

Surveyor Mutation Analysis of Coral Mitochondrial DNA

APPLICATION: This method is used to quantify mitochondrial sequence variations in individual coral colonies within a given species. It assumes familiarity with general molecular biology techniques and applications. Additional information on genomic DNA isolation, nucleic acid quantification, PCR and gel electrophoresis can be found in *Molecular Cloning: A Laboratory Manual* (Green and Sambrook, 2012).

EQUIPMENT AND SUPPLIES:

- DNA purification kit for high quality genomic DNA (example uses Qiagen Genomic-tip 20/G)
- Surveyor™ Mutation Detection kit (Integrated DNA Technologies, or similar)
- Coral tissue homogenate (see CDHC Virtual Protocol 101: *Frozen Tissue Homogenization*)
- Nitrile gloves
- 15 mL polypropylene tubes
- 1.5 mL Eppendorf tubes, sterile
- Liquid nitrogen or dry ice
- Stainless steel micro spatulas (Fisher Scientific #S50822, or similar)
- Water bath to 50 °C
- Isopropanol (molecular biology grade)
- Ethanol, 70 % (molecular biology grade)
- Tris(hydroxymethyl)aminomethane (Tris) base
- Ethylenediaminetetraacetic acid disodium salt (EDTA)
- Glacial acetic acid
- DNA gel loading dye, 6X (New England Biolabs, #B72021, or similar)
- DNA size markers (Lambda-HindIII DNA ladder, 1 kb DNA ladder, 100 bp DNA ladder)
- Agarose, molecular biology grade, low electroendosmosis (EEO range 0.09-0.13)
- Microfuge 6000- 14,000 X g (accommodates 1.5 mL tubes with refrigeration capability)
- Micropipettors and tips (0.1-1000 µL)
- Electrophoresis unit with mold and gel combs
- Power supply
- Gel imaging apparatus with UV light source
- Vortex mixer
- Fluorometric DNA quantification kit (this example uses the Quant-iT HS® kit, Molecular Probes/Life Technologies)
- 0.5 mL optically clear tubes
- Fluorometer for DNA quantification (example uses Invitrogen Qubit® fluorometer)
- Thermocycler
- DNA polymerase with proofreading ability (such as TaKaRa Ex Taq®)
- Oligonucleotide primers for coral mitochondrial DNA (see Supplementary Information, Tables 1 and 2)
- Water, PCR grade
- 0.2 mL thin-walled tubes

- Ice
- Ice bucket
- RNase A (10 mg/mL)
- Proteinase K (20 mg/mL solution)
- Deoxyribonucleotide (dNTP) mixture, 2.5 mM each dNTP
- Magnesium chloride (150 mM), filter sterilized
- Ethidium bromide (10 mg/mL) (★ Ethidium bromide is a mutagenic compound and requires the use of personal protective equipment and appropriate disposal)

STOCK SOLUTIONS


- Tris-EDTA (TE) buffer, 10 mM Tris-1 mM EDTA, pH 7.8, sterile
- Tris-acetate-EDTA (TAE) buffer (40 mM Tris, 20 mM glacial acetic acid, 1 mM EDTA)

PROCEDURE

I. Genomic DNA Purification from Coral Tissue Homogenate

Protocol note: Tissue homogenate should be kept frozen (dry ice or liquid nitrogen) at all times. Purified DNA should be kept on ice while in use or stored frozen (-20 °C) to reduce nuclease activity.

1. Don nitrile gloves.
2. Set water bath to 50 °C.
3. Add to a sterile 15 mL polypropylene tube: 2.0 mL G2 Buffer (from kit, room temperature), 40 µL 10 mg/mL RNase A, and 100 µL proteinase K. Vortex to mix.
4. Pre-chill a small stainless steel spatula.
5. Place one medium scoop (~ 60-80 mg, using a pre-chilled spatula) frozen, homogenized coral in tube and vortex 5-10 s to mix.
6. While tissue is incubating, set up 20/G tips with yellow suspension frames (from kit) over 15 mL polypropylene tubes and equilibrate each filter with 2.0 mL Buffer QBT (from kit). Allow tubes to empty by gravity flow (~5 min).
7. Warm QF Buffer to 50 °C in water bath.
8. Transfer clear lysate (do not disturb sediment at bottom of lysis tube) into a clean 1.5 mL tube and centrifuge at room temperature at 6,000 X g for 2-3 min to remove any particulate matter. Transfer supernatant to 20/G tip and allow to flow through column (gravity, 4-10 drops/min).
9. Wash each 20/G tip 3 times with 1.0 mL Buffer QC (gravity flow). Discard flow-through liquid.
10. Place 20/G tip with DNA into a clean 15 mL tube.
11. Elute DNA with 2 X 0.8 mL warmed Buffer QF (gravity flow).
12. Set water bath to 55 °C.
13. Transfer eluent to two 1.5 mL tubes per sample (TUBE 1 and TUBE 2, 0.8 mL each) and add 0.56 mL room temperature isopropanol to each.
14. Invert to mix and centrifuge immediately at 14,000 x g for 15 min at 4 °C.
15. Carefully aspirate supernatant and discard.
16. Wash DNA pellet (may not be visible) with 500 µL cold 70% ethanol.
17. Vortex briefly and centrifuge at 14,000 x g for 10 min at 4 °C.

18. Carefully aspirate supernatant and discard.
19. Air dry pellet for 15-20 min. Ensure that alcohol has dissipated.
20. Dissolve DNA in each tube in 50 μ L TE buffer (gently mixing by flicking tube with finger).
21. Incubate DNA at 55 $^{\circ}$ C (water bath) for 1-2 h. Following incubation, spin tubes quickly in microfuge, then pool DNA from replicate samples.
22. Verify integrity of genomic DNA by running 10 μ L of each sample plus 2 μ L DNA loading dye on 0.5% TAE-agarose gel containing 0.5 μ g/mL ethidium bromide  at 60 V for 2 h. Use Lambda-HindIII DNA size ladder (0.5 μ g).
23. Image gel for records.

II. Fluorometric DNA Quantification (Molecular Probes Quant-iT[™] HS[®] DNA kit, Invitrogen)

1. Prepare a 1:200 dilution of Quant-iT[™] HS DNA dye in buffer solution.
2. Place 198 μ L Quant-iT[™] buffer solution (with HS DNA dye) into a 0.5 mL clear (PCR) tube.
3. Add 2 μ L of well-mixed genomic DNA sample and vortex to mix. Incubate 2 min in dark.
4. Calibrate instrument with standards as per manufacturer's protocol, then read sample.
5. Multiply Qubit[®] raw values by 100 (dilution factor) for calculation of stock DNA sample. Recovery should be 15-50 ng/ μ L.
6. Store 150-200 ng aliquots of each genomic DNA sample at -20 $^{\circ}$ C.

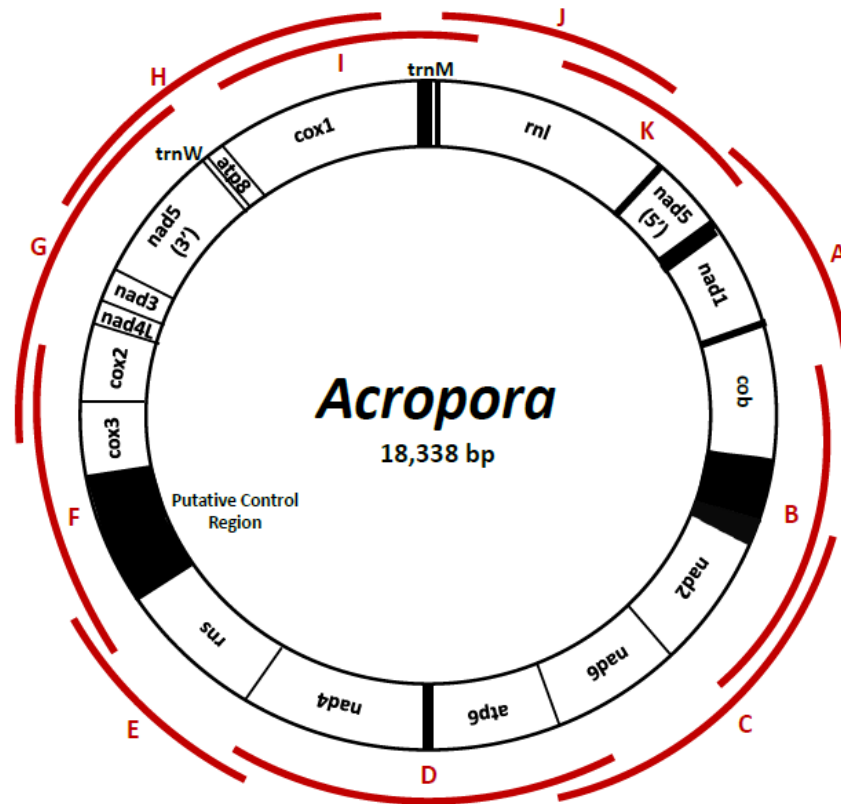
III. Surveyor[™] Mutation Detection Analysis for Coral Mitochondrial Genome

Protocol note: DNA samples and nuclease and DNA polymerase enzymes should be kept on ice while in use or stored at -20 $^{\circ}$ C.


A. Amplification of Mitogenome Regions

Primer sets in Tables 1 and 2 (Supplementary Information) amplify eleven overlapping regions in the circular acroporid or poritid mitochondrial genome (Figure 1).

Figure 1. Example mitochondrial genome map with amplified sections, A-K.



1. Prepare Surveyor Control C and Surveyor Control G reaction tubes according to the manufacturer's protocol, and PCR amplify as directed. This requires different cycling parameters from the coral samples and may be done ahead of time to use for several experiments. Products (632 bp) should be checked on an agarose gel (1.2%) and quantified with the Qubit® fluorometer, as with the coral genomic DNA PCR products.
2. Dilute coral DNA to 0.4-0.5 ng/μL final concentration using sterile water.
3. Set up polymerase chain reaction with selected primer set(s) to amplify 1.0-2.5 Kb regions of the mtDNA genome (See Tables 1 and 2, Supplementary Information).
4. Place 16 μL coral template samples in a thin-walled tube (6-8 ng). Note: prepare 2-4 tubes of reference (or control) sample for each primer set to be analyzed. You will need at least one tube of carrier control for every 8 samples to be analyzed, plus an additional tube for the uncut control.
5. Prepare a negative control for each primer set using 16 μL sterile water.
6. EXAMPLE: These reactions can be done over several days if necessary and stored at -20 °C until ready for the hybridization. There will be 11 tubes per sample, one for each primer set, A-K.
 - a. Reference Sample
 - b. Reference Sample
 - c. Sample 1

- d. Sample 2
 - e. Sample 3
 - f. Sample 4
 - g. Sample 5
 - h. Sample 6
 - i. Sample 7
 - j. Negative Control (no template DNA)
7. Reaction mixture per tube. As per above example, there will be 110 tubes. It is best to prepare a pooled reaction cocktail for the number of tubes required plus 2-3 extra to account for any pipetting errors. Keep all tubes on ice.
- a. 5 μ L 10X ExTaq buffer
 - b. 1 μ L dNTPs (2.5 mM)
 - c. 1.5 μ L 10 μ M Forward primer (15 pmol)
 - d. 1.5 μ L 10 μ M Reverse primer (15 pmol)
 - e. 0.25 μ L ExTaq polymerase
 - f. 24.75 μ L sterile MilliQ water
8. Mix well and add 34 μ L to each thin-walled tube containing template DNA.
9. Cycling parameters:
- a. 94 $^{\circ}$ C, 5 min
 - b. 94 $^{\circ}$ C, 30 s
 - c. 60 $^{\circ}$ C, 30 s
 - d. 72 $^{\circ}$ C, 3 min
 - e. repeat b-d 34 X
 - f. 72 $^{\circ}$ C, 10 min
 - g. 4 $^{\circ}$ C, HOLD
10. Evaluate 5 μ L PCR products on a 1.2% TAE-agarose gel containing ethidium bromide 
Ensure NO secondary amplification products are present for each sample, including primer dimers. Image gel for records.
11. Quantify PCR products using 2 μ L of each sample in the Qubit[®] HS dsDNA assay as detailed in Section II. Target DNA concentration is 25-80 ng/ μ L.
- B. Heteroduplex Formation: There will be a total of 300 ng DNA in each tube in a 12 μ L volume.
1. Prepare 0.2 mL thin-walled tubes with sample DNA PCR:
 - a. Surveyor Nuclease Plasmid Control C (300 ng)
 - b. Surveyor Nuclease Plasmid Control C (150 ng) + Control G (150 ng)
 - c. Reference Sample 1 alone (300 ng) uncut control
 - d. Reference Sample 1 alone (300 ng) homoduplex control
 - e. Reference Sample 1 (150 ng) + Sample 2 (150 ng)
 - f. Reference Sample 1 (150 ng) + Sample 3 (150 ng)
 - g. Reference Sample 1 (150 ng) + Sample 4 (150 ng)
 - h. Reference Sample 1 (150 ng) + Sample 5 (150 ng)
 - i. Reference Sample 1 (150 ng) + Sample 6 (150 ng)
 - j. Reference Sample 1 (150 ng) + Sample 7 (150 ng)

2. Bring volume of all tubes to 12 μL with sterile water.
3. Place tubes in thermocycler and hybridize:
 - a. 95 $^{\circ}\text{C}$, 10 min
 - b. 95-85 $^{\circ}\text{C}$, -2.0 $^{\circ}\text{C}/\text{s}$
 - c. 85 $^{\circ}\text{C}$, 1 min
 - d. 85-75 $^{\circ}\text{C}$, -0.3 $^{\circ}\text{C}/\text{s}$
 - e. 75 $^{\circ}\text{C}$, 1 min
 - f. 75-65 $^{\circ}\text{C}$, -0.3 $^{\circ}\text{C}/\text{s}$
 - g. 65 $^{\circ}\text{C}$, 1 min
 - h. 65-55 $^{\circ}\text{C}$, -0.3 $^{\circ}\text{C}/\text{s}$
 - i. 55 $^{\circ}\text{C}$, 1 min
 - j. 55-45 $^{\circ}\text{C}$, -0.3 $^{\circ}\text{C}/\text{s}$
 - k. 45 $^{\circ}\text{C}$, 1 min
 - l. 45-35 $^{\circ}\text{C}$, -0.3 $^{\circ}\text{C}/\text{s}$
 - m. 35 $^{\circ}\text{C}$, 1 min
 - n. 35-25 $^{\circ}\text{C}$, -0.3 $^{\circ}\text{C}/\text{s}$
 - o. 25 $^{\circ}\text{C}$, 1 min
 - p. 4 $^{\circ}\text{C}$ HOLD
4. During hybridization, pour a 2.0 % TAE-agarose gel containing 0.5 $\mu\text{g}/\text{mL}$ ethidium bromide. Ensure that the gel volume is sufficient to load 20 μL sample.
5. Remove hybridization from the thermocycler and place in a tube rack. Place tube of Reference Sample 1 alone (#1 c, above) on ice. This will be the uncut control (not subjected to nuclease treatment).
6. Treat Plasmid Controls C and C/G with nuclease, per tube:
 - b. 1.2 μL 150 mM MgCl_2
 - c. 1.0 μL Surveyor Enhancer S
 - d. 1.0 μL Surveyor Nuclease S
7. Heat samples at 42 $^{\circ}\text{C}$ for 60 min.
8. Treat coral samples with nuclease (conditions optimized for *Acropora* and *Porites* coral sample). Prepare magnesium chloride, Enhancer and Nuclease (from kit) as a cocktail, and add 2.7 μL to each tube containing 12 μL DNA. Pipet up and down to mix. Per tube:
 - e. 1.2 μL 150 mM MgCl_2
 - f. 1.0 μL Surveyor Enhancer S
 - g. 0.5 μL Surveyor Nuclease S
9. Heat samples at 42 $^{\circ}\text{C}$ for 20 min. Keep samples on ice for 40 min after beginning nuclease treatment of plasmid controls.
10. Following incubation, add 1.5 μL Stop Solution (from kit) to all tubes and pipet up and down to mix.
11. Add 3.5 μL 6X gel loading dye to each tube, including uncut control and mix well. Evaluate all samples (Plasmid Controls, Coral samples, digested and uncut) on a 2.0 % TAE-agarose gel containing 0.5 $\mu\text{g}/\text{mL}$ ethidium bromide and including 100 bp DNA size markers.

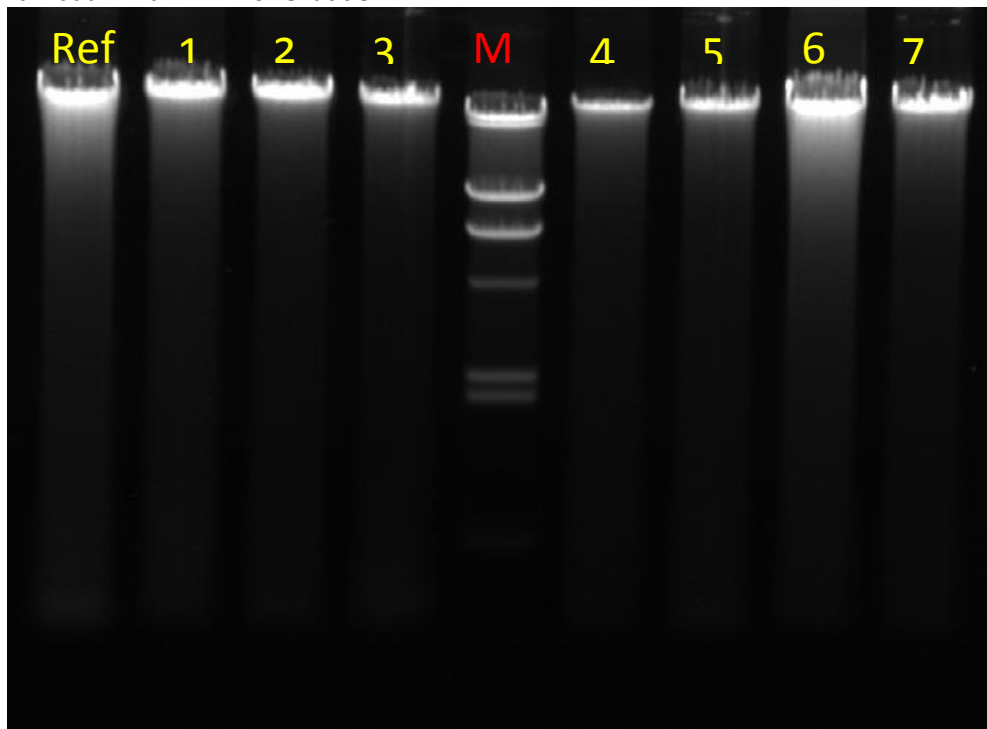
12. Image gel and record the number of fragments for each sample coral as compared to self-hybridized control. The number of fragments (not including the uncut band) equals the number of mutations/sequence variations in the sample.

IV. Expected Results

A. Genomic DNA Isolation

High quality genomic DNA from coral should be verified on a 0.7 % TAE agarose gel (Figure 2). There should be limited breakage of DNA strands as evident from strong high molecular weight bands near the top of the gel above the 23 kb Lambda-HindIII marker.

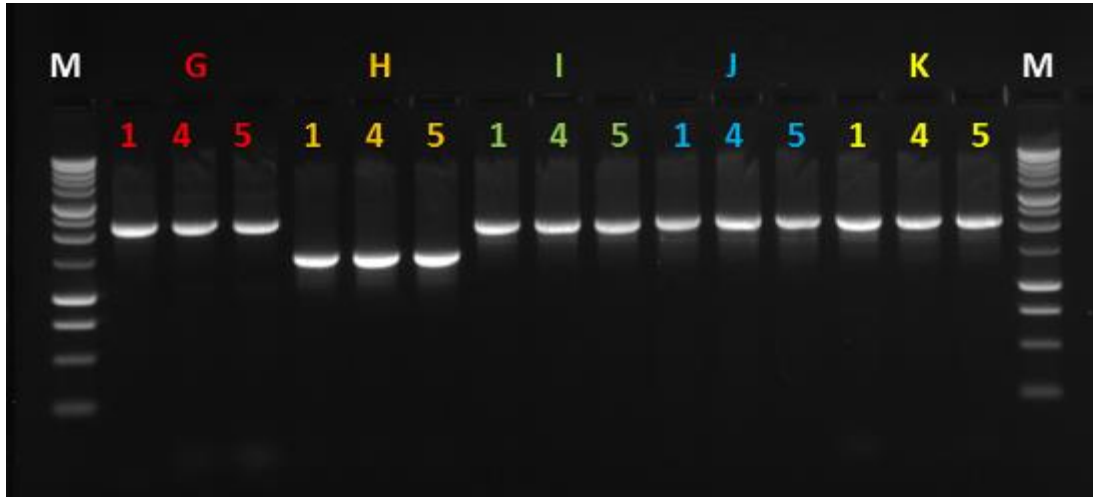
Figure 2. Typical results of the genomic DNA purification for coral samples using Qiagen Genomic Tip 20/G kit. Reference and sample coral in yellow text. M = Lambda-HindIII DNA size ladder.



B. Coral Mitochondrial Genome Fragment Amplification

Amplification should result in single bands with no secondary products, including primer-dimers, observed. Primer dimerization can be eliminated by reducing oligonucleotide concentration in the PCR.

Figure 3. Example of PCR amplification of overlapping mitochondrial DNA fragments G-K from three *Porites lobata* colonies (1, 4 and 5). Samples electrophoresed on a 1.2% TAE-agarose gel containing 0.5 $\mu\text{g}/\text{mL}$ ethidium bromide with 1 kb DNA ladder (lanes M).



C. Surveyor Nuclease Treatment of Hybridized Mitochondrial Genome Fragments

Nuclease-treated heteroduplex DNA is evaluated for mutation sites by simply counting the number of additional bands as compared to the nuclease-treated, homoduplex reference colony (Figure 4). The mutation detection assay is not limited to use with mitochondrial DNA, but can be applied to genomic DNA or targeted mitochondrial regions in the same manner (Figure 5).

Figure 4. Typical results of mutation detection in amplified coral mitogenome sections G and H from eight *Porites lobata* colonies. U = uncut homoduplex control, H = digested homoduplex reference colony, 1-8 = heteroduplex sample colony digest, M = 100 bp DNA size ladder, C = Plasmid Control C homoduplex, C/G = Plasmid Control C/G heteroduplex. Multiple sites of genetic variability (bands) were observed in all heteroduplex DNA samples.

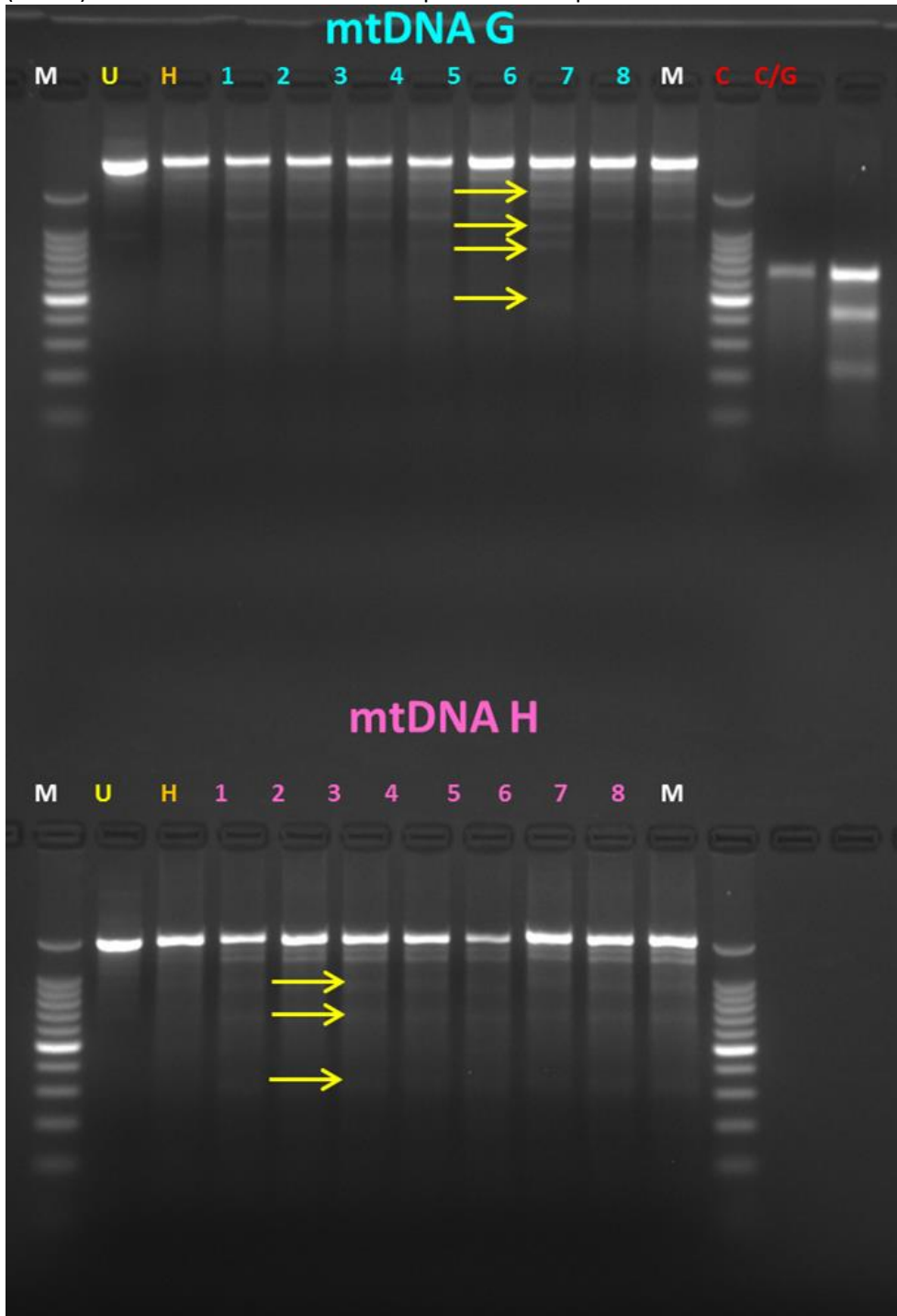
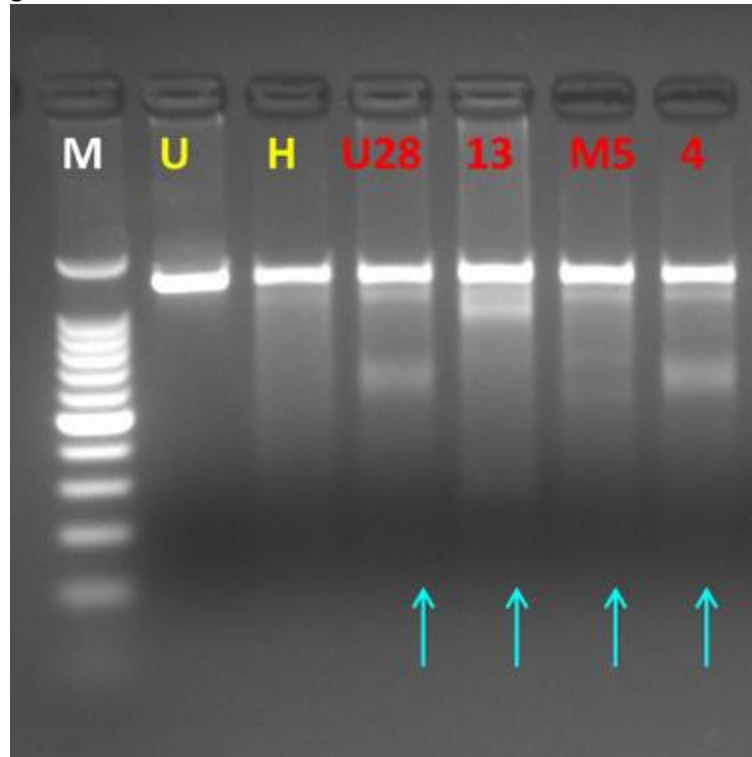


Figure 5. Surveyor mutation analysis of *nad2/nad6* coding region (1.2 kb) in sperm samples from *Acropora cervicornis* genets M10, U28, 13, M5 and 4. M = 100 bp DNA ladder, U = M10 reference uncut homoduplex control, H = M10 reference nuclease-treated homoduplex control. Mitochondrial base mismatches detected in all sample genets as compared to genet M10 reference.



SUPPLEMENTARY INFORMATION

Table 1. Oligonucleotide primer sets for mutation analysis of *Acropora* sp. mitochondrial DNA.

Name	Sect	Sequence (5'-3')	Length (bp)	C+G (%)	Product (Kb)	Seq Range (bp)
<i>Acropora</i> trnMF	J	GAG AGG ACG AAG GGT AAG TCG TC	23	56.5	2.0	5-2024
<i>Acropora</i> rnlR2	J	CAA CAT CGA GGT CGC AAA CAT C	22	50.0		
<i>Acropora</i> rnlF	K	CAG AAG CGA AAA TGT GAA CAT GAG	24	41.7	1.9	1084-2997
<i>Acropora</i> nad5(5')R	K	CGA TCC AAA AGA CCA AAC ATA GCC	24	45.8		
<i>Acropora</i> nad5(5')F	A	CTA CGG CAT ATA TGC TTG GGG AC	23	52.2	1.9	2733-4594
<i>Acropora</i> cobR	A	GCG GAT TCT CTT TAC GCA ATG GC	23	52.2		
<i>Acropora</i> nad1F	B	CCT CGT ATT CGA TAC GAT CAG C	22	50.0	2.5	4339-6859
<i>Acropora</i> nad2R	B	GCT TGA ATA CTC TCA AAT GAC CC	23	43.5		
<i>Acropora</i> intF	C	CCA CGA GCT CCT GTT ACT AAG CC	23	56.5	2.5	5915-8394
<i>Acropora</i> atp6R	C	CCC AGC TAG AGT CAC ACC TAT G	22	54.5		
<i>Acropora</i> nad6F	D	GGA GGT GCT TGA GAT TTG GC	20	55.0	2.5	7748-10266
<i>Acropora</i> nad4R	D	GAG ATA GCC AAT AAC TCA CAC CTG	24	45.8		
<i>Acropora</i> nad4F	E	GTT GGC GCA TGG TTT TGT TAG C	22	50.0	1.7	9901-11563

Acropora rnsR	E	GTT ACG ACT TAC TCT GCC TCG G	22	54.5		
Acropora rnsF	F	CTT GGA TGA AGT CAG GTC TTA TTG TC	26	42.3	2.4	11285-13694
Acropora cox2R	F	GCA ACC ACA ACA CGA CTA TCA C	22	50.0		
Acropora cox3F	G	CAA GTC GCC AAC ATG TTG GGT TTG	24	50.0	2.4	13363-15785
Acropora nad5(3')R	G	CCA CCT CTT TAG TCA AAT AGC CCA C	25	48.0		
Acropora nad5(3')F	H	CTG TCA TCC ATG CTT TGT CTG ACG AGC	27	51.9	2.5	15399-17863
Acropora cox1R	H	CAG AGT ATC GTC TCG GAA AAC CTG	24	50.0		
Acropora cox1F	I	GTA TCC TCC TCT ATC GAG CAT CC	23	52.2	1.9	16923-519
Acropora rnlR1	I	GCC CTC TCG ACT TCT CTT CAC CTA C	25	56.0		

Table 2. Oligonucleotide primer sets for mutation analysis of *Porites* sp. mitochondrial DNA.

Primer Name	Sect	Sequence (5'-3')	Length (bp)	C+G (%)	Product (Kb)	Seq Range (bp)
Porites nad5(5')F	A	GGT GCT GGA ATT TTA ACT TCA AGC	24	41.7	2.1	2640-4741
Porites cobR	A	GAT TCT CTT TGC GCA GTG GCA TAG G	25	52.0		
Porites nad1F	B	CGG TAT GAT CAG CTT ATG GCT C	22	50.0	2.1	4485-6626
Porites nad2R	B	CAG ACC AGA TGA AAG TGC ACC	21	52.4		
Porites intF	C	GAT GGT GGA CAC GGA AAA GC	20	55.0	2.1	6350-8425
Porites atp6R	C	CGA CAC CAT GAA GAT GAT CAT AG	23	43.5		
Porites nad6F	D	CTT GAG ATT TGG CAA CTC CTT GG	23	47.8	2.3	7960-10,238
Porites nad4R	D	CAA ACC CGT GCG CTA ACA TCA TG	23	52.2		
Porites nad4F	E	GCC TCC GAG TAT TTT GCT CCT C	22	54.5	2.3	10,031-12,365
Porites cox3R	E	GGT CAA GCC ACA CCC AAT TCA AC	23	52.2		
Porites cox3F	F	GCG AAC TGT TTT ATG TCA TCC	21	42.9	2.5	12025-14509
Porites nad5(3')R	F	GGA GCT TGT TCA AAA AGA GGA GAA G	25	44.0		
Porites nad3F	G	CTT TGG GTT TAC TCT ATG AGT G	22	40.9	2.2	14,265-16,504
Porites cox1(5')R	G	CAT TGC ACC CAA AAT CGA GGA C	22	50.0		
Porites cox1(5')F	H	CTA CTA ACC ATA AAG ACA TTG GTA CG	26	38.5	1.5	16,044-17,538
Porites coxintR	H	GAG CAC CCT TCT TCC CAC TAT GC	23	56.5		
Porites coxintF	I	CTA GGG TCA ATC AGT GGG AAA C	22	50.0	2.1	17,035-521
Porites rnlR1	I	CTC GAC CTT CTC TTC ACC TAC	21	52.4		
Porites trnMF	J	GTA GAG AAG ACG AAT GGT GAG TC	23	47.8	2.2	2-2193
Porites rnlR2	J	GAA ACC AAG CTG TGT TAC CAC GC	23	52.2		
Porites rnlF	K	GCT TGG TAG TAG AAC AGA CTG	21	47.6	2.2	1090-3246
Porites nad5(5')R	K	CCA ACT GTG CAG ACT TTC CAA CC	23	52.2		

CONSIDERATIONS AND CAVEATS

1. Examples above have used agarose gel electrophoresis for visualization of the nuclease-treated, hybridized samples, however bands smaller than 100 bp can be missed by this method. Polyacrylamide gel electrophoresis or sequence analysis of the fragments may be used for verification of base mismatches.
2. The Dojindo GetPure® DNA kit-cell, tissue is an alternative DNA purification kit that has been tested with acceptable results.

3. It is easier to detect bands in the assay using smaller DNA fragments (~1.0 kb), since a decrease in size will increase copy numbers for any given sample.
4. Customization of the assay requiring design of alternative oligonucleotide primers should include rigorous testing to ensure no secondary amplification products occur. Annealing temperatures in the amplification reaction may need to be optimized with alternative primers.

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