

UC San Diego

Capstone Projects

Title

Methods and S.O.P.s for Quantifying the Unseen: eDNA Insights into the Enigmatic World of Sea Turtle Genetics

Permalink

<https://escholarship.org/uc/item/2ps703w0>

Author

Stasiak, Lena

Publication Date

2024-07-01



NOAA FISHERIES
Southwest Fisheries Science Center

Methods and S.O.P.s for Quantifying the Unseen: eDNA Insights into the Enigmatic World of Sea Turtle Genetics

Lena Stasiak

Masters of Advanced Studies Candidate – Marine Biodiversity and Conservation
Scripps Institution of Oceanography, UC San Diego

Capstone Advisory Committee:

Chair: Ron Burton, PhD, Scripps Institution of Oceanography
Peter Dutton, PhD, NOAA Southwest Fisheries Science Center
Nastassia Patin - Project Scientist

Signature: _____ Date: 6/11/2024

Signature: _____ Date: 7/8/2024

Signature: _____ Date: 7/8/2024

Quantifying the Unseen: eDNA Insights into the Enigmatic World of Sea Turtle Genetics

Lena Stasiak

Masters of Advanced Studies Candidate – Marine Biodiversity and Conservation
Scripps Institution of Oceanography, UC San Diego

Capstone Advisory Committee:

Chair: Ron Burton, PhD, Scripps Institution of Oceanography
Peter Dutton, PhD, NOAA Southwest Fisheries Science Center
Patty Ahn, PhD, Associate Teaching Professor
Nastassia Patin - Project Scientist

There is a growing need to understand sea turtle biology and distribution as changing oceanographic conditions may cause dramatic shifts over time. However, conservation efforts for highly migratory and elusive species such as green and leatherback sea turtles remains a challenge, especially in the marine environment, limiting research to nesting females while the remainder of their life cycle is largely hidden. (1) Green turtles forage in bays and estuaries in Southern California and have been found to originate from tropical nesting beaches in Mexico. (1) On top of logistical challenges, traditional genetic studies on these species often rely on tissue samples, which can be invasive and stressful for turtles, costly, and labor-intensive (3). Environmental DNA (eDNA) offers a non-invasive alternative, potentially revolutionizing the field. This method involves collecting samples of water, sand, air, etc. and essentially extracting DNA straight from the environment rather than the species of interest. (4) Previous studies have shown that eDNA can be extracted and identified from water samples collected directly next to green turtles (5). The next challenge is to be able to quantify this eDNA to reliably determine presence or absence of the species, and to estimate relative abundance. Droplet Digital PCR is a system used to precisely quantify the amount of target DNA in a sample, and has not yet been used for eDNA studies of sea turtles. Without the need to capture or even see the organism itself, eDNA techniques could be vital for the detection and analysis of rare and elusive species. However, there is a need to systematically evaluate the reliability and efficacy of eDNA methodologies for sea turtle research, addressing issues related to sample identification, quantification, and temporal dynamics.

MATERIALS AND METHODS

Sample Collection

For this project, all samples were collected from a known *Chelonia mydas* hot spot in San Diego Bay (see Figure 1 below). The South Bay Power Plant used to attract *C. mydas* with warm effluent flowing from the structure. Even with its decommissioning, the intake channel nearby remains a popular location for the turtles year round (5). This project involved 1 field collection

day in April, and 3 consecutive collecting days in May, for a total of 59 samples. Once the site was reached, researchers spent at least 1 hour surveying the area for turtle heads and kept a record of the number and location of each sighting. Additionally, drones collected aerial survey data when possible to contribute to an accurate count of how many turtles were within the intake channel. Group size estimations ranged from 3-8 individuals. For the water samples, all equipment was disinfected with 10% bleach for 10 minutes, rinsed with deionized water, and dried in a clean room prior to use. Samples were collected in 1 L or 2 L amber bottles (Thermo Scientific) in a distributed pattern to get a true snapshot of the area on 3 of the 4 field days, and right next to individual *C. mydas* captured for further analysis on 1 of the 4 field days. All samples were collected at the air/surface interface (~0.5 m depth) by hand. For each field effort, a field control was filled with 1 L MilliQ deionised water in the lab, and kept on ice in the cooler with the other collected samples while out at the site to monitor for potential contamination. All samples were kept in the cooler with ice packs until getting back to the laboratory at Southwest Fisheries Science Center (SWFSC), La Jolla. All samples were vacuum filtered immediately after collection (within 5 hours).



Figure 1 | a) the South Bay Power Plant located in South San Diego Bay remains a popular location for *C. mydas* year round b) the intake channel is the primary location for the turtles and the study site for this project c) samples were collected in a distributed pattern to get a snapshot of the area on each given sampling day.

Water Filtration

After all surfaces and equipment were disinfected with DNA Away (Thermo Scientific) and fully dry, filtration began with a laboratory blank (1 L MilliQ) to rinse out the vacuum system. Each field blank and experimental sample were then filtered through 0.45 um Whatman cellulose nitrate membrane filters. The filters for the field blank and experimental samples were then

carefully folded/rolled to be stored in a 5 ml tube (CHECK SOP) with 4 ml of Longmire's buffer. The caps were secured with parafilm and the tubes were stored at room temperature in a dark cabinet until DNA extraction.

DNA Extraction

All DNA extractions followed the S.O.P. detailed below in a 2-day procedure. Due to the limitations of a 12-sample incubator, 8 filters went through the extraction process at a time along with 4 non-target controls (NTCs) mixed in each round. The NTCs contained 4 ml of Longmire's buffer only. Briefly, on the first day, samples were heated to 95°C for 5 minutes and allowed to cool to room temperature. 100 ul of Proteinase K was added for every 1 ml Longmire's buffer (ex. 400 ul Proteinase K for 4 ml Longmire's). The samples were then incubated for 2 hours at 56°C at a gentle shaking of 120 rpm in a thermocycler. After the 2 hour incubation, the temperature was reduced to 37°C and the samples remained on the incubator overnight with no shaking. On the following day, 630 ul of 100% Ethanol, 630 ul of buffer AL from the Qiagen DNA Blood and Tissue Kit, and 600 ul of each sample were first combined and mixed in a 2 ml tube. Then, in 4 iterations of 465 ul, this mix was filtered through a spin column tube, followed by washing steps and elution as described by Qiagen's Kit, and further outlined in the S.O.P. for DNA extraction below. Elution was completed in 2 steps, first with 100 ul of Low TE ??? with a room temperature incubation of 5 minutes, and then with 50 ul of Low TE ??? with a room temperature incubation of 1 minute. Therefore, a total of 150 ul was eluted for each sample, including the original field control and all NTC's included in each round of extraction. A total of 40 samples and 20 NTCs have been extracted to date. Each sample was then quantified with a Qubit Flex Fluorometer (Thermo Scientific). All experimental samples ranged from 5-32 ng/ul while all field controls and NTC's were below the limit of detection (<0.05 ng/ul).

ddPCR for *C. mydas* Quantification

To further quantify each sample for *C. mydas* DNA only, Droplet Digital PCR (ddPCR) was used for the first time with specifically designed primers and probes (see Table 1 below). The Bio-Rad QX200 Manual Droplet Digital PCR System at Southwest Fisheries Science Center (SWFSC), La Jolla includes a Manual Droplet Generator that generates ~20,000 droplets from each reaction, and a Droplet Reader for the detection of the target DNA by fluorescence. A fluorescent dye was incorporated into the PCR reaction for amplification of the target DNA and was composed of 1x ddPCR Supermix for Probes (no dUTP), 900 nM a forward primer, 900 nM reverse primer, 250nM the specified TaqMan Probe, and 5 ul sample DNA were mixed into 22 ul reactions. The QXManager software was used to visualize and analyze the target-positive and target-negative droplets. This software used a Poisson distribution to quantify the target DNA within the ~20,000 droplets in each reaction (6). The exact use of the ddPCR follows the S.O.P. detailed below.

To first establish the optimal primer annealing temperature for ddPCR, a gradient PCR was performed as suggested by the ddPCR Applications Guide. The gradient was run from 48 to 62°C, with 48 and 50°C determined to be the best temperatures with the greatest amount of positive droplets. For this experiment, a positive control of 1 ul of a 1/1000 dilution of total cellular DNA extracted from a skin biopsy sample of a *C. mydas*. Based on Qubit Fluorometric Quantification, the positive control yielded 61.2 ng/ul of genomic DNA, resulting in a mass of

61.2 pg/reaction after the 1/1000 dilution (6). The final thermocycling profile for the initial assays is outlined in the S.O.P. below, with the annealing temperature adjusted to 48°C.

To set the lower limit of detection for the eDNA samples, a serial dilution of the positive control was run on the ddPCR (6). This dilution started with the extracted genomic DNA of the *C. mydas* skin sample with an initial dilution of 1/100 (the 1/1000 dilution did not yield nearly as many positive droplets as the Hector’s dolphin used in Baker et. al. 2018). This was the first of 11 total dilutions, outlined below (Figure 2), as well as two negative controls. This serial dilution and negative controls were run in triplicate.

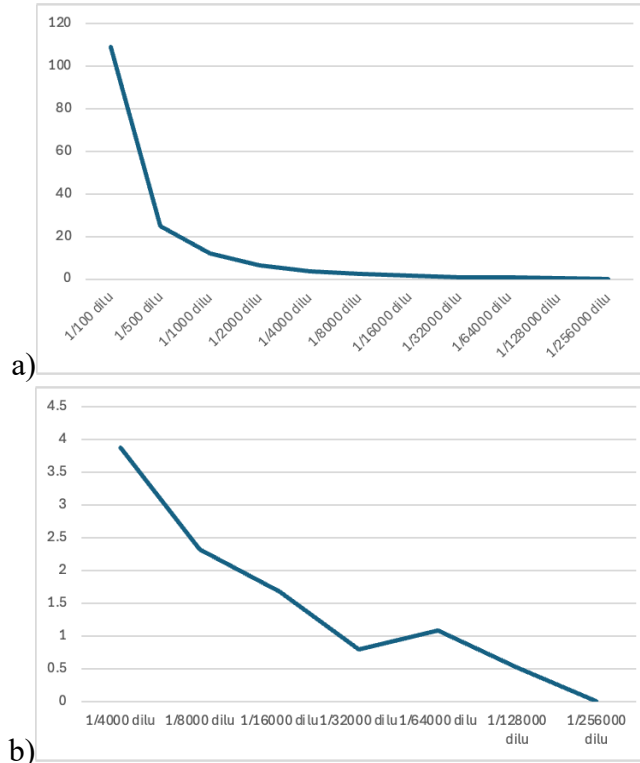


Figure 2 | a) serial dilution of positive control b) zoomed in serial dilution after 1/4000

ddPCR Assay Designs

Different primers and probes were used and designed for this project, but only one assay has been tested thus far (see designs below in Table 2). While primers previously designed for the control region (d-loop) of *C. mydas* were initially chosen for specificity between species (5), difficulty with designing probes that aligned with Bio-Rad’s recommendation of ddPCR shifted focus to universal primers within the *COX1* gene. Additionally, the control region of *C. mydas* is highly variable within species and while that would be an ideal target for intra-specific identification, it did not seem like the best target for just identifying the species as a whole. Instead, *COX1*, which is commonly used for barcoding, is quite conserved within species but could differ enough to be a better marker for species identification. However, a lot of the universal primers used for *COX1* created amplicons >700 bp in length. The ddPCR Applications Guide recommends amplicons <250 bp. Two pairs of primers (Uni-MinibarF1/Uni-MinibarR1 and miniCO1-F/miniCO1-R) were found to be universal “mini-barcodes” for *COX1*, and were initially



tested with regular PCR and several positive control samples of genomic DNA from *C. mydas* skin biopsies (7, 8). Of the two pairs of primers, only the Uni-MinibarF1/Uni-MinibarR1 pair was able to amplify the *C. mydas* samples. The probe was designed between those primers using Primer3Plus and PrimerQuest and perfectly aligned with all *C. mydas* COX1 genes from GenBank. The probe was tagged with a FAM fluorescent dye as well as a nonfluorescent quencher.

Type	Name	Sequence
Forward Primer	Uni-MinibarF1	5'- TCCACTAATCACAARGA TATTGGTAC-3'
Reverse Primer	Uni-MinibarR1	5'- GAAAATCATAATGAAGG CATGAGC-3'
Probe	GreenTurtle	5'- TCTAGGAGATGACCAAA TCTAT-3'

Table 1 | Primers and Probe used for this project



REFERENCES

1. Dutton, Peter H., et al. “Genetic analysis and satellite tracking reveal origin of the green turtles in San Diego Bay.” *Marine Biology*, vol. 166, no. 1, 27 Nov. 2018, <https://doi.org/10.1007/s00227-018-3446-4>.
2. Benson, Scott R., et al. “Large-scale movements and high-use areas of western Pacific leatherback turtles, *Dermochelys Coriacea*.” *Ecosphere*, vol. 2, no. 7, July 2011, <https://doi.org/10.1890/es11-00053.1>.
3. “Sea Turtle Conservation Gets Boost from New DNA Detection Method.” *News*, news.ufl.edu/2022/05/sea-turtle-edna/#:~:text=%E2%80%9CYou%20can%20say%20not%20only,marine%20animals%20C%E2%80%9D%20he%20said. Accessed 26 Feb. 2024.
4. Farrell, Jessica A., et al. “Detection and population genomics of sea turtle species via noninvasive environmental DNA analysis of nesting beach sand tracks and Oceanic Water.” *Molecular Ecology Resources*, vol. 22, no. 7, 22 Apr. 2022, pp. 2471–2493, <https://doi.org/10.1111/1755-0998.13617>.
5. Harper, Kirsten J., et al. “Finding crush: Environmental DNA analysis as a tool for tracking the green sea turtle *Chelonia mydas* in a marine estuary.” *Frontiers in Marine Science*, vol. 6, 20 Jan. 2020, <https://doi.org/10.3389/fmars.2019.00810>.
6. Baker, C. Scott, et al. “Environmental DNA (Edna) from the wake of the whales: Droplet digital PCR for detection and species identification.” *Frontiers in Marine Science*, vol. 5, 19 Apr. 2018, <https://doi.org/10.3389/fmars.2018.00133>.
7. <https://bmcbgenomics.biomedcentral.com/articles/10.1186/1471-2164-9-214>
8. <https://www.nature.com/articles/s41598-020-74918-9>



XX-000.00

Droplet Digital PCR using Bio-Rad's QX200 system

SOP IDENTIFIER:	XX-000
ORIGINAL SOP DATE:	05/24/2024
# OF PAGES IN SOP:	0
REVISION NUMBER:	00
REVISION DATE:	MM/DD/YYYY

WRITTEN BY:	Lena Stasiak	
REVIEWED BY:		
APPROVED BY:		

EFFECTIVE DATE: _____

Droplet Digital PCR using Bio-Rad's QX200 system

Scope and Purpose:

To provide high-precision, absolute quantification of nucleic acid target sequences.

Summary of Method:

This is a 1–2-day process, with the option to stop after the PCR step depending on time. The process includes droplet generation, PCR, and a droplet reader.

Health and Safety Warnings:

- Wear latex or nitrile gloves, closed-toe shoes and a lab coat.
- This procedure uses chemicals that may be harmful. Please read Appendix A before performing this protocol.

Quality Control:

- General Tips
 - Wear latex or nitrile gloves throughout protocol.
 - Include positive and negative controls in plate.
 - Use Rainin LTS pipettes and tips for all pipetting steps – avoids droplet shearing and potential reader clogging.
 - Use Deep well block for thermal cycling – provides even heating and cooling.
- Preparing Reactions
 - Choose dye or probe-based chemistry (specific Supermix, Generator Oil, Buffer Control).
 - Probe-based chemistry: 900nM FP, 900nM RP, 250nM each probe.
 - EvaGreen-based chemistry: 100-250nM each primer (FP conc = RP conc).
 - Make enough Mastermix for 22 uL/reaction (10% overage).
 - Vortex all reagents **thoroughly** prior to use.
 - Use restriction enzymes to separate repeats (CNV), aid droplet loading, open secondary structures.
- Droplet Generation
 - Load samples in full columns of 8.
 - When loading DG8, load sample first (middle row), then droplet generation oil (bottom row).
 - Ensure that the four outer holes of the gasket are oriented over DG8 holder pins.



NOAA FISHERIES

Southwest Fisheries Science Center

- Transfer DG8 to manual droplet generation ASAP after adding sample + oil and positioning gasket.
- Transfer droplets to ddPCR plate ASAP after droplet generation. DO NOT go to pipette second stop.
- Transfer ddPCR plate to thermal cycler within 40 minutes of generating the first column of droplets.
- Thermal Cycling
 - Follow thermal cycling conditions outlined in Supermix product insert.
 - Set ramp rate to 2 °C /s to ensure even heating/cooling of droplets.
 - Optimize annealing temperature (Bio-Rad thermal cyclers have temperature gradient feature).
 - If using existing real-time assay, decrease annealing temp by 2 degrees.
 - For amplicons >250bp, extend annealing time to 2 minutes.
- Droplet Reading
 - Prime the system if it has been left idle >1 week.
 - Add 50 mL of 10% bleach to the waste bottle when changing it out.
 - Ensure that around the lid of the waste container (inside and out) is clean.
 - Do not reuse waste bottles.

Equipment and Supplies: (see Reagent list in SOP Binder for reagent locations)

1. DG8 Manual Droplet Generator Cartridges
2. DG8 Gaskets
3. ddPCR 96-Well Plates
4. PCR Plate Heat Seal, Foil, Pierceable
5. ddPCR droplet reader oil
6. Droplet generation oil for probes
7. Droplet generation oil for EvaGreen
8. Rainin Pipettes (10ul, 100ul, 1000ul)
9. Rainin Pipette tips (10ul, 100ul, 1000ul)
10. Bench Paper
11. Gloves
12. Vortex
13. Mini centrifuge

Reagents:

- QX200 ddPCR EvaGreen supermix
- QX200 buffer control for EvaGreen
- ddPCR supermix for probes (no dUTP)
- ddPCR buffer control for probes

Procedures:

Reaction Setup (Approximate time: 30 mins) – complete in Pre-PCR Lab

1. Thaw all components to room temperature.
2. Mix thoroughly by vortexing (15 seconds on full speed).
3. Centrifuge briefly to collect at the bottom of the tube.
4. Make up Mastermix excluding sample (see below).

Component	Stock concentration	Final concentration	Volume per Reaction	13X Master Mix
2X ddPCR Supermix for Probes (No dUTP)	2X	1X	11 ul	143 ul
FAM assay	20X	900 nM (primers) 250 nM (probes)	1.1	14.3
HEX assay	20X	900 nM (primers) 250 nM (probes)	1.1	14.3
FAM assay template	~20,000 copies/ul	Dilution series	1.1	-
HEX assay template	~7,576 copies/ul	~1,000 copies/ul	2.89	37.57
Diluted restriction enzyme*	-	-	-	-
Nuclease-free water	-	-	4.81 ul	62.53 ul
Total Volume	-	-	22 ul	271.7 ul

*Add enzyme if loading more than 66 ng gDNA or performing CNV assay

5. Mix thoroughly by vortexing (15 seconds on full speed).



6. Prepare reactions (Mastermix + sample) in Bio-Rad ddPCR Plates, adding enough for duplicate reactions + 10% (44 uL) in columns.
7. Seal plate with a removable plate seal.
8. Mix thoroughly by vortexing (15 seconds on full speed).
9. Quickly spin down plate.

Generate Droplets with the DG (Approximate time: 40 minutes for full plate)

1. Place DG8 cartridge in DG8 holder.
2. Load 20 ul of reaction mix into **all 8 wells** of middle row of DG8.
3. Load 70 ul of droplet generation oil into **all 8 wells** of bottom row of DG8.
4. Position gasket over DG8 holder, with holder pins latched onto the gasket.
5. Press DG lid button once to open DG.
6. Load DG8 into DG; press DG lid button once to close DG and begin droplet generation.
7. Transfer droplets from top row of DG8 to ddPCR plate (~40 ul). Be gentle; go to first stop only.
8. After generation, seal plate with pierceable foil seal (**PX1 plate sealer, 180°C, 5 seconds**).

Cycle droplets – PCR (Approximate time: 2-3 hrs)

1. Place plate into deep well C1000 block and cycle as follows:

Cycling Step	Temperature °C	Time	Ramp Rate	# of Cycles
Enzyme Activation	95	10 min	2°C/sec	1
Denaturation	94	30 sec		40
Annealing/Extension	60*	1 min		40
Enzyme Deactivation	98	10 min		1
Hold (optional)	12	Infinite		1

Read Plate (QX200 Droplet Reader) (Approximate time: (2 hrs for full plate)

1. Turn on reader >30 min prior to use.
2. Open *QX Manager* on the laptop attached to the reader.
3. Navigate to the *Initialize Run* tab (top of the lefthand menu).
4. Click *Add Plate* green plus sign.
5. Load plate (in plate holder) into the QX200.
6. Click *Configure Plate*.



7. Under *Plate Information (Required for Run)* tab's *Create New Plate* section, designate plate name, supermix, and data file name.
8. Navigate to *Well Selection (Required for Run)* tab.
9. Select well(s) to include in droplet reading; hit *Include Selected Wells*.
10. Optional: navigate to *Well Information (Optional)* tab.
11. Optional: designate experimental type, sample description(s), sample type, target information, well notes, and/or plate notes. Hit *Apply* to attach that information to the well(s) of interest (may have to scroll to the side to find *Apply* button).
12. Click *Start Run* to start droplet reading.

QX200 Data Analysis

1. Open file in *QX Manager*.
2. Verify Number of Droplets
 1. Select all reaction wells in the Plate Map.
 2. Click on the *Event Counts* tab.
 3. Confirm all wells have >10K droplets
3. Verify Successful Amplification
 1. Click on *1D Amplitude* (for singleplex) or *2D Amplitude* (for multiplex).
 2. Verify absence of positive droplets in negative control sample/NTC.
 3. Verify presence and separation of positive and negative droplets in positive control sample.
4. Set Threshold (1D)
 1. Go to *1D Amplitude* tab.
 2. Auto-threshold automatically applied.
 3. If desired, apply manual threshold (individual/multiple wells) or standard deviation threshold (individual/multiple wells).
 - i. For standard deviation threshold, must click between positive and negative droplets for this threshold to be applied.
 1. Click + drag pink threshold lines to adjust.
5. Set Threshold (2D)
 1. Go to *2D Amplitude* tab.
 2. Auto-threshold automatically applied.
 3. If desired, apply manual threshold (individual/multiple wells) or standard deviation threshold (individual/multiple wells).
 - i. For standard deviation threshold, must click between positive and negative droplets for this threshold to be applied.
 1. Click + drag pink threshold lines to adjust.
6. View All Data
 1. Go to *Data Table* tab.



NOAA FISHERIES

Southwest Fisheries Science Center

2. To adjust the data you see, click the four lines at the top right of the table and select/deselect wells.
7. Generate a Report
 1. Go to *Reports* tab.
 2. Select report template or create a new template by selecting what you want included in the report and clicking *Save*.
 3. Select wells to generate report from; title report; click *Generate Report*.

Waste Management:

Plastics and other liquids can be thrown in the trash. If there are questions on handling chemicals contact the Safety Officer or Lab Manager. Spills, cuts or other accidents should be reported to the Safety Officer and Lab Manager.

References:

QX200 + Manual Droplet Generator (MDG) ddPCR – Workflow

Bio-Rad's Droplet Digital PCR Applications Guide

For QX200 ddPCR Support, go to QX200 ddPCR system resource hub: <http://info.bio-rad.com/QX200-ddPCR-Resource-Hub.html>

Technical Support: 1-800-4-BIORAD option 2

Applications Support: ankita_das@bio-rad.com

Sales Support: april_yu@bio-rad.com

For SDS information, go to C:\\Users\\ljuser\\Desktop\\MMTD Genetics SDS (see Lab Manager for designated computers).

Hazardous substance information: <http://epa.gov/ceppo/pubs/title3.pdf>

Appendix A: Chemical Health and Safety Warnings

List MSDS for all reagents here. Check master list for reagent MSDS.

(Name of chemical here)

Toxic if absorbed through eyes, skin and inhalation. Heating of this reagent may cause explosion.

First Aid

Inhalation: move to fresh air.

Eyes: flush with water

Skin: wash with water.

Ingestion: wash out mouth completely with water if person is conscious. Seek medical attention immediately.



XX-000.00

Extraction of eDNA from filters using the Qiagen DNeasy Blood and Tissue Kit

SOP IDENTIFIER:	XX-000
ORIGINAL SOP DATE:	05/24/2024
# OF PAGES IN SOP:	0
REVISION NUMBER:	00
REVISION DATE:	MM/DD/YYYY



WRITTEN BY:	Lena Stasiak	
REVIEWED BY:		
APPROVED BY:		
EFFECTIVE DATE:		

Extraction of eDNA from filters using the Qiagen DNEasy Blood and Tissue Kit

Scope and Purpose:

To isolate eDNA from filters.

Summary of Methods:

This is a 2 day extraction consisting of separation of eDNA from filter, followed by silica based DNA purification using spin columns.

Health and Safety Warnings:

- Wear latex or nitrile gloves, closed-toe shoes and a lab coat.
- This procedure uses chemicals that are harmful. Please read Appendix A before performing this protocol.

Quality Control:

- Wear latex or nitrile gloves throughout extraction protocol.
- Always use filtered tips and change them each time when adding reagents to samples if tissue is already inside the wells.
- Include a negative control for each batch of extractions (1 negative per 2 samples extracted, so 8 samples and 4 negative controls per batch). Be sure to keep the negative in the same order amongst other samples throughout the extraction process.

Equipment and Supplies (see reagent list in the SOP binder for reagent locations)

1. Bench paper
2. Gloves
3. Bleach
4. MilliQ water
5. 1.7 ml tubes
6. 2 ml tubes
7. 5 ml tubes
8. Pipettes (1000 ul, 100 ul)
9. Pipette tips (1000 ul, 100 ul)

10. Vortex
11. DNEasy columns
12. DNEasy collection tubes
13. Heat block
14. Centrifuge
15. Extraction Reagents:
 - Longmire's buffer for controls
 - Proteinase K (PK) (see SOP GE-001.00)
 - Buffer AL
 - 100% EtOH
 - Buffer AW1
 - Buffer AW2

Procedures:

Day one - Separation of DNA from filter (Approximate time: 2.5-3 hrs)

1. Create extraction grid (see SOP PR-010.00)
2. Make sure thermomixer is on and set to 95°C
3. Make 4 negative controls by adding 4 mL of Longmire's Buffer to 5 mL tubes
4. Heat shock the samples and controls at 95°C for 5 mins in thermomixer
5. Vortex samples and allow them to return to room temperature
6. Add 100 ul of proteinase K per 1 mL of sample (400 uL for 4 mL)
7. Incubate at 56°C and 120 rpm for 2 hours in thermomixer
8. Change temperature to 37°C and leave samples in thermomixer overnight

Day two - Precipitation and Elution (Approximate time: 2 hrs)

1. Ensure that ethanol (EtOH) has been added to Buffers AW1 and AW2
2. Remove 5 mL tubes from thermomixer, and transfer 600 uL of each sample into a new 2 mL tube (the rest of the samples can be stored in -20°C freezer)
3. To the 600 uL of the template, add 630 uL of Buffer AL, and 630 uL of 100% EtOH
4. Invert to mix
5. In 4 iterations, transfer 465 uL of the mix from tubes to a DNEasy Mini spin column placed in a 2 ml collection tube
6. Centrifuge at >6000 x g (8000 rpm) for 1 min. Discard the flow-through and collection tube and repeat until full mix has gone through filter (4 iterations of 465)
7. Place the spin column in a new 2 ml collection tube. Add 500 ul Buffer AW1. Centrifuge for 1 min at >6000 x g. Discard the flow-through and collection tube.
8. Place the spin column in a new 2 ml collection tube. Add 500 ul Buffer AW2 and centrifuge for 3 min at 20,000 x g (14,000 rpm). Discard the flow-through and collection tube.
9. Transfer the spin column to a new 1.7 ml microcentrifuge tube
10. Elute the DNA in two iterations. First, add 100 ul of low TE to the center of the spin column membrane. Incubate for 5 mins at room temperature. Centrifuge for 1 min at >6000 x g.



NOAA FISHERIES

Southwest Fisheries Science Center

11. Second, repeat step 10 with 50 ul of low TE, incubate for 1 min at room temperature, and centrifuge again for 1 min at >6000 x g.
12. Quantify using Qubit and store at -20°C

Waste Management

Plastics and other liquids can be thrown in the trash. If there are questions on handling chemicals contact the Safety Officer or Lab Manager. Spills, cuts or other accidents should be reported to the Safety Officer and Lab Manager.

Buffer AW1 contains guanidine hypochloride, which can form highly reactive compounds when combined with bleach or acidic solutions. If spilt, clean with suitable laboratory detergent and water.

References:

DNeasy Blood and Tissue Handbook. Qiagen, www.qiagen.com/ts/msds.asp

For SDS information, go to C:\\Users\\ljuser\\Desktop\\MMTD Genetics SDS (see Lab Manager for designated computers)

Hazardous substance information: <http://epa.gov/ceppo/pubs/title3.pdf>

Appendix A: Chemical Health and Safety Warnings

Buffer AL – contains guanidine hydrochloride which is a harmful irritant, and can form highly reactive compounds when combined with bleach or acidic solutions.

First Aid

Harmful if swallowed, supply fresh air after contact. Irritating to eyes and skin, after eye contact, rinse affected eye for several minutes with running water. After skin contact, immediately wash with water and soap and rinse thoroughly.

Proteinase K – sensitizer and irritant. Irritating to the eyes, respiratory system and skin. It may cause sensitization by inhalation and skin contact.

First Aid

In case of contact with eyes, rinse immediately with plenty of water and seek medical advice.

100% Ethanol - Causes moderate skin irritation. May cause cyanosis of the extremities and dermatitis. Inhalation may cause respiratory tract irritation, nausea, headaches, dizziness and suffocation.

First Aid

If inhaled, supply fresh air and oxygen. For eye contact, rinse open eyes for at least 15 minutes under running water. If there is skin contact, remove contaminated clothing and immediately wash area with soap and water. If ingested, wash mouth with water and seek medical attention. Do not induce vomit.

Note: highly flammable.

AW1 buffer (concentrate) – contains guanidine hydrochloride which is a harmful irritant, and can form highly reactive compounds when combined with bleach or acidic solutions. Harmful when swallowed, irritant to eyes, skin and the respiratory system.

First Aid

In case of contact with eyes, rinse immediately with plenty of water and seek medical advice. If swallowed, seek medical advice immediately, and show container labels.

AW2 buffer - No MSDS is needed