APPENDIX B

Chemical and reagents for *In vitro* yeast expression assay

1. Luria Bertani (LB)

LB is the media used for growing bacteria. The solution was prepared by dissolving 10 g of Becto-tryptone, 5 grams of Bacto-Yeast Extract and 10 g of NaCl in ddH_2O. Use a magnetic stir bar and stir until all solids have dissolved. Use 10N NaOH to raise pH to 7.5 and then added up to make a final volume of 1 liter. Aliquot 75-100 ml into small bottles. Autoclave 45 mins by steam sterilizing. Do not dry.

2. LB and Ampicillin plate

The plates used to select for bacteria transformed with plasmids carrying ampicillin resistance are made simply by combining LB media with agar and adding the antibiotic ampicillin.

1. Follow the recipe for making 1 liter of LB media
2. Instead of aliquoting the media into bottles for LB stocks pour the liter of LB (including the magnetic stir bar) into a large two liter flask.
3. Add 14.5 g of Bacto Agar to the flask. Be sure not to stir once you've added the agar, it will go into solution upon autoclaving.
4. Wrap mouth with tin foil and tape down securely to avoid evaporation. Autoclave for 45 mins by steam sterilizing. Do not dry.
5. Remove from autoclave and allow to cooling down while stirring to a temperature that is comfortable to the touch. Add 600 µl of a 100mg/ml solution of ampicillin. Stir until the antibiotic has been well mixed into the media.
6. Pour the media into "ten-twenty-nine" Petri dishes. A liter of media make s approximately 50-60 plates.
7. Store at 4^{o}C.

3. YEP and Dextrose

The media used for growing yeast is called YEPD (Yeast Extract, Peptone and Dextrose). Do not autoclave YEP combined with Dextrose, so make each separately and then add one to the other when ready to use the media.

3.1 Yeast extract, Peptone and Dextrose (YEP):

The solution was prepared by dissolving 10 g of Yeast Extract and 20 g of Peptone in 1 liter of ddH_2O. Use a magnetic stir bar and stir until all solids have dissolved. Aliquot 270 ml 0f YEP into large bottles. Autoclave 45 mins by steam sterilizing. Do not dry.

3.2 20% Dextrose:

Weigh out 10 g of Dextrose and add to a small autoclaveable bottle. Add up to 50 ml of ddH_2O to each bottle. Do not mix. Autoclave 45 mins by steam sterilizing. Do not dry.

3.3 YEPD

To make YEPD combine 270 ml YEP with 30 ml 20% Dextrose.

4. 50% PEG:

1. Place 50 grams of polyethylene glycol in a 150 ml glass beaker and add 35 ml of ddH_2O.
2. Use a magnetic stir bar and stir with some heat until all solids have dissolved. (this may take a while)
3. Transfer all liquid to a 100 ml graduated cylinder.
4. Rinse the beaker with a small amount of ddH_2O and transfer this to the graduated cylinder containing the PEG solution.
5. Bring the volume to exactly 100 ml. Place a small piece of parafilm to the top of the graduated cylinder, make sure it is on tight, mix solution well by inverting several times.

6. Transfer 50 ml to two small media bottles.
7. Autoclave 45 mins by steam sterilizing. Do not dry.

Note: Evaporation of the water from the PEG stock solution will result in an increase in the effective concentration of PEG in the transformation reaction and will severely reduce the efficiency.

5. 10XTE

The solution was prepared by dissolving 6 g of Tris-Base(100mM) and 1.8 g of EDTA (10mM) to a 1 liter beaker. Add less than 500 ml of ddH_2O to the beaker. Use a magnetic stir bar and stir until all solids have dissolved. Adjust pH to 8.0 and add up to 500 ml ddH_2O. Transfer to media bottle. Autoclave 45 mins by steam sterilizing. Do not dry.

6. 1 M Lithium Acetate (LiOAc)

Add 51 g of LiOAc and 500 ml ddH_2O to a 1 liter beaker. Use magnetic stir bar and stir until all solids have dissolved. Transfer to media bottle. Autoclave 45 mins by steam sterilizing. Do not dry.

7. Growing bacteria culture

Saturated culture

a. add 3 ml of LB to a small test tube (sterile condition)
b. add 1.8 µl ampicillin.
c. select single small colony from LB-ampicillin plate and inoculate (sterile loop)
d. place the tube in 37^{o}C roller for 16 hrs (O/N) until culture is completely cloudy

8. Plasmid preparation (QIAgen Miniprep)

1. Remove culture from 37^{o}C shaker. Culture should be saturated and cloudy.
2. Pour approximately 1 ml of culture into a microcentifuge tube, spin down at 10,000g for 2 min
3. Take off supernatant with a pipette. Re-spin at 10,000g for 10 second, take off residual supernatant.
4. Resuspend pellet in 250 ul of buffer P1.(make sure that RNase A has been added to beuffer P1)
5. Add 250 ul of buffer P2 and gently invert the tube 4-6 time to mix. Do not vortex. (This is the lysis step so it is important that you do not let this reaction proceed for more than 5 mins. Reaction that go on longer tent to chop up your DNA, cause unreadable sequence data and other major problems)
6. Add 350 ul of buffer N3 and invert the tube immediately but gently 4-6 times, at this point your solution should become cloudy.
7. Centrifuge at maximum speed 14000rpm for 10 mins. You should see a white pellet.
8. Place QIAprep spin column in a 2 ml collection tube (provided). Apply the supernatant from the previous step to the column using your pipitman. Be careful not to draw up any residue.
9. Centrifuge at 10000 rpm for 1 min, discard the flow through
10. Wash the column with 0.75 ml of buffer PE and centrifuge for 1 min at 10000g
11. Discard the flow through and spin at 10000g for 1 min to remove any residual wash buffer
12. Place the column into a new sterile 1.7 ml microcentrifuge tube. To elute the DNA add 50 µl of buffer EB or gdH_2O to the center of the column. Let stand for 1 min and then centrifuge for 1 min
13. Discard the column and store preps in -20^{o}C freezer

9. Growing yeast TH5

Saturated culture: (sterile condition)

a. Add 50 ml of 20% dextrose to the 450 ml YEP to make 2% YEPD.
b. Label one large test tube for each strain name and the date.
c. Add 5 ml of YEPD to each test tube and 12.5 µl dTMP(stock=40mg/ml)
d. Select single colony from YEPD + 50 µl dTMP plate use a sterile long toothpick to pick up one colony (preferably a fat one) and inoculate the YEPD media
e. Put the test tube in the 30^{o}C roller and let them grow overnight. They are saturated when you hold them up to the light and you cannot see your finger through the glass. They should take approximately 20-24 hours to reach saturation, depending on how old the yeast plates are.
f. Put the saturated culture in the fridge. Use these cultures for all the serial inoculations of the next week. After 1 week, make new saturated cultures and dump out the old ones.

10. TH5 inoculation (Serial inoculation, sterile condition)

TH5 cells must be in mid-log phase for successful transformation. To ensure ready cells, perform a serial inoculation of TH5 cells:

Fill 6 large test tubes with:

5 ml YEPD

12.5 µl dTMP

Get the saturated culture from the fridge and vortex to mix the cells.

Add a different amount of saturated liquid culture TH5 to each tube:

2 µl

5 µl

10 µl

20 µl

40 µl

80 µl

Incubate in 30^{o}C roller overnight. (You are aiming to have at least one for each strain in log phase tomorrow when you are ready to perform the assay).

Take OD of the serial inoculation

a. Turn on the spectrometer and allow to warm up 5 min.
b. Set the wavelength to 660 nm.
c. Add 1 ml of YEPD to a plastic 2 ml cuvette and put in the spectrometer. Zero the spectrometer.
d. Dump the YEPD from the cuvette and add 1 ml of the culture.
e. Put the cuvette in the spec in the same orientation as before. Note the reading. Determine which dilution is in the mid-log OD_{660} = 0.5-1.25 or 0.6-1.0 (0.8-0.9 ideal).

11. Reagent preparation for yeast transformation

11.1 TE-LiOAc: (use within 1 month)

2 ml 10XTE

2 ml 1M LiOAc

16 ml gdH_2O

11.2 PEG-TE-LiOAc: (use within 1 week)

0.5 ml 10XTE

0.5 ml 1M LiOAc

4 ml 50% PEG

12. Vector ligation:

Combine the following in a 1.7 ml tube:

37.5 µl GR7 QIAgen prep (~1-2 µg DNA)

75 µl NE Buffer 4

25 µl Nco I

612.5 µl gdH_2O

Total=750 ul

Incubate tube at 37^{o}C for > 5 hours

13. Yeast transformation:

1. Determine which cell inoculation is in mid-log. Pour cells into 15 ml Falcon tube.
2. Centrifuge at 600g (2000 rpm) for 2 min, discard supernatant.
3. Wash cells with 5 ml gdH_2O. Centrifuge at 600g for 2 min. Discard the supernatant.
4. Wash cells with 5 ml TE-LiOAc, Gentle vortex. Centrifuge at 600g for 2 min. Discard the supernatant.
5. Resuspend cells in 0.5 ml TE-LiOAc
6. Incubate in room temperature roller for 1 hour.
7. Incubate on ice for 1 hour. Alternatively, you can leave the cells at this point for 1-2 days.
8. Put ssDNA salmon sperm at 100^{o}C for 10 min
9. Combine the for following in a 1.7 ml tube: (X2)

   200 µl cells

   10 µl ssDNA salmon sperm DNA

   10 µl cut GR7 vector

   15 µl insert (PCR PvDHFR) (this amount can vary depending on PCR success)
10. Incubate in room temperature roller for 30 min.

    (This is the perfect opportunity to begin warming your plates [-trp/+dTMP plate and –trp/-dTMP plate] and making PEG-TE-LiOAc)
11. Add 0.8 ml PEG-TE-LiOAc.
12. Incubate in room temperature roller for 30 min.
13. Heat shock for 10 min at 45^{o}C
14. Plate ~150 µl to appropriate media. [-trp/+dTMP plate and –trp/-dTMP plate]. Incubate at 30^{o}C . 2-4 days
15. Select colony from –trp/-dTMP plate for saturated culture.
16. Saturated culture:

Add 5 ml of YEPD to a large test tube (do not need to add dTMP). Grow up a saturated culture of yeast that has been transformed with plasmid in YEPD. Place the tube in 30^{o}C roller overnight.

17. Serial inoculation:

A. Label 8 large test tubes for each strain. Include the strain name and the amount of saturated culture that you are adding on the label (generally 1, 5, 10, 15, 20, 25, 50 and 75 µl for each strain).

B. Add 5 ml of YEPD to each tube.

C. Get the saturated cultures from the fridge and vortex to mix the cells. Add the 1, 5, 10, 15, 20, 25, 50 and 75 µl amounts of saturated culture to the media in the test tubes.

D. Put the test tubes in the 30^{o}C roller and let them grow overnight. (at least one for mid-log phase).

18. Take OD_{660}

Add 1 ml YEPD to the plastic 2 ml cuvette and put in the spectrometer. Zero the spec. Dump the YEPD from the cuvette and add 1 ml of culture, put in the spectrometer, note the reading OD, and looking up the corresponding cell density on the chart. You want a cell density between $5x10^6$ and $2x10^7$ cells per ml (ideally around 1 to $1.5x10^7$ cells per ml).

19. Use the Growth rates Excel file to determine how much of each saturated culture to add to the little flask of media.

14. Preparation of drug dilutions: (Wear gloves and sterile condition)

Drug stock:

Drug	Stock concentration (in DMSO)
Pyrimethamine	0.05 M ($5x10^{-2}$ M)
WR99210	0.1 M (10^{-1} M)
Chlorcycloguanil	0.1 M (10^{-1} M)

1. Take the drug stock from the freezer and place in the room temperature water bath to thaw. You will need the 10^{-6}, 10^{-5}, 10^{-4}, 10^{-3}, 10^{-2} M stocks.
2. While the drug stocks are thawing, label the 6 Falcon tubes with the desired final drug concentrations (DMSO, 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5} and 10^{-4} M)
3. Add 7.6 ml of YEPD to the DMSO Falcon tube and 3.8 ml of media to each of the others.
4. Add 400 µl of DMSO to the DMSO Falcon tube.
5. Vortex the thawed drug stocks and add 200 µl of each stock to the correct Falcon tube. Vortex the media and drug solution to mix. Return the stocks to the freezer. If doing the dilutions in advance, refrigerate the falcon tubes until needed.
6. Label each little flask with the strain name and add 40 ml of YEPD to each.

Calculation of serial drug dilutions:

$(C_1)(V_1)=(C_2)(V_2)$

(Drug stock conc.)(Volume of stock)=(Drug dilution conc.)(Volume of drug dilution)

Example:

Stock concentration of pyrimethamine at $5x10^{-2}$ M

Recommended convenient concentration for pyrimethamine stock dilution:

$5x10^{-2}$, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} , 10^{-6} M

10^{-2} M dilution = 200 µl of $5x10^{-2}$ M stock + 800 µl DMSO [100%DMSO]

10^{-3} M dilution =100 µl of 10^{-2} M stock + 900 µl of DMSO

10^{-4} M dilution =100 µl of 10^{-3} M stock + 900 µl of DMSO

10^{-5} M dilution =100 µl of 10^{-4} M stock + 900 µl of DMSO

10^{-6} M dilution =100 µl of 10^{-5} M stock + 900 µl of DMSO

Calculation of drug concentrations in media to obtain final concentration of pyrimethamine:

$(C_1)(V_1)=(C_2)(V_2)$

(Drug stock conc.)(Volume of stock)=(Drug dilution conc.)(Volume of drug dilution)

Recommended range of final concentration in wells for Pyrimethamine:

DMSO control, $1x10^{-8}$, $1x10^{-7}$, $1x10^{-6}$, $1x10^{-5}$, $1x10^{-4}$, $5x10^{-4}$ M

Calculation of drug concentrations in media (5X):

$5x10^{-4}$ M drug in media=200 µl of drug dilution $5x10^{-2}$ M +3.8 ml of YEPD media

10^{-4} M drug in media=200 µl of drug dilution 10^{-2} M +3.8 ml of YEPD media

10^{-5} M drug in media=200 µl of drug dilution 10^{-3} M +3.8 ml of YEPD media

10^{-6} M drug in media=200 µl of drug dilution 10^{-4} M +3.8 ml of YEPD media

10^{-7} M drug in media=200 µl of drug dilution 10^{-5} M +3.8 ml of YEPD media

10^{-8} M drug in media=200 µl of drug dilution 10^{-6} M +3.8 ml of YEPD media

DMSO=200 µl of DMSO + 3.8 ml of YEPD media [5%DMSO]

Final drug concentrations in well (1X):

$5x10^{-4}$ M =200 µl of drug + 800 µl of saturated culture

10^{-4} M =200 µl of drug + 800 µl of saturated culture

10^{-5} M =200 µl of drug + 800 µl of saturated culture

10^{-6} M =200 µl of drug + 800 µl of saturated culture

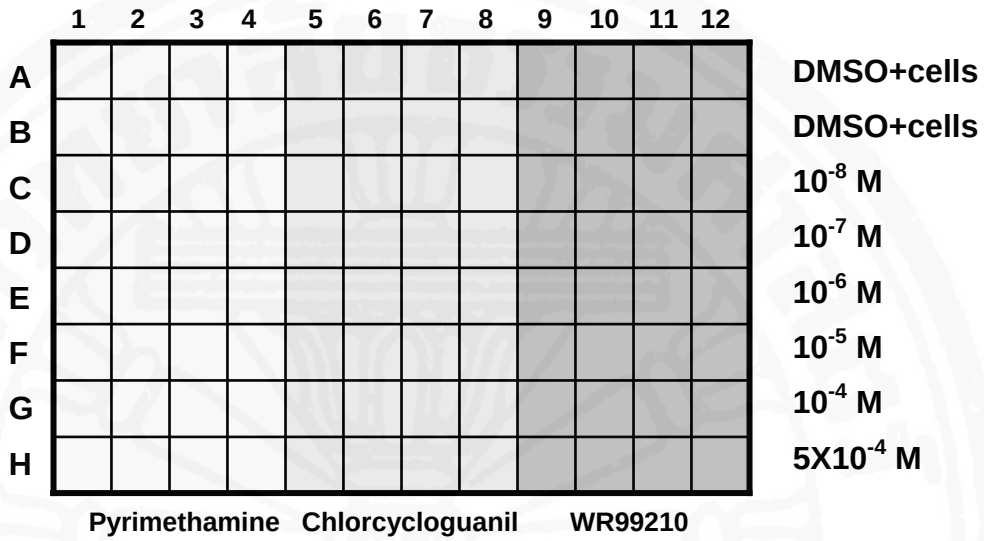
10^{-7} M =200 µl of drug + 800 µl of saturated culture

10^{-8} M =200 µl of drug + 800 µl of saturated culture

DMSO=200 µl of DMSO (5%) + 800 µl of YEPD media [1%DMSO]

Note: Pay attention to the amount of DMSO you will be adding to the wells. The concentration of DMSO in media (in the well) should never exceed 1% as this may inhibit yeast growth

15. IC_{50} set up



1. If the Falcon tubes with the drug dilutions have been refrigerated, take them out to allow them to warm to room temperature.
2. Add the indicated amount of saturated culture (as determine by the Growth rates sheet) to the little flasks of media. Swirl to mix, or add a small stir bar and mix on the blue stir plate.
3. Use the syringe pipetter on setting 4 to add 800 ul of the cells of each strain to 6 columns x 7 rows of the 96 well culture dish (see diagram). Dispense twice into each well to get 800 µl. (there is enough in the syringe to fill 5 ½ wells before refilling). Use a new syringe for each strain.
4. Using either the P1000 pipetman or a new syringe (with the syringe pipetter set to 5), add 1 ml of media alone (no cells or drug) to the bottom row of the 96 well culture dish.
5. Vortex each Falcon tube of drug dilution. Pour the contents of DMSO tube into the plastic boat and use the multi-channel pipetter to add 200 µl of solution to the top 2 rows of the 96-well culture dish (see diagram)

6. Dump the extra into the small waste beaker and pour the 10^{-8} M drug mixture into the plastic boat. Add 200 µl of this to each well of the third row. Lather, rinse, and repeat, progressing from lowest to highest drug concentration until all the wells have a 1 ml total volume.
7. Cover the 96 well culture dish with the breathable membrane, and then with the foil hat. Tuck the dish into a tupperware and snuggle it in with the diaper.
8. Take the dish downstairs to the 37°C shaker and secure it into one of the available 4L clamps. Note the time that the dish went into the incubator 23-24 hours from this time is when you should take the final OD reading.

Reading the final OD

1. Turn on the 96 well plate reader. The power switch is on the back. Start the Genesis program on the PC and make sure the IC_{50} protocol is open.
2. Retrieve the 96 well culture dish from the 37°C shaker. Remove the foil hat and the breathable membrane. The top few rows should be fairly opaque, and you should notice a gradient progressing from most dense at the top of the plate to least dense at the bottom. If the entire plate looks saturated, you may have contamination.
3. Use the multi-channel pipetter to transfer 200 µl of culture from each well to the clear plastic 96 well plate. Try to do this fairly quickly, before the cells begin to sediment.
4. Read the plate on the 96 well plate reader (go to the Reader menu and select Run).
5. Clean up the horrible mess all this has created (wear gloves). The most important steps are to thoroughly clean the glass beads (with water, soap, ethanol, and water again) and the culture dish (with water, soap-scrub each well with the test tube brush-ethanol, and water again). Allow the culture dish to air dry upside down on a paper towel overnight.
6. Transfer the data to a new Excel
7. Enter the information needed to calculate the growth rate for that plate the amount of saturated culture that you added to the little flask, and the starting cell

concentration from the day before, and the amount of time the culture was in the 37^{o}C shaker. Also fill out as much as you can of the first worksheet, and the Excel file called catalogue.xls.

8. Calculate the exact IC_{50}.

Growth rate of yeast cells in culture and absorbance (OD_{660} nm)

OD_{660}	cellsx10^7/ml	OD_{660}	cellsx10^7/ml	OD_{660}	cellsx10^7/ml
0	0	0.36	0.47	0.72	1.093
0.01	0.015	0.37	0.484	0.73	1.116
0.02	0.025	0.38	0.499	0.74	1.14
0.03	0.04	0.39	0.514	0.75	1.6
0.04	0.053	0.4	0.53	0.76	1.18
0.05	0.065	0.41	0.547	0.77	1.2
0.06	0.078	0.42	0.564	0.78	1.22
0.07	0.09	0.43	0.58	0.79	1.24
0.08	0.103	0.44	0.6	0.8	1.26
0.09	0.115	0.45	0.617	0.81	1.283
0.1	0.128	0.46	0.633	0.82	1.306
0.11	0.14	0.47	0.65	0.83	1.33
0.12	0.153	0.48	0.666	0.84	1.353
0.13	0.165	0.49	0.683	0.85	1.376
0.14	0.178	0.5	0.7	0.86	1.4
0.15	0.19	0.51	0.717	0.87	1.43
0.16	0.204	0.52	0.733	0.88	1.46
0.17	0.216	0.53	0.75	0.89	1.49
0.18	0.229	0.54	0.766	0.9	1.52
0.19	0.241	0.55	0.783	0.91	1.55
0.2	0.255	0.56	0.8	0.92	1.58
0.21	0.26	0.57	0.817	0.93	1.61
0.22	0.28	0.58	0.833	0.94	1.64
0.23	0.293	0.59	0.85	0.95	1.67
0.24	0.305	0.6	0.866	0.96	1.703
0.25	0.319	0.61	0.883	0.97	1.736
0.26	0.33	0.62	0.9	0.98	1.77
0.27	0.342	0.63	0.917	0.99	1.81
0.28	0.356	0.64	0.933	1	1.85
0.29	0.37	0.65	0.95		
0.3	0.385	0.66	0.966		
0.31	0.399	0.67	0.983		
0.32	0.412	0.68	1		
0.33	0.426	0.69	1.023		
0.34	0.44	0.7	1.046		
0.35	0.465	0.71	1.07		