Version 2

Jun 22, 2021

Coral Resilience Lab StepOnePlus™ qPCR protocol for *Cladocopium* and *Durusdinium* Symbiodiniaceae detection V.2

Mariana Rocha De Souza[1], Shayle Matsuda[1], Jenna Dilworth[2], Crawford Drury[1], Ross Cunning[3]

[1]University of Hawaii Manoa, Hawaii Institute of Marine Biology;

[2]Florida Department of Environmental Protection Coral Reef Conservation Program;

[3]Shedd's Daniel P. Haerther Center for Conservation and Research

dx.doi.org/10.17504/protocols.io.bvzbn72n

Mariana Rocha De Souza
University of Hawaii Manoa, Hawaii Institute of Marine Biolo...

DISCLAIMER

This a not-for-profit protocol and is designed to be used for educational purposes only. Whilst we endeavour to ensure that the information in this protocol is correct, we do not warrant its completeness or accuracy and we cannot be be held responsible for variable results in qPCR preparations made whilst following this protocol. Any opinions given in this protocol are not those of the University of Hawaii or The Hawaii Institute of Marine Biology.

ABSTRACT

The purpose of this protocol is to quantify *Cladocopium* and *Durusdinium* Symbiodiniaceae cell ratio densities (symbiont to host cell ratios) from individual coral DNA samples using the StepOnePlus™ Real-Time PCR System. It allows for a quick assessment of the symbiont community composition and ratio using targeted primers.

DOI

dx.doi.org/10.17504/protocols.io.bvzbn72n

PROTOCOL CITATION

Mariana Rocha De Souza, Shayle Matsuda, Jenna Dilworth, Crawford Drury, Ross Cunning 2021. Coral Resilience Lab StepOnePlus™ qPCR protocol for Cladocopium and Durusdinium Symbiodiniaceae detection . **protocols.io** https://dx.doi.org/10.17504/protocols.io.bvzbn72n Version created by Mariana Rocha De Souza

KEYWORDS

qPCR, Symbiodiniaceae, Quantitative PCR, Real Time PCR, Coral

IMAGE ATTRIBUTION

Description: Rice coral *Montipora capitata* in Kaneohe Bay - HI. Credit: Mariana Rocha de Souza

CREATED

Jun 22, 2021

LAST MODIFIED

Jun 22, 2021

PROTOCOL INTEGER ID

50947

GUIDELINES

This protocol has been used by Coral Resilience Lab (the legacy of Ruth Gates). Other settings (different machines, coral host, Symbiodiniaceae species, etc), might require protocol optimization.

MATERIALS TEXT

MATERIALS

qPCR primers **Contributed by users**

MicroAmp™ Optical Adhesive Film **Thermo Fisher Catalog #4311971**

qPCR Mastermix (GTMM) **Fisher Scientific Catalog #4346907**

qPCR 96-well plate **Qiagen Catalog #209002**

StepOnePlus™ Real-Time PCR System
Thermofisher **4376600**

DISCLAIMER:

This a not-for-profit protocol and is designed to be used for educational purposes only. Whilst we endeavour to ensure that the information in this protocol is correct, we do not warrant its completeness or accuracy and we cannot be be held responsible for variable results in qPCR preparations made whilst following this protocol. Any opinions given in this protocol are not those of the University of Hawaii or The Hawaii Institute of Marine Biology.

BEFORE STARTING

Introduction

The purpose of this quantitative PCR protocol is to quantify *Cladocopium* and *Durusdinium* symbiont cell ratio densities (symbiont to host cell ratios) from each DNA sample. It measures the abundance of symbionts relative to the total number of host cells at the tissue or colony level. This technique amplifies specific target gene loci in both the symbiont and the host to calculate a ratio of the total number of symbiont cells to host cells (S/H cell ratio).

The primers and probes presented here have been developed for two coral species commonly found in Hawaii: *Montipora capitata* (Cunning et al. 2016) and *Pocillopora damicornis* (Cunning and Baker, 2013,). *Cladocopium* (former clade C) and Durusdinium (former clade D) are the most abundant symbiont species found in Hawaii, and the ones expected to to be found in those coral species. Additional actin primers have been developed for detection of other Symbiodiniaecae species (see Cunning et al. (2015) for *Breviolum* (former clade B) and Winter, R. N.

Citation: Mariana Rocha De Souza, Shayle Matsuda, Jenna Dilworth, Crawford Drury, Ross Cunning (06/22/2021). Coral Resilience Lab StepOnePlusÃ¢ÂÂ¢ÃÂ qPCR protocol for Cladocopium and DurusdiniumÃÂ Symbiodiniaceae detection . https://dx.doi.org/10.17504/protocols.io.bvzbn72n

(2017) for *Symbiodinium* (former clade A)).

The same protocol can be applied to other coral species. In the absense of an specific primer and probe for the coral species of interest, the protocol can be run without host (in this case, qPCR results will not provide S/H cell ratio, but only symbiont relative proportions).

Make sure you have the DNA extracted and quality checked.

Any mistakes?
This protocol is always changing to move with the times, so if you notice any mistakes or issues, please feel free to let us know so we can improve it.

Mastermix preparation

1

Primer and Probe Dilutions

Primers usually come in lyophilized form. We recommend diluting them to a 100µM stock, then diluting the stock to get the desired concentration for the reactions. For the reacions, Forward primers should be 1µM and Reverse primers shold be 1.5 µM. Probes should be diluted to 2µM (**Table 1**).
Stock should be kept at -20 °C and when in use, kept at 4 °C (avoid freezing and thawing). Mastermix should be kept at 4 °C at all times.

Probes for the detection of Symbiodiniaceae *Cladocopium* (former clade C) and *Durusdinium* (former clade D) were labeled by Cunning et al. (2013) with different fluorescent dyes (VIC and FAM respectively), allowing those reactions to me multiplexed. Primers and probes for two coral hosts (*Montipora capitata* and *Pocillopora damicornis*) have been developed by Cunning et al. (2017) and Cunning & Baker (2013) respectively.

Table 1. Primers and probes for symbionts and coral host.

Citation: Mariana Rocha De Souza, Shayle Matsuda, Jenna Dilworth, Crawford Drury, Ross Cunning (06/22/2021). Coral Resilience Lab StepOnePlusÃ¢ÂÂ¢ÃÂ qPCR protocol for Cladocopium and DurusdiniumÃÂ Symbiodiniaceae detection . https://dx.doi.org/10.17504/protocols.io.bvzbn72n

A	B	C	D	E
Target	**Name**		**Working Concentration**	**Sequence**
Symbiodiniaceae				
Cladocopium (C)	CActF	Forward	1µM	5'-CCAGGTGCGATGTCGATATTC- 3'
	CActR	Reverse	1.5 µM	5'-TGGTCATTCGCTCACCAATG- 3'
	CActProbe	Probe	2 µM	5'-VIC-AGGATCTCTATGCCAACG-MGB' 3'
Durusdinium (D)	DActF	Forward	1µM	5'-GGCATGGGGTAAGCACTTCTT- 3'
	DActR	Reverse	1.5 µM	5'-GATCCTTGAACTAGCCTTGGAAAC- 3'
	DActProbe	Probe	2 µM	5'-6FAM-CAAGAACGATACCGCC-MGB- 3'
Coral host				
Montipora capitata	PaxC-For	Forward	1µM	5'-GTGCAGGTGAGATTGAGTCTTATAACA-3'
	PdAct-Rev	Reverse	1.5 µM	5'-CGGTTGAGCTTCGCTAAACAG-3'
	PaxC-Probe	Probe	2 µM	5'-FAM-CAGTTCTTCCAACAATG-MGB-3'
Pocillopora damicornis	PdAct-For	Forward	1µM	5'-GAGAAGCTCTGCTATGTTGCCA- 3
	PdAct-Rev	Reverse	1.5 µM	5'-TCCACGGAATCGCTCGTT- 3'
	PdActProbe	Probe	2 µM	5'-NED-AGACTGCTGCCTCAAC-MGB- 3'

Mastermix preparation

Note:

- Always run duplicates or triplicates of each sample
- The Micro Amp Fast plate has 96 wells, but in our machine, the 4 corners of the plate don't provide a good read, so in a 96 well plate, we only use 92 wells. You might need to test that for the machine you are using.
- Print a 96 well plate template and write the sample ID and the reaction it will be running (S for Symbiodiniaceae and H for coral host) on the printed wells .
- To minimize the time the mastermix will sit on the bench at room temperature, separate the DNA samples you will use in the order they will be plated before you start preparing the mastermix. Leave them in order in a microcentrifuge rack in the freezer.
- It is always recommended to do the whole protocol with the reagents and the samples on ice.

Calculation

Each well should have 9 ul of your qPCR mastermix and 1 ul of DNA sample. Prepare two separate mastermixes, one for the host and one for the Symbiodiniaceae .

qPCR Symbiodiniaceae C and D mastermix (amount per well)

- 5 µl GTMM
- 0.5 µl Symb C primer (F), 0.5 µl Symb C primer (R), 0.5 µl, 0.5ul Symb C probe
- 0.5 µl Symb D primer (F), 0.5 µl Symb D primer (R), 0.5 µl Symb D probe
- 1 µl Hi clone ddH20

qPCR Coral Host master mix (amount per well)

- 5 µl GTMM
- 0.5 µl Host primer (F), 0.5 µl Host primer (R), 0.5 µl Host probe
- 2.5 µl Hi clone ddH20

- Remember to have 3 Controls:

Citation: Mariana Rocha De Souza, Shayle Matsuda, Jenna Dilworth, Crawford Drury, Ross Cunning (06/22/2021). Coral Resilience Lab StepOnePlusÃ¢ÂÂ¢ÃÂ qPCR protocol for Cladocopium and DurusdiniumÃÂ Symbiodiniaceae detection . https://dx.doi.org/10.17504/protocols.io.bvzbn72n

Positive control: coral with Symb C and Symb D (a sample that has been confirmed to have both symbionts you are testing)
Negative control 1: Hi clone ddH20 (will test for external source of DNA)
Negative control 2: control TE buffer (extraction control, replace with the reagent your DNA samples had been eluted with)

- Calculate the quantity of mastermix according to the number of samples you are running and make each mix in a 1.5 µl centrifuge tube, one for the Symbiodiniaceae and one for the coral host.

If doing a full plate, here are the calculations (92 samples):

qPCR Symbiodiniaceae C and D (46 samples)
- 230 µl GTMM
- 23 µl Symb C primer (F), 23 µl Symb C primer (R), 23 µl Symb C probe
- 23 µl Symb D primer (F), 23 µl Symb D primer (R), 23 µl Symb D probe
- 46 µl Hi clone ddH20

qPCR Coral Host master mix (46 samples)
- 230 µl GTMM
- 23 µl Host primer (F), 23 µl Host primer (R), 23 µl Host probe
- 115 µl Hi clone ddH20

2 Plating mastermix

IMPORTANT: Vortex each mix thoroughly before plating.

- Add 9 µl of mastermix to the bottom of each well (remember to check which ones are Symbiont wells and which ones are Host wells). Change pipettes between mastermixes.
- Flick each DNA sample before use, for better mixing
- Add 1 µl of DNA sample to the top side of each well. Change pipette between each DNA sample. As 1 µl is such a small volume, adding to the side makes it easy to track which samples have been loaded already. The droplets will be visible on the side. Plus, adding to the side instead of directly into the mix in the bottom of the well helps prevent bubbles.
- Seal the plate with qPCR sealing tape and use roller to ensure it is well sealed.
- Put plate in centrifuge to spin DNA into mastermix at 10,000 g for about 10s.
- Check that the liquids are at the bottom of each well, that all wells have same amount of liquid, and that there are no bubbles. If there are bubbles, flick the wells and centrifuge again.

3 Running the samples in the Step One Plus machine

Put samples in thermocycler (StepOnePlus machine), and check that the samples are placed in the same orientation you loaded them.

Thermocycler settings have previously been saved with the name TaqMan_Std (**Figure 1**).

Citation: Mariana Rocha De Souza, Shayle Matsuda, Jenna Dilworth, Crawford Drury, Ross Cunning (06/22/2021). Coral Resilience Lab StepOnePlusÃ¢ÂÂ¢ÃÂ qPCR protocol for Cladocopium and DurusdiniumÃÂ Symbiodiniaceae detection . https://dx.doi.org/10.17504/protocols.io.bvzbn72n

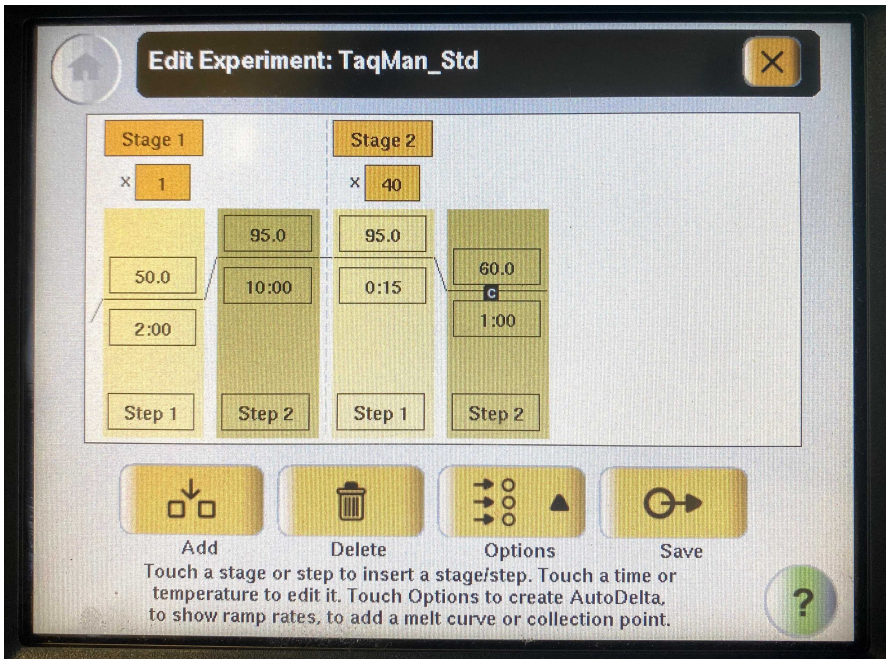

Figure 1. Thermocycler settings

- Check that the settings are correct and press "Save"
- Change volume to your sample volume (10ul)
- Select "Run" and wait for reaction to start to make sure it is running

4 Collecting results and visualizing results on computer

- When reaction is finished, insert a USB thumbdrive into the Step One Plus Machine and select "Save Results", once the file has been saved, remove the USB thumbdrive. Results can also be taken directly from the computer connected to the Step One Machine by selecting "Collect experimental results" on the computer.
- Open your Taq file on the computer (computer should have StepOne software installed).
- Click on "Setup" on the left hand column. In "Define Targets" (your C and D and host) and your samples. Click “Add New Target” into "Define Targets" to get a new row. For reporter, select the fluorescent dye for each reaction (**Table 2**). Choose also the color you would like to display each reporter.

Table 2. Target and fluorescent dyes

A	B
Target	**Fluorescent dye**
Cladocopium (C)	VIC
Durusdinium (D)	FAM
Montipora capitata	FAM
Pocillopora damicornis	NED

- Now on "Define Samples", on the right side, list all your sample names (**Figure 2**).

Citation: Mariana Rocha De Souza, Shayle Matsuda, Jenna Dilworth, Crawford Drury, Ross Cunning (06/22/2021). Coral Resilience Lab StepOnePlusÃ¢ÂÂ¢ÃÂ qPCR protocol for Cladocopium and DurusdiniumÃÂ Symbiodiniaceae detection . https://dx.doi.org/10.17504/protocols.io.bvzbn72n

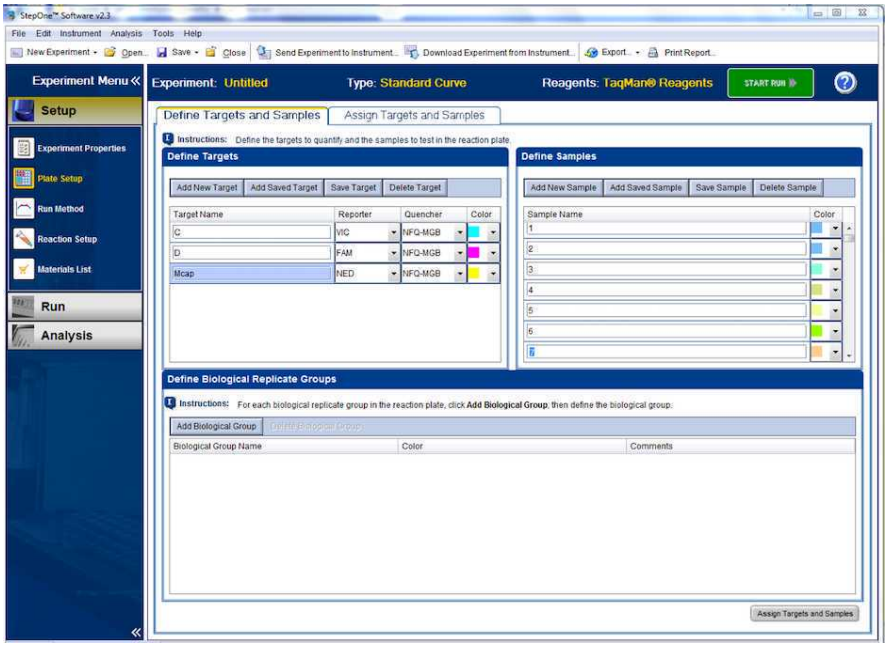

Figure 2. Defining samples

- Now click on the tab "Assign Targets and Samples". Here you will assign targets and samples to each well (**Figure 3**). Highlight the well with your mouse and then check the box next to the targets and the sample names. You can highlight all the wells and assign targets at once, and do the sample names individually.

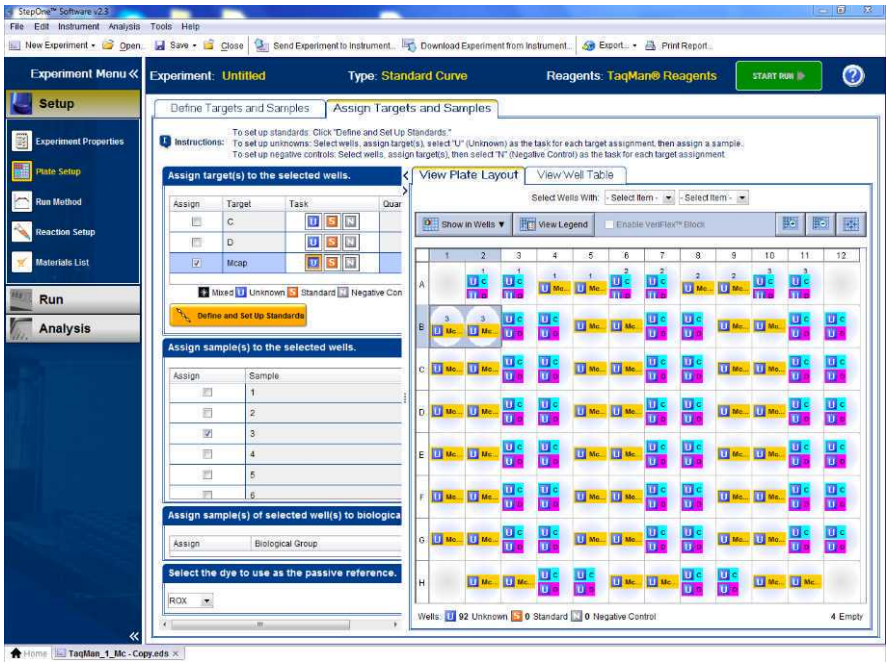

Figure 3. Assigning targets and samples.

- Now click on "Analysis" on the left hand column, then click "Analyze" on the top right corner.

- On left, click "Multicomponent Plot". Here we are looking for the number of cycles before amplification. We want to tell the machine what our baseline is. We want it to be large, but remember, it needs to be the same for all of your runs. On the top right, click on "Analysis settings" (**Figure 4**). Enter the threshold (usually 0.01) and the baseline for both C and D (usually 6-23). Then hit "Apply Analysis Settings" (we tend not to use the "auto" settings and instead

Citation: Mariana Rocha De Souza, Shayle Matsuda, Jenna Dilworth, Crawford Drury, Ross Cunning (06/22/2021). Coral Resilience Lab StepOnePlusÃ¢ÂÂ¢ÃÂ qPCR protocol for Cladocopium and DurusdiniumÃÂ Symbiodiniaceae detection . https://dx.doi.org/10.17504/protocols.io.bvzbn72n

edit them ourselves).

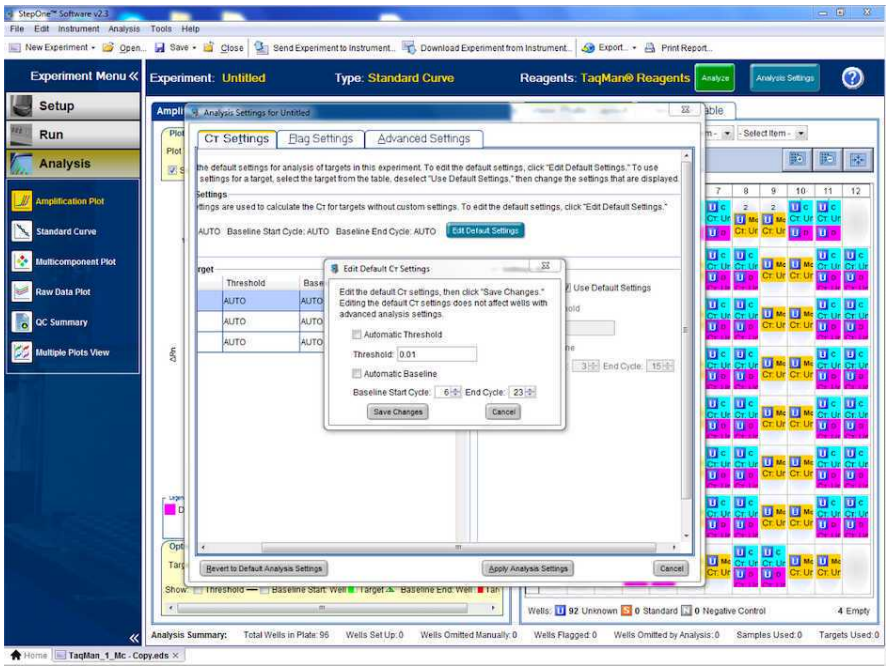

Figure 4. Editing default settings.

- Now the screen should show you the amplification plot (if you don't see the lines, make sure the "Show baseline" and "Show threshold" buttons are clicked at bottom). Also, select "Well" or Target into the Plot Color. Note, you can drag to make the wells larger so you can read the numbers as you would in an Excel sheet. You can also highlight specific wells, or rows (**Figure 5**).

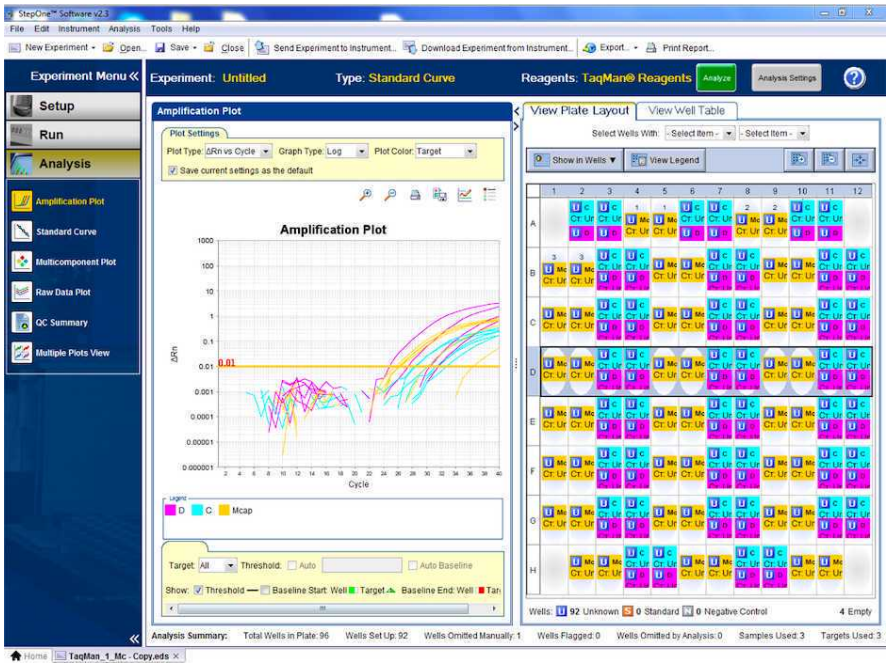

Figure 5. Amplification plot of row D.

- Now we want to export the data as an Excel sheet. Under "File", select "Export". Select "Results" as the data to export. Name your file, and be sure it is exported as an Excel file.
- Also save the StepOne settings and set up (in case you find a mistake, you won't have to re-do everything), by going

Citation: Mariana Rocha De Souza, Shayle Matsuda, Jenna Dilworth, Crawford Drury, Ross Cunning (06/22/2021). Coral Resilience Lab StepOnePlusÃ¢ÂÂ¢ÃÂ qPCR protocol for Cladocopium and DurusdiniumÃÂ Symbiodiniaceae detection . https://dx.doi.org/10.17504/protocols.io.bvzbn72n

into "saving as". Give a name for the settings and this run.
- Then you can copy it to your USB and save to your laptop.

5 Bioinformatics

RStudio Desktop 1.4.1717
by RStudio Public-benefit corporation

- You need to run your data through an R script to see what the values mean. Ross Cunning has developed calculations (Cunning et al. 2012, 2017) and an R script (you can see it in his GitHub). A modified version is below.
- The file "qPCR1.xls" referred to in the script is the Excel file exported from the StepOne machine after doing the qPCR.

The command line below can be copied to clipboard by clicking on the button at the bottom of the command line window. Once copied it can be pasted directly into R.

```
library(tidyverse)
library(janitor)
library(readxl)
library(ResourceSelection)

#Cunning et al. 2012 - Excess algal symbionts increase the susceptibility of reef cc
#Cunning et al. 2017 - Patterns of bleaching and recovery of Montipora capitata in
USA


#---------------------------qPCR1#---------------------------#

######## Uploading data and cleaning ################
setwd("~/Desktop/qPCR_results/")
rawdata<-read_excel("qPCR1.xls")
rawdata<-rawdata[-(1:6),] #deleted first six rows of header information
colnames(rawdata) <- rawdata[1,] #set first row as column names
rawdata <- rawdata[-1, ] #delete first row
format_data<-clean_names(rawdata); colnames(format_data)[7] <- "ct"; colnames(f
"ct_mean"; colnames(format_data)[9] <- "ct_sd" # this renames columns manually,
capital subscripts in column names, so this fixes them
format_data$sample_name <- gsub('\\.',"-", format_data$sample_name);head(format

##_________Set arguments _________##
copy.n.C<-33 #from Cunning et al. 2017 Supplemental Materials
copy.n.D<-3 #from Cunning et al. 2017 Supplemental Materials
copy.n.Mcap<-1 #from Cunning et al. 2017 Supplemental Materials
copy.n.ratioCD<-(copy.n.C/copy.n.D)
copy.n.ratioCM<-(copy.n.C/copy.n.Mcap)
copy.n.ratioDM<-(copy.n.D/copy.n.Mcap)
fluo.C<-2.26827  #from Cunning et al. 2017 Supplemental Materials
fluo.D<-0  #from Cunning et al. 2017 Supplemental Materials
fluo.Mcap<-0.84815   #from Cunning et al. 2017 Supplemental Materials
```


Citation: Mariana Rocha De Souza, Shayle Matsuda, Jenna Dilworth, Crawford Drury, Ross Cunning (06/22/2021). Coral Resilience Lab StepOnePlusÃ¢ÂÂ¢ÃÂ qPCR protocol for Cladocopium and DurusdiniumÃÂ Symbiodiniaceae detection . https://dx.doi.org/10.17504/protocols.io.bvzbn72n

```
##_________Process data _________##
data<-format_data%>%
dplyr::select(sample_name,target_name,ct_mean, ct_sd)%>% #take only the colum
mutate_at(vars(starts_with("ct")),funs(as.numeric))%>%  #set all the ct columns to
are originally stored as characters)
group_by(sample_name,target_name)%>% #group technical replicates
summarise_all(funs(mean(., na.omit = TRUE)))%>%  #take mean of technical replica
filter(!grepl("con",sample_name))%>%
filter(sample_name!="NA")

C<-filter(data,target_name=="C")
D<-filter(data,target_name=="D")
Mcap<-filter(data,target_name=="Mcap")

final_data1<- left_join(left_join (C,D, by="sample_name"), Mcap, by="sample_name")
dplyr::select(-target_name.x,-target_name.y,-target_name)%>%
dplyr::rename(d_mean=ct_mean.y)%>%  #rename columns from rbind
dplyr::rename(d_sd=ct_sd.y)%>%
dplyr::rename(c_mean=ct_mean.x)%>%
dplyr::rename(c_sd=ct_sd.x)%>%
dplyr::rename(mcap_mean=ct_mean)%>%
dplyr::rename(mcap_sd=ct_sd)%>%
mutate(c_mean=c_mean-fluo.C)%>%#from Cunning et al. 2017
mutate(mcap_mean=mcap_mean-fluo.Mcap)%>% #from Cunning et al. 2017
mutate(presence=case_when(d_mean>0~"CD",is.na(d_mean)~"C"))%>%  #set pres
column based on ct values
mutate(dm_ratio=(2^(mcap_mean-d_mean))/copy.n.ratioDM)%>% #from Cunning et
mutate(cm_ratio=(2^(mcap_mean-c_mean))/copy.n.ratioCM)%>% #from Cunning et
mutate(cd_ratio=(cm_ratio/dm_ratio))%>%#from Cunning et al. 2017 #calculate rati
in ct values)
mutate(sh_ratio=sum(cm_ratio,dm_ratio,na.rm=TRUE))%>% #from Cunning et al. 20
ratio + D:host ratio for total symbiont:host ratio #set even numbers as nonbleache
this works by using the remainder command (%%), if 0 it must be an even number
mutate(prop_c=cd_ratio/(cd_ratio+1))%>% #from Cunning et al. 2012
mutate(prop_c=case_when(is.na(prop_c)~"1",TRUE ~ as.character(prop_c)))%>% #
2012
mutate(prop_c=as.numeric(prop_c))%>%
mutate(prop_d=1-prop_c)%>% #set proportion D off of proportion C
mutate(abundance=case_when(prop_d>prop_c~"D>C",prop_c>prop_d~"C>D"))%>
based on which proportion is dominant, create character factor
mutate(sd_warning=case_when((c_sd>1&d_sd>1)~"cd*",c_sd>1~"c*",d_sd>1~"d*"
where standard deviation of tech replicates is >1
mutate(ct_warning=case_when((c_mean>38&d_mean>38)~"cd*",c_mean>38~"c*",
#set warnings where ct values is later than 34
mutate(rep=case_when((is.na(d_sd)&d_mean>0)|(is.na(c_sd)&c_mean>0)~"1replic
warning if only one replicate processed (no standard deviation but mean present)
separate(sample_name,into=c("Timepoint","Fragment"),sep="-")%>%  # customize
names
mutate_at("Timepoint", as.factor)%>%
mutate_at("Fragment", as.factor)%>%
mutate(C=as.integer(prop_c*100))%>%
mutate(D=100-C)%>%
```


Citation: Mariana Rocha De Souza, Shayle Matsuda, Jenna Dilworth, Crawford Drury, Ross Cunning (06/22/2021). Coral Resilience Lab StepOnePlusÃ¢ÂÂ¢ÃÂ qPCR protocol for Cladocopium and DurusdiniumÃÂ Symbiodiniaceae detection . https://dx.doi.org/10.17504/protocols.io.bvzbn72n

```
filter(is.na(rep))

### this is your final file with the result of all your qPCRs. You can now do stats ar
results!
```


Citation: Mariana Rocha De Souza, Shayle Matsuda, Jenna Dilworth, Crawford Drury, Ross Cunning (06/22/2021). Coral Resilience Lab StepOnePlusÃ¢ÂÂ¢ÃÂ qPCR protocol for Cladocopium and DurusdiniumÃÂ Symbiodiniaceae detection . https://dx.doi.org/10.17504/protocols.io.bvzbn72n