CRCP Deliverable 1368

Can telomerase activity serve as an indicator of reproductive senescence?

CRCP Project: 1133

Coral Disease and Health Consortium (CDHC): Diagnostic metrics, Epidemiology and Capacity Building

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January 2023

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Abstract

We evaluated the potential use of telomerase enzyme activity in stony corals as a method for determining reproductive senescence. Since kits for the detection and quantification of telomerase activity were removed from the market prior to project start, we re-engineered the assay using laboratory-cultured corals for assay validation. Coral telomerase activity was detected in HeLa cell extracts (positive control) and in two species of stony corals (*Acopora cervicornis* and *Goniastrea* sp.) using the telomerase random amplification protocol (TRAP) assay, which were visualized on a polyacrylamide gel. For quantification of telomerase activity, a quantitative PCR (qPCR) assay was developed using the products from the TRAP assay, with HeLa cells as a positive control and a synthetic oligonucleotide for standardization. Following validation of the qPCR with protein extracts from laboratory-cultured corals, we then evaluated *Acropora palmata* corals of known ages, with the hope that telomerase activity could be used as a proxy for coral reproductive senescence. No telomerase activity was detected, however. It is possible that sample handling, the length of storage time (due to laboratory closure), or inhibitors (which may co-extract with the protein) negatively affected the results for this coral species. Further research is needed to deduce the reasons for the assay failure with *A. palmata*. Additionally, an alternative method to evaluate reproductive senescence using telomere length was explored.

Introduction

Telomeres are tandem repetitive 6-mer noncoding sequences (in corals, TTAGGG) that cap the ends of chromosomes and which serve to protect against chromosomal sequence loss during replication cycles. The integrity of telomeres is maintained by telomerase, an enzymatic riboprotein (part RNA, part protein). As living organisms age, the function of telomerase decreases, resulting in shortened telomeres. DNA damage from telomere shortening can lead to senescence and a myriad of other cellular dysfunctions. The objective of this research was to optimize assays for telomerase activity and/or telomere length in corals of known ages, to understand if these endpoints could be used to determine senescence in stony corals.

Two telomerase activity assays were explored during this project. A telomerase random amplification protocol (TRAP) assay is a semi-quantitative gel-based assay that uses DNA laddering as an endpoint with longer-laddering associated with increased telomerase enzyme activity. This assay has previously been shown to work with a stony coral species, *Galaxea fascicularis* (Nakamichi et al., 2012). A quantitative PCR

(TRAPeze® PCR) method also was considered, however the kit we anticipated using was removed from the market by the manufacturer. Therefore, we attempted to re-engineer the assay. This involves extracting and quantifying the protein fraction from tissue, then incubating the protein extract with a DNA oligonucleotide, so that the telomeric tandem repeats are added to the oligo 5' ends if active telomerase riboprotein is present in the extract. The products are normalized to protein concentration and the standard curve of oligonucleotides are included for quantitation.

A standard method to measure telomere length (terminal restriction fragment (TRF) assay) using Southern blot analysis of genomic DNA also was explored. The DNA is digested with restriction enzymes, electrophoresed, blotted and probed with labeled oligonucleotides complementary to telomeric repeats representing terminal restriction fragments of chromosomes. The length of telomeres is estimated by comparison with DNA size standards.

Methods

Riboprotein extraction

Riboprotein was extracted from frozen, cryomilled coral tissue samples (~300-400 mg) in 200 µL CHAPS lysis buffer (10 mM Tris-Cl, 1 mM $MgCl_2$, 1 mM EGTA, 0.5 % CHAPS, 10 % glycerol) with 1 µL each of Thermo Fisher RiboLock RNase inhibitor (product #EO0382) and Sigma protease inhibitor cocktail (product # P9599) added. Samples were kept on ice at all times to reduce loss of riboprotein. Following addition of tissue to lysis buffer, samples were briefly vortexed to mix and incubated on ice for 30 min, vortexing briefly every 5 min. Samples were centrifuged at 14K x g for 20 min at 4 °C to remove particulate matter. The supernatant was transferred to a clean tube on ice and the supernatant was recentrifuged to remove any residual particulates. The clarified supernatant was transferred to a new clean tube on ice and a Bradford assay was used to quantify protein. Samples were frozen at -80 °C in 10 µL aliquots for future use. Since cancer cells have elevated telomerase activity, riboprotein was extracted concurrently from a HeLa cell line (~1 x 10^6 cells washed in PBS to remove any culture media) for use as an assay positive control.

TRAP Assay

TRAP extension reactions were performed according to Kim et al. (1994) with minor modifications. Duplicate dilutions of sample riboprotein extracts were made in CHAPS lysis buffer (target range = 0.1 – 2.0 µg protein). One tube of each sample was used to heat inactivate the riboprotein (85 °C for 10 min) as an assay negative control. Tubes for each telomerase extension reaction (several dilutions for active telomerase and one heat inactivated) were prepared containing 5 µL of 10 x TRAP extension buffer, 2 µL 10 mM dNTPs (deoxynucleotide triphosphate), 10 amol (attomole) TS standard oligonucleotide (5'-AAT CCG TCG AGC AGA GTT AGG GTT AGG GTT AGG GTT AGG GTT AGG GTT AGG GTT AGG GTT AG-3'), 0.2 µL Taq polymerase and nuclease-free water to a final volume of 48 µL and held on ice. Two microliters of each riboprotein sample dilution (active) and one heat-inactivated sample, were added to sample tubes containing the TRAP extension buffer. HeLa cell extracts were included

as assay positive controls. Sample tubes were incubated in a 30 °C water bath for 60 min to allow addition of nucleotides (telomeric repeats) to the TS oligonucleotide fragment.

Tubes were removed from the water bath and 1 µL of an equimolar (10 µM) PCR (polymerase chain reaction) primer mixture (ACX primer = 5'-GCG CGG CTT ACC CTT ACC CTT ACC CTA ACC-3'; TS primer = 5'-AAT CCG TCG AGC AGT T-3') was added to each tube. These primers were designed to be complementary to the tandem repeat sequences added by telomerase. Oligonucleotide fragments with tandem repeats added were amplified: 95 °C, 10 min; 40 cycles: 94 °C, 30 s, 58 °C, 30 s, 72 °C, 90 s; extension: 72 °C, 10 min; final hold: 4 °C. Samples were analyzed on a 12.5 % polyacrylamide gel (0.5x TBE, 200 V, 5.5 h at 4 °C). Gels were stained with SYBR Green for 20 min and imaged under ultraviolet lighting (to excite the natural fluorescence in the SYBR Green nucleic acid stain) using a G-Box gel documentation system fitted with a digital camera (Syngene USA, Frederick, MD).

Assay Optimization and Validation

A range of TS oligonucleotide standards were tested to determine the optimal concentrations to be used in the quantitative TRAP assay experiments. Tested ranges included: 50 – 0.08 amol, 40 – 0.0026 fmol, 25 – 0.0016 fmol and 2 – 0.00064 fmol. Varying concentrations of TS oligonucleotide and dNTPs also were evaluated, along with telomerase extension incubation times. All test ranges for the standards were in five-fold dilution series. For testing, a SYBR-primer mixture was synthesized using 10 µL of ABI PowerSYBR Green Master Mix (product #4368706) and 0.2 µL of a TS and ACX primer mixture (equal volumes of 10 µM solutions) and placed in a qPCR plate on ice. Standards in 10 X TRAP extension buffer (10 µL volume) were added to each well and qPCR (quantitative polymerase chain reaction) cycling was performed: 95 °C, 10 min; 40 cycles: 94 °C, 30 s, 58 °C, 30 s, 72 °C, 90 s; dissociation (melt). Once assay parameters had been optimized, the quantitative test was validated using riboprotein extracts from laboratory cultured coral species.

Quantitative TRAP Assay for *Acropora palmata* of Known Ages

Following qPCR TRAP assay validation with coral extracts, *Acropora palmata* colonies with varying relative ages were tested to determine if decreased telomerase activity is associated with increased age. Tissue samples from *A. palmata* colonies of known ages were collected in February 2020 in Curaçao by collaborators at the Caribbean Research and Management of Biodiversity Institute (CARMABI). Samples were shipped to the NOAA Charleston Laboratory and held at -80 °C. The Covid-19 pandemic resulted in research delays due to a 2.5-year laboratory closure beginning in March 2020 and during the hold time, some samples were lost during a laboratory move. The remaining samples along with some laboratory *A. palmata* samples, were cryomilled and riboprotein extracted as detailed above.

Four twofold sample dilutions of the *A. palmata* riboprotein extracts from each colony were prepared in CHAPS lysis buffer. An aliquot of undiluted riboprotein extract from each colony was heat inactivated as described above. Following heat inactivation, all

samples were subjected to the telomerase extension reaction (see above) using 2 µL of diluted sample in a 50 µL total reaction volume. While the extension reaction was proceeding, standards for the telomerase activity qPCR were generated using five-fold dilutions of the TS standard oligo in TRAP extension buffer: 25, 5, 1, 0.2, 0.04, 0.008 and 0.0016 fmol (femtomole). A SYBR-primer mixture was synthesized (see above) and placed in a qPCR plate on ice. Ten microliters of each telomerase extension reaction (active and inactivated telomerase) were added to the SYBR- primer mixture and thermocycling performed as described for standard testing above.

Results and Discussion

TRAP Assay Validation

The original TRAP assay is a semi-quantitative gel-based assay that uses DNA laddering as an endpoint with longer laddering associated with increased telomerase enzyme activity. We used the methods of Kim et al., 1994 for initial tests and optimization. This was a two-step assay involving an extension reaction (in which the cell extracts with active telomerase were incubated with an oligonucleotide), followed by a polymerase chain reaction to amplify products. Polyacrylamide gel electrophoresis was used to visualize the results. Attempts with laboratory-cultured *Acropora cervicornis* (ACER), *Pocillopora damicornis* (PDAM) and *Gonastrea* sp. (GON) were promising, with positive laddering patterns associated with ACER and GON samples (Figure 1).

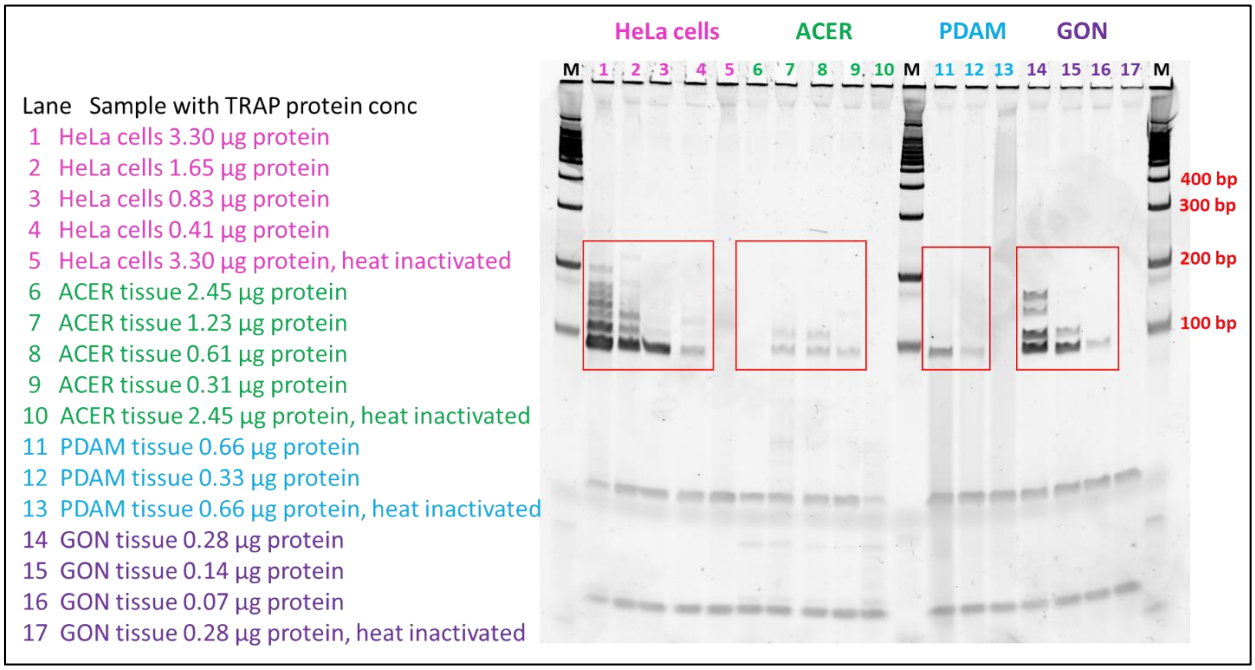

Figure 1. Results of initial TRAP assay validation with HeLa cell extracts (positive control), and *Acropora cervicornis* (ACER), *Pocillopora damicornis* (PDAM) and Goniastrea sp. (GON) coral extracts. DNA size markers (100 bp ladder) are in lanes designated M. Telomerase activity was observed in HeLa cell, ACER coral and GON

coral extracts. Heat-inactivated negative controls for each extract type are in lanes 5, 10, 13 and 17.

Results of Assay Optimization

Before beginning the qPCR analysis, the assay was optimized with respect to TS standard range, TS oligonucleotide template concentration and dNTP concentration. Following initial qPCR with standards, it was determined that the optimal standard range (in a five-fold dilutions series) falls between 25 – 0.0016 fmol (Figures 2 and 3). We should emphasize that standard fluorescence (reported as relative fluorescence units, RFU), Ct value, melt temperature (target 76.5 °C), assay efficiency percent (optimal range 90 -110 %) and R^2 values (optimal = >0.99) may fluctuate depending on the instrument and age of the PowerSYBR Green Master Mix. We observed increased Ct values for the standards following one use of the PowerSYBR Green Master Mix. We also would like to note that any standard not meeting the minimum RFU (baseline) or exact melt temperature (76.5 °C) should be omitted from the standard curve and subsequent telomerase activity calculations made using the adjusted values.

Increasing the telomerase extension time did not improve results. The optimal concentrations of TS oligonucleotide template and dNTPs were determined to be 10 amol and 10 µM each, respectively. The methods optimized for performing the qPCR telomerase activity assay for stony coral species are presented in Supplement 1.

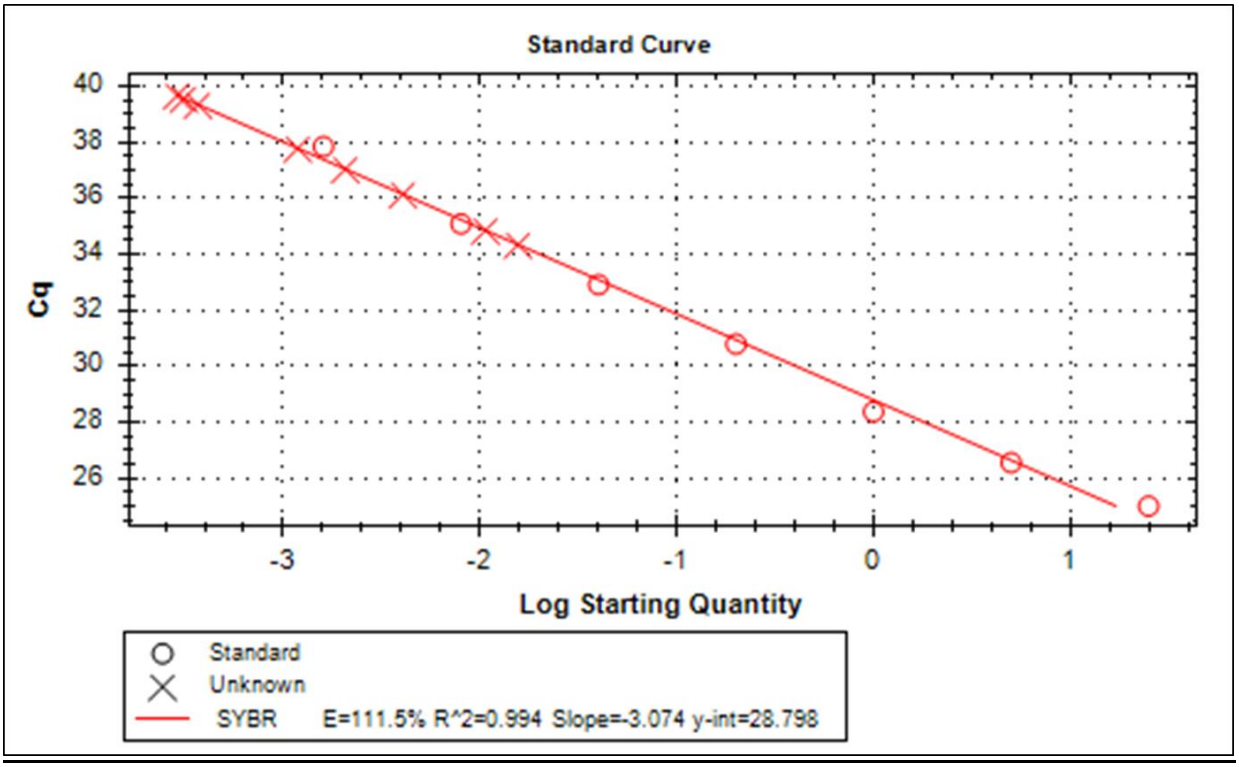

Figure 2. Results of the telomerase qPCR assay standard range optimization with TS standard range from 25 – 0.0016 fmol and including two different telomerase extension reaction times (60 and 90 min) for HeLa cell extracts (Xs on graph). Increasing the telomerase extension time to 90 min did not improve results.

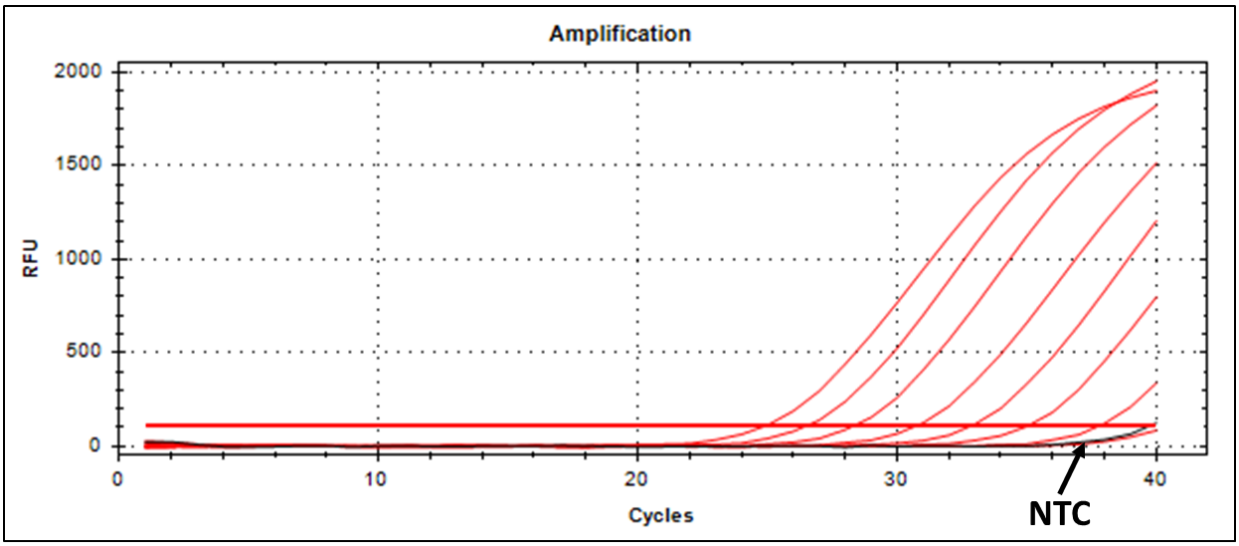

Figure 3. Amplification curves for the TS oligo standards and no template control (NTC, black line).

Prior to sampling Curaçao *Acropora palmata* colonies of known ages, assay validation was performed on laboratory cultured *Acropora cervicornis* and *Pocillopora damicornis* coral samples using HeLa cell extracts as a positive control. The results are presented in Figure 4. We observed that ACER extracts were positive for telomerase activity, while PDAM extracts were not. It is possible that an inhibitor co-extracted with the PDAM riboprotein, thus inhibiting the amplification reaction. Alternatively, telomerase activity was reduced due to the age of the colony since this colony was sourced from the aquarium trade and had been cultured in the Charleston aquaculture facility for more than 10 years.

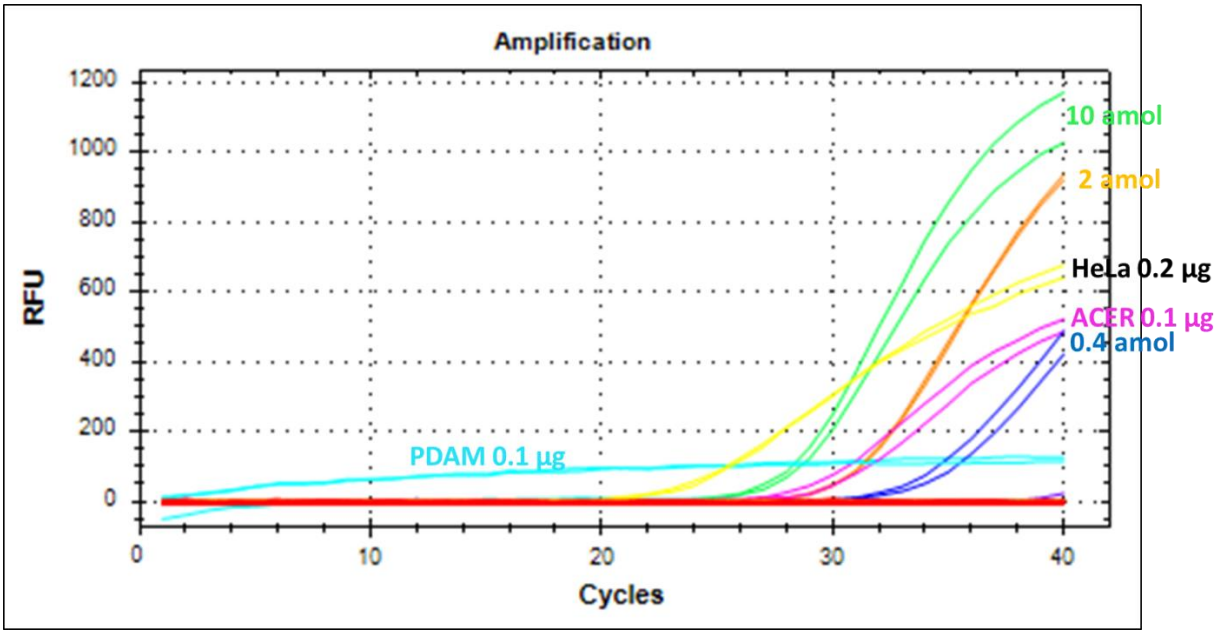

Figure 4. Results of the telomerase qPCR test with laboratory-cultured coral tissue samples from *Acropora cervicornis* (ACER) and *Pocillopora damicornis* (PDAM). All samples, including standards, were run in duplicate. HeLa cell extracts (0.2 µg protein, yellow traces) were used as an assay positive control. ACER extracts (0.1 µg protein) were positive for telomerase activity (Ct = ~27 cycles), while PDAM extracts (0.1 µg protein) were not.

Riboprotein extraction of *Acropora palmata* tissue

Following telomerase assay verification with coral extracts, we proceeded with riboprotein extraction from Curaçao *Acropora palmata* (APAL) coral tissue samples of known ages. The results of the riboprotein extraction for the APAL samples are presented in Table 1. All protein yields were acceptable and ranged from 0.75 – 2.39 µg/µL. One sample from the 8-year-old age group (1025) was accidentally lost during sample processing.

Table 1. *Acropora palmata* samples, amount of extracted protein and colony age used in the quantitative TRAP assay.

Sample number	Sample ID		Age (y)	Protein Concentration of Extract µg/µL
1	TA-SA	83	2	0.75
2	TA-WF	1147	2	1.2
3	TA-WF	1145	2	0.75
4	TA-MR	1242	4	1.38
5	CB-MR	1243	4	1.77
6	TA-MR	1244	4	1.33
7	TA-MR	1245	4	1.33
8	CB-MR	1248	4	1.08
9	TA-SA	1033	7	2.39

10	TA-SA	1037	8	0.91
11	TA-SA	1015	8	1.33
12	TA-SA	1017	8	1.31
13	TA-SA	1018	8	1.60
14	TA-SA	1025 (lost)	8	n/a
15	TA-MR	1235	≥60	0.95
16	TA-MR	1236	≥60	1.60
17	TA-MR	1237	≥60	1.23
18	TA-MR	1281	≥60	1.79
19	TA-MR	1282	≥60	1.87

Quantitative TRAP Assay

Initial telomerase activity PCR assays with ACER, GON and PDAM demonstrated that the assay works better for some stony corals than others, with laddering observed for ACER (weak activity) and GON (robust activity), in the polyacrylamide gel used to visualize results (Figure 1). We did not observe laddering patterns in the PAGE for PDAM, nor did we see positive results for this species in the telomerase qPCR test (Figure 4). While sample handling could be an issue (the nucleic acid on the riboprotein is quite sensitive to degradation if thawed), it is also possible that inhibitory compounds could be isolated with the protein which impede the amplification reaction. Since separate tests failed to amplify telomerase products from PDAM, it is more likely that inhibitory compounds are present in protein extracts from that coral species. Although reduced activity due to colony age cannot be ruled out.

Protein recovery was high for Curaçao samples used to generate the age estimation (Table 1). Only these samples were interrogated to conserve tissue samples with the qPCR TRAP assay. Unfortunately, when the assay was attempted with the Curaçao samples no telomerase activity could be detected (Figure 5). Amplified standards for the assay (5-fold dilutions ranging from 25.0 - 0.0016 fmol) are the curves on the right side of the figure (Ct values between 24 and 38 cycles). Several attempts were made to try to elucidate a reason for the sample failure (increased protein amount in assay, longer telomerase extension time, extractions from laboratory cultured APAL), but again, samples did not amplify. Since ribonucleic acids are notorious for instability, the assay failure could be due to sample handling or the length of storage time elapsed between Curaçao coral sample acquisition and assay (> 2.5 years due to COVID-19 closures). It also is possible that assay inhibitors co-eluted with the riboprotein, as suspected in the case of PDAM. Further work on extraction optimization will need to be done for this coral species.

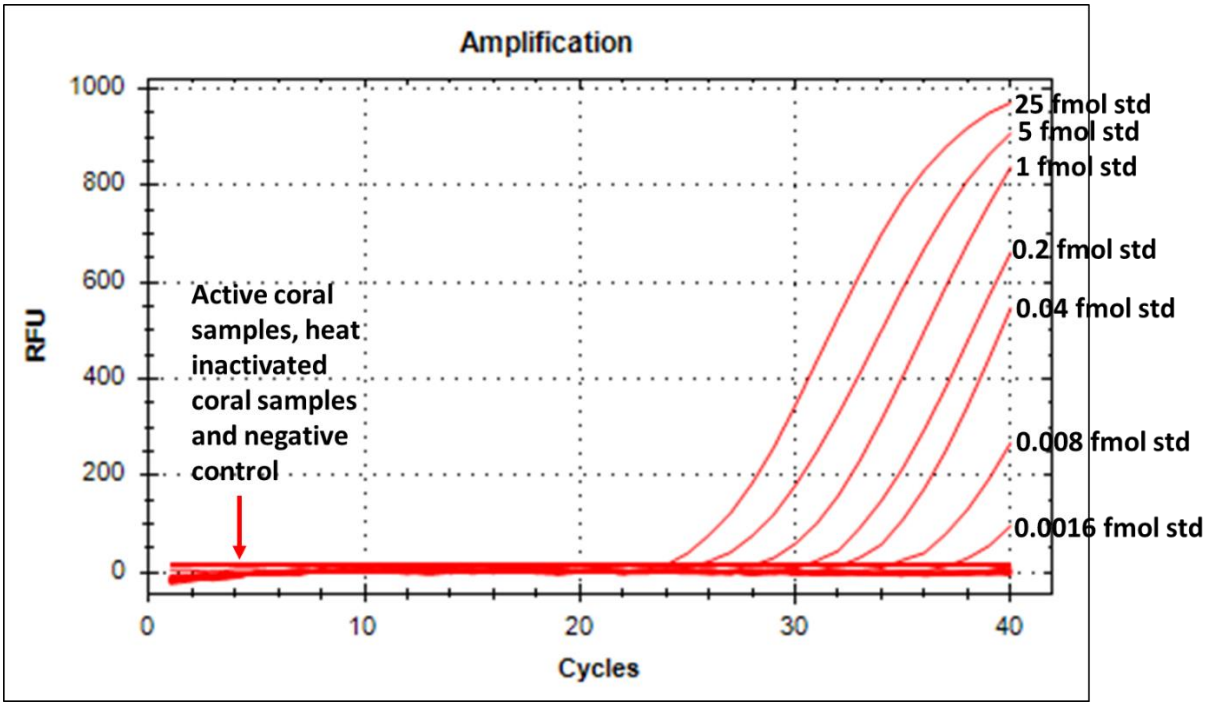

Figure 5. Results of *A. palmata* telomerase activity qPCR. No coral samples amplified. Standard curves are on the right side of the graph and exhibit Ct values of approximately 24 – 38 cycles.

Telomere Fragment Length Assay

The original and standard method to measure telomere length (terminal restriction fragment (TRF) assay) uses Southern blot analysis of genomic that has been digested with frequently-cutting restriction enzymes. The digested DNA is electrophoresed, blotted and probed with labeled oligonucleotides complementary to telomeric repeats representing terminal restriction fragments of chromosomes. The length of telomeres is estimated by comparison with DNA size standards. One of the commercialized products available is the Telo TAGGG assay (Roche). This assay requires relatively large amounts of DNA (>1 µg) that was not feasible with the amount of tissue available. Thus, the Southern-blot based assay platform was not pursued.

We identified one qPCR-based assay for telomere length quantification offered by a small biotech company, ScienCell (here). However, the kits are marketed only for human, rat, mouse and pig, with the specificity largely dictated by specific 'housekeeping' genes used in the quantification and normalization of the assay. In discussing the potential adaptation of this kit to coral the company's technical team indicated that if a coral specific single-copy housekeeping gene could be identified and substituted with the other kit reagents there was a reasonable possibility that it could be used to quantify the average telomere length in coral.

A literature search was undertaken to identify candidate single copy genes in coral. Oshiro et al. (2013) identified and validated a number of internal reference genes for

use in qPCR of gene expression in coral. The closest species to *Acropora palmata* that was evaluated was a Pacific acroporid (*Acropora nasuta*). Two genes from this species were recommended as valid single copy genes, elongation factor 1 alpha and the TATA box binding protein. Our colleague, Dr. Bishoy Hannah of the Joint Genome Institute compared the published primers for these genes with *A. palmata* genes and was unable to find homology. However, Dr. Hannah did send fasta files for these two genes in *A. palmata*. With this information in hand, *A. palmata* specific primers could be designed to amplify a short (75-150 bp) segment and the commercial telomere length qPCR assay will be tested for feasibility with coral telomere DNA. This work is continuing and if the assay can be successfully modified to work with *A. palmata* DNA extracts, the Curaçao samples will be evaluated for differences in telomere length.

Cryomilled tissue samples remain for most of the samples that were collected for age estimation and all of the samples of unknown age remain frozen at -80°C awaiting further analyses (Table 2).

Table 2. Additional samples of *A. palmata* remaining for further testing.			
Sample number	**Sample ID**		
1	TA-SA	1030	
2	TA-SA	1034	
3	TA-SA	1038-1b	
4	TA-SA	1038-1c	
5	TA-SA	1038-2c	
6	TA-SA	1038-3b	
7	CB-SA	1039	
8	TA-SA	1007	
9	TA-SA	1208-1a	
10	TA-SA	1208-2c	
11	TA-SA	1208-3a	
12	TA-SA	1208-3c	
13	TA-SA	1009	
14	TA-SA	1012	
15	TA-SA	1201-1a	
16	TA-SA	1201-1b	
17	TA-SA	1201-1c	
18	TA-SA	1201-2a	
19	TA-SA	1201-2b	
20	TA-SA	1201-2c	
21	TA-SA	1201-3a	
22	TA-SA	1201-3b	
23	TA-SA	1201-3c	
24	TA-SA	1002	
25	TA-SA	1011	
26	TA-SA	1022	
27	CB-SA	1057	
28	TA-WF	90	
29	TA-WF	91	
30	TA-WF	92	
31	TA-WF	93	

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Nakamichi, H., Ojimi, M.C., Isomura, N., Hidaka, M. 2012. Somatic tissues of the coral Galaxea fascicularis possesses telomerase activity. Galaxea 14:53-59.

Oshiro, Y., Kinjo, K., and Nakasone, K. 2013. Validation of internal reference genes for gene expression analysis in *Montipora digitata*, *Pocillopora damicornis* and *Acropora nasuta* by quantitative real-time PCR. *Galaxea.* 15:1-11.

Supplement 1. Telomerase activity qPCR protocol.

GENERAL EXPERIMENTAL OUTLINE: 1) coral homogenization in liquid nitrogen, 2) extracting riboprotein from coral homogenate using CHAPS lysis buffer and protein quantification, 3) telomerase extension of a synthetic oligo using riboprotein extracts and 4) detection and quantification of telomerase activity (extension products) using qPCR with oligonucleotide standard curve.

PREPARATIONS

Chemicals

1. Tris-Cl
2. CHAPS detergent ((3-((3-cholamidopropyl) dimethylammonio)-1-propanesulfonate))
3. EGTA (ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid)
4. Magnesium chloride
5. Glycerol (molecular biology grade)
6. Ribolock RNase inhibitor
7. Sigma Protease inhibitor cocktail (#P9599)
8. Nuclease-free water
9. Bradford protein assay dye reagent concentrate (#Cat# 500-0006)
10. Bovine serum albumin (standard for protein assay and TRAP extension buffer)
11. Potassium chloride
12. Tween 20
13. TS-STD template oligonucleotide, 68-mer, PAGE purification (5' - AAT CCG TCG AGC AGA GTT AGG GTT AGG GTT AGG GTT AGG GTT AGG GTT AGG GTT AGG GTT AG – 3')
14. TS primer, 16-mer, standard desalting purification (5' – AAT CCG TCG AGC AGT T – 3')
15. ACX primer, 30-mer, standard desalting purification (5' –GCG CGG CTT ACC CTT ACC CTT ACC CTA ACC – 3')
16. dNTPs, 10 mM
17. BioRad Power SYBR Master Mix (#4367659, ThermoFisher)
18. Laboratory detergent
19. Isopropanol

Equipment

1. Sterile glass bottles for solutions, 100-250 mL
2. DNase- and RNase-free sterile tubes (1.5 mL, 0.5 mL and 0.2 mL thin-wall)
3. 15 mL sterile polypropylene tubes
4. Small stainless steel spatulas (e.g., Fisher Scientific #50-873-389)
5. Autoclave
6. Sterile filtration apparatus to 0.2 µm
7. Nitrile gloves
8. Aluminum foil
9. Insulated bucket with ice
10. Refrigerated laboratory microfuge
11. Balance (0.00 g)

12. Weigh boat or weigh paper
13. Micropipettors, 1-1000 µL
14. Micropipet filter tips, 1-1000 µL
15. Freezer, -20 °C
16. Ultracold freezer, -80 °C
17. Refrigerator
18. Clear 96 well plate (Costar #3370 or similar)
19. Plate reader capable of reading absorbance at 595 nm
20. Vortex mixer
21. Heat block (0.5 mL tubes) to 85 °C
22. Spray bottle (for 70 % isopropanol)
23. Laboratory markers
24. Tube racks (for 0.2 µL, 0.5 µL, 1.5 µL and 15 mL tubes)
25. Water bath to 30 °C

Prepare stock solutions: All made in nuclease-free water

1. 1 M Tris-HCl, pH 7.5 = 12.1 g Tris base in 80 mL nuclease-free water, adjust pH to 7.5. To 100 mL final volume with nuclease-free water. Autoclave.
2. 1 M Tris-HCl, pH 8.3, as in #2 but adjust pH to 8.3. Autoclave.
3. 100 mM EGTA = 3.8 g EGTA in 20 mL nuclease-free water. Adjust pH to 11 with NaOH, then to pH 8.0 with HCl. Add nuclease-free water to 100 mL. Autoclave.
4. 100 mM $MgCl_2$ = 1.02 g $MgCl_2$ hexahydrate in 50 mL nuclease-free water. Filter sterilized.
5. 2.5 M KCl = 18.64 g in 100 mL nuclease-free water, autoclaved
6. 10 mg/mL bovine serum albumin = 0.1 g in 10 mL nuclease-free water, filter sterilized and stored at -20 °C
7. Glycerol, molecular biology grade, autoclaved

Make fresh 10% CHAPS = 1 g CHAPS in 9 mL nuclease-free water

Make 10 mL CHAPS Lysis buffer in sterile 15 mL polypropylene tube:

8.2 mL nuclease-free water
0.1 mL 1 M Tris-Cl, pH 7.5 (10 mM)
0.1 mL 100 mM $MgCl_2$ (1 mM)
0.1 mL 100 mM EGTA, pH 8.0 (1 mM)
0.5 mL 10 % CHAPS (0.5 %)
1.0 mL glycerol (autoclaved) (10 %)
(Store at -20 °C up to 1 year)

1. RIBOPROTEIN EXTRACTION

NOTE: Telomerase is a riboprotein responsible for adding protective repetitive DNA segments to the end of chromosomes. This extraction process keeps the RNA segment of the riboprotein intact. RNA is easily degraded, however. Sample extracts and reagents should be kept on ice

while in use. Care should be taken to keep RNase contamination low (wear gloves, spray surfaces with RNase inhibitors, include RNA stabilizers in extraction buffer).

Coral homogenate extraction protocol:

1. Don gloves.
2. Clean spatulas with detergent, rinse well and soak in 70% isopropanol for 15 min. Dry on foil sprayed with 70% isopropanol. Wrap in foil and place in liquid nitrogen or ultracold freezer to chill.
3. Change gloves and spray them with 70% isopropanol. Allow to dry.
4. Label sterile 1.5 mL tubes with sample name and place on ice.
5. Thaw CHAPS buffer on ice.
6. Aliquot 200 µL CHAPS buffer/sample tube. Keep on ice.
7. Add 1 µL Ribolock and 1 µL Sigma Protease Inhibitor Cocktail (Cat. #P9599) to each sample tube and mix well.
8. Using cleaned, chilled spatula, place frozen tissue homogenate in tube. Final weight of homogenate should be ~300-400 mg. Vortex to mix.
9. Keep samples on ice 30 min, vortexing quickly every 5 min or so.
10. Centrifuge at 14K x g for 20 min at 4 °C.
11. Transfer supernatant to clean tube with micropipettor and filter tip.
12. Centrifuge samples at 14K x g for 5 min at 4 °C to remove any residual particulates from sample.
13. Transfer clarified supernatant to clean tube, taking care not to aspirate any detritus.
14. Aliquot protein samples into clean, sterile 0.5 mL tubes (10 µL/tube), reserving 20 µL for a protein assay. Keep the protein assay tube on ice and immediately archive the remaining tubes in an ultracold freezer (-80 °C).
15. Perform protein assay. Use straight extract and 2-3 dilutions (1:2) to ensure that it will be in the standard curve range (target >0.1 µg/µL).
16. Store unused CHAPs buffer at -20 °C.

2. BRADFORD PROTEIN ASSAY

1. Prepare dye reagent by diluting one part dye reagent concentrate (BioRad Cat# 500-0006) with 4 parts nuclease-free water in a 15 mL polypropylene tube. (BioRad protocol states to filter through #1 Whatman, I haven't included this and haven't had problems). Make enough for all samples/dilutions and standards to be analyzed (will need 200 µL per well).
2. Prepare a stock of bovine serum albumin, 1 mg/mL. Perform dilutions indicated below. Use buffer or nuclease-free water as needed, be sure to use appropriate blank. The linear range for the plate assay is approximately 0.05 mg/mL to 0.5 mg/mL.
3. Pipet 10 µL of each standard or sample into wells of a clear 96 well microtiter plate (suggested plate layout below)
4. Add 200 µL diluted dye reagent to each well.
5. Mix by pipetting or plate mixer.
6. Incubate at room temp for at least 5 min (1 h max).
7. Measure absorbance at 595 nm. Standard curve is linear (r^2 = >0.97).

Standard preparation:

1. Stock BSA standard solution is 1 mg/mL
2. Dilute 1 mg/mL stock 1:2 = 500 µL of 10 mg/mL plus 500 µL of sterile water (0.5 mg/mL)
3. Using this 0.5 mg/mL solution, make the following dilution curve:
    a. 0.5 mg/mL (no dilution)
    b. 0.4 mg/mL (80 µL plus 20 µL water)
    c. 0.3 mg/mL (60 µL plus 40 µL water)
    d. 0.2 mg/mL (40 µL plus 60 µL water)
    e. 0.1 mg/mL (20 µL plus 80 µL water)
    f. 0.05 mg/mL (10 µL plus 90 µL water)

Suggested plate layout:

	1	2	3	4	5	6	7	8	9	10	11	12
A	STD1 0.5	STD1 0.5	SPL1	SPL1	SPL2	SPL2	SPL3	SPL3	SPL4	SPL4	SPL5	SPL5
B	SPL6	SPL6	STD2 0.4	STD2 0.4	SPL7	SPL7	SPL8	SPL8	SPL9	SPL9	SPL10	SPL10
C	SPL11	SPL11	SPL12	SPL12	STD3 0.3	STD3 0.3	SPL13	SPL13	SPL14	SPL14	SPL15	SPL15
D	SPL16	SPL16	SPL17	SPL17	SPL18	SPL18	STD4 0.2	STD4 0.2	SPL19	SPL19	SPL20	SPL20
E	SPL21	SPL21	SPL22	SPL22	SPL23	SPL23	SPL24	SPL24	STD5 0.1	STD5 0.1	SPL25	SPL25
F	SPL26	SPL26	SPL27	SPL27	SPL28	SPL28	SPL29	SPL29	SPL30	SPL30	STD6 0.05	STD6 0.05
G	SPL31	SPL31	SPL32	SPL32	SPL33	SPL33	SPL34	SPL34	BLK	BLK	SPL35	SPL35
H	SPL36	SPL36	SPL37	SPL37	SPL38	SPL38	SPL39	SPL39	SPL40	SPL40	SPL41	SPL41

3. TELOMERASE EXTENSION OF SYNTHETIC OLIGO TEMPLATE

TS-STD oligo template (68-mer): 5' - AAT CCG TCG AGC AGA GTT AGG GTT AGG GTT AGG GTT AGG GTT AGG GTT AGG GTT AGG GTT AG – 3'

The TS-STD oligo should be diluted to 10 µM in nuclease-free water and stored at -20 °C.

Make fresh 0.5 % Tween 20 = 50 µL in 10 mL nuclease-free water

Make up 10X TRAP Extension buffer in sterile 15 mL polypropylene tube:

2.0 mL 1 M Tris-Cl, pH 8.3, sterile
1.5 mL 100 mM $MgCl_2$, sterile
2.5 mL 2.5 M KCl, sterile
1.0 mL 0.5% Tween 20 (50 µL Tween 20 in 10 mL n-f water)
1.0 mL 10 mg/mL BSA fraction V, in nuclease-free water, filter sterilized
1.0 mL 100 mM EGTA, sterile (10 mM)
1.0 mL nuclease-free water
(Store at -20 °C for up to 3 months)

1. Turn on 85 °C heat block (for heat inactivation of samples).
2. Heat inactivate enough protein extracts for assay. Ideally this should be done for every dilution or sample. Place at least one tube of protein extract at 85 °C for at least 10 min. CHAPS lysis buffer with inhibitors (RiboLock and protease inhibitor cocktail) should be used for any dilutions. Samples for telomerase activity should be held at -80 °C until immediately before use and kept on ice as much as possible. Target for coral extracts is 0.1-0.4 µg/µL, but initial samples should be evaluated undiluted, until activity can be gauged. Best to run straight extract and 2-3 dilutions in CHAPS buffer, with corresponding heat-inactivated samples.
3. While HI samples are incubating, make up Sample Master Mix in a sterile 1.5 mL tube for the telomerase extension reaction (make up enough for all protein sample tubes (no standards), active and heat killed, 48 µL total volume/tube):

   5 µL 10X TRAP extension buffer
   1 µL TS template oligo, 68-mer (10 amol/µL)
   2 µL dNTPs (10 mM each)
   40 µL nuclease-free water
4. Aliquot 1-2 µL of each sample extract to be tested (unheated and heat inactivated samples) into 0.2 mL thin walled tubes (target is 0.1-0.4 µg per tube). Add 48 µL of Sample Master Mix to each tube.
5. Float sample tubes in a water bath at 30 °C for 60 min. Remove from water bath and place on ice.

4. TELOMERASE ACTIVITY qPCR

1. While telomerase oligo extension is in progress, dilute 10X TRAP buffer 1:10 in nuclease-free water (1X) in a 15 mL sterile polypropylene tube.
2. Dilute TS-STD oligo in 1X TRAP buffer for standard curve in 1.5 mL sterile tubes. Keep on ice, or archive at -20 °C. TS-STD STOCK in nuclease-free water = 10 µM = 10 pmol/µL = 10,000 fmol/µL.
   a. 2.0 µL STOCK + 798 µL TRAP Buffer = 25 fmol/µL
   b. 100 µL (A) + 900 µL TRAP buffer = 2.5 fmol/µL; using 10 µL = 25 fmol STD
   c. 100 µL (B) + 400 µL TRAP buffer = 0.5 fmol/µL; using 10 µL = 5 fmol STD
   d. 100 µL (C) + 400 µL TRAP buffer = 0.1 fmol/µL; using 10 µL = 1 fmol STD
   e. 100 µL (D) + 400 µL TRAP buffer = 0.02 fmol/µL; using 10 µL = 0.2 fmol STD
   f. 100 µL (E) + 400 µL TRAP buffer = 0.004 fmol/µL; using 10 µL = 0.04 fmol STD
   g. 100 µL (F) + 400 µL TRAP buffer = 0.0008 fmol/µL; using 10 µL = 0.008 fmol STD
   h. 100 µL (G) + 400 µL TRAP buffer = 0.00016 fmol/µL; using 10 µL = 0.0016 fmol STD (Standards can be stored frozen (-20 °C) for re-use.)
3. Dilute primers to 100 µM in nuclease-free water, if not already done. Store these at -20 °C when not in use. Thaw on ice before use.
4. Make primer mixture, 10 µM each: 50 µL TS primer (100 µM), 50 µL ACX primer (100 µM) and 400 µL nuclease-free water. This can be stored at -20 °C and reused by thawing on ice.
5. Make up SYBR-primer mixture enough for all tubes (samples, heat-inactivated and standards):

10 µL SYBR Green Mix + 0.2 µL TS+ACX primer mixture (10 µM each) per tube. Make up extra to account for any pipetting errors.

6. Add 10 µL TRAP incubation products or standards to each well of a 96 well PCR plate on ice. For standards this will be 10 µL of dilutions B-H above (0.0016-25 fmol standard range).
7. Add 10.2 µL of SYBR-primer mix to each well and pipet up and down a couple of times to mix.
8. Place plate in qPCR machine: heat kill telomerase and amplify products (~3 h):
   95 °C, 10 min (heat kill telomerase)
   40 cycles: 94 °C, 30 s
   58 °C, 30 s
   72 °C, 90 s
   Dissociation (melt)

5. CALCULATIONS

Determine relative telomerase activity for each sample: fmol product/µg protein used in the assay.

6. PAGE ANALYIS OF qPCR PRODUCTS (OPTIONAL):

1. Pour 12.5% acrylamide:bis (29:1) gel in 0.5X TBE (36 mL volume, 16cm X 16cm X 1 mm) and let polymerize 1 h:
   1.8 mL 10X TBE
   15 mL 30% acrylamide:bis (29:1)
   18.9 mL di water
   0.3 mL 10% ammonium persulfate
   30 µL TEMED
2. Mix 20 µL PCR sample with 4 µL loading dye (30 µL + 6 µL dye for straight PCR –not qPCR)
3. Mix 4 µL 100 bp markers with 16 µL 0.5X TBE and 4 µL loading dye.
4. Rinse wells and add sample to each.
5. Make up 3 L 0.5X TBE.
6. Electrophorese in cold, 0.5X TBE, 400 V for 1.5-2.0 h.
7. Stain in 200 mL SYBR green in 0.5X TBE for 20 min and image.