**Protocol title: Bacterial liquid culture serial transfer for ecology**

**Aim of the protocol:** To understand how bacterial communities with different species richness resist during and recover from a 40\*C heat shock of variable length.  
We use 4 *Pseudomonas* species (***P. putida* BSC001, *P. protegens* CK101, *P. grimontii*, and *P. veronii* BSC005**). The basic idea of the protocol is as follows: After growing up overnight cultures from plates with single colonies, the monoculture cell densities are measured by flow cytometry. The cells are diluted to equal starting densities then combined in all 15 possible combinations of communities (i.e., monocultures as well as pairs, triplets, and the quad community). Liquid batch culture, serial transfer is used to grow the bacterial cells every day (these dynamics approximate slow chemostat growth and thus Lotka-Voltera type dynamics). We transfer the cells every day by diluting them to 5% in fresh media then we allow them to grow up to stationary phase (~22h). Starting from the end of the first day, a 40\*C heat shock of variable length is given (either 6, 12, 24, or 48h): this is the resistance phase. Then cells are allowed to grow for 2 days at the normal temperature (30\*C): this is the recovery phase. Each day, after transferring the cells, we also measure the relative and absolute bacterial cell densities for each community.

*As indicated in the title, this protocol can be used for any bacterial liquid culture serial transfer experiment.* It is intended for ecology (i.e., as opposed to evolution) because we use relatively large inoculum sizes each day. This is meant to maximize interactions and minimize evolutionary time. Assuming little cell death, 6 days’ worth of serial transfers should be <30 generations. The protocol is split up into 3 main parts: inoculation (which only happens on day 0), serial transfer, and measurement by flow cytometry. The measurement by flow happens each day (including day 0) and the serial transfers happen each day except for day 0 and the last day.

## **Inoculation** Day –2

**Materials:**

* Biosafety cabinet (or Bunsen burner)
* 5 LB agar petri dishes
* 70% ethanol
* Inoculation loop
* Cryostocks (use the –20 frozen rack to bring them up from the –80\*C)

**Step by step procedure**

1. Get safety cabinet ready (remove unnecessary stuff, disinfect, and add needed materials. Let the cabinet run for ~15 minutes before using it.)
2. While the cabinet is getting ready, bring the cryostocks up from the basement.
3. Label the petri dishes.
4. Streak each cryostock to its own separate petri dish. The 5th petri dish is for the negative control.  
   **To minimize contamination, it is preferable to always work in the following order: BSC005, P. grim, BSC001, then CK101.**
5. Incubate (without shaking) at 28\*C.  
   *The fast growers (BSC001, CK101) will take just under 12h to grow while the slow growers (P. grim, BSC005) will take ~24h.*  
   *Colonies can sit in the fridge at 4\*C for <3 weeks.*

## Day –1

**Materials:**

* Biosafety cabinet (or Bunsen burner)
* 50% LB media (sterile)
* 4x 50mL Erlenmeyer flasks (sterile) + 1 test tube (sterile)
* 70% ethanol
* Inoculation loop

**Step by step procedure**

1. Get safety cabinet ready (remove unnecessary stuff, disinfect, and add needed materials. Let the cabinet run for ~15 minutes before using it.)
2. Label Erlenmeyer flasks and test tube.
3. Fill Erlenmeyer flasks with 10mL of media and test tube with 1mL.
4. Pick single colonies from each petri dish and inoculate respective monocultures.

**To minimize contamination, it is preferable to always work in the following order: BSC005, P. grim, BSC001, then CK101.**

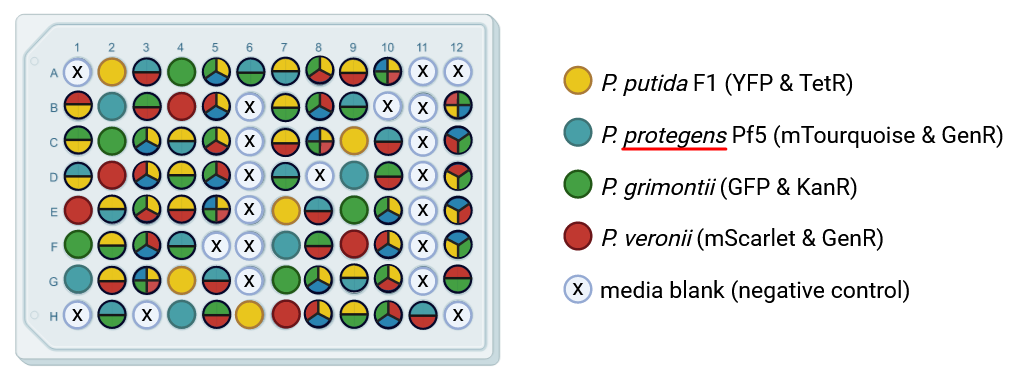
1. Incubate at 28\*C with shaking (225rpm) overnight.

## Day 0

**Materials:**

* Biosafety cabinet (or Bunsen burner)
* ~100mL 50% LB media (sterile)
* 5mL of STERILE 1x NaPPi (final concentration: 2g/L, pH 7.5, filter sterilized)
* ~100mL of non-sterile 1x NaPPi
* Two non-sterile flat bottom 96-wells (250uL)
* Reservoir (for multi-channel)
* Deep 96-well plate (2mL)
* P10 multi-channel pipette
* P100 multi-channel Pipette
* P300 Multi-Chanel Pipette
* Single channel pipettes: P10, P200, P1000
* Sterile U-bottom 95-well plate with lid (250uL)
* Wide parafilm (and scissors)
* Flow cytometer
* Microplate spectrophotometer
* **Depending on the cell densities:** sterile 50mL centrifuge (“falcon”) tubes and large centrifuge **OR** sterile 1.5mL microcentrifuge (“eppendorf”) tubes

**Step by step procedure**

1. Get safety cabinet ready (remove unnecessary stuff, disinfect, and add needed materials. Let the cabinet run for ~15 minutes before using it.)
2. While you’re waiting, turn on the flow cytometer.  
   *Remember to check that you have the fluorescent protein filter set!!*
3. Aliquot **900uL** of non-sterile NaPPi to top 3 rows of the 96-well deep well plate (this is 36 wells in total).
4. For each of the 4 monocultures, aliquot **3x100uL** of overnight culture to the top row in the following order: BSC001, CK101, P. grim, BSC005.  
   **To minimize contamination, it is preferable to always work in the following order: BSC005, P. grim, BSC001, then CK101.**  
   *This yields a 1:10 dilution.*
5. Using the P100 multichannel, pipette up and down to mix. Then, aliquot **100uL** from the top row to the 2nd row.  
   *This yields a 1:100 dilution.*
6. Repeat the previous step to dilute from the 2nd row into the 3rd row.  
   *This yields a 1:1000 dilution.*
7. Aliquot **200uL** of 1:1000 diluted cells into the top row of a flat bottom plate.
8. Aliquot **180uL** of NaPPi into the 2nd row of the same flat bottom plate.
9. Run the samples on the flow cytometer.  
   *Note: this can take up to an hour to run... That is very annoying.*
10. Record the concentrations (events/uL in the appropriate gate) in the Excel spreadsheet to calculate the mean density of each overnight culture.  
    *The Excel spreadsheet will also tell you how much to dilute or concentrate the cells for the desired inoculation density of ~10^3 cells/uL (i.e., the inoculum itself is at 2\*10^4 cells/uL but you will dilute it 0.05x in LB).*
11. Follow part *a* if the cells need to be diluted (i.e., the concentration of the diluted overnight cultures are at >20 cells/uL on the cytometer) and follow part *b* if the cells need to be concentrated (i.e., the concentration of the diluted overnight cultures are at <10 cells/uL on the cytometer).  
    **To minimize contamination, it is preferable to always work in the following order: BSC005, P. grim, BSC001, then CK101.**
    1. Label the 1.5mL eppendorf tubes.  
       Then add the appropriate volume of overnight culture.  
       Finally, add the appropriate volume of STERILE NaPPi.
    2. Label the 50mL falcon tubes.  
       Then add the appropriate volume of overnight culture.  
       Pellet the cells by running the big centrifuge at maximum speed for 10minutes. *Remember to include appropriate balance tube(s)!*  
       Discard the supernatent by pipetting it out carefully.  
       Aliquot 1.0mL of STERILE NaPPi. Pipette up and down to resuspend.
    3. *Regardless of whether you use part* a *or part* b*, this will result in a final volume of 1.0mL of each monoculture at a final density of ~2\*10^4 cells/uL.*
12. Now you are ready to make the 15 different communities:  
    Label the 96 deep well in 2 columns each of 8. You can load 200uL of sterile NaPPi as a control in the last well of the 2nd column.
13. Following the order indicated in columns 2 and 3 of the plate layout, pipette **200uL** of each species to the monocultures, **100uL** of each species to the pairs, **66.8uL** of each species to the triplet, and **50uL** of each species to the quad.
14. Open sterile U-bottom plate with lid from its sterile blister pack.  
    Label the plate with the date and, if you have more than one plate, the machine it will be incubated in.
15. Use the P200 multi-channel to add media to the sterile U-bottom 96-well plate: **118.8 uL**.
16. Using the P10 multi-channel and following the plate layout,  
    inoculate **6.25 uL** to each well.
17. Cut a piece of the wide parafilm to 3 dotted lines in length.  
    Cover evenly with parafilm and then plate lid.
18. Incubate in the microplate reader.  
    *Be careful not to jostle the plate!! And remember to rotate the plate so that the A1 well is in the indicated position in the microplate reader.*

## **Transfer**

**Materials:**

* Biosafety cabinet (or Bunsen burner)
* ~100mL 50% LB media (sterile)
* Reservoir (for multi-channel)
* STERILE 50mL Combi Tips with its respective pipette
* Deep 96-well plate (2mL)
* P100 multi-channel Pipette
* P300 Multi-Chanel Pipette
* Sterile U-bottom 95-well plate with lid (250uL)
* Wide parafilm (and scissors)
* Microplate spectrophotometer

**Step by step procedure**

1. Get safety cabinet ready (remove unnecessary stuff, disinfect, and add needed materials. Let the cabinet run for ~15 minutes before using it.)
2. Fill Reservoir with 50% LB.
3. Assemble Combi Tips (setting: 0) and fill the syringe with 50mL of 50% LB
4. Fill deep 96-well plate with **500µl** of 50% LB
5. Take the stationary phase culture out of the microplate reader and bring it into the safety cabinet.  
   *Be careful not to jostle the plate!!*
6. Carefully remove the parafilm from the plate.  
   *It’s important to be careful here to prevent contamination!*
7. Use the P100 multi-channel to inoculate **26.3uL** from the stationary phase plate to the fresh media in the deep 96-well plate.
   1. Resuspend the cells before collecting them and when releasing them
8. Open sterile U-bottom plate with lid from its sterile blister pack.  
   Label the plate with the date and, if you have more than one plate, the machine it will be incubated in.
9. Use the P200 multi-channel to aliquot **125uL** from the deep-well plate to the empty, new U-bottom plate.
10. Cover with parafilm and then plate lid.
11. Incubate in the microplate reader.  
    *Be careful not to jostle the plate!! And remember to rotate the plate so that the A1 well is in the indicated position in the microplate reader.*

## **Measurement by flow**

**Materials:**

* Biosafety cabinet
* 1x NaPPi (final concentration: 2g/L, pH 7.5)
* 50mL Combi Tips (does not have to be sterile) with its respective pipette
* Two non-sterile flat bottom 96-wells (250uL)
* Reservoir (for multi-channel)
* Deep 96-well plate (2mL)
* P100 multi channel Pipette
* P300 Multi-Chanel Pipette
* Flow cytometer

**Step by step procedure**

1. Prepare sample plan for wells
2. Get safety cabinet ready (remove unnecessary stuff, disinfect, and add needed materials. Let the cabinet run for ~15 minutes before using it.)
3. Fill Reservoir with 1x NaPPi
4. Assemble Combi Tips (setting: 0) and fill the syringe with 50mL of NaPPi
5. Fill deep 96-plate wells with **500µ**l of NaPPi
6. Label two non-sterile flat bottom 96-plates: “**plate 1**” and “**plate 2**”
7. **180µl** of NaPPi in each well of the two non-sterile flat bottom 96-plate
8. **55.6µl** cells into deep 96-plate wells (look at the prepared sample plan)
   1. If you haven’t already removed the parafilm from the plate, be careful while removing parafilm from the 96-plate, to prevent contamination
   2. Resuspend the cells before collecting them and when releasing them

*This results in a 1:10 dilution.*

1. Aliquot **20µl** (NaPPi + Cells) of the wells from the deep 96-plate to the 96-wells plate (look at the prepared sample plan: load only odd columns & leave the even columns as blanks.)  
   *This results in a 1:100 dilution in total.*
2. Start up Cytometer
3. Make sure you’re using the fluorescent protein filters kit (check 3 filters on blue laser and 1 filter on yellow laser).
4. Copy the template for plate 1 and rename it with the correct date, day of the experiment, and the machine.
5. Load plate 1 into the flow cytometer.
6. Start the programme for plate 1.
7. Make sure to check that well A3 is *P. putida*. It should show up in the YFP histogram gate.  
   If you forgot to switch the filter set, then it will show up in the GFP historgram gate (i.e., it will look like *P. grimontii*). This is okay! You need to stop the run and switch the filters to be the correct ones. You can re-aliquot 200 or 180uL of the appropriate contents to wells A1, A2, and A3. Delete the wrong data that you acquired. And re-start the cytometer.
8. When plate 1 finishes, check that all wells were completed successfully.  
   You may sometimes have bubbles that prevent some wells from completing. In this case, re-aliquot 200 or 180uL of the appropriate contents to those wells, delete the incomplete data, and re-run the cytometer just for those wells.
9. Copy the template for plate 2 and rename it with the correct date, day of the experiment, and the machine.
10. Load plate 2 into the flow cytometer.
11. Start the programme for plate 2.
12. When plate 2 finishes, check that all wells were completed successfully.  
    You may sometimes have bubbles that prevent some wells from completing. In this case, re-aliquot 200 or 180uL of the appropriate contents to those wells, delete the incomplete data, and re-run the cytometer just for those wells.
13. Finally, you can dispose of the NaPPi + cells dilution that is in the deep well plate. Cover it with aluminum foil, add a bit of autoclave tape, and label it as “waste”. This goes in the bin marked “liquid waste to autoclave”.  
    *Do NOT dispose of this 96-well plate sooner because you may have issues with the cytometer and then you will have to re-aliquot the cells from this plate!!!!*

INSERT A COPY OF THE FLOW CYTOMETERY PLATE LAYOUT HERE!