

A Standard Field Protocol for Testing Relative Disease Resistance in *Acropora palmata* and *Acropora cervicornis*

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ABSTRACT:

Coral disease has been identified as an ongoing threat to the recovery of sustainable coral populations. This document provides a standard field protocol for screening disease resistance traits in the imperiled coral species, *Acropora palmata* and *Acropora cervicornis*, particularly genotypes under nursery culture for population enhancement. It is intended to be implemented by practitioners and researchers in coral restoration and provide standardized information on disease resistance that can aid in the design and success of population enhancement efforts.

BACKGROUND:

Lethal disease remains a severe and ongoing threat to the recovery of both *Acropora palmata* and *Acropora cervicornis* throughout their range. Vollmer and Klein (2008) reported that 6% of *A. cervicornis* genotypes tested in Panama showed innate resistance to disease within both a year-long surveillance study and replicated challenge experiments ($n \geq 5$), providing a hopeful indication that the disease threat is not insurmountable.

Growing investment is being made in culturing and restocking for both species and there is wide recognition that disease can affect the ultimate success of restored populations. The need to quantify disease resistance (along with other performance-related) traits in cultured stocks was recently identified in a prioritization of research needs to support coral restoration in Florida (Hunt and Sharp 2014). Information on extent of natural disease resistance for *A. cervicornis* in areas other than Panama, and for *A. palmata* are also needed. Identifying disease resistant phenotypes is the first step to leveraging them to enhance resilience of restored populations and to understanding their underlying biological mechanisms.

The protocol for challenge experiments described in this document is intended to be implemented in a standardized way, with the goal of providing comparable information regarding *relative disease resistance/susceptibility* among cultured genotypes from different nurseries and regions. One factor that is particularly difficult to standardize is the identity or etiology of the inoculants used in the challenge experiments, given poor characterization of disease etiology in these species. This uncertainty must be acknowledged and standardized to the degree possible in terms of observable signs and in taking care to randomize the pairing of inoculants with test genets. However, it should be remembered that the goal of this protocol is to test the *susceptibility* of coral stocks to disease, not to test or describe the process or mechanisms of *transmission* per se. Hence, perfect control of the nature of the inoculant challenge, while desirable, is not necessary to make conclusions about susceptibility.

It is also important to stress that the testing of cultured genotypes/fragments can be accomplished with minimal risk by performing challenge assays in quarantined areas and always transporting the healthy test fragment TO the site of the diseased inoculant; no translocation of diseased corals between sites should be undertaken. Additional standard precautions with regard to biosecurity should also be observed, such as at least daily dis-infection of tools and dive gear (5% bleach solution) and planning daily work to progress from sites unaffected to sites affected by disease. Additional biosecurity considerations for field disease investigations are discussed in Chapter 5 of Woodley et al. (2008).

PROCEDURES:

Note that field testing of this protocol was conducted under permits from the Florida Keys National Marine Sanctuary and the Florida Fish and Wildlife Conservation Commission

STEP 1: Staging

At least 2 weeks prior to the experiment, multiple replicate ~ 10cm length fragments are snipped from each genotype to be tested. These replicate ‘test’ fragments should be staged within the nursery and allowed to heal and stabilize. Minimal contact with surfaces, sediments, or the bottom is desirable (e.g., suspension from lines or floating ‘tree’ structures; Fig 1) as these can be additional sources of infection. For *A. palmata*, the required healing time may be longer as the intent is that no exposed tissue margins are present on the fragment.

STEP 2: Determine appropriate disease inoculants

The intent is to utilize active/rapidly progressing diseased coral branches (inoculants) to provide a disease exposure (or ‘challenge’) to the test fragments by cable-tying them in direct contact. Availability of truly ‘active’ diseased inoculants may be limiting. Nonetheless, fewer, more-active replicates are more desirable than more replicates of questionable virulence. Replicates of each test genet can be conducted over time (i.e., multiple trials) as diseased inoculants are available, though some care is needed in combining multiple trials (see discussion). Active disease is identified as a tissue front adjacent to relatively extensive areas of white skeleton (indicating rapidity of tissue loss) and often displaying tissue sloughing (i.e. small patches of brown tissue clinging to the skeleton along the tissue margin; Fig 2). If time and effort are available, a cable tie can be applied to the tissue margin of each candidate inoculant a day or two prior to implementation to ensure the real-time progression of active tissue loss.

For disease present within the nursery population, inoculant fragments are snipped from diseased colonies and directly applied in the challenge experiments. Although it is not appropriate to transport diseased fragments between sites, nursery operations often ‘cull’ diseased fragments as a routine husbandry measure (i.e. excise them from their growth position and either pile them at a distance from the healthy stocks or remove/sacrifice them completely). In this case, the diseased fragments are simply being ‘culled’ temporarily to the experimental assay area. Separate nursery structures should be established as a quarantine area for the challenge assays at some distance from the main nursery population and staging area. There is yet no evidence to determine what a ‘safe’ distance may be, though it may be affected by currents and potential predator movement.

If disease is not present within the nursery population, diseased colonies within neighboring reef populations can be used as inoculants. Additional logistic and scientific challenges are involved in conducting wild trials. Logistically, suitable wild actively diseased colonies need to be located before test fragments are moved to minimize transit time, and additional permitting will likely be required. Establishing these replicates generally requires calm sea conditions both to minimize damage in transit and to achieve adequate contact without damaging the inoculant colonies (difficult in surge). Scientifically, there is likely to be inherently more variability in the conditions of a natural reef environment than a nursery (e.g., proximity and diversity of macroalgae or other microbially-rich surfaces, potential contact with predators or other animals, diversity of disease inoculant present). These concerns should be acknowledged and addressed as best as possible (especially by the randomization of test genets

with inoculants), but should not be paralyzing. Once active inoculant colonies have been identified, healthy test fragments can be transported from the staging area and physically attached to these ‘wild’ colonies for the duration of the assay. If multiple diseased margins are available on a single inoculant colony, different test genotypes should be applied (rather than several replicate fragments of a single test genet).

In our experience, *A. palmata* and *A. cervicornis* disease conditions are mutually transmissible (Pontes et al., Pers. Obs; Williams and Miller 2005) so either species can be considered for use as an inoculant. However, consistency across replicates in terms of inoculant type and virulence is desirable. If possible, consider fixing a few additional inoculant fragments in formalin-based fixative for histopathological analyses and/or flash frozen for molecular analyses to better characterize (post-hoc) the inoculant condition(s) being used in each trial.

STEP 3: Establish challenge assays

There is ample evidence that disease outbreaks are more common during or after warm thermal stress or bleaching. This may present some dilemma in that disease may be more available in warmer times of year, but there may also be reduced resistance that is discernable when fragments are under warm temperature stress (E. Muller, pers. comm). For this reason, it is recommended that resistance assays be targeted in temperatures below 29-30°C if disease is available. If disease is only available in conditions where ambient temperatures are higher than this, assays can be conducted, but results should be interpreted cautiously (i.e., some degree of disease resistance of the test genotypes may be overcome by the warmer conditions). Monitoring of temperature is also desirable during the challenge assays.

Due to the uncertainty in the etiology and virulence of each individual inoculant, it is important to randomize or distribute inoculants among test genet replicates. (For example, it would be undesirable to gather the ‘best’ inoculants first and apply them to replicates of test genets 1 and 3, leaving only more marginally active inoculants available to apply to replicates of test genets 2 and 4.) On Day 0 of an assay trial, the test fragments are moved, if needed, to the quarantine area structures or transported to the reef location if using *in situ* reef-colony inoculants. It is always good to perform any handling or transport of healthy frags first (or by an individual diver) and of diseased fragments later (or by a second person). Separate, designated containers and implements should be used in handling diseased and healthy fragments, and disinfected in 5% bleach solution at the end of each day. Disposable lab nitrile gloves should be worn (and changed between handling diseased and healthy fragments).

Each fragment should be applied in random/haphazard sequence to its inoculant. Each assay replicate should have an individual designation (tag or identifier of some kind) as each test fragment will be scored individually over multiple time points. The designation should include both the genotype and the replicate fragment within that genotype (e.g., K3-a for genotype “K3” and replicate “a”).

The test and inoculant fragments should be fit together so that there is direct contact between the tissue margin of the diseased inoculant and the healthy tissue of test fragment. If good contact is not possible due to irregular shape of the inoculant, attempt to pair it with a different test fragment or exclude it. While holding them in this position a cable tie should be secured tight enough to prevent wobble between the fragments but no tighter. This procedure can result in a very small amount of abrasion to calices of the test fragment, but based on past observation of non-diseased controls and many non-transmitting replicates, this abrasion does not result in tissue loss. For small fragments, small beaded cable ties are preferred as their

flexibility allows them to ‘nest’ more easily among the calices of the fragment providing a snug hold with minimal abrasion (e.g., Fig 3, 4, 5). In cases of attaching to large wild inoculant colonies, heftier cable ties will likely be needed (e.g., Fig 4)

An initial scaled photograph should be taken of each pair with its tag.

All dive and other equipment should be soaked in a 5% bleach solution for decontamination after handling diseased corals, either at the end of the dive day or prior to visiting another dive site where disease is not present.

STEP 4: Maintenance and monitoring of challenge assays

Although there is some flexibility, we recommend monitoring the assay on days 1, 3, 5, and ending on day 7. If you wish to observe the assay for a longer period of time, we encourage an observation to be made on day 7 (or 8 if 7 is impossible) so as to have a standard observation among trials and practitioners/stocks. On day 1 it is occasionally necessary to tighten the cable ties of pairs that appear slack once fragments have had a chance to ‘settle’ in order to prevent ongoing abrasion. However, we do not recommend any additional manipulations to the assay (e.g., replacement or repositioning of inoculants). As stated before, there is inherent variability in inoculant quality. The strategy is to standardize the application of the challenge treatment (i.e., implementing a single direct contact between actively regressing tissue and the test fragment) and observing the response of the test fragment for 7 days. In this way, variability in inoculant quality (e.g., death or remission during the trial) should be distributed among treatments and not confound the conclusions.

On each monitoring day, each assay should be examined carefully for the development of incipient tissue loss on the test fragment with particular attention to the point of contact with the inoculant (Fig 4, Day 1). Each test fragment should be scored for presence/absence of tissue loss. When tissue loss is observed, the size of the lesion(s) can also be measured (either length and width, if the lesion is confined to one surface, or width and circumference if the lesion wraps all the way around a branch) with a ruler and recorded. Alternatively, the size of the lesion can be measured just at the end (day 7). A scaled photograph can be taken of each replicate on each monitoring day as a record.

All dive and other equipment should be soaked in a 5% bleach solution for decontamination after handling diseased corals, either at the end of the dive day or prior to visiting another dive site where disease is not present.

STEP 5: Assay completion

On the last day (targeted as day 7), each replicate should have observations recorded as in previous days (presence/absence of tissue loss and size of scar). Afterward, the pair can be cut apart and a final, unobstructed scaled photograph and measurement of lesion (if present) of the target fragment should be made. If the inoculant fragments and transmitted test fragments are still alive, they can be sampled for histopathological or molecular characterization, or sacrificed. If inoculant material is limiting, transmitted test fragments that are still alive and appear to have active tissue loss (same considerations as described in Step 2 above) can be used as inoculants in a subsequent trial. Un-transmitted test fragments can be considered for several alternate fates

such as export for other beneficial uses (e.g., lab experimentation), sampling for histological or molecular characterization, or reintegration to the nursery stocks after a longer quarantine period without development of disease signs. Deciding among these fates should be undertaken in consultation among practitioners and permitting agencies.

STEP 6: Data analysis

Several parameters can be developed from the scores that have been recorded. These parameters can be averaged within and compared between genotypes.

Proportion of fragments transmitted: also can be thought of as the risk of transmission (upon exposure)

Transmission Time (d): Number of days for tissue loss to initiate in transmitted test fragments

Rate of tissue loss (cm^2/d for fragments which have transmitted): total ending lesion size/# days since tissue loss first occurred. Note that the sample size for this parameter will be less than the number of assays because not all replicates will transmit. Additional replicates may need to be conducted to obtain a robust sample size for this parameter.

DISCUSSION:

The protocol for challenge experiments described in this document is intended to be implementable in a standardized way, with the goal of providing comparable information regarding relative disease resistance among cultured genotypes from different nurseries and regions. It is not designed to investigate the likelihood or process of transmission, itself. For this reason, we consider the implementation of experimental controls as optional. In direct transmission assays, controls may be implemented to discern potential artifactual disease transmission (i.e., not caused by the challenge treatment). For example, physically-caused lesions such as abrasion may lead to disease signs regardless of an inoculant challenge. Also, there is sometimes concern regarding potential physiological responses to physical contact with non-self tissue (so called allo-graft response). If molecular or physiological responses are being examined, both of these situations (spurious transmission or allo-response) would require controls (i.e., inclusion of test samples that experienced either no direct contact or direct contact with a 'healthy' inoculant). However, neither of these situations impede the interpretation of relative disease resistance among genets as spurious transmission would still be a contraindication of resistance. Similarly, in our experience, allo-response in healthy control assays, though sometimes manifesting gross observable signs, does not yield tissue loss on the scale of one-week assays (see Fig 5 for example). Thus, for the purpose of discerning resistance, we recommend prioritizing effort and coral material to challenge assays, rather than controls.

There is inherent variability in the implementation of field challenge assays, especially as the lack of diagnostic tests for Atlantic *Acropora* disease makes variation in the inoculant disease condition unavoidable. There is also some variation in the effectiveness of contact between an individual pair, the ambient temperature, etc. Along with random distribution of inoculants among test genets, this unavoidable variation can be partially addressed with replication.

Although there is no fixed target number for replication, based on the Vollmer & Klein (2008) published standard we recommend a minimum of five replicates. Generally the more replicates that are conducted, the greater the confidence in the conclusion. In some cases, genets that have proved resistant in a moderate number of standardized replicates might show a low level of transmission at a higher level of replication.

Table 1 below shows some illustrative preliminary data from two trials with a set of four genets of *A. cervicornis*. The first trial involved five replicates each using inoculants snipped from resident diseased conspecific colonies within the nursery. One of these genet showed no transmission among 5 replicates in the first trial. This, along with a lack of disease observed in field surveys on its native reef, would have qualified it as a ‘resistant’ genet in the Vollmer & Klein (2008) study. We have not conducted field surveys of disease occurrence on these genets. However, in a subsequent trial, one of three fragments of this genet did transmit, yielding an overall risk of transmission of 12%. Indeed, the overall risk of transmission was higher in Trial 2 (8/13) than in Trial 1 (7/20). Perhaps the temperature was more stressful or the background disease virulence was higher, and these factors should definitely be considered in interpretation. Nonetheless, the RELATIVE resistance among these genets was fairly consistent between trials and this is the conclusion we draw: Genet C4 is RELATIVELY disease-resistant (risk of 12%) compared to the other three tested (risk of 50-62%) at a level of 8 replicates conducted in a nursery environment. Additional challenge assays with a different type of disease inoculant may be contemplated as a next step, as well as targeting additional ecological studies to discern potential tradeoffs (e.g., does this disease-resistant genet show relatively high or low reproductive effort? thermal tolerance?) or molecular studies to discern mechanistic differences.

Table 1: Example challenge assay results from two trials testing four *Acropora cervicornis* genets.

	TRIAL 1		TRIAL 2		POOLED		
Genet	# tested	# transmitted	# tested	# transmitted	# tested	# transmitted	RISK
C1	5	2	4	3	9	5	55.6%
C2	5	2	3	2	8	4	50.0%
C3	5	3	3	2	8	5	62.5%
C4	5	0	3	1	8	1	12.5%

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Atmospheric Administration NOAA Technical Memorandum NOS NCCOS 80 and
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288 **Figure 1** Step 1: Test fragments of both species recently staged prior to conducting challenge assays. In
 289 this case, replicate fragments of each genotype (designated by the white numbered goat tag) are staged on
 290 the same tree branch. Fragments should heal/stabilize for at least two weeks prior to initiating assays.



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Figure 2 Examples of ‘active disease’ with tissue sloughing on *A. cervicornis* and *A. palmata* which would serve as suitable inoculants for disease challenge assays.

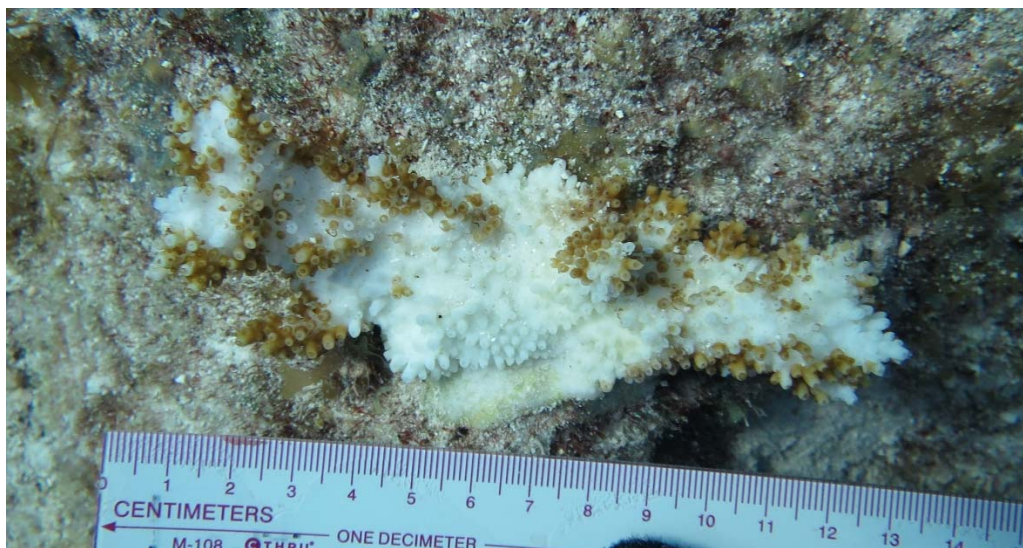
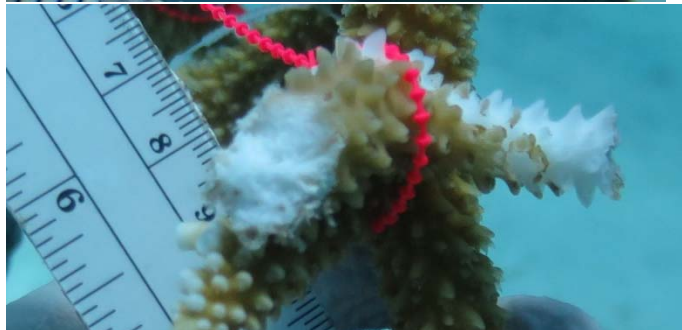
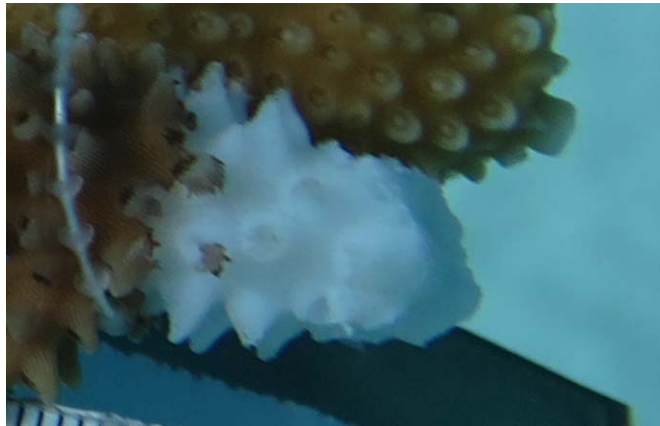
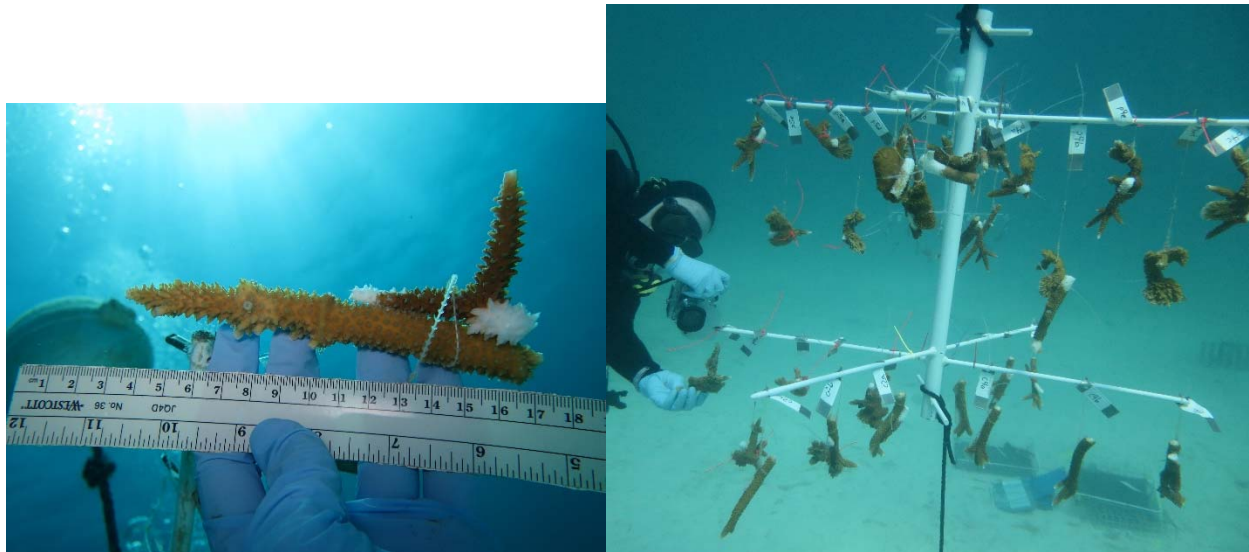
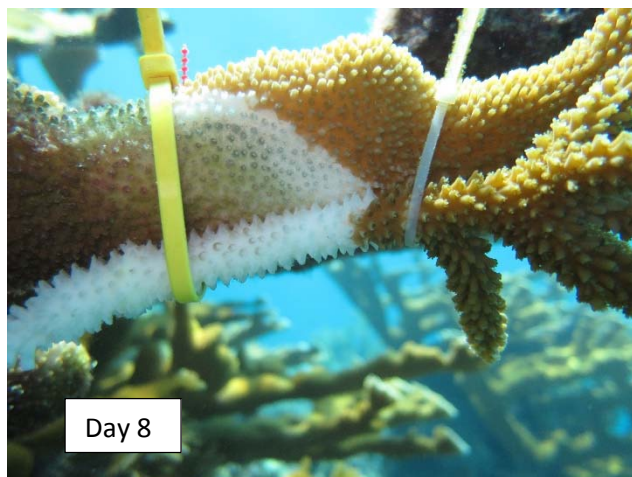
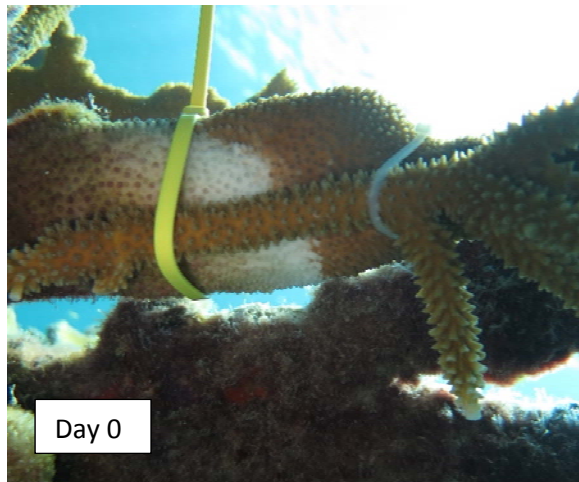


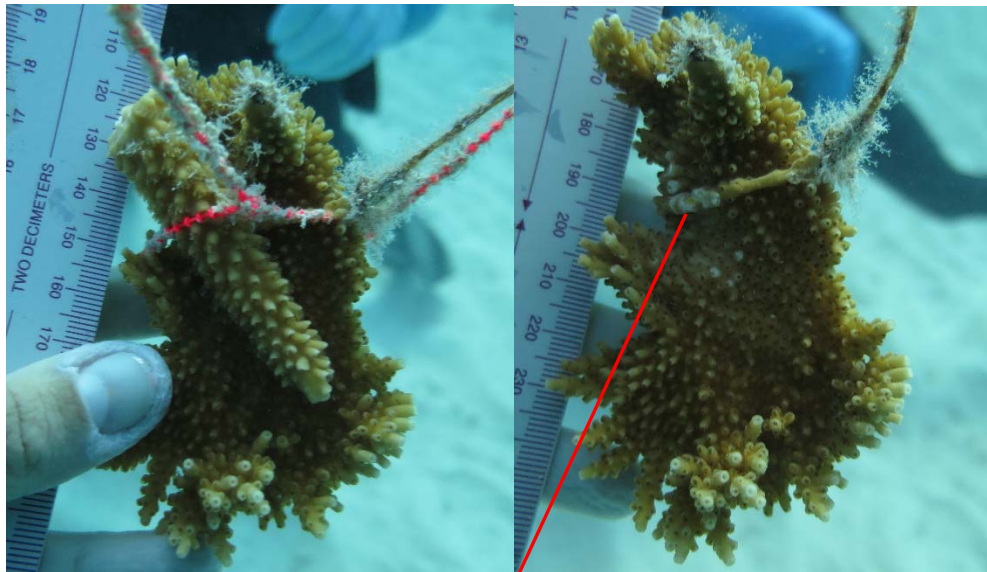
Figure 3 Scaled photo of initiated assay (day 0) and replicate fragment pairs on the nursery tree. Each assay is tagged with a unique identifier allowing for repeated/sequential observations for each test fragment.



310 **Figure 4** Completed heterospecific trial using a wild reef colony of *A. palmata* (typical white band
311 disease) as the inoculant. Transmission (indicated by red arrow) was evident by Day 1, and tissue loss
312 was rapid in this case.



317 **Figure 5** Healthy heterospecific control assay with no transmission: As is typical for healthy control
 318 assays, a small degree of abrasion is visible after 7 days exposure, but no signs of progressive tissue
 319 loss/disease are evident



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