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U. S. DEPARTMENT OF COMMERCE
National Oceanic and Atmospheric Administration
National Marine Fisheries Service
Southeast Fisheries Science Center

75 Virginia Beach Drive
Miami, FL 33149

Cruise Report

Date Submitted: 11/17/2023

Platform: NOAA Ship GORDON GUNTER

Cruise Number: GU23-02

Project Title: Marine Mammal

Cruise Dates: 05/04/2023 - 06/04/2023

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Gulf of Mexico *Stenella attenuata* Stock Structure (GSASS) Research Cruise Report

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November 2023

CRUISE REPORT

NOAA Ship *Gordon Gunter* Cruise GU23-02

04 May – 04 June 2023

GSASS Research Cruise

U.S Department of Commerce
National Oceanic and Atmospheric Administration
National Marine Fisheries Service
Southeast Fisheries Science Center
75 Virginia Beach Drive
Miami, Florida 33149

November 2023

On 12 May 2016, an estimated 1,926 barrels of crude oil from Green Canyon Block 248 were discharged into the northern Gulf of Mexico (GoMx) off Louisiana. The Natural Resources Damage Assessment (NRDA) for the Green Canyon oil spill documented pantropical spotted dolphins (*Stenella attenuata*) swimming in oil. *S. attenuata* is the most abundant cetacean species in oceanic waters of the U.S. GoMx with an estimated abundance of over 50,000 dolphins. These dolphins occur in single-species groups as large as 650 dolphins with an average of about 70 dolphins. *S. attenuata* occur throughout all U.S. GoMx oceanic waters with densities that are highest in the eastern GoMx and that decline to the west. This species is currently managed as a single GoMx-wide stock, but no efforts to examine its stock structure in the GoMx have ever been conducted. Therefore, as part of the Green Canyon NRDA settlement, a genetic assessment of *S. attenuata* in the GoMx was planned. *S. attenuata* skin biopsy samples have been collected on SEFSC marine mammal surveys since the early 1990s. The primary goal of this survey was to collect new skin biopsy samples that would reflect the current stock structure and also fill spatial gaps in previous sample collections. A large vessel survey aboard NOAA Ship *Gordon Gunter* was conducted to collect samples from *S. attenuata* in the eastern and central GoMx.

The cruise was segmented into two legs, totaling 29 planned sea-days (staffing – Table 1):

Leg	Date	Location	Days at Sea
1	DEP: 04 May 2023	Pascagoula, MS	14
	ARR: 17 May 2023	Pascagoula, MS	
2	DEP: 21 May 2023	Pascagoula, MS	15
	ARR: 04 June 2023	Pascagoula, MS	

Due to an Able Seaman (AB) shortage to staff NOAA Ship *Gordon Gunter*, eight sea days were lost (4 on Leg 1, 4 on Leg 2) when AB positions could not be filled. Actual departure dates and accomplished days at sea on each leg are shown in Table 2.

Cruise objectives

The specific objectives of this survey were to:

1. Conduct a one-team visual line transect survey to locate groups of *S. attenuata* in U.S. GoMx waters
2. Collect tissue samples for genetic and other analyses from *S. attenuata*
3. Collect water samples for environmental DNA (eDNA) from *S. attenuata*

Visual Survey Design

The objectives of the survey design were to cover distinct regions of the GoMx, maximize the encounter of *S. attenuata,* and sample across the geographic range within each region. Based upon *S. attenuata* sightings from previous surveys and the available samples from prior years, two operational areas were defined to focus biopsy collection efforts (Figure 1). These areas

were intended to spatially separate the sample collection into “eastern” and “central” GoMx areas with the demarcation between these aligning roughly with the Mississippi River delta. This is an important biogeographic break in the GoMx and the hypothesis we are testing is that population structure in *S. attenuata* is aligned with this break. Vessel tracklines were planned within each operational area along an evenly spaced, zig-zag design to provide even coverage of each region. The tracklines generally covered waters deeper than 1,000m and corresponded to areas with high predicted density of *S. attenuata*. The actual tracklines covered were adjusted during the survey as sea days were lost and in response to environmental and weather conditions. The accomplished survey effort is shown in Figure 1.

Visual Survey Operations

Surveys were conducted by a team of three observers stationed on the vessel’s flying bridge (height above water = 13.9 m) and consisted of two observers using two pedestal-mounted 25x150 mm “bigeye” binoculars located on the port and starboard sides and a central observer/data recorder performing naked-eye observations.

Observers used the bigeye binoculars to determine the bearing and radial distance of observed cetacean groups (i.e., “sightings”). The location of groups sighted close to the ship without bigeye binoculars were estimated in degrees and meters. Marine mammal sightings were defined as systematic records of cetacean groups consisting of one or more individuals observed at the same location and time.

Visual survey effort commenced daily at approximately 0700 (CST) and ended at 1730 (CST) for depending on operational requirements and survey conditions. Survey speed was typically 18 km hr^{-1} (10 kt) but varied with ship traffic and sea conditions such as ocean currents. Data were recorded by the data recorder using a custom written visual data acquisition program (VisSurvey) installed on a networked laptop.

Observers scanned the water using the bigeye binoculars from 10° right and left of the ship’s bow to the beam (90° left or right depending on the side); i.e., the left observer scanned from 10° right to 90° left and the right observer scanned from 10° left to 90° right. Whenever an observer suspected or had in fact seen a marine mammal, a cue (marine mammal, splash, blow, etc.) was immediately entered in the data program. A cue is a time and location stamp in the database that captures the spatial and temporal data of a sighting. If the sighting was potentially of *S. attenuata*, the ship was diverted to close on the group to make an identification. Once an identification was confirmed, if the group was *S. attenuata*, it was approached to elicit bow riding behavior to collect biopsy samples from the ship’s bow. If it was not, the ship returned to the trackline. Except for diverting from the trackline to identify and biopsy *S. attenuata*, the survey was primarily conducted in “passing mode” whereby the ship maintained a steady course and speed along the trackline while the visual teams identify all sightings to species level if possible and count the number of individuals in the sighting. After sightings were identified to the lowest taxonomic level possible and group size enumerated, the sighting was entered in the visual data program by the data recorder. Group size estimates were recorded independently by each observer. Observers were instructed to only enter values

for sightings they observed entirely. Group size was counted as the minimum, maximum, and best number of animals for each sighting.

For each encounter, time, position, bearing and reticle, species, group size, behavior, and associated animals (e.g., seabirds, fish) were recorded. An attempt was made to photograph animals that closely approached the ship.

Based input from observers, basic survey parameters were automatically recorded by the survey program every minute and included the effort status and observer position. At the start of the survey day and at 20-minute time intervals thereafter, the survey program prompts observers for an update of the subjective sighting condition variables (e.g., glare, sea state, cloud cover, etc.).

Environmental Data

Environmental data including water temperature, salinity, and weather conditions (e.g., wind speed, wind direction), ship's position, and heading were continuously collected *in situ* via the ship's Scientific Computer System (SCS) and recorded in the visual marine mammal sighting database.

Marine mammal biopsy sampling and eDNA

Biopsy samples were collected from the bow of the ship with an air-rifle and a tethered dart. The dart was fitted with a 7x25mm sampling tip. Depending on the sample size, samples were subsampled for the following analyses: skin – genetics, microbiome swab, stable isotopes, transcriptomics, tissue culture; blubber – persistent organic pollutants, hormones. Each subsample was stored according to protocols (e.g., 20% DMSO/saturated NaCl, -80°C, -20°C). A maximum of two to four samples were collected per group, depending on group size. Water samples were also collected for eDNA. Biopsy and eDNA sample collection and processing protocols are provided in Appendix 1.

RESULTS

Visual Survey

During this cruise, 3144 km of trackline were visually surveyed (Table 2, Figure 1). Sighting conditions were fair to good throughout most of the survey, with sea states of 3-5 on most survey days. There were 90 marine mammal sightings from 10 confirmed species during the survey (Table 3). Twenty groups of *S. attenuata* were sighted. Additionally, a diverse suite of oceanic dolphin and small whale species were encountered including, Risso's dolphins (*Grampus griseus*), rough-toothed dolphins (*Steno bredanensis*), beaked whales (Ziphiidae), and shortfin pilot whales (*Globicephala* macrorhynchus.; Table 3, Figures 2). There were a total of 34 sperm whale (*Physeter macrocephalus*) sightings.

Marine mammal biopsy sampling and eDNA

A total of 33 *S. attenuata* biopsy samples for genetic analysis were collected (Figure 3). Subsamples for additional analyses are shown in Table 4. Two biopsy samples were also

collected from rough-toothed dolphins. To partially offset the days-at-sea lost during GSASS, the SEFSC was able to collect an additional 7 *S. attenuata* samples on a *Deepwater Horizon*-supported marine mammal abundance and distribution survey that immediately followed the GSASS survey (Figure 3). All of the biopsy samples are stored and archived at the SEFSC Marine Mammal Molecular Genetics Laboratory in Lafayette, Louisiana.

On two occasions, water samples filtered for eDNA analyses were collected from a small boat in areas where *S. attenuata* were present or shortly after they were present (Table 5).

Data Disposition

All data collected during GU23-02 including visual survey data and SCS data are archived and managed at the Southeast Fisheries Science Center (SEFSC) in Miami, Florida with backup copies at the SEFSC Pascagoula Laboratory in Mississippi. The data presented here are preliminary and subject to change as further auditing and analyses continue.

Permit and Funding Source

The Southeast Fisheries Science Center was authorized to conduct marine mammal research activities during the cruise under MMPA Research Permit No. 21938, issued to the SEFSC by the NMFS Office of Protected Resources. This study was funded in part with Shell Green Canyon Block 248 Oil Spill settlement funds.

Table 1. List of Participants

Name	Legs	Affiliation	Duty
Anthony Martinez	1, 2	SEFSC	Field Party Chief
Jesse Wicker	1, 2	CIMAS	Marine mammal observer
Matt Maiello	2	SEFSC	Marine mammal observer
Carrie Sinclair	1, 2	SEFSC	Marine mammal observer
Amy Brossard	1	CIMAS	Marine mammal observer
Nicole Vollmer	1, 2	CIMAS	Marine mammal observer
Mary Applegate	1, 2	CIMAS	Marine mammal observer
Heidi Malizia	1, 2	CIMAS	Marine mammal observer
Juan Carlos Salinas	1, 2	CIMAS	Marine mammal observer
Ellie Hartman	1	CIMAS	Marine mammal observer
Sascha Cushner	2	SEFSC	Marine mammal observer

Affiliations: SEFSC = NOAA Southeast Fisheries Science Center; CIMAS = Cooperative Institute for Marine and Atmospheric Studies

Table 2. Daily survey operations during GU23-02 including the visual effort, number of marine mammal (cetacean) sightings, the number of *S. attenuata* biopsies collected and the average Beaufort sea state (AB = Able Seaman).

Survey Leg	Date	Sea Day	Event	Effort (km)	Cetacean Sightings	S. attenuata Biopsies	Avg Sea State (OnEffort)
Leg 1	4-May	Lost	Sailing delayed, lack of AB				
	-May	Lost	Sailing delayed, lack of AB				
	6-May	Lost	Sailing delayed, lack of AB				
	7-May	Lost	Sailing delayed, lack of AB				
	8-May	1	Depart Pascagoula, MS	0			
	9-May	2	Marine Mammal Survey	144	11	2	2.9
	10-May	3	Marine Mammal Survey	174	11	4	2.9
	11-May	4	Marine Mammal Survey	198	6	1	3.7
	12-May	5	Marine Mammal Survey	196	2	1	3.9
	13-May	6	Marine Mammal Survey	197	3	0	4.7
	14-May	7	Marine Mammal Survey	220	5	2	3.7
	15-May	8	Marine Mammal Survey	190	4	1	2.9
	16-May	9	Marine Mammal Survey	129	9	10	2.1
	17-May	10	Arrive Pascagoula, MS	0			
	18-20 May		In port				
	19-May		In port				
	20-May		In port				
Leg 2	21-May	Lost	Sailing delayed, lack of AB				
	22-May	11	Depart Pascagoula, MS	0			
	23-May	12	Marine Mammal Survey	132	7	0	2.8
	24-May	13	Marine Mammal Survey	190	7	3	2.7
	25-May	14	Marine Mammal Survey	224	1	0	3.7
	26-May	15	Marine Mammal Survey	218	4	0	4.6
	27-May	16	Marine Mammal Survey	209	1	1	4.6
	28-May	17	Marine Mammal Survey	190	3	2	2.8
	29-May	18	Marine Mammal Survey	157	3	3	3.0
	30-May	19	Marine Mammal Survey	163	10	3	2.2
	31-May	20	Marine Mammal Survey	214	3	0	4.6
	1-Jun	21	Arrive Pascagoula, MS	0			
	2-Jun		Return early, lack of AB				
	3-Jun		Return early, lack of AB				
	4-Jun		Return early, lack of AB				

Table 3. Marine mammal sightings during each leg of GU23-02.

Species	Leg 1	Leg 2	Total
Common bottlenose dolphin	2	5	7
Clymene dolphin	3	0	3
Cuvier's beaked whale	2	1	3
Melon-headed whale	0	1	1
Pantropical spotted dolphin	14	6	20
Short-finned Pilot whale	0	1	1
Risso's dolphin	2	1	3
Rough-toothed dolphin	1	1	2
Sperm whale	20	14	34
Stenella spp.	1	1	2
Striped dolphin	2	3	5
Unid. Dolphin	3	2	5
Unid. Mesoplodont	0	1	1
Unid. Odontocete	1	1	2
Unid. Ziphiid	0	1	1
Total	**51**	**39**	**90**

Table 4. Biopsy samples and subsamples collected from *S. attenuata* during each leg of GU23-02 (DMSO – 20% dimethyl sulfoxide/saturated NaCl, M-biome – microbiome, SI – stable isotope, T-omics – transcriptomics). Sample GU230516-07 was also subsampled for tissue culture and provided to the Frozen Zoo at the San Diego Zoo Wildlife Alliance.

Leg	Date	Sighting No.	Sample No.	2/3 Skin (DMSO, Genetics)	Swab (Frozen, M-biome)	Blubber (Frozen, POPs)	1/3 Skin (Frozen, SI/M-biome)	1/3 Skin (RNAlater, T-omics)	Eligible for skin microbiome
1	05/09/2023	11	GU230509-01	1		1	1		yes
1	05/09/2023	11	GU230509-02	1	1	1	1		no
1	05/10/2023	22	GU230510-01	1		1	1		yes
1	05/10/2023	22	GU230510-02	1	1	1	1		no
1	05/10/2023	22	GU230510-03	1		1		1	no
1	05/10/2023	22	GU230510-04	1		1	1		yes
1	05/11/2023	23	GU230511-01	1	1	1	1		no
1	05/12/2023	30	GU230512-01	1		1	1		yes
1	05/14/2023	38	GU230514-01	1	1	1	1		no
1	05/14/2023	38	GU230514-02	1		1		1	no
1	05/15/2023	40	GU230515-01	1		1	1		yes
1	05/16/2023	43	GU230516-01	1	1	1	1		no
1	05/16/2023	43	GU230516-02	1		1	1		yes
1	05/16/2023	43	GU230516-03	1	1	1	1		no
1	05/16/2023	44	GU230516-04	1		1		1	no
1	05/16/2023	44	GU230516-05	1		1	1		yes
1	05/16/2023	47	GU230516-06	1		1	1		yes
1	05/16/2023	47	GU230516-07	1					no
1	05/16/2023	47	GU230516-08	1	1	1	1		no
1	05/16/2023	51	GU230516-09	1		1		1	no
1	05/16/2023	51	GU230516-10	1		1	1		yes
			Leg 1 total	**21**	**7**	**20**	**16**	**4**	
2	05/24/2023	61	GU230524-01	1		1	1		yes
2	05/24/2023	62	GU230524-02	1	1	1	1		no
2	05/24/2023	62	GU230524-03	1		1	1		yes
2	05/27/2023	71	GU230527-01	1	1	1	1		no
2	05/28/2023	74	GU230528-01	1		1		1	no
2	05/28/2023	74	GU230528-02	1		1	1		yes
2	05/29/2023	77	GU230529-01	1	1	1	1		no
2	05/29/2023	77	GU230529-02	1		1	1		yes
2	05/29/2023	77	GU230529-03	1	1	1	1		no
2	05/30/2023	80	GU230530-01	1		1		1	no
2	05/30/2023	80	GU230530-02	1		1	1		yes
2	05/30/2023	80	GU230530-03	1	1	1	1		no
			Leg 2 total	**12**	**5**	**12**	**10**	**2**	
			Grand total	**33**	**12**	**32**	**26**	**6**	

Table 5. Environmental DNA (eDNA) water samples collected during GU23-02.

Field #	Date	Sample Type	# Animals Present at Time of Collection	Water Collection Time	Latitude	Longitude
control #1	05/16/23	control	n/a	11:43	n/a	n/a
GU230516-01e_rep1	05/16/23	Stenella	35	10:52	27.01996	-90.0075
GU230516-01e_rep2	05/16/23	Stenella	35	10:52	27.01996	-90.0075
control #2	05/28/23	control	n/a	17:55	n/a	n/a
GU230528-01e_rep1	05/28/23	Stenella	15-20	17:29	27.15252	-85.3987
GU230528-01e_rep2	05/28/23	Stenella	15-20	17:29	27.15252	-85.3987

Figure 1. Survey boxes and effort during GU23-02.

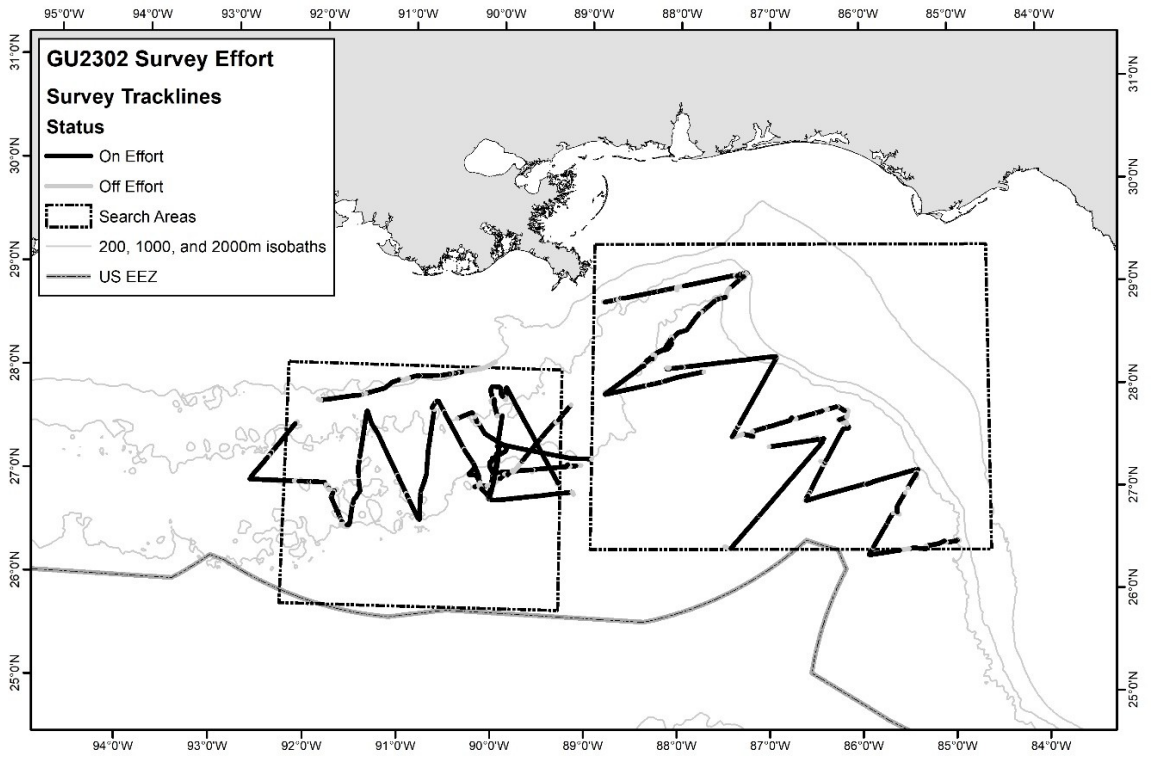


Figure 2. Locations of cetacean species sighted during GU23-02.

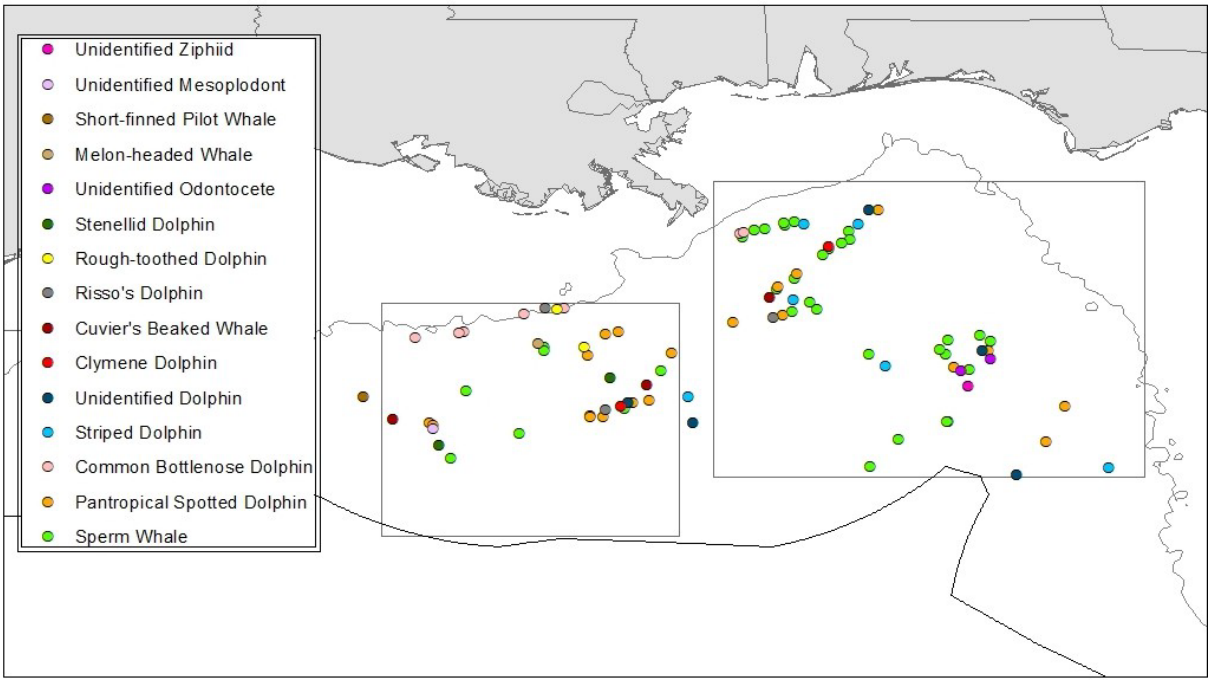
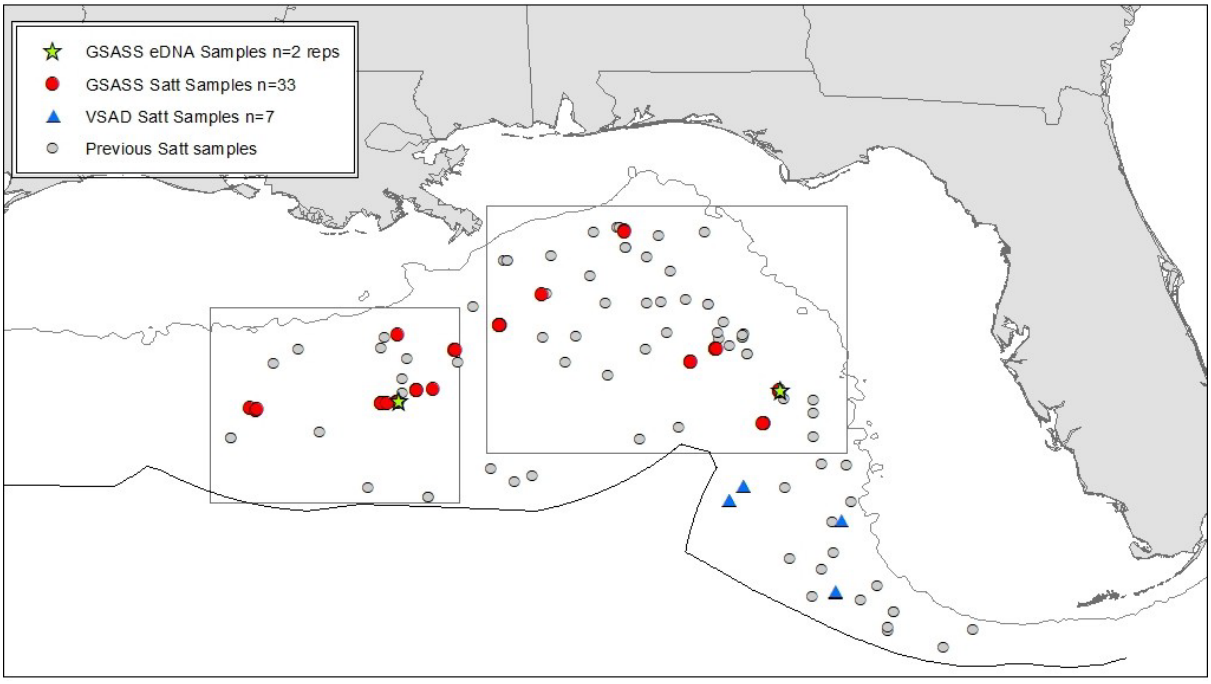


Figure 3. Location of *S. attenuata* biopsy samples collected in the Gulf of Mexico by the SEFSC during GU23-02 and other surveys. (VSAD - Vessel Survey for Abundance and Distribution; marine mammal survey immediately following GSASS)



Appendix 1

Marine Mammal Biopsy and eDNA Sampling and Processing Protocols

Gulf of Mexico *Stenella attenuata* Stock Structure (GSASS)

NOAA Ship Gordon Gunter Cruise GU23-02

04 May – 04 June 2023

Biopsy Sample Processing for GSASS 2023
(adapted from Sinclair *et al.* 2015)

1. Put on clean gloves (nitrile or powder-free latex)
2. First, wipe down all surfaces you will be working on with soap or bleach and dry.
3. Prepare cutting surface: lay down some no-slip matting, then place clean bench coat paper on top. Place the glass cutting board on top of the bench coat. Once set up, this can stay in place throughout the cruise.
4. If bringing back the entire dart shaft with the biopsy head attached with the sample, place a kimwipe next to the cutting board (on top of the bench coat) to set the dart(s) on while waiting to be processed. Replace kimwipe after each set of "dart shaft" samples is processed. If the biopsy head with the sample was removed from the shaft on the bow, and the head/sample is in the cooler with ice packs, leave it in the cooler until ready to process.
5. Prepare subsample vials according to subsample spreadsheet; these may include:
    a. Whole blubber - one 7.0 mL Teflon vial
    b. 2/3 skin - one salt-saturated 20% DMSO vial
    c. 1/3 skin - one blue-capped 2.0 mL cryo-vial -or- one 2.0 mL RNA*later* vial
    d. Skin swab - one yellow-capped 2.0 mL cryo-vial
    e. 1/3 skin - one tissue culture media vial
6. Label each sample vial with the sample number in vessel, year, month, day, number format; VVYYMMDD-nn (*e.g.*, GU230509-02; GU = Gordon Gunter, 230509 = 09 May 2023, 02 = sample #2). Note that NIST provided labels that have FreezerWorks ID numbers on them, with a space underneath for the sample number, that are for the Teflon contaminants vials.
7. Remove gloves and put on another pair of clean gloves (nitrile or powder-free latex)
8. Retrieve all needed instruments: forceps, scalpel handle (for GSASS these are individually foil-wrapped by NIST), and scalpel blade. If doing a swab for microbiome you will also need a pair of cleaned scissors (Alconox washed, freshwater rinsed, bleached, DI water rinsed, EtOH rinsed, dried, and covered in foil).

9. Retrieve one Teflon bag and carefully open lengthwise keeping interior-side up and place on the cutting board
10. Unwrap scalpel handle, attach blade, and set on Teflon bag
11. Unwrap forceps (and scissors if needed) and place on Teflon bag
12. If working with the entire dart (shaft + head), carefully unwrap the foil around the dart head and remove the sampling head from the dart using forceps. Set the shaft aside. Using the forceps, remove the sample from the sampling head and place it on the Teflon bag. It is ok to touch the sampling head, but not the sample, with gloved hands and/or place it on the Teflon bag.
13. If the sample is to be swabbed for microbiome analysis, first swab the skin (See GSASS 2023 Cruise Skin Microbiome Swab Protocol) then begin cutting the sample into appropriate subsamples and place into appropriate vials (see #5 above). If not swabbing for microbiome, then you can start cutting right away. It is usually easiest to first cut off the blubber, then subsample the skin. If the sample is to be used for tissue culture, after cutting off the blubber and partitioning the skin, follow the GSASS 2023 Cruise Tissue Culture Sample Protocol. BE CAREFUL not to cut through the Teflon!
14. After the skin has been subsampled (and swabbed):
    - ➔ First put the blubber in the Teflon vial, being careful not to touch the sides of the vial with the forceps
    - ➔ Next put the ⅓ skin either in the blue-capped stable isotope vial **or** in the RNA*later* vial **or** in the tissue culture vial
    - ➔ Finally put the ⅔ skin into the DMSO vial
15. Store subsamples using appropriate storage method:
    a. Place Teflon vial in -80°C freezer
    b. Place DMSO vial in the dark at room temperature
    c. Place blue-capped stable isotope vial in -80°C freezer
    d. Place RNA*later* vial in 4°C refrigerator overnight then move to -20°C freezer
    e. Place yellow-capped vial in vapor shipper for a minimum of 1 hour then move to -80°C freezer

f. Place tissue culture vial in 4°C refrigerator

16. Mark on the Biopsy Sheet the time of tissue preservation and the number and type of subsamples that were taken
17. Remove scalpel blade from handle and place into sharps container
18. Place all used sampling and processing implements (scalpel handle, forceps, scissors, sampling tip) in container for cleaning
19. Dispose of used foil, gloves, wrappers, teflon bag, and other waste in the trash
20. Record all appropriate information in the Biopsy Log

Directions for Cleaning Processing Implements and Sampling Equipment

1. Collect necessary cleaning supplies (Table 1)
2. Put on gloves (nitrile or powder-free latex)
3. At the beginning of each day prepare two jars of 10% bleach/water solution (can use fresh tap water from the sink). Follow the markings on jar A to add the appropriate amount of water and bleach. From jar A, pour a small amount of bleach/water solution into jar B (see step 8).
4. Place all processing implements, dart shafts, and sample tips in warm fresh tap water (from the ship) with a drip or two of soap (Alconox)
5. Use toothbrushes to clean processing implements, micro-brushes to clean sampling tips, and the small hand brush to clean dart shafts especially around the sampling end
6. Rinse well with warm tap water from the ship
7. Place washed processing tools and sampling tips in jar A with 10% bleach/water solution and let soak for 10 minutes.
8. For the dart shafts, after rinsing with warm ship tap water, shake quickly to remove excess water before putting it into jar B with a small amount of 10% bleach/water solution and let soak for 10 min. The bleach/water solution should cover the metal tip attachment but NOT touch the tape wrapped around the float.

9. Rinse dart shafts, sampling heads, and scissors well with multiple fresh water (deionized) rinses followed by a 200% ethanol rinse (both in Teflon wash bottles). Both bottles are stored covered with a Teflon bag that will need to be carefully taken off to use. Collect the waste 200% ethanol in an appropriate catch container. Shake the dart shaft through the air quickly to remove excess ethanol before placing it on the large aluminum tray and covering with a kimwipe. Place scissors and tips on the large aluminum tray and cover the tray with a large kimwipe. Carefully place the Teflon bags back onto the Teflon ethanol and deionized water bottles.

10. Rinse forceps and scalpel handles well with fresh tap water then place on the small aluminum tray to dry (these are single use and will be sent back to NIST and further cleaned).

11. For forceps and scalpel handles, once dry place in the labeled tupperware to be sent back to NIST.

12. When sample tips and dart shafts are dry, reassemble the sampling tip to a dart shaft and wrap the entire sampling end in cleaned/sterilized foil (hexane/acetone rinsed - provided by NIST) and return to quiver.

13. When scissors are dry, wrap in clean foil (does not have to be hexane/acetone rinsed) and place with processing equipment.

14. At the end of each day, scrub all cleaning equipment (trays, toothbrushes, etc) with Alconox and warm ship tap water, rinse trays with 200% ethanol (collect in an appropriate catch container), shake out excess water and let air dry

15. Dispose of gloves and pour ethanol from the catch container into the waste disposal container stored in the chemical cabinet.

<u>Note About NIST Blanks</u>

During the cruise, on three occasions, blanks for NIST will need to be collected. For each substance being blanked, you will need 3 replicates. Blanks of 4 substances will be collected: seawater, DI water, Alconox, and 200% Ethanol. This will be a total of 36 blanks for the entire cruise. See NIST Dart Biopsy pre-sampling protocol May 2023 (provided by NIST) to see the protocol for collecting blanks.

Table 1 (adapted from Sinclair *et al.* 2015 Table 4)

Sample Implements	**Cleaning Supplies**	**Chemicals**
Biopsy dart tips	Teflon wash bottles (2)	200% Ethanol
Biopsy dart shafts	Gloves (nitrile or powder-free latex)	Bleach (not no-splash)
Forceps	Micro brushes	Alconox soap
Scalpel handles	Toothbrushes	Hexane (at NIST)
Scalpel blades	Small hand brush	Acetone (at NIST)
Probes (optional)	Kimwipes	Reagent/deionized water
Scissors	Heavy duty aluminum foil	
Swabs	Glass jars (2)	
	Aluminum trays (2)	
	Ultra clean Teflon bags	
	EtOH catch container	
	EtOH disposal container	

*Note the Hexane and Acetone are chemicals used by NIST to prep some of the sampling implements

References

Sinclair, C., J. Sinclair, E. S. Zolman, A. Martinez, B. Balmer and K. P. Barry. 2015. Remote biopsy field sampling procedures for cetaceans used during the Natural Resource Damage Assessment of the MSC252 Deepwater Horizon Oil Spill. NOAA Technical Memorandum NMFS-SEFSC-670. 28pp. doi:10.7289/V5CC0XN0

Dart Biopsy Protocol for Blubber

Preface: Inadvertent contamination during sampling and sample handling is the major concern during the biopsy procedure. There are two major sources of contamination:

1. Hydrocarbons from sources other than the animal. These include:
- Sunscreen
- Boat fuel
- Engine exhaust
- Insect repellent
- Other oils associated with the sampling platform
- Cigarette smoke

2. Carry-over contamination from the previous animal. The concentrations of organohalogen pollutants in dolphins can vary by nearly two orders of magnitude between old reproductively-active females (very low) and juveniles or old males (very high). Ensure that instruments are not reused between animals.

Materials:

- Scalpel handle, pre-cleaned with acetone then hexane, wrapped in hexane-rinsed aluminum foil, and autoclaved by NIST staff (2/animal)
- Sterile stainless steel scalpel blades opened from manufacturer's foil wrapper when the biopsy is performed or sub-sampled (2/animal)
- Forceps, pre-cleaned with acetone then hexane, wrapped in hexane-rinsed aluminum foil, and autoclaved by NIST staff (2/animal)
- Teflon bag for cutting surface
- NIST pre-cleaned Teflon jar (1/animal)
- Barcoded lid labels
- Freezer (LN_2 shipper and/or -80ºC freezer)

Procedure

Prior to biopsy collection, kits containing a scalpel blade and handle, forceps, and Teflon bag/sheet prepared/provided by NIST staff. All instruments were rinsed in acetone, then hexane, covered with hexane-rinsed aluminum foil, and autoclaved.

1.OCs (NIST) (1, 7 mL Teflon jar) **(full depth blubber**)

Biopsy sub-sample summary – in order of collection

sample order	tissue	sample container	analyte(s)	researcher(s)	storage
	blubber	**Teflon jar**	**OCs**	**NIST**	**-80° C**

Note: Avoidance of sample contamination is crucial. This includes contamination from one vial to another. Hence, it is important to adhere to the proposed sample order, and if possible, to avoid touching the forceps to sample containers or any other item that might contaminate the sample.

Special Considerations:

- Blubber Sampling:
    - Organohalogen/Organochlorine Compounds (NIST) - Using forceps, place the OC (NIST) blubber biopsy samples in their respective 7mL Teflon jar. Check to make sure the jar is properly labeled with a lid label and that the label is secure. Do not use common lab tape to attempt to label the jar. This will either disintegrate or fall off in the liquid nitrogen vapor shipper. Be aware that many types of plastic will not withstand the temperatures present in the liquid nitrogen vapor shippers. If you have questions, please contact Amanda Moors or Jennifer Ness. Place the jar containing the biopsy in the liquid nitrogen vapor shipper, or -80°C freezer.

BIOPSY BLANKS: After tissue has been sub-sampled, sample blanks should also be taken on board the boat. Take blanks of each sample below for each researcher until at least 3-4 blanks of each sample is collected.

*Blanks should be made in the same manner as samples were collected or handled. (example: If the veterinarian does not wear gloves during the lidocaine injection, gloves do not need to be worn to make the lidocaine blanks.)

Boat Blanks

analyte(s)	sample container		researcher(s) (# blanks/day)	storage
Seawater collected in Teflon jar	**7 mL Teflon jar**		**NIST**	**-80° C**
Seawater collected in cryovial	**2 mL cryovial**		**NIST**	**-80° C**
Alconox collected in Teflon jar	**7 mL Teflon jar**		**NIST**	**-80° C**
Alconox collected in cryovial	**2 mL cryovial**		**NIST**	**-80° C**
Ethanol collected in Teflon jar	**7 mL Teflon jar**		**NIST**	**-80° C**
Ethanol collected in cryovial	**2 mL cryovial**		**NIST**	**-80° C**
DI water collected in Teflon jar	**7 mL Teflon jar**		**NIST**	**-80° C**
DIwater collected in cryovial	**2 mL cryovial**		**NIST**	**-80° C**

*Storage of blanks in liquid nitrogen is not necessary, but doing so maintains consistency between samples and blanks and will aid in keeping appropriate blanks & samples together.

Seawater collected in <u>Teflon jar</u>: Using the same device to collect eDNA, dip one 180 mL Teflon jar into seawater to collect a sample. After collection, pour in/fill the provided 7 mL Teflon jars with collected seawater. Document where sample was physically collected/poured; on bow of boat, in sample processing area, etc.

Date: (A1, A2, A3) ___ May 2023 (B1, B2, B3) ___ May 2023 (C1, C2, C3) ___ May 2023

Blank A collection location: _____

Blank B collection location:_____

Blank C collection location:_____

Seawater collected in <u>plastic cryovial</u> : Using the same device to collect eDNA, dip one plastic jar into seawater to collect a sample. After collection, pour in/fill the cryovials used for sample collection with collected seawater. Document where sample was physically collected/poured; on bow of boat, in sample processing area, etc.

Date: (A1, A2, A3) ___ May 2023 (B1, B2, B3) ___ May 2023 (C1, C2, C3) ___ May 2023

Blank A collection location: _____

Blank B collection location:_____

Blank C collection location:_____

Alconox blanks in Teflon jar: Pour Alconox solution into the Teflon jars provided prior to washing dart tips.
Date: (A1, A2, A3) ___ May 2023 (B1, B2, B3) ___ May 2023 (C1, C2, C3) ___ May 2023

Alconox blanks in plastic cryovial jar: Pour Alconox solution into the cryovials prior to washing dart tips.
Date: (A1, A2, A3) ___ May 2023 (B1, B2, B3) ___ May 2023 (C1, C2, C3) ___ May 2023

Ethanol blanks in Teflon jar: Squirt DI water from bottle into the provided Teflon jars provided.
Date: (A1, A2, A3) ___ May 2023 (B1, B2, B3) ___ May 2023 (C1, C2, C3) ___ May 2023

Ethanol blanks in plastic cryovial jar: Squirt DI water from bottle into plastic cryovials used for sample collection.
Date: (A1, A2, A3) ___ May 2023 (B1, B2, B3) ___ May 2023 (C1, C2, C3) ___ May 2023

Di Water blanks in Teflon jar: Squirt ethanol from bottle into the provided Teflon jars provided.
Date: (A1, A2, A3) ___ May 2023 (B1, B2, B3) ___ May 2023 (C1, C2, C3) ___ May 2023

DI Water blanks in plastic cryovial jar: Squirt Di Water from bottle into plastic cryovials used for sample collection.
Date: (A1, A2, A3) ___ May 2023 (B1, B2, B3) ___ May 2023 (C1, C2, C3) ___ May 2023

Storage & Shipping NIST Samples

All samples collected for NIST will be stored and shipped in LN2 vapor shippers supplied by NIST. Prepaid FedEx labels will be provided for shipping.

Labeling

Labels for NIST samples, including Blanks, will be provided with the sampling kits.

Labels for Teflon jars and cryovials, including blanks, will contain the following information:

Initial ID
Date sampled (dd/May/yy)
Tissue Type (if multiple samples/tissue collected, 1, 2 or 3 should follow)
Institution/Location of Animal (SRQ)

Shipping

A label will be provided for shipping. All samples must be shipped overnight to:

[redacted]

Please provide a copy of the data sheets along with the shipment.

[redacted]

Directions for Cleaning Processing Implements and Sampling Equipment

1. Put on gloves (nitrile or powder-free latex)

2. Prep two jars of 10% bleach/water solution (can use fresh tap water from sink). Follow the markings on jar A to add the appropriate amount of water and bleach. From jar A, pour a **small amount** of bleach/water solution into jar B (see step 7).

3. Place all processing implements, dart shafts, and sample tips in warm fresh tap water (from ship) with a drip or two of Alconox

4. Use toothbrushes to clean processing implements, micro-brushes to clean sampling tips, and the small hand brush to clean dart shafts especially around the sampling end

5. Rinse well multiple times with warm tap water from the ship

6. Place washed processing tools and sampling tips in jar A -> let soak for 10 minutes.

7. For the dart shafts, after rinsing with warm ship tap water, shake quickly to remove excess water before putting it into jar B -> let soak for 10 min.

   [The bleach/water solution should cover the metal tip attachment but NOT touch the tape wrapped around the float.]

8. Rinse dart shafts, sampling heads, and scissors well with multiple fresh water (deionized) rinses followed by a 200% ethanol rinse (both in Teflon wash bottles). Both bottles are stored covered with a Teflon bag that will need to be carefully taken off to use. Collect the waste 200% ethanol in an appropriate catch container. Shake the dart shaft through the air quickly to remove excess ethanol before placing it on the large aluminum tray and covering with a kimwipe. Place scissors and tips on the large aluminum tray and cover the tray with a large kimwipe. Carefully place the Teflon bags back onto the Teflon ethanol and deionized water bottles.

9. Rinse forceps & scalpel handles well with fresh tap water then place on the small aluminum tray to dry

10. For scalpel handles and forceps, once dry place in the labeled Tupperware.

11. When sample tips and dart shafts are dry, reassemble the tip to a dart shaft and wrap the entire sampling end in cleaned/sterilized foil (hexane/acetone rinsed - provided by NIST) and return to quiver.

12. When scissors are dry, wrap in clean foil (does not have to be hexane/acetone rinsed) and place with processing equipment.

13. At the end of each day, scrub all cleaning equipment (trays, toothbrushes, etc) with Alconox soap and warm ship tap water, rinse trays with 200% ethanol (collect in an appropriate catch container), shake out excess water and let air dry

14. Dispose of gloves and pour ethanol from the catch container into the waste disposal container stored in the chemical cabinet.

NVollmer & CSinclair May2023

GSASS 2023 Cruise Skin Microbiome Swab Protocol

([redacted] modified from Robles-Malagamba et al. 2020)

1. Using sterile forceps (provided by NIST), carefully remove the sample from the biopsy dart
2. Using a sterile polyurethane foam swab (we used Puritan 6" Sterile Standard Foam Swab w/Polystyrene Handle - SKU#: 25-1506 1PF 100) to gently scrape the surface of the biopsy skin sample making sure the entire skin surface area of the biopsy sample has been scraped by the entire area of the swab (excluding the blubber surface). Swabbed for 10-15 sec.
3. Place the swab into a yellow-capped 2mL cryovial with the appropriate sample ID label from NIST
4. Cut the swab with previously sterilized scissors, so the cryovial can be properly sealed
5. Place the sample in a vapor shipper (charged with liquid N) for the remainder of the field day, or if collected at the end of the day for a minimum of 1 hr.
6. Transfer swab samples from vapor shipper to the appropriately labeled box in the -80°C freezer
7. In the event of -80°C failure – RNALater can be added to the vial and then refrigerated or frozen at -20°C.
8. Transfer samples on dry ice to the facility for -80°C storage once the ship returns to shore.

GSASS 2023 Cruise Tissue Culture Sample Protocol
26 April, 2023
(adapted from protocol received by [redacted] Curator of the Frozen Zoo)

1. Remove biopsy from dart and cut the blubber off with sterile scalpel and place in Teflon vial. Cut off 2/3 of the skin and place in DMSO vial as per normal.

2. Set the remaining 1/3 skin biopsy on the inside of the sterile Teflon bag. Holding with a sterile forceps, use a sterile cotton swab drenched in 70% ethanol to gently clean the sample surfaces.

3. Continue cleaning with fresh cotton swabs and alcohol until the cotton tip appears free of debris/epithelial cells, and **all** surfaces have been wiped. Do NOT let the biopsy sit in a pool of ethanol, and rinse with a small amount of fresh biopsy media if it appears to be drying.

4. Once it is clean, do a final rinse with 70% ethanol on all sides. We want the entire piece to be sterile.

5. Get a fresh biopsy vial ready and loosen the cap. Be sure that the medium is clear and pink. If it is cloudy or yellow, it is contaminated and cannot be used. Place the tissue for cell culture immediately into the biopsy vial and close cap tightly.

6. Label the vial with the species name, sex, date, animal identification number (field ID) and biopsy location (or tissue type). Also please fill in the sample form

7. Wrap the top of the vial and lid in ParaFilm to ensure a leak-proof seal, and store upright in a storage container.

8. Refrigerate the sample until shipping (4-8°C). DO NOT FREEZE. DO NOT SHIP FROZEN OR WITH ICE PACKS. Include their sample form in the box.

Ship via Fedex to:

[redacted]

eDNA Water Sampling Protocol for 2023 Gulf *Stenella attenuata* Stock Structure (GSASS) Cruise for Green Canyon Restoration (GU23-02)

Please read through entire protocol before starting

April 2023

eDNA Sampling Protocol 2023 GSASS Cruise (GU23-02)

Maintaining sterile technique while filtering is critical. Keep Clorox bleach wipes handy for wiping down bags, glove fingertips, etc. between each handling

Please read through entire protocol before starting

This filtering ***must be performed as far away as possible from the location that biopsy samples are processed and trawl samples are processed to avoid any possible chance of contaminating the water filtering process. If using same sink or space as biopsy processing/cleaning please do not do them at the same time***

Sampling Goals

eDNA sampling is focused on:

1) Collect water in tail "wake" of any large baleen whales that are encountered so we can do a genetic species identification.
2) Collect water where a school of *Stenella attenuata* is encountered to test the newly developed dolphin eDNA assay.

Water samples should be collected in the presence of any large whale(s) and within or very close to the fluke print and/or in water that has just experienced a school of pantropical spotted dolphins. Water samples need to be stored on ice in the dark until filtering and need to be filtered within 2 – 4 hours of collection.

Each eDNA water sample is comprised of a 96oz Nalgene bag filled as close to the lip as possible.

Metadata

Metadata should be recorded to accompany each sample:

Sample Field Number

For a Single Sample use standard biopsy field number format with 'e' at the end:
GUYYMMDD-01e *e.g.*, GU230425-01e

For Duplicate water samples (multiple bags from same time and location) use:
GUYYMMDD-01e_rep#
e.g., GU230425-01e_rep1, GU230425-01e_rep2

Please collect the following additional metadata in spreadsheet

- Field number
- Sighting number
- Date
- Waypoint from site of collection
- Time of water collection and who it was collected by
- Latitude and longitude
- Number of animals thought to be present where water was collected
- Start and Stop times of filtration and who did the filtering
- Approximate amount of water filtered per filter/sample** Goal is to filter 2.5-3 L (~96oz)
- Image/frame numbers from photos with good ID shot of sampled animal(s) taken during sighting
- Water characteristics (blue, blue-green, turbid)
- Sample Type (i.e., whale, group of Stenella, Control)
- Onboard location of where filtering is occurring (e.g., wetlab)

***Make note if more than one filter is needed to filter a water sample due to clogging. Record the amount of water filtered through each filter, and distinguish the filters with the field number followed by 'a', 'b', 'c',* etc.

Water Sampling

Items to have on the ship

- Clorox Wipes
- Latex/Nitrile Gloves
- Nalgene Bags for water collection
- Cooler with ice or ice packs to keep water bags cold and in the dark after collection
- Tape and Marker for labeling

Water Collection Protocol

- Make sure the Nalgene bags are properly labelled using tape with temporary ID numbers (whale Field ID and date/time or numerical order when multiple water samples are collected from same whale(s)/group)
- Wearing clean gloves, with the lid on the bag, wipe the exterior of the lid & bag with a Clorox wipe. **Let dry completely**. Avoid touching the bag mouth to any human or boat surfaces or equipment
- Remove lid and collect the water sample off the side of the RHIB
- Fill each bag with near surface water, pull up, and cap. Avoid touching the bag mouth to any human or boat surfaces or equipment
- Collect two bags (replicates) of water per sampling site (i.e., at the same place and time collect two bags of water)
- Store the bag in a cooler with ice packs or ice so that it stays cold and out of direct sunlight until filtering. UV radiation is the enemy of DNA. Keep sample bags out of the light as much as possible.

Control Water Samples to Collect

Filtered water Control samples can be named Control 1, Control 2, etc. with appropriate comment in metadata spreadsheet about the water source and date

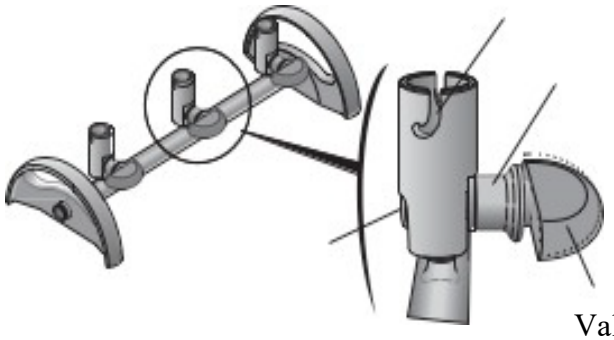
1) First day of water sampling: Collect 2-3 L of Galley water (= water from filtered water fountain) in a *dedicated* Control water Nalgene bottle (2L or 4L) that will be used for cleaning of any eDNA equipment. Treat the water sample as you would a field sample, i.e., use good clean technique while handling the bottle. Filter it prior to filtering any field samples.
2) *Filter a Control Galley water sample on any subsequent day that field samples are collected*. Always filter Control water before field water.

Initial Filtration Setup on the Ship

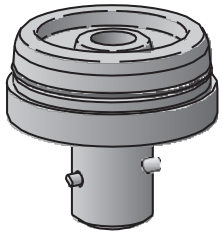
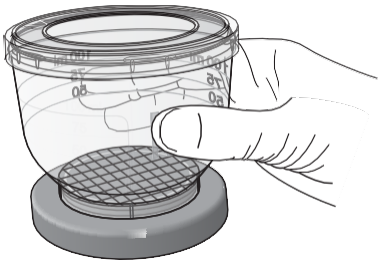
To be done on the first day samples are collected.
See graphics below for pump set up diagrams and images.

Terms [See Appendix for more views]

Filtration Manifold



Valve Button, Closed

Filtration Head	Filtration Head	Filter Cup/Funnel	EZ-Stream Vacuum Pump
1-cup manifold	3-cup manifold		
			

Process

The first step is to set up and rinse the manifold system.

- Put on a fresh pair of clean gloves.
- First, **wipe down all surfaces** you will be working on with a Clorox wipe.
- Surface needs to be flat and not slippery, and near a sink so the filtered waste water can go down the sink. Ideally, the sink should be on the left of the vacuum pump because that is where the outflow port is located. Please place it away from any biopsy/trawl sample processing areas.
- Lay down a piece of white bench paper on the surface.
- Place EZ-Stream vacuum pump on clean, stable flat surface. If ship has some non-slip matting, you could cut a piece and place it under the pump for increased safety and stability. Make sure

it can easily connect to a power supply. Remove the two protective caps from the tubing connections and keep in a safe place (*e.g.*, in a baggie back in a cooler). Please don't lose the caps.

- Before you connect any of the tubing to the filter apparatus, with a Clorox wipe, wipe down the ***exterior*** of 1) the vacuum pump, 2) tubing, 3) filtration head, and 4) manifold. Take extra care to **never** get any Clorox inside the manifold pipe or inside the filtration head(s).
- Immediately rinse these parts with fresh water. All can be placed under running water *except* the pump, for which you can use a wet kimwipe for rinsing off.
- Dry off all excess water using clean kimwipes.
- Attach the filtration head(s) to the manifold.
- Place the manifold unit next to the pump (on the right side of it) and connect the two with the short piece of tubing provided. NOTE the pump has an IN side (right side) and an OUT side (left side). Manifold must be attached to the IN side.
- Connect water disposal tubing to pump OUT side and place other end of tubing in sink so water can go down the drain. Be careful that while this setup is in place no water, or other material, will be able to get up into the disposal tubing from the sink.
- Connect the pump to a power supply.
- Make sure the valve button is in the closed position on all filtration heads (open = valve in vertical position; closed = valve in horizontal position. See Figure 5d).
- For each filter head on the manifold, get a filter-less 250 mL filter cup pre-labeled "For Distilled Water Cleaning ONLY". For the three-cup manifold, clean as many heads as you are going to use for filtering, and use only those heads for filtering.
- Place a filter cup on each filtration head (see Figure 5).
- For each filtration head on the manifold you are going to use, obtain ~500 mL (2 filter cups full) of filtered water from the water fountain in the Galley in the provided 500 mL or 2 L Nalgene bottles (labeled "Clean Water").
- Fill each filter cup with the Galley water (~250 mL).
- Replace filter funnel cover/lid(s).
- Start the vacuum pump (lightly tap the circled space on top of pump).
- Start filtration by slowly rotating all valve buttons counter clockwise (vertical position) until they stop turning.
- Once ~20 mL remains in each cup, close each valve button, take off each lid, pour in the remaining water, replace lids and open the valve buttons.
- Continue filtering until all of the water has run through each filter.
- **Keep a close eye**, as soon as the last of the water is filtered through close the valve button for each filtration head.
- Turn off the pump, remove the filter cups (place in baggie), and set aside for future distilled water cleaning.
- Open all valve buttons and turn the pump on for ~10 sec to suck through any excess water in the tubing/manifold. You may have to slightly lift up the manifold and tilt toward the pump to get all excess water out.

- Close the valve buttons, run pump another 10 seconds then turn the pump off.

Filtering Samples on the Ship

Once you have done the above steps, and you are ready to filter a Control water sample and/or field water sample, proceed with the following. See graphics below for pump set up diagrams and images.

Keep eDNA water sample bags on ice and in the dark at all times until ready to filter

***Maintaining sterile technique while filtering is critical ***

If you feel you have spilled sample water on your gloves, change them for new gloves

Keep Clorox wipes handy for wiping down bottles, gloves and workspace. Be sure to keep lids closed on both the bags and filter cups when not directly adding water.

General Instructions

- Wear clean gloves.
- Connect the tubing to the pump and manifold as described above and check the filtration set up to make sure everything is set up in correct direction for filtering.
- If necessary, remove the baggie covering the filtration head(s). **Wipe down all surfaces** you will be working on with a Clorox wipe, including the exterior of the manifold and filtration heads, but not inside the pipe/the small hole the air is going through.
- Immediately rinse exterior of manifold (via wet kimwipe) and filtration head with fresh water and dry with kimwipes.
- If necessary, connect the pump to a power supply.
- ►For each sample you are going to process, obtain a new filter cup and label with the sample bottle field number.
- Attach the filter cup to a filtration head on the manifold. **See Figure 5b of the Appendix.**
- For each sample being processed, included water Control samples, label a 4 mL tube (screw cap tubes with Longmire's Lysis Buffer already inside) with your initials, date, and the sample bottle field number. If this is a Control water sample, label tube with initials, date, Control number and water source.
- Keep tubes upright so they don't touch any surfaces and in the dark.
- **Wipe down the exterior of the sample bag with Clorox wipe before removing the lid**. *Be sure the Clorox has completely dried on the bag* prior to proceeding. We do not want Clorox to get into the water sample for it will destroy the already fragile eDNA.
- Make sure all valve buttons are in the closed position (open = valve in vertical position; closed = valve in horizontal position. See Figure 5d).

  - ❖ If this is the first filtering of the day, start by obtaining and filtering a Control water sample (2.5-3L Galley water collected in a Control water bottle)- fill the labelled filter cup with the Galley water (Figure 5c) and replace the filter cup cover. Follow all the instructions

below for filtering a sample and collecting the filter into Longmire's solution before filtering field water samples.

- ❖ If a Control water sample has already been filtered, then fill the labelled filter cup with water from the corresponding sample bottle (Figure 5c) and replace filter cup cover and follow filtering instructions below.

- ❖ If using the 3-cup manifold and are going to process more than one sample at a time, fill up the first labelled filter cup with water from the corresponding sample bottle and replace filter funnel cover/lid, and sample bottle lid. Then proceed to do the same with samples/cups 2 and 3 before starting the vacuum pump.

- Start the vacuum pump (lightly tap the circled space on top of pump) and then slowly open the valve button(s) (turn counterclockwise until vertical) on the manifold port(s) to start filtration (open = valve in vertical position; closed = valve in horizontal position. See Figure 5d).
    - Record Start Time
    - Filtration may slow as the filters fill with marine debris. Be patient.
    - The volume of the filter cup is 250 mL, so as filtration proceeds, the water level will need to be topped off from the corresponding 96oz water sample. See Figures 5c and d.
    - Once the water gets to ~20 mL, close the valve button, take off the filter cup lid, pour in more water, replace lid and then open the valve button.
    - <u>When not pouring water keep the lid on the sample bags closed to help prevent cross contamination.</u>
- Continue filtering as described until the 2.5-3 L of sample has run through the filter. **<u>Do not let the filter run dry</u>** as you filter. *If you notice that the filtration has significantly slowed due to the filter getting clogged, let all of the liquid that is currently in the cup filter through. Get new gloves and place the filter in Longmire's solution as described below. Put on new gloves again, and place a new filter in the filter cup to filter the remainder of the sample or as much as possible; place subsequent filters in a different tube of Longmire's (be sure to label with the volume that was filtered) using more new gloves and new sterile forceps. If after clogging two filters, you still have sea water left, just stop filtering that sample. Make sure each filter is placed in a separate Longmire's tube and note approximately how much liquid was filtered through each and label with 'a', 'b', 'c', etc.
- If all bags filter at the same pace, once all water has been filtered note Stop Time and let the vacuum pull 3-5 additional seconds to remove any remaining liquid from the filter(s). Close filter head valve. *If some water samples are filtering faster than others, you will have to continue filtering some, while removing the filter from those that are finished*. Please note each sample's Stop Time.
- Then, working with each sample:
    - Put on a new set of clean gloves and remove the cover/lid from the filter funnel.

- Lightly pinch the top half of the filter funnel to remove it, exposing the filter laying on the lower part of the filter funnel. See Figures 5e and f. Close the valve button.
- With sterile forceps (cleaned with 10% bleach, rinsed with water and dried), remove and carefully roll/fold the filter, with the sample surface enclosed to the interior of the folds, and slide the folded filter into the labelled 4 mL tube of Longmire's Lysis Buffer (one filter per tube of buffer)
    - NOTE: one way to fold the filter to fit in these tubes is to use two pairs of forceps, fold it gently in half, then half again lengthwise so it is a long thin strip. Pick it up with one set of forceps and curl it into the vial. You can also use the base of the filter funnel (where the filter was stuck originally) as a kind of table to use to help fold the filter.
- Filter must be fully submerged in the solution. If for some reason there is not enough liquid in the tube, open a second vial of Longmire's and use it to top off the tube with the filter in it. NOTE: use a clean set of forceps for each filter then clean the batch of forceps at the end of the day, or clean forceps well between each sample with a 10 min soak in 10% bleach and rinse well with distilled water (or drinking water from the filtered fountain from the Galley) and allow to air dry. Place the 4 mL tubes containing filters and lysis buffer back in the storage box and store upright AT ROOM TEMPERATURE in the dark.
- Then put on a new pair of gloves before repeating these steps with the next sample.

- Once all filters have been removed, shut off the vacuum pump.
- Throw the clear plastic filter cups and bases in the trash.

If you are done filtering field samples for the day, go to the "When you are done filtering for the day" section. If you have more field water samples to filter, you must next clean the system with fresh water to prevent cross-contamination of samples as follows:

- Get a new pair of gloves.
- Get "For Distilled Water Cleaning ONLY" filter cup(s).
- Place each filter cup on a filtration head (see Figure 5).
- Fill each filter cup with the distilled water (or the filtered water from the water fountain in the Galley) that you obtained in the provided 500 mL or 2 L Nalgene bottles (with white lids).
- Replace filter funnel cover/lid(s).
- Start the vacuum pump (lightly tap the circled space on top of pump) and then open all valve buttons on the manifold to start filtration (open = valve in vertical position; closed = valve in horizontal position. See Figure 5d).
- Once ~20 mL remains in each cup, close the valve buttons, take off the lids, pour in the remaining water, replace lids and open the valve buttons.
- Continue filtering until all of the water has run through each filter.

- **Keep a close eye**, as soon as the last of the water is filtered through each filter cup close the valve button.
- Turn off the pump, remove all filter cups, place in clean baggie and set aside for later distilled water cleaning.
- Open all valve buttons and turn the pump on briefly to suck through any excess water in the tubing/manifold (you may have to slightly lift up the manifold and tilt toward the pump to get all excess water out) then close valve and pump for another 10 seconds.
- Turn the pump off.
- Take off each filtration heads and clean exterior with a cotton swab dipped in 10% bleach solution (but don't get any bleach inside the pipe/the small hole the air is going through).
- Rinse each filtration head with fresh water.
- Wipe the outside of the manifold with a Clorox wipe and then thoroughly wipe with kimwipe wet with fresh water.
- Re-attach filtration head(s) to manifold.
- Get a new pair of gloves.
- Obtain new filter cups and proceed as detailed above (starting at on page 8) with prepping for and filtering next samples.

When you are done filtering for the day

- After the last sample is completed, get a new pair of gloves.
- Get "For Distilled Water Cleaning ONLY" filter cups.
- Place the filter cups on the filtration heads (see Figure 5).
- Fill each filter cup with ~ 250 mL Galley water.
- Replace filter funnel cover/lids.
- Start the vacuum pump (lightly tap the circled space on top of pump) and then open all valve buttons on the manifold port to start filtration (open = valve in vertical position; closed = valve in horizontal position. See Figure 5d).
- Once ~20 mL remains in each cup, close the valve buttons, take off the lids, pour in the remaining water (~ 250 mL), replace lids and open the valve buttons.
- Continue filtering until all of the water has run through each filter.
- **Keep a close eye**, as soon as the last of the water is filtered through each filtration cup close the valve button.
- Turn off the pump, remove the filter cups, place in clean baggie and set aside for later distilled water cleaning.
- Open all valves and turn the pump on briefly to suck through any excess water in the tubing/manifold. You may have to slightly lift up the manifold and tilt toward the pump to get all excess water out.
- Close all valves and run the pump for another 10 sec. Turn off pump.

- Gently detach the tube between the pump and the manifold – this is most easily accomplished by detaching at the pump end, leaving the tubing connected to the manifold.
    - Run the pump ~20 – 30 sec to make sure it is dry. Disconnect the pump from the power supply. Then detach the OUT tubing from the pump.
- Detach tubing from the manifold and drain any water that remains (you may have to pick the manifold up and carefully tip it over sink to pour out excess water). Rinse all tubing inside and out thoroughly with fresh water.
- Detach all filtration heads.
- Wipe exterior of pump, manifold, and each valve button and filtration head with Clorox wipe.
- Immediately do a fresh water rinse for all of those same parts and let air dry. All can be placed under running water *except* the pump, for which you can use a wet paper towel or kimwipe for rinsing off.
- Place tubing and filtration heads on kimwipe and cover with another kimwipe. Cover where each filtration head attaches to the manifold with a baggie loosely held in place with a rubber band.
- Let all parts dry overnight, making sure the entire set up/apparatus is secure.
- Place caps on the pump's tubing connectors.

In the morning, to minimize the number of loose parts, reassemble filtration heads on the manifold. Cover each filtration head with a baggie loosely held in place with a rubber band.

Equipment list

In Blue Coolers sent from Lafayette

- Vacuum pump
- Filter manifolds (1-cup and 3-cup manifolds)
- 3/8" vacuum tubing (Two precut pieces – one short piece for manifold to pump connection and one longer piece cut as needed for water waste), plus bag of spare tubing if needed
- 4L plastic Nalgene bottles (n=11) for collecting seawater
- 10 clean, sterile forceps
- 4 mL screw cap storage vials with Longmire's Lysis Buffer
- Lidded storage box for vials. **Keep at Room Temperature in dark**
- Two empty 250 mL bottles with white screw-cap lids for making 10% bleach and for distilled water for cleaning forceps
- Four clear 500 mL bottles with small white screw cap lids for getting clean ship water for cleaning the filtering system
- Bag of sterile Cotton Swabs for cleaning inside manifold with 10% bleach
- Box of large kimwipes
- Bench coat paper cut in squares to put down on working surface used for filtering to keep things clean
- Boxes of 1-quart and 1-gallon baggies
- Rubber bands
- 2 Boxes of 49 DMSO tubes each
- 2L Nalgene Bottles (n=2)
- 250 mL filter funnels with MCE filters (n=24)
- Box of MCE filters
- 96oz Nalgene bags (n=6)

PLEASE RETURN EVERYTHING TO LAFAYETTE AT END OF CRUISE

- **Need to purchase separately**
  - Bleach. Be sure this is regular Bleach and NOT the no-splash bleach
  - Clorox wipes
  - Aluminum foil
  - Non-slip bench mat
  - Boxes of 1-quart and 1-gallon baggies

Equipment clean-up & sterilization

It is important that all water sample bags and Control water bottles, forceps and other equipment are sterilized between uses. Note: 10% bleach can be re-used for several days before losing efficacy. Just replace lid on bottle when not using.

- **Cleaning forceps (metal or plastic)**
  - Make a 10% bleach solution in the small 250 mL plastic bottles with white lids (20 mL bleach in 180 mL of fresh water). Be sure this is regular Bleach and NOT the no-splash bleach.
  - Soak in 10% bleach solution for 10 min in provided container.
  - Rinse well by soaking in fresh water (distilled water or filtered water from the water fountain in the Galley) for 5 min in provided container to remove all traces of bleach.
  - Air dry on a kimwipe and loosely covered by a kimwipe in a safe place away from disturbance by people or air flow.
  - Once dried, wrap each forcep in clean aluminum foil for future use.
- **Cleaning Nalgene bottles and bags**
  - Pour ~200 mL of 10% bleach solution (20 mL bleach plus 180 mL fresh water) into the bottle/bag, tighten cap, invert and swirl several times over 10 minutes to cover all internal surfaces with sanitizer. Be sure this is regular Bleach and NOT the no-splash bleach.
  - Let bottles/bags sit with bleach in them for ~10 min, rotating liquid around occasionally.
  - Pour bleach out (can re-use several times).
  - Rinse well inside & outside FIVE times using tap water and lots of swishing of the liquid around the bottle/bag. Finally, perform a final rinse with Galley water. Any remaining bleach, even after dried will destroy eDNA so PLEASE be sure you have super thoroughly rinsed the bottles/bags.
  - Allow to air dry (can prop bottles upside down on piece of clean benchcoat paper but allow air to get in, or use a strung up bungie cord to hang open bags) in a clean location away from disturbance by people or air flow.

Appendix

Figure 1. Vacuum pump with 3 filter cup holder manifold.

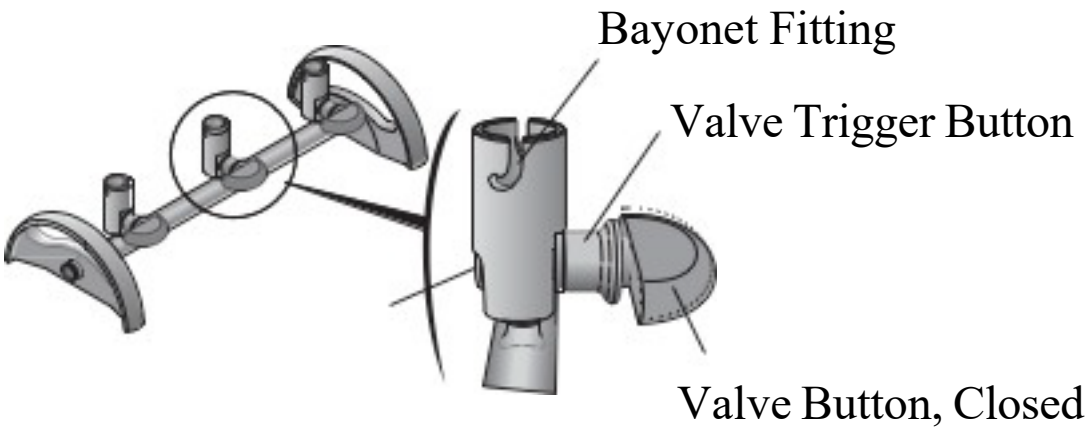


Figure 2. Close up of manifold connector. Vacuum will pull when valve button is in vertical position and will stop pulling when valve button is horizontal as shown.

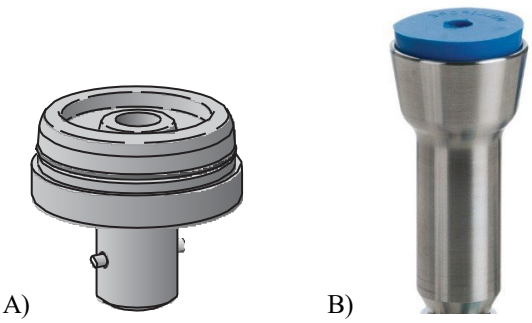


Figure 3. Filtration head that goes into Bayonet Fitting shown above. Use with A) 1-cup or B) 3-cup manifold.

The EZ-Fit™ filtration device consists of:

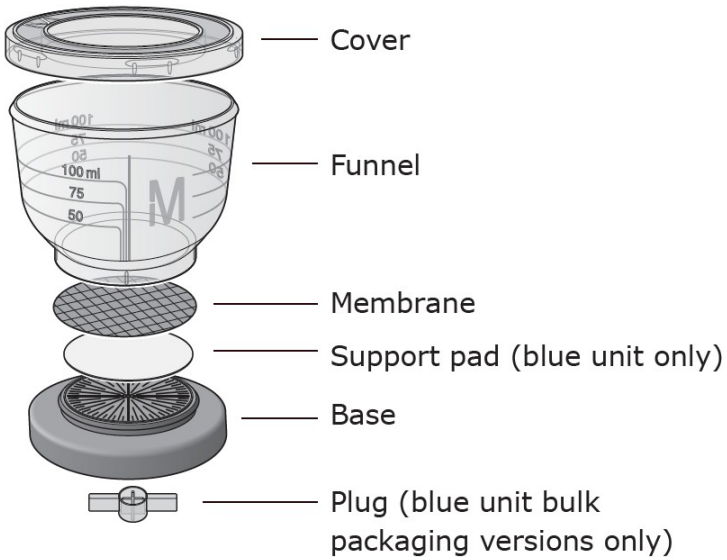
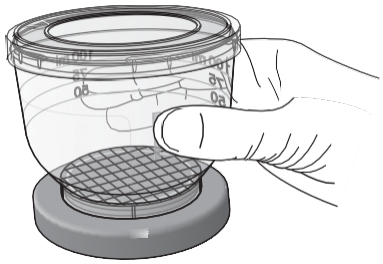


Figure 4. The filter funnel with filter membrane pre-installed. ***Our units are pink and will not have a plug.***

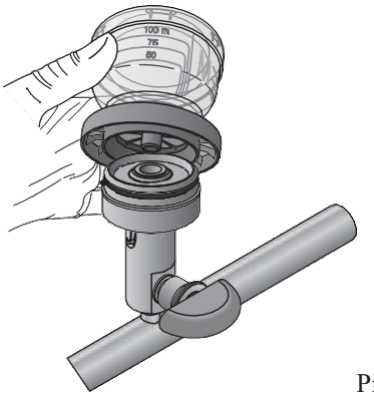
Figure 5. Instructions for using filter funnel and removing filter membrane from funnel after filtering water

a) With clean gloves on, remove the device from the packaging by grasping the funnel.



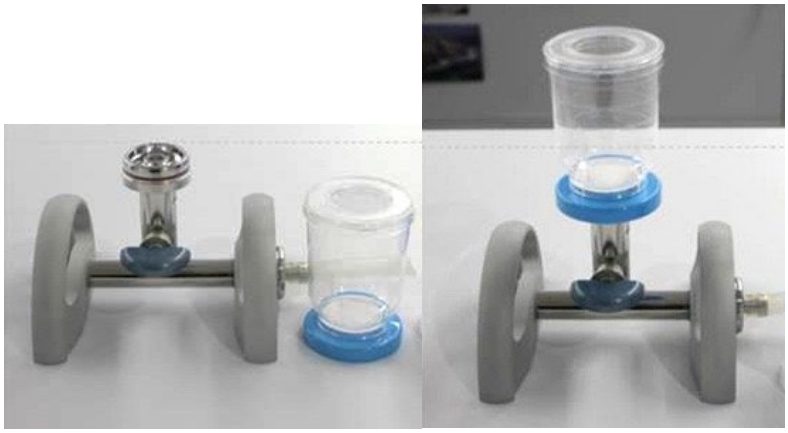
CAUTION Grasping the cover could result in removing the cover from the funnel.

b) Place/snap the filter cup onto the filtration head of the 1-cup EZ-Fit™ Manifold or tulip head with blue stopper of the 3-cup manifold.



Pictures of setups continued next page

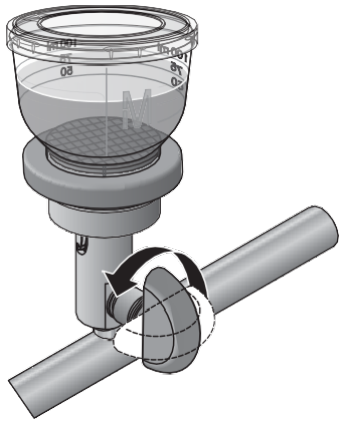
One-cup EZ-Fit manifold example:



Three-cup manifold Tulip head with blue stopper example:

c) Remove the cover and pour the sample into the funnel. Replace cover after each refill.

d) Turn the knob to the upright position to apply vacuum to perform filtration.

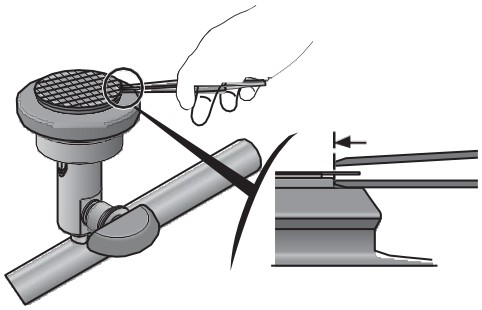


e) Once all water has been filtered, allow air to pull through the filter for 3-5 seconds to ensure all water has been removed from the filter.

f) Remove the cover from the funnel and slightly pinch the upper part of the filter funnel and remove it as shown above.

g) Turn the knob on the manifold to the horizontal position to close the vacuum and then shut off the vacuum pump.

h) Gently remove the membrane using clean DRY forceps, carefully fold it into a curl and place it in a 4 mL tube with Longmire's Lysis buffer.



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