

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 *H. 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 *H. volcanii* undergoes a striking density-dependent morphological transition: during early logarithmic growth (OD_{600} 0.05), cells are motile rods, but as they reach higher densities ($< OD_{600}$ 0.3), 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 *H. volcanii* transposon (tn) mutants in a 96-well plate¹

1. Spread with a spreader onto Hv-Cab solid agar plates (see below for recipe) 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 45°C 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 plates²
 - ii. Transfer ~22 mL Hv-Cab per 96-well plate into reservoirs
3. Using a multi-channel pipette, add 200 μ L of filter-sterilized Hv-Cab into each well
4. Lightly tap a sterile toothpick onto the top of a single colony and inoculate each well³
 - 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 to avoid evaporation from the wells
6. Incubate 96-well plate at 45°C overnight
 - i. Use a shaking microplate reader (BioTek Epoch 2) – settings:
 - a. Temperature 45°C, Gradient 2
 - b. Double orbital, continuous shake
 - c. Optional: Read absorbance OD₆₀₀ 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 (250 rpm), placed inside a sealed Tupperware box to prevent evaporation. This setup is particularly useful when running multiple plates simultaneously. At the final timepoint, measure OD₆₀₀ values using the microplate reader for use in subsequent steps⁴

B. Glycerol stock of the arrayed tn-library

1. Remove Breathe-Easy breathable membrane
2. Pipette 75 μ L culture from each well into prepared 96-well plate with 25 μ L glycerol solution (see below for recipe) and mix by pipetting up and down
3. Store glycerol stocks at -80°C 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.
 - i. For determining the ideal cell concentration in the 96-well plate for use in the Guava easyCyte instrument, OD₆₀₀ 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).
 - ii. Dilute with Hv-Cab to achieve OD₆₀₀ reading of wells to be between 0.1 - 0.15, which corresponds to an optimal cell concentration for the Guava easyCyte instrument.
3. Run the InCyte program to collect cell samples
 - i. Edit worklist as follows:
 - a. Check “Mixing Required”
 - b. Set Concentration Warning Limit (cells/ μ L) 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 the Forward Scatter (FSC-HLog) versus Side Scatter (SSC-HLog)⁵, 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 compares the FSC-SSC plots between experimental wells and wells containing medium control (no cells), and manually gates for data points that are distinct from the control; this represents the cellular populations in the experimental wells. Then, averaging the Forward Scatter HLog values makes an x-coordinate and averaging the Side Scatter HLog makes a y-coordinate, allowing for an x-y coordinate plot of all the wells in one experiment (Fig. 1). 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.1664897](https://zenodo.org/record/1664897)

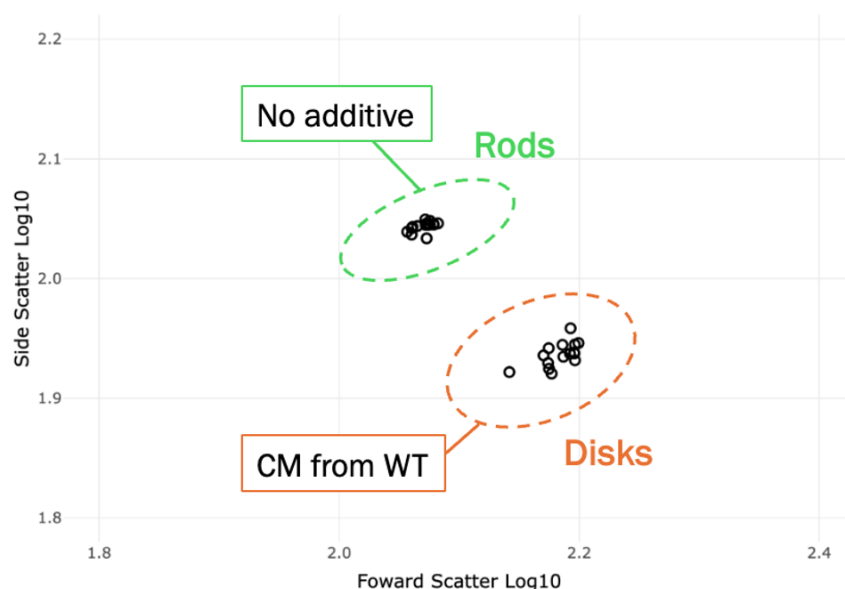


Figure 1. Averages of forward scatter (FSC) and side scatter (SSC) values from each well plotted on $\log_{10}$ scale. Wells had wild-type *H. volcanii* cells grown to early log. Plot indicates wells with either no additive or conditioned medium (CM) from wild type (WT) at 5% total volume. Cells in “No additive” wells were rods while cells in “CM from WT” wells were disks.

Recipes

500 mL of Hv-Cab medium

1. 200 mL MilliQ 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 solid agar plates: 7.5 g Difco bacteriological agar (BD)
6. Autoclave, cool to 65C
7. 1.5 mL CaCl₂ (1 M), sterile filtered
8. 450 µL solution of thiamine (0.88 mg/mL) & biotin (0.11 mg/mL), sterile filtered
9. 5 mL of trace element solution (de Silva et al., 2021, sterile filtered)
10. any additional additives required by the specific *H. volcanii* strain (Allers et al., 2010, 2004).

150 mL salt water glycerol solution

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 forms 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, the forward and side scatter analysis of control samples in wells from the outer circumference is indistinguishable from that of inner wells.
- ⁴ Continuous OD₆₀₀ readings are useful for tracking the growth of the tn-mutants, since some may have growth defects. Final OD₆₀₀ 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 FSC-HLog and SSC-HLog values are depicted on the software as showing the log value of the pulse signal height. These parameters can be changed in the program.

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 *H. volcanii* autofluorescence was attempted, but *H. volcanii* autofluorescence was not distinguishable from background signal

Competing interests

The authors declare that they have no conflict of interest

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