Identification and Optimization of qPCR Standards for *Aiptasia pallida*

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Six housekeeping genes were selected as potential qPCR standards based on their prior use in coral studies. Gene names used here are those assigned to the *Aiptasia pallida* genes and differ in most cases from those used in the other organisms. The genes encoding ribosomal protein L11 (*RPL11*), NADH-dehydrogenase subunit 5 (*NDH5*), and glyceraldehyde-3-phosphate-dehydrogenase (*GPD1*) were reported by Kenkel *et al*. (2011) to be stable in *Porites astreoides* during heat stress, settlement induction, and metamorphosis. The genes encoding ribosomal protein S7 (*RPS7*) and adenosylhomocysteinase (*AHC1*) were used as standards during studies of thermal stress in *Acropora aspera* by Leggat *et al*. (2011). The β-actin gene (*ACT1*) was used by Rodriguez-Lanetty *et al*. (2006) to explore modulation of host-gene expression and was used as the standard for early qPCR studies in our lab.

Primers were developed and tested for these six potential standard genes. The aposymbiotic *A. pallida* transcriptome (see this website under "Projects") was searched using tblastx with sequences from *Porites lobata* for *NDH5*, *P. astreoides* for *RPL11*, *Urticina eques* for *GPD1*, *Acropora millepora* for *RPS7*, and *Nematostella vectensis* for *AHC1*. The loci identified in the *A. pallida* transcriptome were searched using blastx in NCBI and all top hits were indeed the genes of interest. The identified loci were then translated using ORFPredictor and the longest ORFs were used to identify conserved sequences by performing protein alignments in MacVector with sequences available from NCBI. Conserved sequences were then used to develop primers using PrimerQuest from Integrated DNA Technologies (IDT).

Primers were tested on *A. pallida* cDNA and gDNA. Primers that spanned an exon-intron junction were preferentially identified for further use (Table 1). PCR products were cloned into a TA cloning vector and electroporation-competent *E. coli* cells were transformed with the plasmids. Transformed cells were plated on Ampicillin/X-Gal plates and white/light-blue colonies were selected for colony PCR using M13 forward and reverse primers. PCR products were sequenced, and the sequences were aligned with the expected sequences from the transcriptome. All primer pairs accurately selected the sequences of interest.

Table 1. Primer sequences

Gene	Primer Sequences
RPL11	F: AGCCAAGGTCTTGGAGCAGCTTA
	R: TTGGGCCTCTGACAGTACAGTGAACA
RPS7	F: ACTGCAGTCCACGATGCTATCCTT
	R: GTCTGTTGTGCTTTGTCGAGATGC
NDH5	F: AGCAGTTGGTAAGTCTGCACAA
	R: GTAACCATGGTAGCAGCATGAA
GPD1	F: AACAGCTTTGGCAGCACCTGTAGA
	R: TGCTTTCACAGCAACCCAGAAGAC
AHC1	F: CCATTACAGCAACAACACAGGCCA
	R: GCATCAAACGTTGGCAGATGAAGC
ACT1	F: ACACCGTCTTGTCAGGAGGTTCAA
	R: TCCACATCTGTTGGAAGGTGGACA

The six genes were then tested for their expression levels across 11 experimental conditions (Table 2). RNA was extracted from 3-4 medium-sized anemones from each condition using a Trizol/RNeasy hybrid protocol (details available upon request). RNA integrity was checked both by using a Nanodrop and by running samples on a 2% agarose gel. For all RNA samples used, 260/280 readings were >1.9, and two clear rRNA bands were visible. For each condition, 300 ng of RNA was reverse transcribed using the Maxima® First Strand cDNA-synthesis kit for RT-qPCR (Fermentas). 17 μL of RT product was then diluted with 23 μL of H_2O. 2 μL of this cDNA solution was then used for the qPCR reaction. Each qPCR well had 2 μL of cDNA, 2 μL of H_2O, 5 μL of *Power* SYBR® Green PCR Master Mix (Applied Biosystems), and 1 μL of a primer mix containing 1.5 μM forward (F) primer and 1.5 μM reverse (R) primer.

Table 2. Experimental conditions used to test gene-expression levels

Conditions [a]	**CC7** [b] **Sym** [c]	**CC7** [b] **Apo** [d]
Room Temperature (27ºC)	x	x
1 h heat shock (35ºC)	x	x
1.5 h heat shock (37ºC)	x	x
1 h cold shock (8ºC)	x	x
1 h incubation with 500 μg/mL dsRNA[e] (27ºC)	x	x
Kept in the dark for 1 month (27ºC)	x [f]	not done

[a] Except for the sample incubated in the dark, all anemones were incubated on a 12L:12D cycle with 25 μmol photons m^{-2} s^{-1} from Cool White fluorescent bulbs, and the manipulations indicated were performed during the light period.
[b] CC7 clonal line of *A. pallida* (Sunagawa *et al*., 2009).
[c] Symbiotic CC7 with endogenous Clade A dinoflagellate symbiont population.
[d] Aposymbiotic CC7 animals that had been cured of their endogenous endosymbionts by a combination of cold shock, DCMU treatment, and extended growth in the dark. All anemones were screened for absence of dinoflagellates prior to use in these experiments.
[e] dsRNA (477 bp) synthesized for *A. pallida* nematogalectin gene knockdown.
[f] Represents a partially aposymbiotic condition.

The primer efficiency of each primer pair was tested across a dilution series of 1:1, 1:10, 1:100, 1:1000, and 1:10000 cDNA; the calculated efficiencies were 95-105%. Possible gDNA contamination in RNA samples was tested by running RNA-only controls; these samples showed no amplification. Standard qPCR settings were used, and an additional dissociation stage was added to test for the presence of multiple products. The dissociation stage showed only one clear peak in every case.

Ct values for each of the six genes under each of the 11 conditions were analyzed using geNorm (Vandesompele *et al*., 2002) to determine the relative expression stabilities of the prospective standard genes; the M-values are inversely proportional to the stabilities of the genes (Table 3). *ACT1* (M = 0.625) and *AHC1* (M = 0.775) were considerably less stable in expression than the four genes shown in the table.

Statistical analysis of the qPCR results also indicates that *ACT1* should not be used as an expression standard in the study of symbiosis in *Aiptasia* due to the large expression difference between aposymbiotic and symbiotic animals: there was a significant ($p = 0.002$) up-regulation

Table 3. Assessment of gene-expression stability under various conditions [a]

Gene	Function	geNorm M	Product Sequence	Product Length	Primer Efficiency
RPL11	Encodes a component of the 60S ribosomal subunit	0.357	AGCCAAGGTCTTGGA GCAGCTTACAGGCCA ACAGCCTGTGTTTTCAA AAG **(INTRON – 236bp)** CTCGCTACACTGTGAGA TCTTTTGGAATCAGAAG GAACGAGAAGATCTCT GTTCACTGTACTGTCAG AGGCCCAA	cDNA 125 bp gDNA 361 bp	98%
RPS7	Encodes a component of the 40S ribosomal subunit	0.380	ACTGCAGTCCACGATGC TATCCTTGAAGATCTTG TCTTTCCTAGTGAAATT GTTGGCAAAAGGATAA GAGTTAAACTTGATGGT TCACGTCTCGTTAAAGT G **(INTRON - 411bp)** CATCTCGACAAAGCAC AACAGAC	cDNA 125 bp gDNA 536 bp	97%
NDH5	Encodes NADH-dehydrogenase subunit 5	0.423	AGCAGTTGGTAAGTCTG CACAATTAGGCTTACAC ACTTGGTTACCGGATGC AATGGAAGGT **(INTRON – 1729bp)** CCAACTCCGG TGTCTGCCTTGATTCAT GCTGCTACCATGGTTAC	cDNA 105 bp gDNA 1834 bp	95%
GPD1	Encodes glyceraldehyde-3-phosphate-dehydrogenase	0.530	AACAGCTTTGGCAGCAC CTGTAGAGGCTGGGAT GATATTCTGATTGGCAC CTCTACCATCACGCCAT TTCT **(INTRON - 567bp)** TCCCACTAGGTCCATCT ACAGTCTTCTGGGTTGC TGTGAAAGCA	cDNA 114 bp gDNA 681 bp	95%

[a] Tested across the 11 experimental conditions described in Table 2.

in *ACT1* expression in aposymbiotic (or mostly aposymbiotic) anemones compared to symbiotic anemones across all conditions. This was determined by normalizing qPCR Ct values with the two most stable standard genes (*RPL11* and *RPS7*) and performing a Mann-Whitney statistical test on *ACT1* expression levels in aposymbiotic and symbiotic anemones.

References

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