Dilution-to-Extinction Experiment Protocol V.2

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VERSION 2

JUL 10, 2023

OPEN ACCESS

DOI: dx.doi.org/10.17504/protocols.io.36wgq7d2ovk5/v2

Protocol Citation: Shelby J Barnes, Michael Henson, Celeste Lanclos, Jordan Coelho, Cameron Thrash 2023. Dilution-to-Extinction Experiment Protocol. **protocols.io** https://dx.doi.org/10.17504/protocols.io.36wgq7d2ovk5/v2 Version created by Shelby J Barnes

Protocol status: Working
We use this protocol and it's working

Created: May 30, 2023

Last Modified: Jul 10, 2023

PROTOCOL integer ID: 82655

ABSTRACT

Dilution-to-Extinction Experiment Protocol

MATERIALS

Medium Preparation

Defined Media

Acid-washed and autoclaved Pyrex screw-top bottles (Corning)
See SJB Artificial Media Protocol DOI:
dx.doi.org/10.17504/protocols.io.rm7vzy615lx1/v1

Collection and Preparation

10% HCl in a large volume (~25 L) for acid washing of collection carboys, medium preparation bottles, and incubation PTFE plates and flasks.

2.7um Whatman GF/D glass fiber filters (25 mm) and housings (see Note 1) OR Pall PES filters Supor (25mm) and housings or vacuum filtration.

Inoculum Sample Enumeration

BD Accuri C6 Plus Flow Cytometer systems and software from BD Biosciences (San Jose, California, United States); with associated computer/reagents(BD).

SYBR Green (Life Technologies) diluted to 100x (from 10,000x stock) in TE for DNA staining.

Corning non-sterile, round-bottom 96-well assay plates

Repeater pipette (Gilson) with 2 µL capability for rapid dispensation of SYBR Green into 96-well plates.

Sterile cabinet (for dark incubation)

Medium Inoculation, Distribution and Incubation

PTFE 2.1 mL, 96-well plates (Radleys, Essex) for incubation of the cultivation experiment.
(Acid-washed and autoclaved)

PTFE-coated silicon 96-well plate mats (Thermo Scientific) for sealing the cultivation plates to prevent contamination and evaporation.
(Acid-washed and autoclaved)

PIPETMAN L Multichannel P8x1200L, 100-1200 µL (Gilson)

Laminar flow hood or biosafety cabinet for inoculation/transfer with reduced contamination risk (see Note 5). The hood is wiped down with 70% EtOH before and after use to improve sterility. UV light sterilization in between uses, if available, can be added according to the manufacturer's instructions.

Large Format Enumeration

Incubator capable of maintaining in situ temperatures.

PIPETMAN L Multichannel P8x200L, 100-1200 µL (Gilson)
Multichannel pipette (Gilson) for efficient transfer of 200 µL volumes from 96-well incubation plates to 96-well counting plates.

Repeater pipette (Gilson) with 2 µL capability for rapid dispensation of SYBR Green into 96-well plates.

Sterile Cabinet (for dark incubation)

Culture Filtering and DNA Extraction

Supor PES Membrane Disc Filters

GenElute™ Bacterial Genomic DNA Kit

PCR Preparation and Purification

PCR Prep

Taq DNA Polymerase, recombinant **Thermo Fisher Catalog #10342020**
Invitrogen

Integrated DNA Technologies 16s rRNA Primer 27F (aka S-D-Bact-0008-d-S-20): 5'-AGAGTTTGATCMTGGCTCAG

Integrated DNA Technologies 16s rRNA Primer 1492R (aka S-*-Univ-1492-a-A-21): 5'-GGTTACCTTGTTACGACTT

MilliporeSigma, dNTP Mix, 10mM, 0.2 mL, 71004-3

Molecular Biology Water, 500mL, VWRL0201-0500

PCR Master Mix (per sample)
(for Invitrogen or OneTaq)
Buffer: 5uL
Forward Primer (10uM) : 5uL
Reverse Primer (10uM): 5uL
MgCl2: 1.5uL
dNTP: 1uL
taq Polymerase: 0.2uL

GoTaq PCR Master Mix
GoTaq Green Master Mix **Promega Catalog #M7122**

Gel

1.5% agarose and 1xTAE Buffer solution (50mL makes one gel)
Microwave in 30 second intervals until all agarose is dissolved
Swirl intermittently

PCR Product Purification

GeneJET PCR Purification Kit **Thermo Fisher Catalog #K0702**

Ampure XP beads **Beckman Coulter Catalog #A63881**

Medium Preparation

1 **Defined Medium**

1.1 Can be prepared in advance according to SJB Artificial Media Protocol dx.doi.org/10.17504/protocols.io.rm7vzy615lx1/v1 or using medium of choice.

Collection and Filtration

2 **Culturing Hardware Preparation**

2.1 All culturing hardware (carboys, flasks, glassware, PTFE plates, sealing mats, stir bars) are washed first with dishwasher or lab-grade detergent; rinsed with hot tap water, cold tap water, and then DI water; and let dry.

2.2 They are then soaked in a 10% HCl acid bath overnight, rinsed six times with DI water and MilliQ water, lids applied or wrapped in foil, and autoclaved prior to storage or use.

3 Collect samples at any source of interest with sterile, acid-washed carboys appropriate to the

required volumes, taking as many precautions as practical to avoid contamination.

3.1 Collectors should wear gloves.

3.2 Samples should be transported back to the laboratory with the shortest delay possible to reduce potential bottle effects that can alter the community prior to inoculation.

4 Filter sample(s) prior to enumeration using a (2.7um) Whatman GF/D glass fiber filter to exclude large aggregates/particles and increase the proportion of planktonic cells in the seawater.

Inoculum Sample Enumeration

5 **Sample Preparation**

5.1 Enumerate DTE inoculum sample prior to dilution with artificial medium and PTFE plate inoculation.

Total cells μL^{-1} needs to be between 1,000 and 5*10^6 for a given sample to stay within the accurate counting range of the BDAccuri. If samples are higher, make appropriate dilutions in sterile medium.

5.2 Add two artificial medium controls to count (one stained and one unstained).

Controls. Unstained controls (artificial medium) are used to set a green fluorescence threshold for exclusion of autofluorescence and system noise, respectively. Stained controls (one stained and one unstained artificial medium sample) are used for gating results around standard noise of the artificial media and identify the potential area for positive cell growth. Control samples are counted along with the experimental samples.

5.3 Transfer 198 µL of each sample, in triplicate, into a round- bottom 96-well plate.

5.4 Transfer 400 µL of each of the desired controls into a 0.5 mL microcentrifuge tube and add 198uL of each to 96-well plate. Typically, controls consist of stained and unstained medium and stained and unstained E. coli.

5.5 Stain samples and one control with 1x final concentration SYBR Green (2 µL 100x SYBR Green added to 198 µL sample).

5.6 Incubate samples for 30 minutes in the dark.

6 Run Settings and Worklist Setup

6.1 The following is used with BD Accuri C6 Plus Software for the BD Accuri C6 Plus Flow Cytometer systems from BD Biosciences (San Jose, California, United States).

6.2 Adjust Thresholds. Run control samples in manual collect tab using no thresholds and the general run settings: "Run with Limits" , volume: 10uL , flow rate: medium. The green fluorescence (FL1 or FITC) and forward scatter (FSC)/ side scatter (SSC) thresholds are set to exclude noise based on the unstained controls (Fig. 2). These settings are used for counting the samples in the auto collect tab.

6.3 Worklist need to be tailored by application and stain used. General run settings (w/SYBR Green): "Run with Limits"; volume: 10uL; flow rate: medium; Thresholds - FSC or SSC: 10, FL1 or FITC: 100 (thresholds may vary depending on media type and culture signature).

6.4 Plots. Forward scatter (log scale) vs. green fluorescence (log scale), side scatter (log scale) vs. green fluorescence (log scale), and green fluorescence (log scale) vs. yellow fluorescence

(log scale) are used to evaluate the counts.

6.5 SIP Settings

SIP Rinse: 1 cycle per sample
SIP Clean after completed run

7 **Analysis and Gating**

7.1 Cell concentration is calculated using a gated region, which is selected based on the side scatter vs. green fluorescence plot. Forward scatter vs. green fluorescence can also be used but may reduce resolution depending on the sample.

7.2 Initially, a global gate is applied to plots using the stained artificial medium such that the gate excludes the stained medium signal.

7.3 However, especially in the case of isolates, but sometimes with whole seawater, individual samples must have the gate customized to account for variations in signal. In practice, this will be empirically defined, as different media will give different amounts of background noise.

Medium Inoculation

8 All steps except incubation are performed inside a biosafety cabinet or laminar flow hood.

8.1 Based on enumeration (Sect. above), cells are diluted in medium such that they can be

inoculated into deep 96-well plates using minimal volume. The average number of estimated cells per well should be 1–3 after inoculation.

8.2 Example. With a medium plus inoculum final volume of 900mL, an inoculum cell density of $5*10^3$ cells/mL and final concentration of 1-3 cells/1.7 mL after distribution ; the inoculation volume should be approximately 0.1062 mL or 106 µL.

(5*10^3 cells/mL)V1 = (1 cell/1.7mL)(900 mL)
V1 = 0.1062mL

Inoculated Medium Distribution and Incubation

9 Distribute with a P1200 multichannel pipette or the Eppendorf epMotion 5075 automated liquid handling system.

10 **Manual Distribution**

Inoculated medium is distributed into sterile, acid-washed deep 96-well plates (2.1 mL PTFE) at 1.7 mL per well in a biosafety cabinet using a P100-1200 µL multichannel pipette.

10.1 Swirl inoculated medium thoroughly and aliquot into sterile liquid reservoir for ease of distribution.

10.2 Top full plates with sterile silicon lids. Incubate plates at room temperature (~25C°) for 2 - 4 weeks.

11 **Automated Distribution**

Eppendorf epMotion 5075 can be set up following the manufacturer's instructions.

11.1 Distribution and incubation protocol is consistent between distribution types.

Large Format Enumeration

12 Carried out in the same manner as enumeration for medium inoculation with the following adaptations for high-throughput counting with 96-well plates.

12.1 Aliquoting samples for enumeration can be done with the Eppendorf epMotion 5075 automated liquid handling system or P200 multichannel pipette.

12.2 In a biosafety cabinet or laminar flow hood, using a multichannel pipette, transfer 198 µL from the deep 96-well incubation plates to 96-well round-bottom counting plates.

Transferring Plate Isolates and Culture Maintenance

13 Wells with cell densities of 10^4 and higher are considered positive and should be transferred.

13.1 The four corners of each plate containing a negative control should have very little signature apart from the media signal and a cell density of 10^3 or less. If a biotic signature is seen in a negative control, consider the plate contaminated and should not be used.

14 Transfer 200uL of each positive well to a 250mL flask filled with 50mL of medium of choice.
- transferring inoculum and culture volume can vary with preferred starting culture cell density.

14.1 Prepare medium before day of transferring.

Identification through 16S rRNA PCR Amplification

15 Either extracted genomic DNA or cells from maintained cultures can be used as the template for the PCR section below.
Step 15 includes a Step case.
DNA Extraction Pipeline
Direct PCR Pipeline

step case

DNA Extraction Pipeline

This pipeline is for using gDNA as the PCR template.

16 **Culture Filtering**

16.1 Transfer cultures prior to filtering. When cultures are at late log phase create a new transfer group using 200uL of culture into 50mL of fresh medium. (volumes can vary depending on preference)

17 Once transferred, filter 40-45mL of late log phase culture using a 0.1-0.2 Supor PES filter. Filter either by hand or with vacuum filtration.

17.1 Store filters with cells in the -20°C until ready for DNA extraction.

18 **DNA Extraction**

18.1 Extract DNA from filtered cells using the GenElute™ Bacterial Genomic DNA Kit or Phenol Chloroform Extraction Protocol dx.doi.org/10.17504/protocols.io.b5iiq4ce
Quantify the gDNA concentration after extraction using the Qubit Fluorometer.

18.2 Follow GenElute kit protocol.

16S rRNA PCR Preparation and Purification

19 Once DNA is extracted, prepare the PCR master mix for the number of samples

Volume of gDNA will vary depending on concentration.
Can use taq Polymerase kit (Invitrogen, OneTaq, etc) and dNTP's.
All PCR's require a positive and negative control to verify the run as successful. The positive control consists of gDNA of an already verified bacterial strain and the negative control is a sample with the gDNA replaced with molecular H2O.

19.1 To each PCR tube add(50uL total volume):
Master Mix: 15uL
gDNA: 3uL
H20: 32uL

19.2 Run PCR's using the following Thermocycler conditions:

Lid Temp: 105°C
Sample Volume: 50uL
1. 94°C, 3:00 mins
2. 94°C, 0:30 mins
3. 50.8°C, 0:30 mins
4. 72°C, 2:00
5. GoTo step 2, 34x
6. 72°C, 10:00
7. 4°C, infinite hold

20 Store PCR products in 4°C or continue to gel and purification.

21 Run a 1.5% agarose gel to verify success of PCR amplification at the correct band length (usually 1500 bp).

Gel reagents in Materials section of protocol.

21.1

Set up gel casting tray in stabilizer and use leveler.
Add a 50mL solution of 1.5% agarose and 1x TAE Buffer to the designated gel pyrex bottle.
Microwave in 30 second intervals until all agarose is dissolved (swirl intermittently).
Add 30ul of 100x SYBR Green to molten agarose solution, swirl and pore into gel mold.

Remove any bubbles with a pipette tip by either sucking up bubble or moving it to the side. Place gel mold in a dark place(SYBR Green is light sensitive) for at least an hour for gel to harden.

22 Purify PCR product with either GeneJet PCR purification kit or Ampure XP beads.

22.1 Follow GeneJet PCR purification kit protocol.
OR
Follow Ampure XP bead protocol (lab protocol).

Sanger Sequencing Preparation

23 Quantify purified PCR product using Qubit assay (following kit instructions) and set up sequencing samples as follows:

23.1 For each PCR product there will be two sequencing samples made (one for 16S rRNA 27F Forward and one for 1492R Reverse sequences).

23.2 Sequencing Sample Setup
(follow sequencing service guidelines)

23.3 Once samples are set up, submit sequencing request through a sequencing facility (Genewiz, Laragen, etc).