

FACs-based analysis for competition experiments

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Aim:

This protocol describes the method for performing competition experiments in the chemostat. We compete an unlabeled clonal isolate (or unlabeled heterogeneous population) against a reference strain labeled with a constitutively expressed fluorescent protein. By taking regular samples of the competing strains over a number of culture generation (>10 generations), it is possible to follow the relative abundance of the two subpopulations and compute a fitness coefficient.

Considerations:

In order to compete two strains of interest it is essential that they are in the same physiological state. Thus, the two competing strains (labeled reference and unlabeled strain of interest) must be grown individually in chemostat vessels and only mixed after steady-state is attained. (We have tried mixing both strains prior to batch growth, but this yields unpredictable results). Only ~25% of the unlabeled culture is used in the competition, so prior to mixing samples can be taken for RNA/dry weight etc.

Before you begin:

1. Determine which labeled reference strain you want to use. We use FY4 labeled with d-Tomato DBY11249 and have demonstrated here [<link>](#) in control experiments that this strain is well-behaved. This needs to be demonstrated for any new labeled strain.
2. Grow up the labeled reference in YPD and aliquot it into cryo-tubes in 1 ml aliquots with 15% glycerol. Freeze a number of these samples to ensure that you are using the same reference for every experiment (typically we freeze down 20-30 samples)
3. Equipment required:
 - 1 chemostat vessel per competing strain (200mL volume). We often do groups of three clones in the same media (i.e. three chemostats)
 - 1 chemostat for reference (450ml volume)
 - Autoclaved funnels wrapped in foil
 - Sterile graduated cylinders (250mL)
 - Appropriate media (if 1 reference and 3 clones we make 2 x10L)
 - 1x phosphate buffered saline (PBS) + 0.01% Tween-20

Starting competition

1. Grow up the labeled reference and competing strain in individual chemostats using the same media.
 - Inoculate one chemostat with labeled reference sample using the entire contents of a thawed 1mL cryo-tube. Set working volume to 450mL.

- Inoculate chemostat with competing strain grown in 5mL overnight in chemostat media. We run 3 chemostats off the same media carboy for 3 clones isolated from an evolution
 - Allow 2 days batch growth to grow cultures to saturation
 - Turn on chemostat pumps for desired dilution rate. Allow 6-10 generations to pass and take culture parameter measurements to confirm establishment of steady-state (i.e. two consecutive measurements separated by ~4 generations).
2. Prior to mixing samples and reference is a good opportunity to sample cells for RNA.
 - Take two steady state RNA samples (2 x ~10mL samples; refer to the chemostat manual for method)
 3. Mixing strain and reference
 - Before mixing take a 1mL pre-sample of each chemostat (refer to sampling method below)
 - Remove 150mL of culture from the unlabeled clone chemostat using a graduated cylinder (if you sampled for RNA remove ~130mL)
 - Reset drop tube on chemostat to appropriate level (i.e. 200mL)
 - Remove 150mL of the labeled reference into sterile graduated cylinder
 - Remove one of the port plugs on the clone chemostat and insert funnel
 - Pour the 150mL of labeled cells into the chemostat containing the unlabeled sample
 - Replace port plug. If the chemostat volume is too low (i.e. top of culture doesn't reach drop tube) add media to ensure that continuous phase is re-established
 - Repeat this for each sample
 - Note the time and empty the effluent vessels. This is the beginning of the competition
 - Save any remaining media in the reference sample carboy. This can be used for the competition media when it is depleted.

Sampling

1. Sample every few hours over ~ 4-5 days, record the time and the volume of effluent—this is used to determine how many generations have passed
 - Take a 1mL sample using passive sampling directly into Eppendorf Tube. This tends to result in some spillage. Therefore, place tube in a rack that can subsequently be washed
 - Spin Cells 1 min at 6000g (10,000rpm in microcentrifuge).
 - Remove supernatant
 - Resuspend cells in 1mL PBS + 0.01%Tween
 - Place in fridge until you are ready to perform FACS (4C deg)
2. Prepare samples for FACS
 - On the day of FACS analysis, sonicate the samples using Program 1 on the Botstein sonicator (5 sec pulse, pulse on 0.5sec, off 0.5 sec, output level 5)

- Dilute cells to $\sim 1 \times 10^6$ cells/mL in 1x PBS + 0.01% Tween (for samples recovered from chemostats at $\sim 2 \times 10^7$ cells/mL we add 200uL sample to 2mL PBS)
- Fill FACS tubes (FALCON #2054 12x75) with 2mL of 1x PBS + 0.01% Tween. Label the tubes clearly.
- Vortex tube once sample is added
- Store at 4C until FACS

FACs analysis

1. FACs appointments need to be booked with Tina
2. In order to use established protocol copy a previous session (DG####) and delete all samples from copy (duplicate without data, leave one tube listed on the protocol and rename it)
3. Measure the pre-samples – i.e. 100% unlabeled sample and 100% labeled sample in order to set gates appropriately in FACS software.
4. Count 50,000 cells to obtain accurate measure of relative proportions.

Analysis

1. Tina provides an excel spreadsheet of values
2. In order to compute a per generation selective coefficient regress $\ln(\text{clone/reference})$ against generations.
3. Rcode for performing linear fit and automated plotting is available here [<link>](#).
 - a. File format:
Header: <Gen> <Evo> <Anc>
Values: <Computed generations> <P unlabeled> <P labeled>
4. Inspect regression for outliers using regression diagnostics – often the first point is an outlier as the culture takes some time to equilibrate after mixing.
5. Selective coefficients are the slope of the regression
6. Compute 95% CI of linear fit using this function in R [<link>](#).