American Museum of Natural History
Center for Biodiversity and Conservation
Network of Conservation Educators & Practitioners

What's in the Water? Using environmental DNA for Marine Monitoring and Planning

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Figure 4. Filtering seawater samples in a lab. Photo credit: NOAA fisheries.

Barcoding
Scientists find diagnostic DNA sequences called barcodes that they use like fingerprints to identify individual species.

Metabarcoding
Metabarcoding is a technique to efficiently find many barcodes from many different organisms at one time.

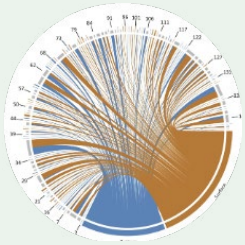
eDNA
Scientists collect "environmental DNA" in water samples and use metabarcoding to identify what animals have been there.

Figure 5. What is eDNA? DNA barcodes are akin to genetic fingerprints for a species. The process of DNA barcoding involves samples collected directly from the species of interest and builds a genetic database of known species against which new samples can be compared. Metabarcoding is a process where genetic information from many *different organisms is sequenced at the same time, and species are identified comparing these to a database of known sequences. Environmental DNA uses metabarcoding to identify the genetic material of organisms in an environmental sample. Image credit: Natalie Renier and Eric S. Taylor, WHOI Creative (www.whoi.edu/oceanus/feature/round-up-the-unusual-suspects/).*

Box 1: Example Applications of eDNA in Marine Conservation

Environmental DNA can be used to monitor individual species of management interest, such as the crown-of-thorns seastar (COTS; *Acanthaster planci*), which decimates reefs as it feeds directly on corals. During the most recent COTS outbreak in the Great Barrier Reef, ecological monitoring failed to detect early outbreak stages, leading to 42% loss of all coral cover. Researchers have since developed a digital droplet PCR-based method for early detection of outbreaks, providing managers with more time for intervention against this ecologically disruptive species (Uthicke et al. 2018). Photo credit: Johan J.Ingles-Le Nobel/Flickr (CC BY-ND 2.0).

Environmental DNA can be used to survey groups of species. In a species-rich sea off the coast of Japan, researchers using eDNA detected 62.5% of fish species that were observed by visual census, while also detecting another 23 species not documented by visual census (Yaramoto et al. 2017). When comparing the 6-hour collection of water samples to the 14-year period of visual census, eDNA shows considerable advantages in time investment compared to conventional methods. Image credit: Yamamoto et al. 2017.



Environmental DNA can be used for broad biodiversity assessments. Researchers in the Red Sea detected a diverse set of small, often well-hidden species while characterizing the rich animal community of these reefs (Carvalho et al. 2019). The ability to distinguish among elusive and seemingly identical species provides researchers and managers with valuable new information. Photo credit: Derek Keats/ Flickr (CC BY 2.0).

Detecting Priority Taxa for Conservation: The Case of Hawaiian Coral Reef Fishes

Fishes represent one of the success stories for eDNA in terms of detecting target taxa and estimating relative abundance (Takahara et al. 2012; Kelly et al. 2014; Valentini et al. 2016; Yamamoto et al. 2017). However, despite its utility, eDNA technology is not yet widely used to support marine protected area selection. Visual surveys conducted by divers are still the go-to method for collecting census data on fish species.

The Hawaiian Islands offer a natural laboratory for detecting target fish and investigating patterns of coral reef fish diversity through eDNA approaches because previous research and monitoring in Hawai'i (Grigg 1994; Friedlander et al. 2002, 2003) provide a comprehensive baseline against which to fine-tune eDNA analysis.

As mentioned earlier in this exercise, you have been invited to a fictional meeting in Hawai'i to conduct marine reserve planning. While there are many working groups at this meeting considering a wide variety of dimensions within marine reserve planning, your working group is focused on ensuring that the 13 priority coral reef fish species identified are included in the site selection process. Your working group is tasked with designing a marine protected area system that:

1. Enhances species representation by ensuring that all 13 priority species are included at least once; and
2. Minimizes the total number of sites in the reserve system due to budgetary constraints.

The basic procedure you will follow below (Parts B and C) is a simplified example of a more complex process of marine reserve planning and site selection, which is often carried out with the help of software programs and is typically influenced by the priorities and constraints of the decisionmakers involved.

Part A: Learning about Hawaiian Coral Reef Fishes

The instructor will first assign priority fish species. Students will work in pairs or in groups to familiarize themselves with their assigned target species by researching and summarizing in table format information that addresses: Where do they occur? What is their conservation status on the US Endangered Species list or the IUCN Red List, if applicable? What are their habitat preferences? Why were they targeted for monitoring and management?

These sites may be useful in your research:

- fishbase.org: A global fish species database with taxonomy, geographical distribution, biometrics and morphology, behavior and habitats, ecology and population dynamics as well as reproductive, metabolic, and genetic data.
- iucnredlist.org: A comprehensive inventory of the global conservation status of biological species, including information similar to FishBase.
- obis.org: Ocean Biogeographic Information System, a web-based portal for information about the distribution and abundance of ocean species.

The first species has been filled out for you:

Acanthurus achilles (Achilles tang) **Pākuʻikuʻi**	1. Central & Western Pacific, limited in eastern Pacific, concentrated in south-central Pacific; concentrated in Main Hawaiian Islands, but also present in Northwestern Hawaiian Islands. 2. IUCN Least Concern; listed by Hawaiʻi DLNR as a Regulated Species under Oʻahu Aquarium Fishing Rules. 3. Benthopelagic, occurring mostly in groups in clear seaward reefs, often in shallow water (<5m) exposed to wave action. 4. Exploited extensively as ornamental catch in aquarium trade; popular food fish in western Hawaiʻi. *Reason for Priority Status:* control of turf algae; catch for the aquarium trade.

Part B. Designating Sites for Protection Using Visual Survey Data

At the meeting, researchers from the National Oceanic and Atmospheric Administration (NOAA) have provided your working group with occurrence data for the 13 priority fish species from a survey effort conducted during their coral reef ecosystem monitoring program (Table 1). To create this occurrence data table, NOAA divers skilled in taxonomic identification performed stationary-point-count visual surveys (Figure 2) at the nine sites flagged as potential locations for protected designation (Figure 6).

Review the occurrence data from the following nine sites (A through I) outlined in Table 1. Can you achieve the two above goals of minimizing the total number of sites while also making sure all species are represented in your final site selection?

Which sites would you select for MPA designation and why?

Part C: Using eDNA Data

A PhD student from a local university is in attendance at the meeting, just back from spending the field season collaborating with NOAA scientists on reef monitoring projects. Eager to test out her lab's newly acquired thermocycler (a machine used to amplify segments of DNA via PCR) and sequencer, she collected water samples at each of the nine sites while the visual fish surveys were occurring. She then isolated the DNA and promptly sent the samples back to her lab for amplification and sequencing analysis. She hasn't had a chance to fully analyze the resulting data yet—at this point, she just has sets of sequences from each of the sites—but she's happy to share these data with your working group (see Appendix I: eDNA Hawaiʻi Fish Sequences). Could her data give us new information?

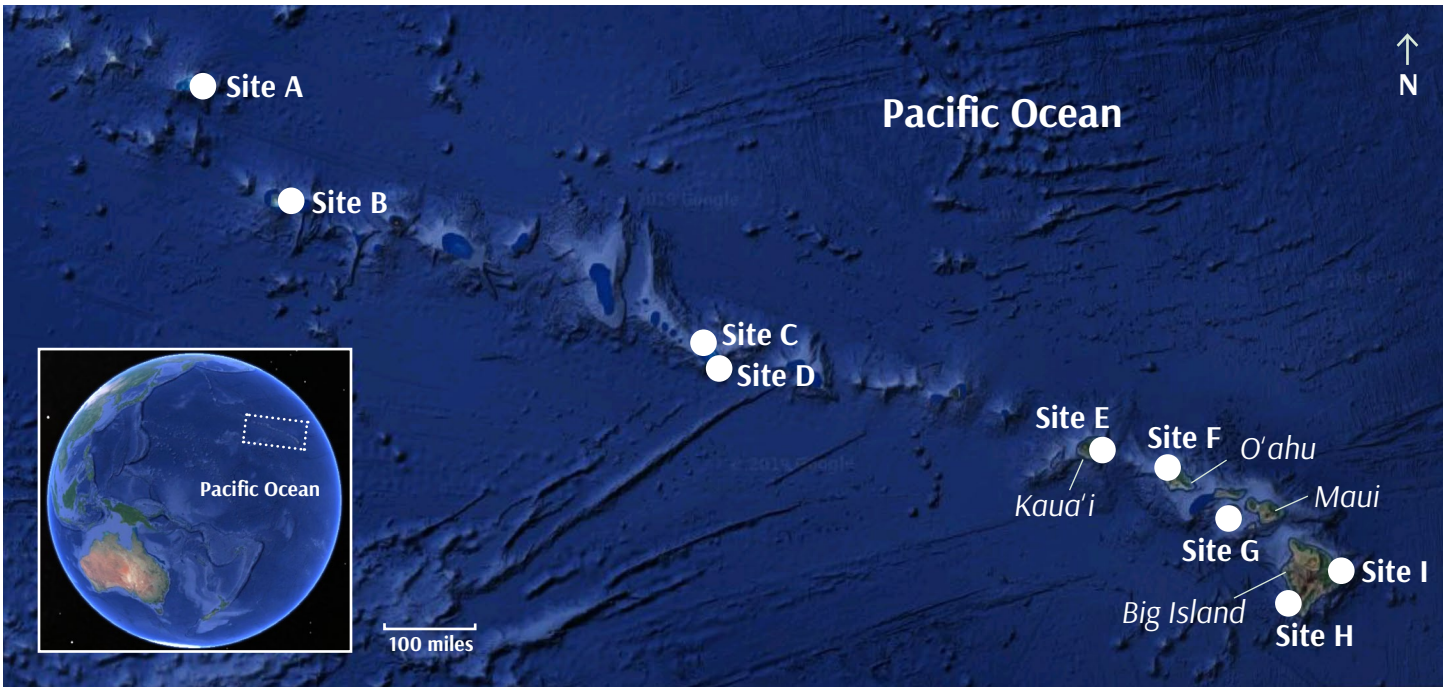


Figure 6. Locations of fictional sites A through I, identified as possible locations for protected designation throughout the Hawaiian Archipelago. Sites A-D are located in the Northwestern Hawaiian Islands, while sites E-I are located in the Main Hawaiian Islands. Names of several Main Islands are provided for reference. Global view (inset image) shows archipelago location in the Central Pacific. Image credit: Google Earth.

Table 1. Characteristics of the nine fictional marine sites, as determined by stationary point count (SPC) visual survey. The occurrence of each priority fish species at every site is entered as "Y" if the group is found there, and "N" if not.

FISH SPECIES	**SITE**								
	A	**B**	**C**	**D**	**E**	**F**	**G**	**H**	**I**
Achilles tang (*Acanthurus achilles*)	Y	N	Y	N	N	N	Y	N	Y
Eyestripe surgeonfish (*Acanthurus dussumieri*)	N	N	Y	Y	N	Y	Y	Y	Y
Convict surgeonfish (*Acanthurus triostegus*)	Y	N	N	Y	Y	N	Y	Y	Y
Bluefin trevally (*Caranx melampygus*)	Y	Y	Y	N	N	N	N	N	N
Bullethead parrotfish (*Chlorurus spilurus*)	Y	N	Y	N	Y	Y	N	N	N
Yellowfin goatfish (*Mulloidicthys vanicolensis*)	N	Y	N	Y	N	Y	N	N	N
Bluespine unicornfish (*Naso unicornis*)	Y	N	N	Y	N	N	Y	N	N
Whitesaddle goatfish (*Parupeneus porphyreus*)	N	N	N	N	Y	N	N	N	N
Sixfinger threadfin (*Polydactylus sexfilis*)	N	Y	N	N	N	Y	N	N	N
Ember parrotfish (*Scarus rubroviolaceus*)	Y	N	N	N	Y	Y	N	Y	N
Green jobfish (*Aprion virescens*)	Y	N	N	Y	Y	N	Y	Y	Y
Island trevally (*Carangoides orthogrammus*)	Y	N	N	N	N	N	Y	Y	N
Scalloped hammerhead (*Sphyrna lewini*)	N	Y	Y	Y	N	Y	N	N	Y
Total number of species present at each site	**8**	**4**	**5**	**6**	**5**	**6**	**6**	**5**	**5**

BLAST the Sequences!

In order to determine if the eDNA is helpful, you will use the reference sequence data derived from eDNA samples to identify the fish species present at each site. The National Center for Biotechnology Information's (NCBI) Basic Local Alignment Search Tool (BLAST) is a useful tool for this task: BLAST identifies regions of similarity between

sequences and compares the provided sequence with sequence databases to calculate the statistical significance of matches. To get started, visit: blast.ncbi.nlm.nih.gov/Blast.cgi (Figure 7).

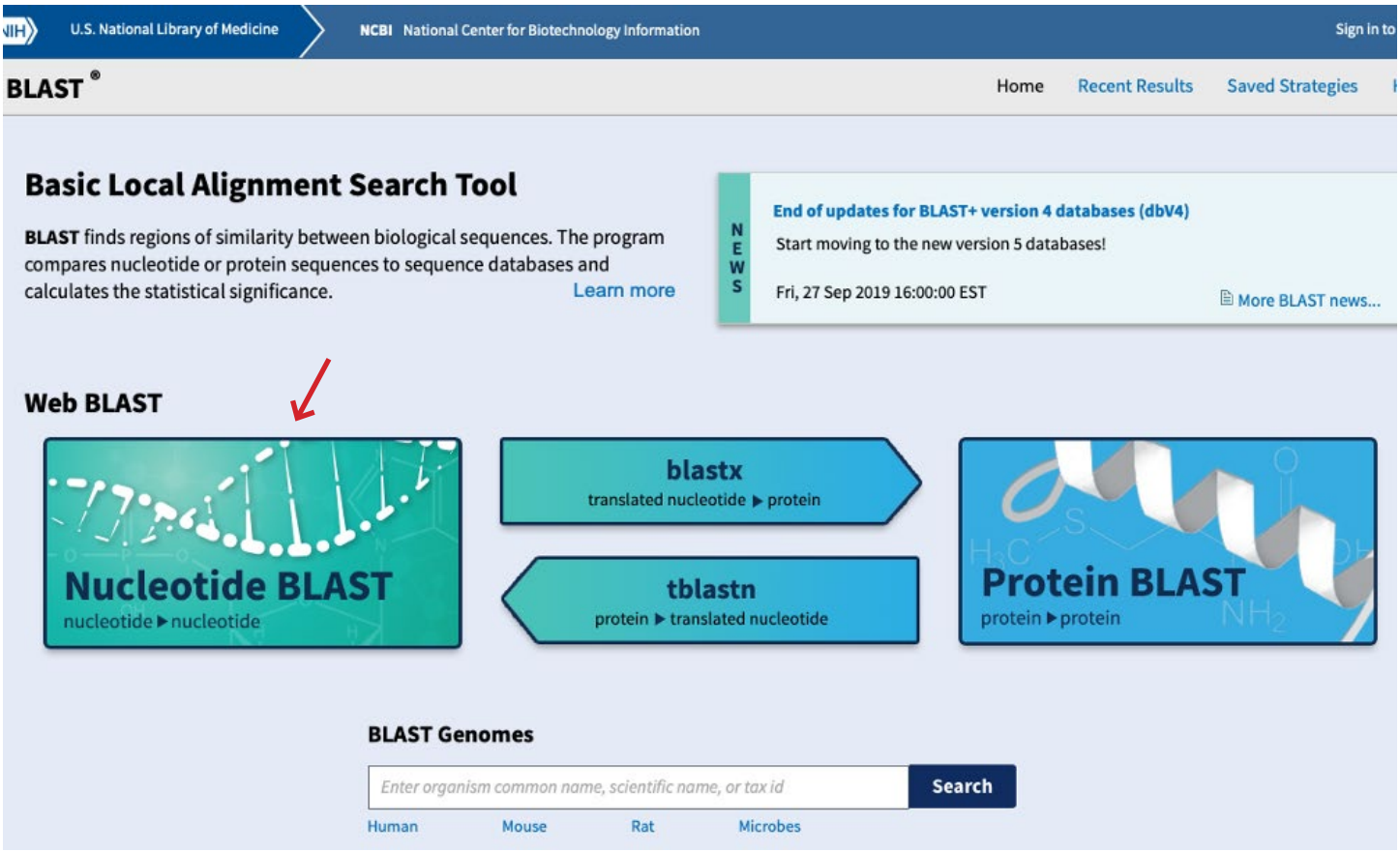


Figure 7. The National Center for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST) landing page. Click on "Nucleotide BLAST" to access the Standard Nucleotide BLAST page.

Using the provided reference sequence data in Appendix I, identify all of the fish species present from the water sample collected from each of the nine sites to create a new occurrence table (Table 2). **Note**: water samples may not include DNA from every species that is present at the site, it is just an inventory of the DNA that was in the sample. The absence of a species' DNA in a sample doesn't necessarily mean the absence of that species at a site; the species could be there, but just not detected in that sample. In this way, eDNA results can represent a minimum list of species that are actually present at the site. On the other hand, the presence of DNA from a particular species is a good indicator that that species is actually present—with the understanding that contamination could be possible in the collection and lab process, and therefore researchers should follow strict laboratory methods and perform re-sampling to confirm results.

Each group of students will receive a set of reference sequences found in the water sample from one of the nine sites. As a group, perform a BLAST on each of the eDNA sequences found at your site to determine which species were present in the sample, one sequence at a time (Figure 8). Results may take a few moments to load, but this step should only take a few minutes.

Once you BLAST your sequence, a new results page will display (Figure 9). You'll know it's a match if the Percent Identity is 100% (or whichever reference sequence returns the highest percent identity). In order to confidently obtain a species-level ID for an eDNA sequence, there should be at least a 97–98% similarity to a reference sequence. At lower levels of similarity (<97%), you may identify the genus of the organism, but likely not the species. Use this information to complete your site's column in Table 2.

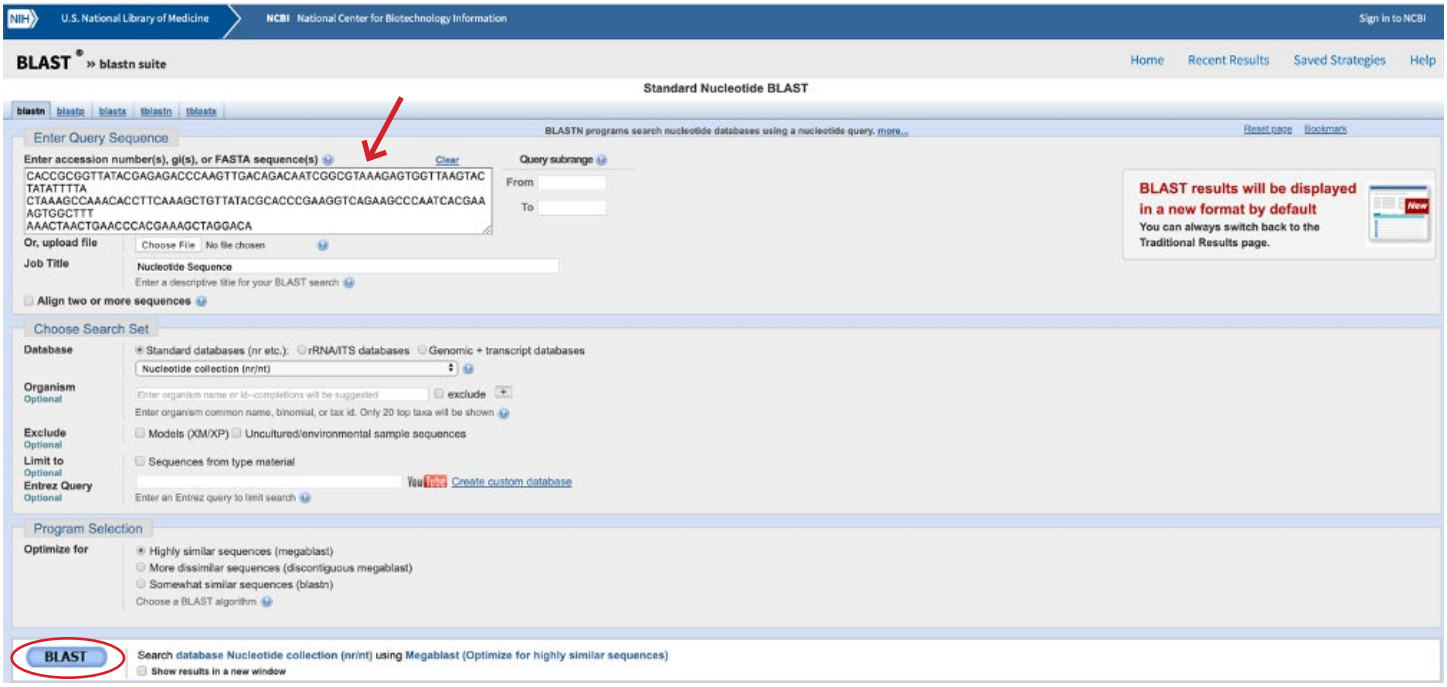


Figure 8. Enter each sequence individually in the "Enter Query Sequence" box. Then, click the "BLAST" button at the bottom of the page. All other default settings can be maintained.

Figure 9. Results page. Locate the "Percent Identity" column to select the best match (100% or whichever reference sequence returns the highest percentage).

It is possible that you may get more than one species with a 100% Percent Identity for a reference sequence. This could be due to many reasons. For example, the gene region that you are testing may be similar across multiple species. For the purpose of this exercise, make note of any time multiple species get 100%—but if one of these results is a priority species, record it as an occurrence in Table 2. If you were going to investigate these results

further, then a possible approach could be to compare the amount of DNA associated with each of those 100% matches (which is data not included in this exercise). Alternatively, you may only be able to identify the sequence at the genus level.

Once you've identified which species are present at your site, complete a new occurrence table as a class (Table 2).

Table 2. Characteristics of the nine fictional marine sites, as determined by eDNA data. Mark the occurrence of each priority fish species with a "Y" if the species is found there, and "N" if not.

FISH SPECIES	**SITE**								
	A	**B**	**C**	**D**	**E**	**F**	**G**	**H**	**I**
Achilles tang (*Acanthurus achilles*)									
Eyestripe surgeonfish (*Acanthurus dussumieri*)									
Convict surgeonfish (*Acanthurus triostegus*)									
Bluefin trevally (*Caranx melampygus*)									
Bullethead parrotfish (*Chlorurus spilurus*)									
Yellowfin goatfish (*Mulloidicthys vanicolensis*)									
Bluespine unicornfish (*Naso unicornis*)									
Whitesaddle goatfish (*Parupeneus porphyreus*)									
Sixfinger threadfin (*Polydactylus sexfilis*)									
Ember parrotfish (*Scarus rubroviolaceus*)									
Green jobfish (*Aprion virescens*)									
Island trevally (*Carangoides orthogrammus*)									
Scalloped hammerhead (*Sphyrna lewini*)									
Total number of species present at each site									

Do you reach a different conclusion about which sites to prioritize when using eDNA data only? What differences did you find between the two datasets? Why might this be the case?

Finally, what sites would you prioritize if you had access to both datasets, and why?

Part D: Discussion Questions

1. What are some pros and cons of representing a target species only once among these sites? How would your analysis be affected if, for example, there was a rare or threatened species at site G?
2. In some case(s), no definitive species match was found in the database. Why might this be the case?
3. In real life, sequencing data is never as clean and clear as the sequencing data presented here. Typically, sequencing machines do not read eDNA sequences perfectly, occasionally mistaking a C for a T, for instance (though this is often <1% of the time). Additionally, there will even be some variability of the barcode among individuals of the same species! How can sequences without 100% identity to a reference still be useful for biodiversity assessment?
4. Are there additional biological factors beyond fish presence information that you think should be considered to provide a sound scientific basis for reserve selection?

SECTION 2. BROADENING PERSPECTIVES: AN EXPLORATION OF OTHER APPROACHES TO PROTECTING MARINE ECOSYSTEMS

The first section of this exercise took a site-based approach to designating an MPA and your working group was focused specifically on identifying sites based on a suite of fish species. However, protected areas can fall under many types of conservation and management methods, and MPAs as we have defined them here are just one approach to mitigating threats to marine ecosystems and conserving biodiversity. For example, around the main islands of Hawai'i, there are more than eight types of marine managed areas. These areas can range from species-specific conservation areas like the federally managed Hawaiian Islands Humpback Whale National Marine Sanctuary, to state managed areas with very specific fishing regulations, like Lay Net Fishing Prohibited Areas (Figure 10). To explore the locations and different types of marine managed areas in Hawai'i, check out the interactive map produced by the Hawaii Statewide GIS Program: histategis.maps.arcgis.com/apps/webappviewer/index.html?id=87c5df3d6519482fa860769e33475a26.

The largest area of marine conservation in Hawai'i is also the world's largest: Papahānaumokuākea National Monument. To protect a significant part of the unique biological and cultural diversity found here, the Papahānaumokuākea National Monument was first established in 2006 and was later expanded to its current size—582,578 square miles of the Pacific Ocean—in 2016 (Figure 10).

This large-scale approach to marine ecosystem protection differs from the criteria laid out in this exercise. Instead of minimizing the area under protection while maximizing the number of priority fish protected, a more holistic approach was used to argue for the National Monument designation. Papahānaumokuākea is a sacred place;

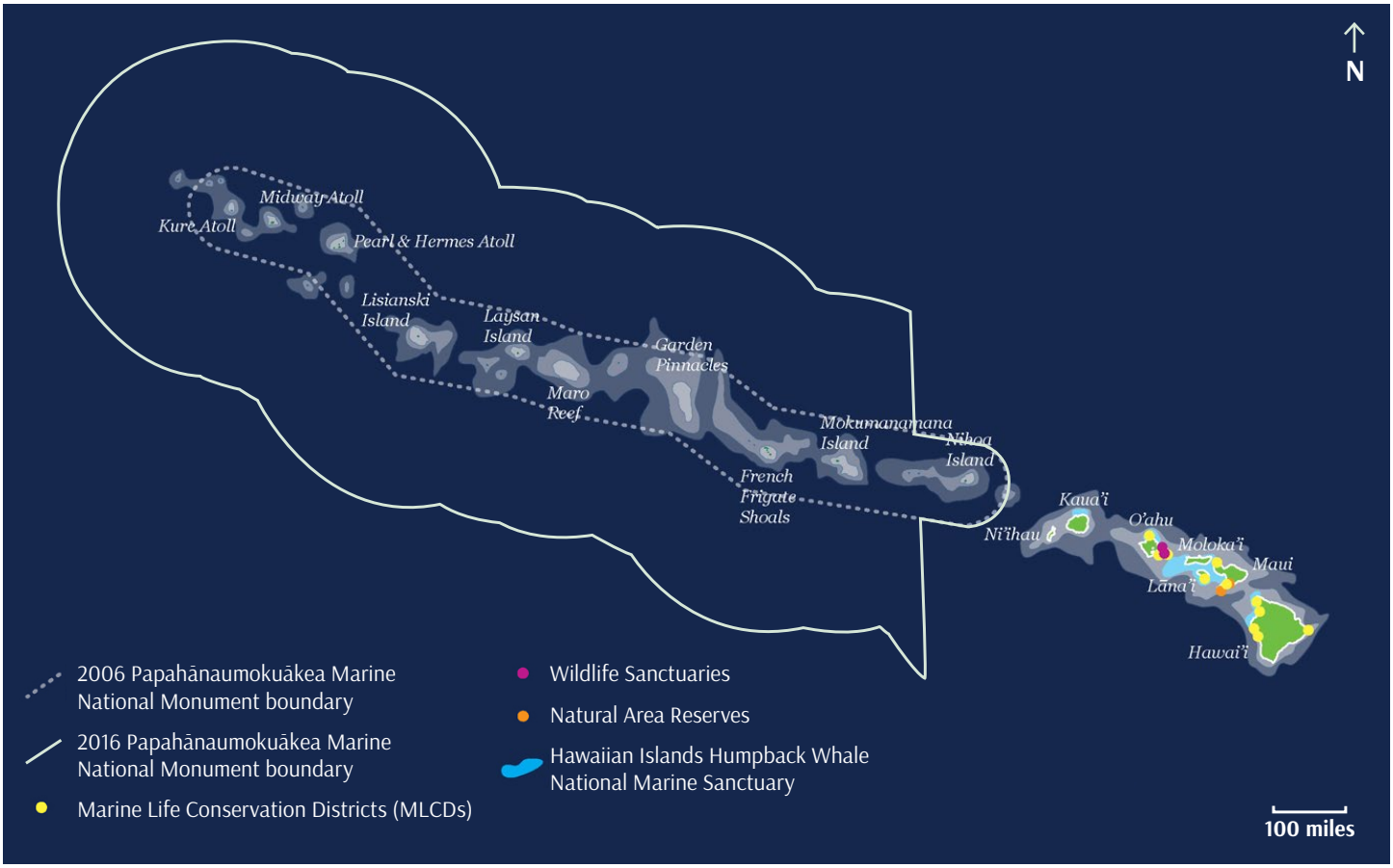


Figure 10. Map showing the different types of marine managed areas in Hawai'i, including the original extent of Papahānaumokuākea Marine National Monument and its 2016 expansion. Image credit: NOAA and the State of Hawai'i (adapted).

its islands, reefs, atolls, and waters hold great traditional and spiritual importance for Native Hawaiians. Over 7,000 marine species reside here, including, but not restricted to, the priority species listed in this exercise. The Monument is administered jointly by four co-trustees, including federal and state agencies as well as the Office of Hawaiian Affairs, a public agency responsible for improving the well-being and representing the interests of the Native Hawaiian community. Access to the Monument is limited to specific purposes (e.g., conducting cultural practices, habitat restoration, or scientific work). The grand size of Papahānaumokuākea's protected waters is atypical; marine spatial planning and reserve design often operates on a much smaller scale, where connectivity, adjacency, complementarity, and metapopulation structure are key elements of reserve design, as is linking reserves into a network—as you've practiced here in this exercise.

Although most government-managed marine conservation areas are still more focused on specific species or practices at individual sites, the more holistic approach of Papahānaumokuākea National Monument builds off of a long history of sophisticated, complex coastal and marine resource management systems that Hawaiians have been practicing (and continue to practice) since before Western contact. The Hawaiian kapu system—a system of rules and regulations embedded in society norms—includes marine customary stewardship practices, such as guidelines for fishing activity. Other traditional practices include harvest management focused on maintaining habitats and basic processes of important food resources (e.g., not interfering with fish spawning), instead of focusing on specific amounts of fish (Friedlander et al. 2013). Additionally, at the local level, land and marine resources were and are still managed together under a land unit that stretches from mountain to sea (ahupuaʻa).

Currently, many communities are reviving traditional knowledge to strengthen their influence on marine resources (Friedlander et al. 2013). Recent surveys in Hawaiʻi show that community-managed areas (using customary stewardship practices) can be just as effective as no-take MPAs that are government managed (Vaughan et al. 2016), and that a mix of traditional and modern scientific approaches can be used successfully and cost-effectively to monitor many aspects of fish ecology, such as lunar spawning cycles and seasonal spawning peaks (Schemmel et al. 2016). Communities, researchers, and agencies are actively collaborating to explore better ways to harmonize customary and state management (Vaughan et al. 2016).

The success of areas designed, governed, and protected in diverse ways is being recognized outside of Hawaiʻi as well. The International Union for Conservation of Nature (IUCN), the international body that categorizes protected areas and tracks endangered species, has recently recognized that many areas outside national and regional protected area networks also contribute to effective biodiversity conservation. This new approach, called "other effective area-based conservation measures" (OECMs), recognizes areas where effective long-term conservation is implemented by diverse actors, including local communities, Indigenous Peoples, and the private sector (IUCN-WCPA 2019). In Hawaiʻi, OECMs may help provide better recognition to sites where traditional coastal management practices are making a difference for coral reef and coastal conservation.

Reflection Questions

1. List other considerations (beyond diversity of species) that might impact reserve selection. What might account for the emphasis on minimizing the number of sites in conservation planning, in this exercise?
2. If you wanted to consider other approaches to protecting the marine sites you investigated in the first section of this exercise, what questions would you ask? What other information would you need?
3. Can you think of some ways that eDNA techniques could contribute to the alternative management approaches described in this section?

RECOMMENDED READING

For further reading on MPA design, refer to NCEP's modules: Marine Protected Areas and MPA Networks and Building Marine Reserve Networks to Fit Multiple Needs: An Introduction to Marine Spatial Planning Using SeaSketch.

Papers on Hawaiian customary marine knowledge and management:

- Vaughan, M.B., B. Thompson, and A.L. Ayers. 2016. Pāwehe Ke Kai aʻo Hāʻena: creating state law based on customary Indigenous norms of coastal management. Society & Natural Resources 30:31–46.
- Friedlander, A., J.M. Shackeroff, and J.N. Kittinger. 2013. Customary marine resource knowledge and use in contemporary Hawaiʻi. Pacific Science 67(3):441–460.

Video on "Hawaiian Culture, Responsibilities of an Ahupua'a, Waimea Valley" by the Hi'ipaka LLC. This video covers the functions of an ahupua'a, and provides specific information for one area Waimea, O'ahu, where the shoreline is designated as a Marine Life Conservation District.
www.youtube.com/watch?v=ygD5ozXMZsc

NOAA Fisheries blog: "Sea Tales: Monitoring Coral Reef Ecosystems Throughout the Hawaiian Archipelago"
www.fisheries.noaa.gov/science-blog/sea-tales-monitoring-coral-reef-ecosystems-throughout-hawaiian-archipelago

NOAA Fisheries: Survey Methods
origin-apps-pifsc.fisheries.noaa.gov/cred/survey_methods.php

Marine National Monuments in the Pacific: NOAA Fisheries
www.fisheries.noaa.gov/pacific-islands/habitat-conservation/marine-national-monuments-pacific

More information on Papahānaumokuākea Marine National Monument and its co-management:
www.papahanaumokuakea.gov/new-about/management/

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Grigg, R.W. 1994. Effects of sewage discharge, fishing pressure and habitat complexity on coral ecosystems and reef fishes in Hawaii. Marine Ecology Press Series 103:25–34.

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APPENDIX I: eDNA HAWAIʻI FISH SEQUENCES

Reference sequence data from each of the nine sites.

Site A — Eight Sequences

CACCGCGGTTATACGAGAGGCTCAAGTTGACAGACAACGGCGTAAAGCGTGGTTAAGGAAAACATACAACTAAAGCGGAA
CCTCCTCCTAGCTGTTATACGCTTCCGAGGAAGTGAACCCCAACTACGAAAGTGGCTTTATCTAACCTGAACCCACGAAAGC
TAAGAAA

CACCGCGGTTATACGAGAGACCCAAGTTGTTAGACGCCGGCGTAAAGAGTGGTTAGGGAAACTCATTCAACTAGAGCCGAA
CACCTTCAAAGCTGTCATACGCACCCGAAGGTAAGAAGCCCAACTACGAAAGTGGCTTTATGTTAACTGAACCCACGAAAG
CTAGGGCA

CACCGCGGTTATACGGGTGACTCAAGTTGTTGGTCATCGGCGTAAAGAGTGGTTAAGACAAACTATATATTAAAGCCGAACG
CCCTCAGAGCCGTTATACGCTTCCGAGGGTAAGAAGTCCAATCACGAAAGTGGCTTTATTGTGTCTGATCCCACGAAAGCTA
TGGCA

CACCGCGGTTATACGAGAGACCCAAGTTGACAGACAATCGGCGTAAAGAGTGGTTAAGTACTATATTTTACTAAAGCCAAAC
ACCTTCAAAGCTGTTATACGCACCCGAAGGTCAGAAGCCCAATCACGAAAGTGGCTTTAAACTAACTGAACCCACGAAAGC
TAGGACA

CGACGAGGGCTTAACTGTCTCCTTTCCTAAGTCAATGAAATTGATCTCCCCGTGCAGAAGCGGGGATACACTCATAAGACG
AGAAGACCCTATGGAGCTTTAGACACTAAAACAGCTCATGTTAAAACCCCTCCCACAAGAGGCCAAACTAGATGACCCCTG
TCCTATTGTCTTTGGTTGGGGCGACCACGGGGCAACAAAAAACCCCCG

CACCGCGGTTATACGAAAGGCCCAAGTTGAAAAACATTCGGCGTAAAGGGTGGCTAAGGACCTATTTCAAACTAGAGCTGA
ATTTCTTCAAAGCTGTTATACGCTCATGAAAACTAGAAAATCAACCACGAAAGTGGCTCTAATCCCTCCTGACACCACGAAA
GCTATGACA

CACCGCGGTTATACGAGAGGCTCAAGTCGACAGACAACGGCGTAAAGAGTGGTTAAGGAAAATATTTAACTAAAGCGGAA
CACCCTCATAGCTGTTATACGCTTCCGAGGGCATGAACCTCAACTACGAAGGTGGCTTTATACTACCTGAACCCACGAAAGC
TAAGAAA

CACCGCGGTTATACGAGAGACCCAAGTTGACAGACAATCGGCGTAAAGAGTGGTTAAGTACTATACCCTACTAAAGCCAAA
CACCTTCAAAGCTGTTATACGCACCCGAAGGTTAGAAGCCCAATCACGAAAGTGGCTTTAAACTTACTGAACCCACGAAAG
CTAGGGCA

Site B — Seven Sequences

CACCGCGGTTATACGAGAGACCCAAGTTGACAGACAATCGGCGTAAAGAGTGGTTAAGTACTATACCCTACTAAAGCCAAA
CACCTTCAAAGCTGTTATACGCACCCGAAGGTTAGAAGCCCAATCACGAAAGTGGCTTTAAACTTACTGAACCCACGAAAG
CTAGGGCA

CACCGCGGTTATACGAGAGGCCCAAGTTGATAGGTACCGGCGTAAAGGGTGGTTAGGGATAACTATAAAATAAAGCCGAAT
ATCTTCAACGCTGTTATACGCCCTCGAAGATTCGAAGCCCCATTACGAAAGTAGCTTTACCTTCCCCCGAACCCACGAAAGC
CAGGGTA

CACCGCGGTTATACGAAAGGCCCAAGTTGAAAAACATTCGGCGTAAAGGGTGGCTAAGGACCTATTTCAAACTAGAGCTGA
ATTTCTTCAAAGCTGTTATACGCTCATGAAAACTAGAAAATCAACCACGAAAGTGGCTCTAATCCCTCCTGACACCACGAAA
GCTATGACA

CACCGCGGTTATACGAGAGACCCAAGTTGACAGATAATCGGCGTAAAGAGTGGTTAAGTAATACATTTCACTAAAGCCAAAC
ACCTTCAAAGCTGTTATACGCACCCGAAGATCAGAAGCCCAATCACGAAAGTGGCTTTAAACCAACTGAACCCACGAAAGC
TAGGGCA

CACCGCGGTTATACGAGAGGCTCAAGTTGACAGACAACGGCGTAAAGCGTGGTTAAGGAAAACATACAACTAAAGCGGAA
CCTCCTCCTAGCTGTTATACGCTTCCGAGGAAGTGAACCCCAACTACGAAAGTGGCTTTATCTAACCTGAACCCACGAAAGC
TAAGAAA

CACCGCGGTTATACGAGAGGCCCGAGTTGACAGTCCACGGCGTAAAGGGTGGTTAGGGGATTCTATTAAACTAAAGCCGA
ACGCTTACAAAGCTGTTATACGCGCACGAAAGTATGAAGATCAACAACGAAAGTAGCTTTATTATACCTGAACCCACGAAAG
CTAAGAGA

CACCGCGGTTATACGAGTGACTCACATTAACACACCACGGCGTAAAGAGTGATTAAAGAATGACCTCAAACTTACTAAAGTT
CAGACCTCATAAAGCCGTTATACGCATCCATGAGTAGAATAAACAACAACGAAAGTGACTTTATAAATATAAGAAACCTTGAT
GTCACGACAGTTGGGACC

Site C — Five Sequences

CGACGAGGGCTTAACTGTCTCCTTTCCTAAGTCAATGAAATTGATCTCCCCGTGCAGAAGCGGGGATACACTCATAAGACG
AGAAGACCCTATGGAGCTTTAGACACTAAAACAGCTCATGTTAAAACCCCTCCCACAAGAGGCCAAACTAGATGACCCCTG
TCCTATTGTCTTTGGTTGGGGCGACCACGGGGCAACAAAAAACCCCCG

CACCGCGGTTATACGAGAGACCCAAGTTGACAGACAATCGGCGTAAAGAGTGGTTAAGTACTATATTTTACTAAAGCCAAAC
ACCTTCAAAGCTGTTATACGCACCCGAAGGTCAGAAGCCCAATCACGAAAGTGGCTTTAAACTAACTGAACCCACGAAAG
CTAGGACA

CACCGCGGTTATACGAGAGGCTCAAGTTGACAGACAACGGCGTAAAGCGTGGTTAAGGAAAACATACAACTAAAGCGGAA
CCTCCTCCTAGCTGTTATACGCTTCCGAGGAAGTGAACCCCAACTACGAAAGTGGCTTTATCTAACCTGAACCCACGAAAG
CTAAGAAA

CACCGCGGTTATACGAGTGACTCACATTAACACACCACGGCGTAAAGAGTGATTAAAGAATGACCTCAAACTTACTAAAGTT
CAGACCTCATAAAGCCGTTATACGCATCCATGAGTAGAATAAACAACAACGAAAGTGACTTTATAAATATAAGAAACCTTGAT
GTCACGACAGTTGGGACC

CACCGCGGTTATACGAGAGACCCAAGTTGACAGATAATCGGCGTAAAGAGTGGTTAAGTAATACATTTCACTAAAGCCAAAC
ACCTTCAAAGCTGTTATACGCACCCGAAGATCAGAAGCCCAATCACGAAAGTGGCTTTAAACCAACTGAACCCACGAAAG
CTAGGGCA

Site D — Six Sequences

CACCGCGGTTATACGGGTGACTCAAGTTGTTGGTCATCGGCGTAAAGAGTGGTTAAGACAAACTATATATTAAAGCCGAACG
CCCTCAGAGCCGTTATACGCTTCCGAGGGTAAGAAGTCCAATCACGAAAGTGGCTTTATTGTGTCTGATCCCACGAAAGCT
ATGGCA

CACCGCGGTTATACGAGAGACCCAAGTTGACAGACAATCGGCGTAAAGAGTGGTTAAGTACTATACCCTACTAAAGCCAAA
CACCTTCAAAGCTGTTATACGCACCCGAAGGTTAGAAGCCCAATCACGAAAGTGGCTTTAAACTTACTGAACCCACGAAAG
CTAGGGCA

CACCGCGGTTATACGAGAGACCCAAGTTGTTAGACGCCGGCGTAAAGAGTGGTTAGGGAAACTCATTCAACTAGAGCCGA
ACACCTTCAAAGCTGTCATACGCACCCGAAGGTAAGAAGCCCAACTACGAAAGTGGCTTTATGTTAACTGAACCCACGAAA
GCTAGGGCA

CACCGCGGTTATACGAGTGACTCACATTAACACACCACGGCGTAAAGAGTGATTAAAGAATGACCTCAAACTTACTAAAGTT
CAGACCTCATAAAGCCGTTATACGCATCCATGAGTAGAATAAACAACAACGAAAGTGACTTTATAAATATAAGAAACCTTGAT
GTCACGACAGTTGGGACC

CACCGCGGTTATACGAGAGACCCAAGTTGACAGATAATCGGCGTAAAGAGTGGTTAAGTAATACATTTCACTAAAGCCAAAC
ACCTTCAAAGCTGTTATACGCACCCGAAGATCAGAAGCCCAATCACGAAAGTGGCTTTAAACCAACTGAACCCACGAAAG
CTAGGGCA

CACCGCGGTTATACGAGAGGCCCAAGTTGATAGGTACCGGCGTAAAGGGTGGTTAGGGATAACTATAAAATAAAGCCGAAT
ATCTTCAACGCTGTTATACGCCCTCGAAGATTCGAAGCCCCATTACGAAAGTAGCTTTACCTTCCCCCGAACCCACGAAAGC
CAGGGTA

Site E — Four Sequences

CGACGAGGGCTTAACTGTCTCCTTTCCTAAGTCAATGAAATTGATCTCCCCGTGCAGAAGCGGGGATACACTCATAAGACG
AGAAGACCCTATGGAGCTTTAGACACTAAAACAGCTCATGTTAAAACCCCTCCCACAAGAGGCCAAACTAGATGACCCCTG
TCCTATTGTCTTTGGTTGGGGCGACCACGGGGCAACAAAAAACCCCCG

CACCGCGGTTATACGGGTGACTCAAGTTGTTGGTCATCGGCGTAAAGAGTGGTTAAGACAAACTATATATTAAAGCCGAACG
CCCTCAGAGCCGTTATACGCTTCCGAGGGTAAGAAGTCCAATCACGAAAGTGGCTTTATTGTGTCTGATCCCACGAAAGCT
ATGGCA

CACCGCGGTTATACGAGAGACCCAAGTTGACAGACAATCGGCGTAAAGAGTGGTTAAGTACTATACCCTACTAAAGCCAAA
CACCTTCAAAGCTGTTATACGCACCCGAAGGTTAGAAGCCCAATCACGAAAGTGGCTTTAAACTTACTGAACCCACGAAAG
CTAGGGCA

CACCGCGGTTATACGAAAGGCCCAAGTTGAAAAACATTCGGCGTAAAGGGTGGCTAAGGACCTATTTCAAACTAGAGCTGA
ATTTCTTCAAAGCTGTTATACGCTCATGAAAACTAGAAAATCAACCACGAAAGTGGCTCTAATCCCTCCTGACACCACGAAA
GCTATGACA

Site F — Five Sequences

CACCGCGGTTATACGAGTGACTCACATTAACACACCACGGCGTAAAGAGTGATTAAAGAATGACCTCAAACTTACTAAAGTT
CAGACCTCATAAAGCCGTTATACGCATCCATGAGTAGAATAAACAACAACGAAAGTGACTTTATAAATATAAGAAACCTTGAT
GTCACGACAGTTGGGACC

CACCGCGGTTATACGAGAGGCCCAAGTTGATAGGTACCGGCGTAAAGGGTGGTTAGGGATAACTATAAAATAAAGCCGAAT
ATCTTCAACGCTGTTATACGCCCTCGAAGATTCGAAGCCCCATTACGAAAGTAGCTTTACCTTCCCCCGAACCCACGAAAGC
CAGGGTA

CACCGCGGTTATACGAAAGGCCCAAGTTGAAAAACATTCGGCGTAAAGGGTGGCTAAGGACCTATTTCAAACTAGAGCTGA
ATTTCTTCAAAGCTGTTATACGCTCATGAAAACTAGAAAATCAACCACGAAAGTGGCTCTAATCCCTCCTGACACCACGAAA
GCTATGACA

CGACGAGGGCTTAACTGTCTCCTTTCCTAAGTCAATGAAATTGATCTCCCCGTGCAGAAGCGGGGATACACTCATAAGACG
AGAAGACCCTATGGAGCTTTAGACACTAAAACAGCTCATGTTAAAACCCCTCCCACAAGAGGCCAAACTAGATGACCCCTG
TCCTATTGTCTTTGGTTGGGGCGACCACGGGGCAACAAAAAACCCCCG

CACCGCGGTTATACGAGAGGCCCGAGTTGACAGTCCACGGCGTAAAGGGTGGTTAGGGGATTCTATTAAACTAAAGCCGA
ACGCTTACAAAGCTGTTATACGCGCACGAAAGTATGAAGATCAACAACGAAAGTAGCTTTATTATACCTGAACCCACGAAAG
CTAAGAGA

Site G — Six Sequences

CACCGCGGTTATACGAGAGACCCAAGTTGACAGATAATCGGCGTAAAGAGTGGTTAAGTAATACATTTCACTAAAGCCAAA
CACCTTCAAAGCTGTTATACGCACCCGAAGATCAGAAGCCCAATCACGAAAGTGGCTTTAAACCAACTGAACCCACGAAA
GCTAGGGCA

CACCGCGGTTATACGGGTGACTCAAGTTGTTGGTCATCGGCGTAAAGAGTGGTTAAGACAAACTATATATTAAAGCCGAACG
CCCTCAGAGCCGTTATACGCTTCCGAGGGTAAGAAGTCCAATCACGAAAGTGGCTTTATTGTGTCTGATCCCACGAAAGCT
ATGGCA

CACCGCGGTTATACGAGAGACCCAAGTTGACAGACAATCGGCGTAAAGAGTGGTTAAGTACTATACCCTACTAAAGCCAAA
CACCTTCAAAGCTGTTATACGCACCCGAAGGTTAGAAGCCCAATCACGAAAGTGGCTTTAAACTTACTGAACCCACGAAAG
CTAGGGCA

CACCGCGGTTATACGAGAGGCTCAAGTCGACAGACAACGGCGTAAAGAGTGGTTAAGGAAAATATTTAACTAAAGCGGAA
CACCCTCATAGCTGTTATACGCTTCCGAGGGCATGAACCTCAACTACGAAGGTGGCTTTATACTACCTGAACCCACGAAAGC
TAAGAAA

CACCGCGGTTATACGAGAGACCCAAGTTGTTAGACGCCGGCGTAAAGAGTGGTTAGGGAAACTCATTCAACTAGAGCCGA
ACACCTTCAAAGCTGTCATACGCACCCGAAGGTAAGAAGCCCAACTACGAAAGTGGCTTTATGTTAACTGAACCCACGAAA
GCTAGGGCA

CACCGCGGTTATACGAGAGACCCAAGTTGACAGACAATCGGCGTAAAGAGTGGTTAAGTACTATATTTTACTAAAGCCAAAC
ACCTTCAAAGCTGTTATACGCACCCGAAGGTCAGAAGCCCAATCACGAAAGTGGCTTTAAACTAACTGAACCCACGAAAGC
TAGGACA

Site H — Five Sequences

CACCGCGGTTATACGAAAGGCCCAAGTTGAAAAACATTCGGCGTAAAGGGTGGCTAAGGACCTATTTCAAACTAGAGCTGA
ATTTCTTCAAAGCTGTTATACGCTCATGAAAACTAGAAAATCAACCACGAAAGTGGCTCTAATCCCTCCTGACACCACGAAA
GCTATGACA

CACCGCGGTTATACGAGAGACCCAAGTTGACAGATAATCGGCGTAAAGAGTGGTTAAGTAATACATTTCACTAAAGCCAAAC
ACCTTCAAAGCTGTTATACGCACCCGAAGATCAGAAGCCCAATCACGAAAGTGGCTTTAAACCAACTGAACCCACGAAAG
CTAGGGCA

CACCGCGGTTATACGGGTGACTCAAGTTGTTGGTCATCGGCGTAAAGAGTGGTTAAGACAAACTATATATTAAAGCCGAACG
CCCTCAGAGCCGTTATACGCTTCCGAGGGTAAGAAGTCCAATCACGAAAGTGGCTTTATTGTGTCTGATCCCACGAAAGCTA
TGGCA

CACCGCGGTTATACGAGAGACCCAAGTTGACAGACAATCGGCGTAAAGAGTGGTTAAGTACTATACCCTACTAAAGCCAAA
CACCTTCAAAGCTGTTATACGCACCCGAAGGTTAGAAGCCCAATCACGAAAGTGGCTTTAAACTTACTGAACCCACGAAAG
CTAGGGCA

CACCGCGGTTATACGAGAGGCTCAAGTCGACAGACAACGGCGTAAAGAGTGGTTAAGGAAAATATTTAACTAAAGCGGAA
CACCCTCATAGCTGTTATACGCTTCCGAGGGCATGAACCTCAACTACGAAGGTGGCTTTATACTACCTGAACCCACGAAAGC
TAAGAAA

Site I — Five Sequences

CACCGCGGTTATACGAGAGACCCAAGTTGACAGACAATCGGCGTAAAGAGTGGTTAAGTACTATATTTTACTAAAGCCAAAC
ACCTTCAAAGCTGTTATACGCACCCGAAGGTCAGAAGCCCAATCACGAAAGTGGCTTTAAACTAACTGAACCCACGAAAGC
TAGGACA

CACCGCGGTTATACGAGAGACCCAAGTTGACAGACAATCGGCGTAAAGAGTGGTTAAGTACTATACCCTACTAAAGCCAAA
CACCTTCAAAGCTGTTATACGCACCCGAAGGTTAGAAGCCCAATCACGAAAGTGGCTTTAAACTTACTGAACCCACGAAAG
CTAGGGCA

CACCGCGGTTATACGAGTGACTCACATTAACACACCACGGCGTAAAGAGTGATTAAAGAATGACCTCAAACTTACTAAAGTT
CAGACCTCATAAAGCCGTTATACGCATCCATGAGTAGAATAAACAACAACGAAAGTGACTTTATAAATATAAGAAACCTTGAT
GTCACGACAGTTGGGACC

CACCGCGGTTATACGAGAGACCCAAGTTGACAGATAATCGGCGTAAAGAGTGGTTAAGTAATACATTTCACTAAAGCCAAAC
ACCTTCAAAGCTGTTATACGCACCCGAAGATCAGAAGCCCAATCACGAAAGTGGCTTTAAACCAACTGAACCCACGAAAGC
TAGGGCA

CACCGCGGTTATACGGGTGACTCAAGTTGTTGGTCATCGGCGTAAAGAGTGGTTAAGACAAACTATATATTAAAGCCGAACG
CCCTCAGAGCCGTTATACGCTTCCGAGGGTAAGAAGTCCAATCACGAAAGTGGCTTTATTGTGTCTGATCCCACGAAAGCTA
TGGCA