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High-throughput flow cytometry-based morphology screen in *Haloferax volcanii*

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Abstract

This protocol describes a high-throughput approach to screen cell shape mutants of *Haloferax volcanii* using flow cytometry (forward and side scatter combined). Compared to traditional microscopy, this method enables more rapid detection of shape differences and can be further adapted to incorporate fluorescent markers or applied to other organisms with distinct cell shapes during their life cycles.

Background

Many archaeal proteins are annotated as hypothetical proteins, whose functions are unknown. When homology-based functional predictions fall short, phenotype-based genetic screens offer a powerful alternative to understanding fundamental aspects of archaeal biology. In the model archaeon *Haloferax volcanii*, various screens have been used to uncover novel protein functions. For example, motility screens in *Hfx. volcanii* have identified key proteins involved in both motility and its regulation (Chatterjee et al., 2024; Collins et al., 2020). These motility screens also uncovered proteins linked to cell shape, since *Hfx. volcanii* undergoes a striking density-dependent morphological transition: during early logarithmic growth, cells are motile rods, but as they reach higher densities, they become non-motile disks in response to a secreted quorum sensing–like signal (Chatterjee et al., 2025; Duggin et al., 2015; Schiller et al., 2024).

To identify additional shape mutants independent of motility, we developed a high-throughput 96-well plate screen using flow cytometry to analyze forward and side scatter patterns to distinguish between rods and disks. This method has enabled rapid detection of transposon (tn) mutants (Kiljunen et al., 2014) unable to transition to the disk state, and further analysis of those tn-mutants can lead to identification of novel proteins

important for the shape transition. Because the shape change is induced by a secreted signaling molecule (Chatterjee et al., 2025), this screen can also be adapted to identify mutants defective in signal production or response—thus offering a platform to dissect the molecular basis of archaeal quorum sensing–mediated morphological regulation.

Materials

Product name	Brand	Manufacturer	Catalogue number	Notes
96 well plate	Corning	Corning Incorporated	3370	
Syringe filters, 0.22µm, PES	Millipore	MerckMillipore	SLGPR33RS	
Breathe-Easy membrane	Millipore	Diversified Biotech	BEM-1	
Hv-Cab medium	See below for recipe			Store at 4°C, up to 1 month

Equipment

Equipment name	Brand	Manufacturer	Catalogue number	Notes
Cytek Guava easyCyte Flow Cytometer	Cytek Bioscience			
BioTek Epoch 2 Microplate Spectrophotometer	BioTek	Agilent	EPOCH2NS-SN	

Software and bioinformatics tools

Software/tool/script	Company/Developer	Version	Web address	Github repository
Guava easycyte software	Luminex	guavaSoft 3.4		
Biotek Gen6	Agilent	Gen6 1.04.11		
R				

Protocol

A. Preparing overnight culture of *Hfx. volcanii* transposon (tn) mutants in a 96-well plate[1]

1. Spread Hv-Cab (see below for recipe) solid agar plates with 50µL of a 1:20,000 dilution of the pooled tn-mutant library (Kiljunen et al., 2014) to achieve single colonies of tn-mutants. Incubate plates at 45ºC for 3-4 days until colonies are visible

2. Filter-sterilize Hv-Cab liquid medium using a syringe-driven filter unit
    i. Filter-sterilize the media fresh each time for overnight cultures in 96-well plates[2]
    ii. Transfer ~22 mL Hv-Cab per 96-well plate into reservoirs
3. Using a multi-channel pipette, add 200 µL of filter-sterilized Hv-Cab into each well
4. Lightly tap a sterile toothpick onto the top of a single colony and inoculate each well[3]
    i. Inoculate control wells with wild type or strains with known shapes, for example known rod-only or disk-only strains (Schiller et al., 2024)
5. Cover the 96-well plate with Breathe-Easy breathable membrane by removing both sides of plastic, to avoid evaporation from the wells
6. Incubate 96-well plate at 45ºC overnight
    i. Use a shaking microplate reader (BioTek Epoch 2) – settings:
        a. Temperature 45ºC, Gradient 2
        b. Double orbital, continuous shake
        c. Optional: Read absorbance OD_{600} every 30 min to track growth
    ii. Since continuous absorbance measurements are not required, an alternative is to incubate the 96-well plates in a large shaking incubator, placed inside a sealed Tupperware box to prevent evaporation. This setup is particularly useful when running multiple plates simultaneously. At the final timepoint, measure OD_{600} values using the microplate reader for use in subsequent steps[4]

B. Glycerol stock of the arrayed tn-library
1. Remove Breathe-Easy breathable membrane
2. Pipette 75 µL culture from each well into prepared 96-well plate with 25 µL glycerol solution (see below for recipe) and mix by pipetting up and down
3. Store glycerol stocks at -80ºC by sealing with parafilm and keeping plate in a Ziploc bag to prevent evaporation

C. Determine cell shape of each well using flow cytometry
1. Prepare the Guava easyCyte instrument by running the Cleaning and the easyCheck method
2. Dilute cells from the overnight 96-well plate in a fresh plate with sterile-filtered Hv-Cab[5]
3. Run the InCyte program to collect cell samples
    i. Edit worklist as follows:
        a. Check “Mixing Required”
        b. Set Concentration Warning Limit (cells/µL) to 500
        c. Select quick clean for every 12 samples
        d. Park capillary in T09 (ensure water is in tube meant for T09)
        e. Highlight desired wells and select “Acquire this sample”
        f. Set events to acquire to maximum
        g. Set time to acquire to 20 seconds

h. Mix sample for 3 seconds at high rpm

ii. Set output plots to show Forward Scatter (FSC-HLog) versus Side Scatter (SSC-HLog), and any other preferences for output plots (can include fluorescence channels where necessary)

iii. Make sure to remove lid from 96-well plate before loading onto instrument

4. Export "List Mode Data" after collection
5. Run the Cleaning method before powering off the instrument

Data analysis

The CSV data from the Guava can be processed in many different ways. The Pohlschroder lab averages the Forward Scatter HLog to make an x-coordinate and the Side Scatter HLog to make a y-coordinate, and plots these coordinates as one data point per well. This has worked well to determine a 2-D visualization of cell shape. The R code for this method can be found at 10.5281/zenodo.16648977

Recipes

<u>500 mL of Hv-Cab medium</u>

1. 200 mL Millipore water
2. 2.5 g Casamino Acids (Acid Casein Peptone, Fisher)
3. 1.17 mL KOH (1 M)
4. 300 mL concentrated salt water stock solution, 30% (w/v) (Dyall-Smith et al., 2009)
5. For plates: 7.5 g Difco bacteriological agar (BD)
6. Autoclave
7. 1.5 mL $CaCl_2$ (1 M)
8. 450 µL solution of thiamine (0.88 mg/mL) & biotin (0.11 mg/mL)
9. 5 mL of trace element solution (de Silva et al., 2021)
10. any additional additives required by the specific *Hfx. volcanii* strain (Allers et al., 2010, 2004).

<u>150 mL salt water glycerol solution</u>

1. Final concentration: 80% glycerol and 20% of the 30% (w/v) salt water stock (Dyall-Smith et al., 2009)
2. 120 mL glycerol (invitrogen)
3. 30 mL 30% (w/v) salt water stock
4. Shake vigorously to homogenize
5. Autoclave

Additional Notes

[1] This protocol can be used with EMS mutagenesis or other form of mutation screening instead of tn-library. Adjust A.1 to inoculate a plate of the respective mutant library.

[2] Filter sterilizing Hv-Cab medium before growing 96-well plate cultures minimizes precipitates and debris for use in the Guava easyCyte instrument.

[3] It is not necessary to maintain an outer circumference of wells of the 96-well plate filled with just medium to offset evaporation because the Breathe-Easy breathable membrane prevents evaporation. Through empirical experimentation, forward and side scatter analysis of control samples in wells from the outer circumference is indistinguishable from that of inner wells.

[4] Continuous OD_{600} readings are useful for tracking the growth of the tn-mutants, since some may have growth defects. Final OD_{600} reading is important to ensure that there is growth of the tn-mutant as well as to determine the dilution necessary for the Guava easyCyte instrument.

[5] For determining the ideal cell concentration in the 96-well plate for use in the Guava easyCyte instrument, OD_{600} of the plate should be taken after overnight growth with the plate lid on and after removal of Breathe-Easy breathable film (Biotek Epoch 2). Dilute with Hv-Cab to achieve OD_{600} reading of wells to be between 0.1 - 0.15, which corresponds to an optimal cell concentration for the Guava easyCyte instrument.

Trial and error

- Without the breathable film, evaporation occurred during incubation leading to excess precipitation and debris that was indistinguishable from cells.
- Gating between cells and precipitation/debris based on *Hfx. volcanii* autofluorescence was attempted, but *Hfx. volcanii* autofluorescence was not distinguishable from background signal

Competing interests

The authors declare that they have no conflict of interest

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