Treatments for the Preservation of Seawater Samples

Tucson Marine Phage Lab

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Purpose: This protocol consists of several methods for the preservation of seawater samples to later perform various techniques in the lab. It is meant as a sampling treatment in the field and is written as comprehensive; however, depending on needs and scientific questions, any or all of the methods may be used.

Materials: Listed here are the consumables needed for all methods. Please check individual methods for specific material needs and quantities.

- Seawater
- 10μm nylon mesh or Nitex screen
- 200μm nylon mesh or Nitex screen
- 0.2μm Nucleopore™ filter (25mm diameter) – Whatman 110606
- 0.2μm polyethersulfone (PES) filter
- 0.22μm Sterivex™ filters – Millipore
- 0.02μm Anotop® syringe filters (25mm diameter) – Whatman 6809-2102
- 0.45μm cellulose nitrate support filter (25mm diameter)
- 0.45μm cellulose nitrate support filter (47mm diameter)
- 0.2μm polycarbonate membrane filter (25mm diameter) – Millipore GTTP 025 00
- 0.2μm polycarbonate membrane filter (47mm diameter) – Millipore GTTP 047 00
- Gluteraldehyde, 25%
- DMSO
- Chloroform
- Betaine anhydrous
- Formaldehyde (fresh, unopened)
- Milli Q water
- TM buffer (1M Tris pH8, 0.45M NaCl, 0.1M $MgCl_2$)
- TE buffer (10mM Tris pH8, 1mM EDTA)
- 50ml centrifuge tubes
- 1.2ml cryovials
- 5ml cryovials
- 1.5ml screw-cap tubes with o-ring gasket
- Kimwipes
- Parafilm
- Ziplock bags
- Petri dishes (60mm) or PetriSlides™ Dish – Millipore PDMA04700
- Tube storage boxes

Procedures:

***Pre-filter Seawater*:** This step is optional depending on sampling conditions and scientific questions to be answered.

1) Filter the seawater using a 10μm or 200μm nylon mesh or Nitex filter. Amounts needed for each protocol given below.

Flow Cytometry (FCM): Samples are used for counting bacteria and for sorting different groups of microbes (eg, sorting synechococcus and prochlorococcus and non-fluorescent bacteria). Glutaraldehyde fixes microbes and renders them unculturable whereas microbes preserved in DMSO and Betaine can usually be cultured after thawing and rinsing. Betaine was developed for single cell sorting and may be a better preservative than DMSO.

1) *Gluteraldehyde, 25%*:
   - Requires 5ml of the 10μm or 200μm seawater filtrate.
   a) Dispense 1ml filtrate into 1.2ml cryovial. Make 3 replicates.
   b) Add 5μl of 25% glutaraldehyde to each (final 0.125%). Invert to mix.
   c) Incubate dark for 15 min. Flash freeze in liquid nitrogen (can use nylon sock to submerge and retrieve samples). Store at -80ºC.
2) *DMSO:*
   - Requires 5ml of the 10μm or 200μm seawater filtrate.
   a) Dispense 1ml filtrate into 1.2ml cryovial. Make 5 replicates.
   b) Add 70μl of DMSO to each (final 7%). Invert to mix.
   c) Flash freeze in liquid nitrogen (can use nylon sock to submerge and retrieve samples). Store at -80ºC.
3) *Betaine:* (method from R. Stepanauskas, 10/17/09)
   - Requires 5ml of unfiltered or 200μm filtered seawater.
   a) Prepare betaine stock, 48%: Dissolve 48g betaine anhydrous in 80 ml MilliQ water, bring volume up to 100ml. Filter with 0.2μm PES filter. Store refrigerated. Re-filter every month.
   b) Dispense 1ml unfiltered (or 200μm filtered) seawater into 1.2ml cryovial. Make 5 replicates.
   c) Add 143μl prepared betaine stock. Invert to mix.
   d) Flash freeze in liquid nitrogen (can use nylon sock to submerge and retrieve samples). Store at -80ºC.
   e) Betaine method notes:
      - The method was found to work well on diverse marine and freshwater samples: both the numbers and the optical properties of prokaryote cells were well preserved and the downstream single cell FACS-MDA-16SPCR success rate was slightly better than for fresh samples.
      - The method was found to work poorly on hypersaline samples (~350 psu): almost 50% cells were lost after the cryopreservation.
      - The method was not tested for the preservation of protists.

SYBR staining: Samples are used for counting bacteria (unfiltered or pre-filtered seawater) and viruses (unfiltered, pre-filtered or 0.2um filtered seawater) microscopically after staining with SYBR-Gold (or SYBR-Green).

- Requires 12ml of unfiltered or 10μm or 200μm filtered seawater. If only viruses are to be examined, the pre-filtrate should be filtered through a 0.2µm PES filter to eliminate bacteria and other protists.

1) Dispense 3.92ml seawater into 5ml cryovial. Make 3 replicates.
2) Add 80μl 25% glutaraldehyde to each. Invert to mix.
3) Flash freeze in liquid nitrogen (can use nylon sock to submerge and retrieve samples). Store at -80ºC.

Transmission Electron Microscopy (TEM): Samples are used for determining frequency of visibly infected cells (FVIC) and determining types of microbes or viruses present, and can be quantitative.

- Requires 12ml of unfiltered or 10μm or 200μm filtered seawater. If only viruses are to be examined, the pre-filtrate should be filtered through a 0.2μm PES filter to eliminate bacteria and other protists.

1) Dispense 3.92ml seawater into 5ml cryovial. Make 3 replicates.
2) Add 80μl 25% glutaraldehyde to each. Invert to mix.
3) Flash freeze in liquid nitrogen (can use nylon sock to submerge and retrieve samples). Store at -80ºC.

Quantitative PCR (qPCR): Samples are used for quantitative PCR of microbes. They can also be used for quantitative PCR of viruses.

- Requires 150ml (coastal) or 300ml (open-ocean) filtrate for 3 qPCR filters.

1) Filter 50ml (coastal) or 100ml (open ocean) through 0.2μm nucleopore filter (25mm diameter). Note: Can save filtrates for Viral qPCR and Culturing Viruses preservation methods.
2) Pass 3ml TE buffer through filter to rinse.
3) Place filter membrane into 1.5ml screw-cap tubes with o-ring gasket.
4) Repeat to prepare 3 filters per sample.
5) Store at -80ºC.

Viral qPCR: Samples are used for quantitative PCR of viruses.

- If 0.2μm filtrate from qPCR is not available, filter 330ml of 10μm or 200μm filtrate through 0.22μm Sterivex filters.

1) Filter 100ml 0.2μm filtrate though 0.02μm Anotop syringe filters (25mm diameter) into cleaned container.
2) Pass 10ml TM buffer through filter to rinse.
3) Label syringe filter (date, location & depth).
4) Wrap filter in parafilm (using a 4-square length), wrap in aluminum foil, label outside (date, location & depth) and put into ziploc bag with other filters from that depth profile.
5) Repeat to prepare 3 syringe filters per sample.
6) Store at -80ºC.

Culturing Viruses: Samples are used for cultivating viruses by solid or liquid plaque assay on susceptible hosts. Samples can also be used for viral tagging (using flow cytometry) and most probable number (MPN) assays.

- If 0.2μm filtrate from qPCR is not available, filter 165ml of 10μm or 200μm filtrate through 0.22μm Sterivex filters.

1) Dispense 50ml 0.22μm filtrate into sterile 50ml centrifuge tubes. Make 3 replicates.
2) Add 20μl chloroform (optional to keep down bacterial growth). Invert to mix.
3) Store at 4ºC.

Fluorescence in situ hybridization (FISH): Samples are used with fluorescent probes to determine what populations of bacteria or other microbes are present. The filters may also be used to simply count the number of microbes in a sample.

- Requires varying amounts of 10μm or 200μm filtrate (up to 11.5L for open ocean depth profile) as shown in Table I.

Table I. Samples from open ocean depth profile for FISH. Goal cell concentrations of about 10^6/ml.

Depth	Volume seawater	Volume 37% formaldehyde	# of Reps
10m	150ml	8ml	2
500m	1000ml	54ml	2
1000m	2000ml	108ml	2
2000m	2600ml	140ml	2
Total with reps	11.5 L	620ml	8 filters

1) Add formaldehyde at a final concentration of 1-2% to filtrate. Fix for 12-24 hr at 4°C.
2) *Cell counts* (not needed if using a different counting method, e.g. flow cytometry):
    a) Setup frittered glass support (25mm diameter) in filtration manifold.
    b) Place a drop of Milli Q water (~250-500µl) on support, float 0.45µm cellulose nitrate support filter (25mm diameter) on top, turn on vacuum (~ 5 in of Hg) to lay filter flat with no air bubbles.
    c) Turn off vacuum and release pressure from chamber.
    d) Add another drop of Milli Q to support filter, float 0.2µm polycarbonate membrane filter (25mm diameter) on support filter (shiny side facing up!). Save filter separator sheets for storage later.
    e) Turn on vacuum (~ 5 in of Hg) to lay filter flat with no air bubbles. Leave vacuum on.
    f) Place filter tower on membrane and clamp.
    g) Filter 1ml of fixed sample by applying gentle vacuum (~5 inch Hg); filter 2 ml of the fixed sample onto a second filter. The support filter may be utilized for both samples. Note: Pipette sample directly onto membrane in a timely manner to achieve even spread.
    h) Remove filter from filter holder and put it on Kimwipes to dry. Cover, e.g. with the lid of a cryo box or a Petri dish. Allow to air-dry.
    i) Label membrane filter with pencil; place membrane filter between separator sheets (will prevent the membrane filters from sticking to each other or to the Petri dish). Seal Petri dish with parafilm, prevent dish from opening with tape, and put Petri dishes into a Ziploc bag
    j) Store at -20°C until processing. Filters can be stored frozen for several months without apparent loss of hybridization signal.
3) *Samples for FISH:*
    a) Setup frittered glass (47mm diameter) support in filtration manifold.
    b) Place a drop of Milli Q water (~1ml) on support, float 0.45µm cellulose nitrate support filter (47mm diameter) on top, turn on vacuum (~ 5 in of Hg) to lay filter flat with no air bubbles.
    c) Turn off vacuum and release pressure from chamber.
    d) Add another drop of Milli Q to support filter, float 0.2µm polycarbonate membrane filter (47mm diameter) on support filter (shiny side facing up!). Save filter separator sheets for storage later.
    e) Turn on vacuum (~ 5 in of Hg) to lay filter flat with no air bubbles. Leave vacuum on.
    f) Place filter tower on membrane and clamp.
    g) Filter appropriate volume (see Table I) of fixed sample by applying gentle vacuum (~5 inch Hg). The support filter may be utilized for several samples. Note: Pipette sample directly onto membrane in a timely manner to achieve even spread.

h) After complete sample filtration, wash filter and tower with 20-30 ml of sterile H_2O; remove H2O by vacuum.

i) Remove filter from filter holder and put it on Kimwipes to dry. Cover, e.g. with the lid of a cryo box or a Petri dish. Allow to air-dry.

j) Label membrane filter with pencil; place membrane filter between separator sheets (will prevent the membrane filters from sticking to each other or to the Petri dish). Seal Petri dish with parafilm, prevent dish from opening with tape, and put Petri dishes into a Ziploc bag

• Note: If available, use Petrislides™ Dish. These seal better than Petri dishes and are easier to store.

h) Store at -20°C until processing. Filters can be stored frozen for several months without apparent loss of hybridization signal.

Protocol Revision History:

Version	Revision	Date
4	SYBR and TEM sampling methods updated	06 January 2014
5	Added in betaine single cell sampling method	09 January 2014
6	Reformatting for a cleaner read	30 January 2014