ImageStream® Bacteria Live/Dead Protocol

Samples: (~1x10^8 bacteria/ml) per sample

Bacteria	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5
Live:Dead	100% to 0%	75% to 25%	50% to 50%	25% to 75%	0% to100%
Volume	100ulL:0ulD	75ulL:25ulD	50ulL:50ulD	25ulL:75ulD	0ulL:100ulD

Materials

1.) **0.85% NaCl** solution in dH2O (Sigma #)
2.) **LB-Media** (Sigma #)
3.) **70% Isopropanol** (Sigma #)
4.) **Propidium Iodide** 20mM Stock (Invitrogen #)
5.) **E. Coli** starter Culture (Invitrogen #)

Bacteria Preparation

1.) Add 100ul LB media to sterile erlenmyer flask using sterile technique.
2.) Innoculate with a single colony from E. Coli starter culture.
3.) Place on shaker and incubate room temperature overnight.

Live, Dead Cell Preparation

1.) To 2x 1.5ml eppendorph tubes add 1ml of growing culture.
2.) Centrifuge 10,000rpm for 5min.
3.) Wash 1x in 1ml 0.85% NaCl buffer.
4.) Centrifuge 10,000rpm for 5min,
    a. To one tube resuspend in 0.85%NaCl buffer (this is the live sample)
    b. To the second: disrupt the pellet by vigorous mixing and resuspend in 100ul of 0.85% NaCl mixing thoroughly to prevent clumps. Next add 1ml of 70% Isopropanol and vortex vigorously (this is the dead sample).
5.) Incubate 30min at room temperature mixing every 15min.
6.) Centrifuge both samples 10,000rpm for 5min, and resuspend in 1ml 0.85% NaCl.

Bacteria Staining Protocol

1.) Prepare 5x 1.5ml eppendorf tubes with 890ul of 0.85%NaCl.
2.) Add 10ul of 20mM (or equivalent) PI.
3.) Mix each according to the dilution factors listed in the table below:

Bacteria	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5
Live:Dead	100% to 0%	75% to 25%	50% to 50%	25% to 75%	0% to100%
0.85% NaCl	890ul	890ul	890ul	890ul	890ul
20mM PI	10ul	10ul	10ul	10ul	10ul
Volume	100ulL:0ulD	75ulL:25ulD	50ulL:50ulD	25ulL:75ulD	0ulL:100ulD
Live:Dead Ratio					

4.) Incubate for 15min in the dark at room temperature.
5.) Vortex vigorously and aliquot 100ul into a 1.5ml eppendorph tube for running on ImageStream.
6.) Dilute 10ul of the 100% Live and 100% Dead samples 1 to 10 with 0.85%NaCl for counting on a hemocytometer.
7.) Analyze data in IDEAS and generate a live:dead ratio plot in excel.

ImageStream® Bacteria Live/Dead Protocol

Instrument Settings

Load INSPIRE template named “Bacteria PI”. To have the fluidics changes load properly a sample must be loaded and running on the system, otherwise an error is thrown and the fluidics portion is entered manually. Key changes to the INSPIRE template are listed below.

1.) Turn off un-used channels (collect channels 1, 4, and 6 only for PI).
2.) Magnification is 40x.
3.) 488 laser power to 100mw (or a level that maximizes PI fluorescence without saturation).
4.) 785 laser power to 70mw.
5.) 4um core diameter (runs 1ul every ~5min)
6.) 10% Beads.
7.) 60mm/second core velocity.
8.) Turn off auto bead detect.
9.) Set squelch to -10.
10.) Remove all cell classifiers to collect everything.
11.) Collect 50,000 total events to get more than 10,000 bacteria.

Counting Bacteria With the Hemocytometer

1.) To 90ul of 0.85% NaCl add 10ul of bacteria in single cell suspension.
2.) Vortex vigoursly
3.) Place 10ul under hemocytometer cover slip.
4.) Let the sample settle for 20min.
5.) Use 40x and count bacteria being aware that some cells may be out of the normal plane of focus and may require some focus adjustment.
6.) Count 10 of the smallest squares (these are 1:16th of the 1x10^4th square).
7.) Average all 10 counts.
8.) Calculate the final concentration:

Concentration = Average x dilution x factor x 1x10^4th cells per ml
Concentration = [Ave x 10 x 16 x 1x10^4th] cells/ml