

# PROTOCOL ITS2-HRM FOR *Symbiodinium*

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This protocol describes the use of high-resolution melting, HRM, for the genotyping of *Symbiodinium* using the internal transcribed spacer, region 2, ITS2. HRM is a technique based on the melting properties of double-stranded DNA. As temperature increases, dsDNA is denatured and forms single-stranded DNA. The dyes used in this technique will specifically bind to dsDNA, thus, as DNA denatures, less fluorescence will be detected resulting in a melting curve. HRM is performed immediately after PCR amplifications, which makes it a fast technique. It also reduces cross-contamination, does not require the use of hazardous materials, is sensitive, and very straightforward. For further details of the technique please refer to:

Wittwer et al. (2003) Clinical Chemistry; Reed et al. (2007) Pharmacogenomics; Montgomery et al. (2007) Nature Protocols; Taylor (2009) Biochemical Society Transactions; Vossen et al. (2009) Human Mutation.

\* This protocol is based on the Type-it HRM PCR Handbook (Qiagen). \*

## **Reagents for running HRM in the Rotor-Gene 6000 (original concentrations)**

- ★ 2x HRM PCR Master Mix (Type-it HRM PCR Kit, Qiagen)
- ★ 10  $\mu$ M forward primer (ITSIntfor2)
- ★ 10  $\mu$ M reverse primer (ITS2rev)
- ★ RNase-free water (comes with the Type-it HRM PCR Kit, Qiagen)

## **Before starting:**

- ✓ It is recommended to use all your samples at the same template concentration. For HRM, your concentration may vary between 1-50 ng/ $\mu$ l.
- ✓ Depending on your concentration the number of cycles for PCR will vary: 1-9 ng/ $\mu$ l 45 cycles or 10-50 ng/ $\mu$ l 40 cycles (see below). For good-quality DNA, 10 ng/ $\mu$ l is a good concentration to use.
- ✓ Regardless of template concentration, you must make sure you add the same volume to all your reactions.
- ✓ It is important to remember that you always run replicates per sample, thus reducing the number of samples that you can run at a time.

- ✓ You don't have to optimize any of the concentrations of the reagents that come in the kit e.g.  $MgCl_2$ , Master Mix, or the final concentration of the primers i.e.  $0.7 \mu M$  each primer.
- ✓ Since the tubes used for the 72-tube rotor are small, make sure you write everything in your notebook. Otherwise, you will be "lost" and mix up tubes when loading. An easy way to do so is to write the numbers 1-72 in one column and the number of your samples in a second column. Keep in mind that you are doing replicate per sample. The rack provided has clearly labeled de numbers 1-72 for easy guidance.

## OK, let's do some HRM!

1. Completely thaw the HRM PCR Master Mix, the RNase-free water, the primers, and the DNA in ice.
2. Prepare a reaction mix as follows

Reagent	$V_f$ per reaction = 25 $\mu l$	$V_f$ per reaction = 10 $\mu l$	Final concentration
<b>2x HRM PCR Master Mix (Qiagen)</b>	12.5 $\mu l$	5 $\mu l$	1x
<b>10 <math>\mu M</math> forward primer</b>	1.75 $\mu l$	0.7 $\mu l$	0.7 $\mu M$
<b>10 <math>\mu M</math> reverse primer</b>	1.75 $\mu l$	0.7 $\mu l$	0.7 $\mu M$
<b>RNase-free water</b>	Varies depending on the volume of template DNA you are using *	Varies depending on the volume of template DNA you are using **	----
<b>Template DNA</b>	Varies; must be equal for all reactions; can not exceed 9 $\mu l$	Varies; must be equal for all reactions; can not exceed 3.6 $\mu l$	Eukaryotic: 1-50 ng DNA / reaction Microbial: 1-50 pg DNA / reaction

\* For example, if you are using 2  $\mu l$  of template, you would add 7  $\mu l$  of RNase-free water per reaction.

\*\* For example, if you are using 1  $\mu l$  of template, you would add 2.6  $\mu l$  of RNase-free water per reaction.

3. Mix your reaction mix and add the appropriate volume into the PCR tubes.
  - ✓ Please note that the volume you add of reaction mix is  $V_f$  reaction minus  $V_{template}$ . For example, if you are using  $V_f = 10 \mu l$  and  $V_{template} = 1 \mu l$ , you should add 9  $\mu l$  of your reaction mix.
  - ✓ The Taq contained in the HRM PCR Master Mix is HotStartTaq Plus DNA Polymerase, so it will only activate by a heating step in the PCR run.

4. Add your template, mix, and close your tubes.
5. Place in Rotor-Gene 6000 (see the “Loading in the Rotor-Gene 6000” and the “Programming the Rotor-Gene 600 for HRM” sections below).
6. Done!
7. Analyze you data (see “Data analysis” below)

## Loading in the Rotor-Gene 6000

- Transfer the tubes from the rack to the rotor. MAKE SURE YOU PLACE THEM IN THE CORRECT ORDER.
- Place “lid” securely on rotor. Make sure it “clicks”.
- Turn on computer and Rotor-Gene 6000.
- Open Rotor-Gene and place rotor. Put a little pressure until it “clicks”.
- Close the Rotor-Gene.

## Programming the Rotor-Gene 600 for HRM

- Initial PCR activation step: 5 min 95°C
- 3-step cycling:
  - Denaturation 10 s 95°C
  - Annealing 30s 55°C
  - Extension 10s 72°C
- Number of cycles:
  - 40 for template DNA between 10-50ng or 10-50pg for microbial DNA
  - 45 for template DNA between 1-9ng or 1-9pg for microbial DNA
- HRM:
  - 2s 65-95°C for initial run. After you have results, you can reduce this ramp between 5°C below the lowest  $T_m$  of all expected PCR products and 5°C above the highest  $T_m$ . This reduces the time require for HRM analysis.
- Once the program starts running, a Samples window will show up. Label your samples accordingly. This is why it is also important to write in your notebook the loading order of your samples.

## Data analysis

- Click “Analysis”
- Click “Other” in the Analysis little window.

- Click “HRM”
- In the tab of HRM, click “Show”
- Three windows will pop-up: 1) HRM Analysis 2) HRM Normalized Graph 3) HRM Results
- Define your genotypes by clicking “Genotypes” in the HRM Analysis window. You decide the name of your genotype and pick a reference sample. Do this for all your genotypes.
- The “Normalization Region 1” and “Normalization Region 2” can/must be moved closer to the melting curves. You will notice how the HRM Normalized Graph changes.
- In the HRM Results you have a table with all your samples and the results of the genotyping. This is based on a confidence percentage, which you can modify at the right bottom of the screen. The percentage of confidence is calculated as follows:

The reference genotype is specified and the fluorescence readings for the melt curve are recovered (*REFFluorescence*). For each sample, the fluorescence readings for the melt curve are also retrieved (*SampleFluorescence*). For each reading/point X in the respective melt curves, an error value is calculated as the square of the difference between the readings:

$$ERROR(X) = ((SampleFluorescence(X) - REFFluorescence(X))^2$$

The Error values are then summed over the entire set of readings Y:

$$SumError = \sum_{X=1}^Y Error(X)$$

The percentage of confidence (ConfidencePercentage) is then calculated as:

$$ConfidencePercentage = C\_SPREADFACTOR^{(-C\_SHARPNESSFACTOR \times SumError)}$$

where C\_SPREADFACTOR = 1.05, C\_SHARPNESSFACTOR = 0.02.

It is recommended by the technical support of the manufacturer (Qiagen) to use a percentage of confidence above 85%.

- An easier way to visualize your different genotypes is by having a fluorescence difference plot. This plot is different from conventional derivative plots (-dF/dT). Instead, these are fluorescence differences between normalized melting curves, which allow discerning more easily melting curves changes between genotypes. For this, one melting curve was chosen as a standard, and the differences between each curve and the standard was plotted against temperature to give a “fluorescence difference” plot.
  - To view your fluorescence difference plot, click the “Difference Graph” tab in the HRM Normalized Graph window. Select a genotype as reference and all other genotypes/samples will be plotted against it.

- **Save your results.**

## Edit figures

You can edit the melting profiles or the fluorescence difference plots by doing click in the *tool* icon on the bottom-left of each plot. Select “Edit Chart in TeeChart Office”. Here you can change labels, colors, and export image, among others.