day 3:

1. **24hrs** after the first fluconazole dose-slowly remove media **(put in 96-well plate for dispersion)** and dose all wells with fluconazole a second time-try not to disrupt biofilm. **(DOSE 2)**
2. Parafilm; **incubate at 37°C without shaking for 24 hr.**

Day 4:

1. Slowly remove media **(put in 96-well plate for dispersion)** and wash each well carefully (unless fragile biofilm-then don't wash), so as not to disturb the biofilm, with **100µL of 1x PBS one time**.
2. Combine **1mL PMS+9mL XTT solution** (aliquots kept in -20°C-both are light sensitive)**-only enough for one plate.**
Add **100µL of XTT+PMS** solution to each well.
3. Wrap plate in foil and incubate at **37°C without shaking for 30 min**.
4. Read absorbance at λ: 490 nm-**4 sample Fluconazole XTT.prt** (protocol to use with Gen5)

Dosing

1. Slowly remove media from all wells.
2. Add **180µL of drug+RPMI** (1:2 dilution-that's why final concentration is half of stock) to wells slowly-try not to dirupt biofilm. **(if using same tips plate lowest concentration to highest)**
3. When Finished parafilm plate and place in **37°C incubator**.

Reagents:

XTT: (Sigma X4626; kept at 4°C).
Prepare at 0.83 mg/mL in 1x PBS.
Add 7.5 mg of XTT salt to 9mL 1x PBS
Centrifuge (3000 RPM for 1 min at RT) or filter to remove sediment
Collect supernatant
Foil tube and store at -20°C

Phenazine methosulfate (PMS): (Sigma P9625; kept at -20°C)
Prepare at 0.32 mg/mL in dH20.
Add 3.2 mg of PMS to 10mL ddH_2O (filter-sterilize)
Shake to dissolve and place in foil and store at -20°C.

RPMI + MOPS: (RPMI-1640 Medium HyClone SH30011.04; kept at 4°C, MOPS BP308; kept at RT)
Add 10.4 g RPMI to 700 mL of ddH_2O into a 1-2L beaker with a magnetic stir bar. Add 34.5 g of MOPS and mix well (with magnetic stir bar). Adjust pH to 7.0 using 5N liquid NaOH or NaOH pellets. Bring volume to 1 L and filter sterilize using Polyethersulfone Membrane (PES) 0.22 µm pore size filters into 500 mL bottles. Store at 37°C or RT.

Plate Layout (Fluconazole)

	1	2	3	4	5	6	7	8	9	10	11	12
A	PBS 200µL											
B	RPMI 100µL		Highest Drug Concentration	Wild Type 100µL			Ctrl Wells (No Drug)	Highest Drug Concentration	Sample B 100µL			PBS 200µL
C		Ctrl Wells (No Drug)										
D												
E				Sample A 100µL					Sample C 100µL			
F				Decreasing Drug Concentration →					Decreasing Drug Concentration →			
G												
H	PBS 200µL											

Dosing Plate (Fluconazole)

	1	2	3	4	5	6	7	8	9	10	11	12
A			**1000µg/ml F.C.**	**500**	**250**	**125**		**1000**	**500**	**250**	**125**	
B	RPMI 100µL		Fluconazole 200µL (2 mg/mL) (2000µg/ml)	RPMI 100µL				Fluconazole 200µL (2 mg/mL)	RPMI 100µL			
C												
D												
E												
F												
G												
H			**100µL**	**100µL**	**100µL**			**100µL**	**100µL**		**100µL**	

Discard 100µL **Discard 100µL**

+100 µL RPMI before plating

Plate Layout (Amphotericin, Micafungin, Biocides, Etc.)

	1	2	3	4	5	6	7	8	9	10	11	12
A	PBS 200µL											
B	RPMI 100µL		Highest Drug Concentration			Wild Type 100µL						PBS 200µL
C		Ctrl Wells (No Drug)										
D												
E						Sample A 100µL						
F												
G				Decreasing Drug Concentration →								
H	PBS 200µL											

Dosing Plate (Amphotericin, Micafungin, Biocides, Etc.)

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B	Saline 100µL		Drug Being Tested 200µL (Highest Concentration)	Saline 100µL								
C												
D												
E												
F												
G												
H				100µL	100µL	100µL	100µL	100µL	100µL	100µL	100µL	

Discard 100µL

Addendum

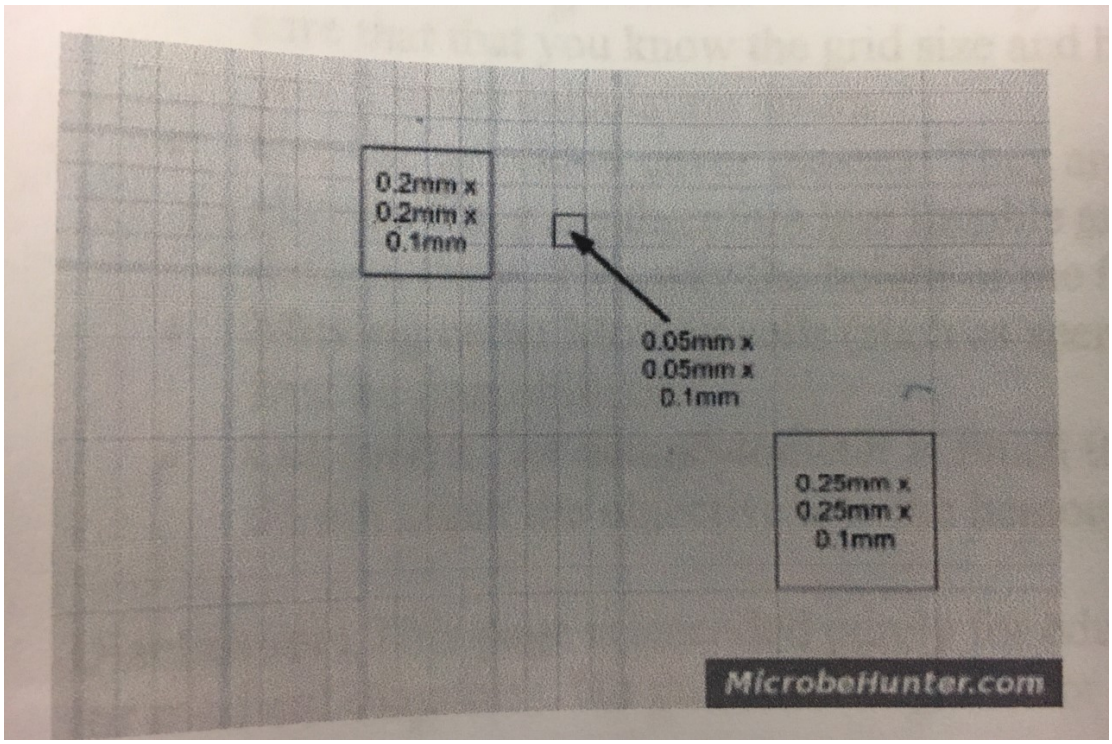
Cell counting with hemocytometer

1- **Prepare 5 ml of a 1:100 dilution of your overnight culture (50 µl of overnight culture into 4,950 µl of RPMI Media) mix well.**
2- **Take 10 µl of the cell dilution and place it in the hematocytometer.**
3- **Count the number of cells in 4 large squares (you can also count the cells in 4 medium and 4 small size squares but all you need is 4 of the same size, you decide what to use) see Fig 1.**
4- **Average the number of cells in all of the four squares and multiply the average by 1000 µl.**
5- **Then divide that number by one of these three;**
    a. **If large squares were used divide by 0.00625 mm^3**
    b. **If medium squares divide by 0.004 mm^3**
    c. **If small squares divide by 0.00025 mm^3**

For example:
Dilution was 1:100 and you counted the cells in four large squares, in each individual square you counted 15,19,18 & 17, when added and divided by 4, you end up with 69/4 = 17.25
17.25 is multiply by 1000 µl = 17,250. Since you counted the cells in the large square you need to divide the 17,250 by 0.00625. 17,250/0.00625 = 2760000 which is the same as 2.76 x 10^6 cell/ ml in your 1:100 dilution. The total number of cells in your original overnight culture is 2.76 x 10^6/ ml X 100 = 2.76 x 10^8 cells/ml.
So for your experiment you need 1 X10^6 cell/ml, so follow this;
(Number of cells you want) x (the volume in ml you need)
Total number of cells you have in your (1:100 dilution)

= (1 X10^6) x (5 ml) / 2.76 x 10^6 per ml = 1.81 ml
Add 1.81 ml of your 1:100 dilution to 3.18 ml of media to make 5 ml at 1 X10^6 cell/ml.

Fig.1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