**Virulence Index Protocol**

Based on Storms. et al. (2019) “The Virulence Index: A Metric for Quantitative Analysis of Phage Virulence.” PHAGE 1:27-36 ([link](https://www.liebertpub.com/doi/10.1089/phage.2019.0001)).

The aim of this assay is to compare the fitness of phage SPO1 on the 2 different hosts that will be used in the coevolution assay: B. subtilis 168Δ6-WT and 168Δ6-ΔspoIIE (erm resistance removed). In this assay the virulence index is derived from measuring the phage-induced reduction in bacterial growth over a range of multiplicities of infection (MOI; ratio of phage to host). This is achieved by mixing a fixed number of hosts with a series of phage lysate dilutions.

**Media**

The coevolution experiment will be conducted in DSM, and so this will also be the media in the experiment. However, we may want to laer repeat the assay in LB. All host culturing below should be done in media with CaCl­2 (1mM in DSM, 10mM in LB) and chloramphenicol (5μg/ml).

**Preparation of lysate**

For this assay a large range of MOIs is needed and therefore a high titer lysate is required. This is best achieved by preparing a fresh lysate. Prepare lysate by plate flooding method from phagesdb:

1. Plaque assay previous SPO1 lysate on a lawn of *B. subtilis* Δ6-ΔspoIIE. (10-1 – 10-8)
2. Identify plate on which web pattern has formed and flood it with 5-10ml of phage buffer.
3. plates stand for ~4 hours at room temperature (or overnight in the cold room). Gently swirl plates occasionally during this period.
4. Collect Phage Buffer into falcon tube, centrifuge at max speed for 10 min and filter (0.2μm) into a sterilized tube (preferably glass).
5. Store in 4°C.

**NOTE**: I typically allow the lysate to sit in the fridge for a day or two before tittering it. This is because titers can drop significantly in the first day due to phage adsorption to the tube walls.

**Titer and determine efficiency of plating (EOP)**

To determine the lysate titer a plaque assay needs to be done. This can be also used to evaluate the relative plaquing efficiency of the phage on the 2 host strains. This is used as a first pass at estimating relative fitness of the phage on the hosts. Growth and plating in this part should be done using LB medium.

1. Grow an overnight culture of 168Δ6-WT and 168Δ6-ΔspoIIE.
2. Prepare 3 serial dilution series (10-fold) of the SPO1 lysate up to 10-9.
3. From each series plaque assay 10-5 – 10-9 dilutions on a lawn of each host.
4. EOP = titer on 168Δ6-ΔspoIIE /168Δ6-WT.

**NOTE:** If SPO1 lysate titer is lower than 108 PFU/ml do not continue to Virulence Index assay. Rather a new lysate should be prepared with a higher titer.

**Virulence Index Assay**

1. Inocula culture preparation
   1. Streak out colonies of the 2 strains on LB or DSM plate. Grow overnight at 37°C.
   2. Distribute 1.2 ml DSM+Cm into each of 12 green microtubes.
   3. Pick 5 colonies of each strain and place each colony in a separate DSM tube. 2 tubes are left as no culture controls. (4 colonies will be used for the experiment. 5 are picked in case there are problems with any of the colonies.)
   4. Close tubes tightly and incubate overnight at 37°C without shaking.
   5. Set WT culture for lawn in plaque assay.
2. Growth to late exponential

We would like to grow the cultures to OD600 ~ 1. To facilitate OD measurements, we will track OD by measuring 200μl samples in the Synergy plate reader. We will aim for an OD value of 0.5 in that reader.

* 1. Transfer 200µl of each tube to a well of 96-well plate
  2. Measure OD600 of overnight oxygen-limited culture (200μl) in Synergy plate reader.
  3. Choose 4 colonies of each strain to proceed with. Avoid outliers if possible.
  4. Subculture: From each of the selected cultures transfer 200μl into 10 ml DSM in 50ml flask. Adjust inoculation volume if there are great discrepancies in OD.
  5. Incubate in 37°C, 200RPM and monitor growth by plate reader.
  6. Stop incubation at OD ~ 0.5.

1. Make phage dilutions (10-2 – 10-7) in DSM (150μl => 1350μl)
2. Plate setup (see layout below)
   1. Distribute cultures into wells or DSM into blank wells (100µl/well)
   2. Add to wells 100µl of phage dilution or DSM into no-phage columns.
   3. Read OD600 in synergy 2 (37°C, 1200RPM, every 2min, 10hr)
3. Titer hosts and phages
   1. Plaque assay: use leftovers from phage dilutions to do a plaque assay with WT host lawn. Plate phage dilutions 10-4 – 10-7. Make replicates.
   2. Make serial dilutions of host cultures used to setup plate and spread plate 100μl of 10-5 and 10-6 dilutions onto LB plates.

To facilitate serial dilutions of multiple cultures I make such dilutions in a 96-well plate using multi-channel pipettes:

* Distribute 135μl PBS into wells of plate.
* Transfer 15μl into first well to make 10-1 dilution.
* Mix using plate mixer at 1200RPM for ~5 seconds
* For each of the following dilutions use multi-channel to transfer 15μl of previous well to new wells with PBS and repeat mixing as above.

**Plate layout**

|  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  | **1** | **2** | **3** | **4** | **5** | **6** | **7** | **8** | **9** | **10** | **11** | **12** | SPO1 dilution |
| **A** | Blank | 100 | 100 | 100 | 100 | COL1 | COL1 | 100 | 100 | 100 | 100 | Blank | 100 |
| **B** | Blank | 10-1 | 10-1 | 10-1 | 10-1 | COL2 | COL2 | 10-1 | 10-1 | 10-1 | 10-1 | Blank | 10-1 |
| **C** | COL1 | 10-2 | 10-2 | 10-2 | 10-2 | COL3 | COL3 | 10-2 | 10-2 | 10-2 | 10-2 | COL1 | 10-2 |
| **D** | COL2 | 10-3 | 10-3 | 10-3 | 10-3 | COL4 | COL4 | 10-3 | 10-3 | 10-3 | 10-3 | COL2 | 10-3 |
| **E** | COL3 | 10-4 | 10-4 | 10-4 | 10-4 | COL1 | COL1 | 10-4 | 10-4 | 10-4 | 10-4 | COL3 | 10-4 |
| **F** | COL4 | 10-5 | 10-5 | 10-5 | 10-5 | COL2 | COL2 | 10-5 | 10-5 | 10-5 | 10-5 | COL4 | 10-5 |
| **G** | Blank | 10-6 | 10-6 | 10-6 | 10-6 | COL3 | COL3 | 10-6 | 10-6 | 10-6 | 10-6 | Blank | 10-6 |
| **H** | Blank | 10-7 | 10-7 | 10-7 | 10-7 | COL4 | COL4 | 10-7 | 10-7 | 10-7 | 10-7 | Blank | 10-7 |
| **host** | WT | WT | WT | WT | WT | WT | spoIIE | spoIIE | spoIIE | spoIIE | spoIIE | spoIIE |  |
| **colony** | All | COL1 | COL2 | COL3 | COL4 | All | All | COL1 | COL2 | COL3 | COL4 | All |  |
| **phage** | no phage | SPO1 | SPO1 | SPO1 | SPO1 | no phage | no phage | SPO1 | SPO1 | SPO1 | SPO1 | no phage |  |