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# Quantification of fungal ITS Gene Copies Using ddPCR (EvaGreen-based assay: ITS1f-ITS2)

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**Protocol status:** Working

**We use this protocol and it's working**

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**Keywords:** Absolute quantification, ddPCR, 16S rRNA

## Abstract

The protocol was designed to quantify microbial eukaryotic fungi using ITS region copy number evaluation by Droplet Digital PCR technology (ddPCR) from Bio-Rad company.

For the assay, we are using a universal fungal ITS rRNA primer pair:

ITS1f 5'- CTT GGT CAT TTA GAG GAA GTA A -3', 38 bp upstream of ITS1 from White et al., 1990  
ITS2 5'- GCT GCG TTC TTC ATC GAT GC -3', identical to ITS2 from White et al., 1990 until now because of its extreme length variability among the fungi species. In fact, this variability introduced a bias to final copy numbers estimated by qPCR.

Amplicon size: ~250–600 bp (Bokulich & Mills, 2013; Hoggart et al., 2018)

The same ITS1f and ITS2 primers are recommended for use for fungi identification by the Earth Microbiome Project.

### Note

Internal Transcribed Spacer (ITS) has not been possible to use for fungi quantification until now because of its extreme length variability among the fungi species. In fact, this variability introduced a bias to final copy numbers estimated by qPCR, which was overcome here with the ddPCR method.

## Attachments



[Li et al 2012 Enviro...](#)

1.1MB

## Guidelines

1. The crucial steps that can influence the final assay results a lot are **precise pipetting, mixing and dilutions!**
2. Keep in mind that ddPCR technique do not posses as wide dynamic concentration range as qPCR does. You can easily "overload" the reaction with too much template DNA putting in. In that case you will see only positive droplets at the end and no negative and system will not be able to calculate copy number for them. As a consequence, you usually have to dilute your template DNA more than for qPCR experiments. Ideally fit inside the range of  $10^1$  -  $10^4$  copies of target gene. That is why we usually test several dilutions of few samples in advance to see "where we are".
3. One have to also keep in mind that using ddPCR technology you must work within the format of eight. If you do not fulfil all the wells in a cartridge you still have to put the reagencies inside the empty wells as well. So, think economically before you start.

## Materials

### MATERIALS

- ☒ PCR Plate Heat Seal foil piercable **Bio-rad Laboratories Catalog #1814040**
- ☒ ddPCR 96-well plates **Bio-rad Laboratories Catalog #12001925**
- ☒ QX200™ ddPCR™ EvaGreen Supermix **Bio-rad Laboratories Catalog #1864033**
- ☒ Automated Droplet Generation Oil for EvaGreen **Bio-rad Laboratories Catalog #1864112**

### Protocol materials

- ☒ PCR Plate Heat Seal foil piercable **Bio-Rad Laboratories Catalog #1814040** Step 4
- ☒ PCR Plate Heat Seal foil piercable **Bio-Rad Laboratories Catalog #1814040** Step 9
- ☒ PCR Plate Heat Seal foil piercable **Bio-Rad Laboratories Catalog #1814040** Materials
- ☒ ddPCR 96-well plates **Bio-Rad Laboratories Catalog #12001925** Materials
- ☒ QX200™ ddPCR™ EvaGreen Supermix **Bio-Rad Laboratories Catalog #1864033** Materials, Step 2
- ☒ Automated Droplet Generation Oil for EvaGreen **Bio-Rad Laboratories Catalog #1864112** Materials, Step 7
- ☒ ddPCR 96-well plates **Bio-Rad Laboratories Catalog #12001925** Step 2

### Safety warnings

- ❗ Protect probe from light.

### Before start

Take all the reagencies out of a freezer and let them temperate to room temperature.

## ddPCR reaction mixture

20m

- 1 All reagents must be equilibrated to RT (do not keep them on ice). Mix each of them properly before use.

20m



2

Reagent	Final conc.	1 tube (22 µl)	plate (22 µl x 100)
PCR H2O		8.6	860
QX200 ddPCR EvaGreen Supermix	1x	11	1100
ITS1f (10 µM)	0.1 µM	0.2	20
ITS2 (10 µM)	0.1 µM	0.2	20
Template		2	2 x 100

Prepare the master mix according to the number of samples and mix several seconds by vortexing. Transfer mix into 96-well plate à 20 µl.

20 µL master mix per well

ddPCR 96-well plates **Bio-rad Laboratories Catalog #12001925**

QX200™ ddPCR™ EvaGreen Supermix **Bio-rad Laboratories Catalog #1864033**

#### Note

Tip: use a mechanical or electronic dispenser (for ex. Multipette, Pipettman, or multichannel pipette) during this step to speed up the work.

- 3 Add 2 µl of your DNA sample into each well

2 µL of examined DNA per well

4

Seal the plate (180°C, 5s) with pierceable aluminium foil.

PCR Plate Heat Seal foil piercable **Bio-rad Laboratories Catalog #1814040**

 00:00:05 sealing at 180°C

- 5 Let the foil cool down and mix the plate vigorously by vortexing 30 s - 1 min.

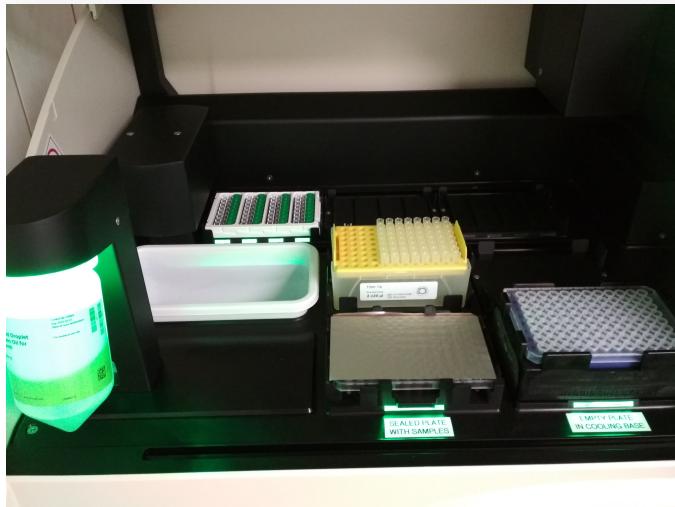
 00:00:30 vortexing

## Droplets generation by AutoDG

- 6 Put all the staff (cartridges, tips, sealed plate with samples and empty 96-well plate in cooling stand) in corresponding amount inside the **AutoDG machine** (Bio-Rad).

### Note

Per one sample there is a need of two pipette tips!

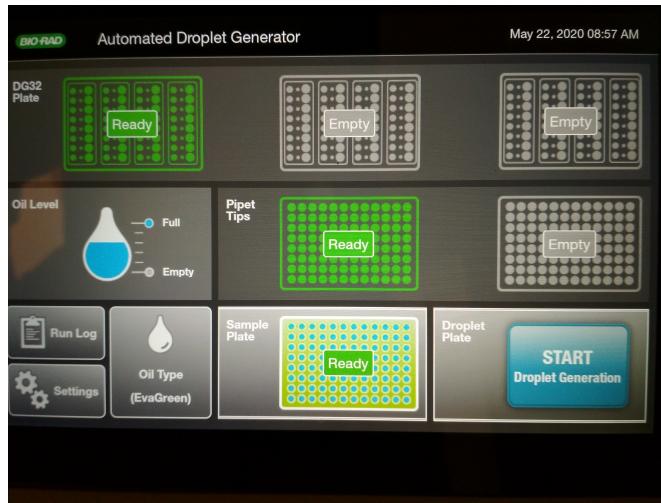


This is an example how it should look like inside the AutoDG before strating droplets generation.

- 7 Make sure there is a right oil bottle (**Automated Droplet Generation oil for Probes**) connected to the system.



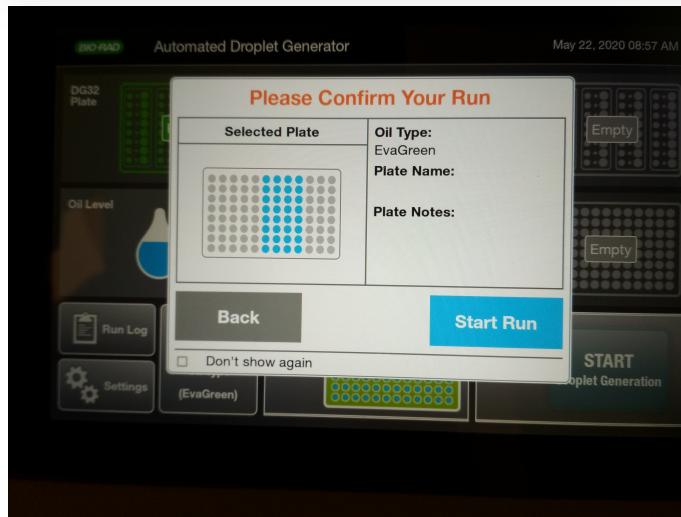
Automated Droplet Generation Oil for EvaGreen **Bio-rad**  
**Laboratories Catalog #1864112**



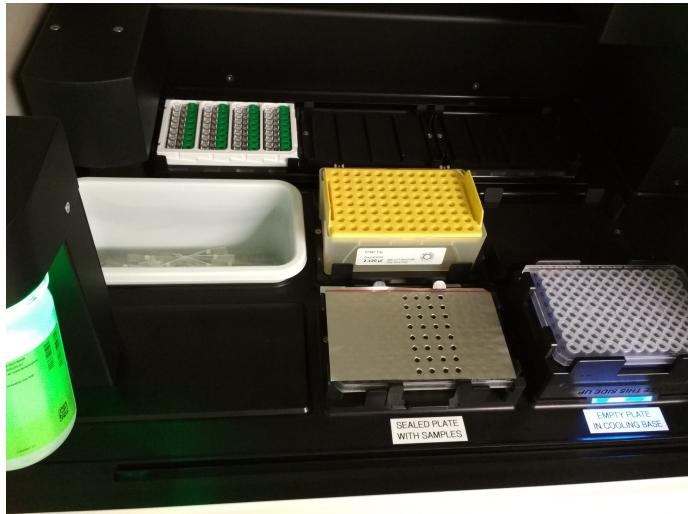
- 8 Choose a position of samples on touch screen and start droplets generation. Wait after its finished.

#### Note

System will you announce automatically about success or failure of droplets creation after procedure is finished. Nevertheless, every time make also a visual inspection of droplets. Two separated phases should be visible. Upper part with droplets and lower clear oil phase.



selection of a columns within the plate where the samples are positioned



This is how it should look like inside the AutoDG after droplets generation.

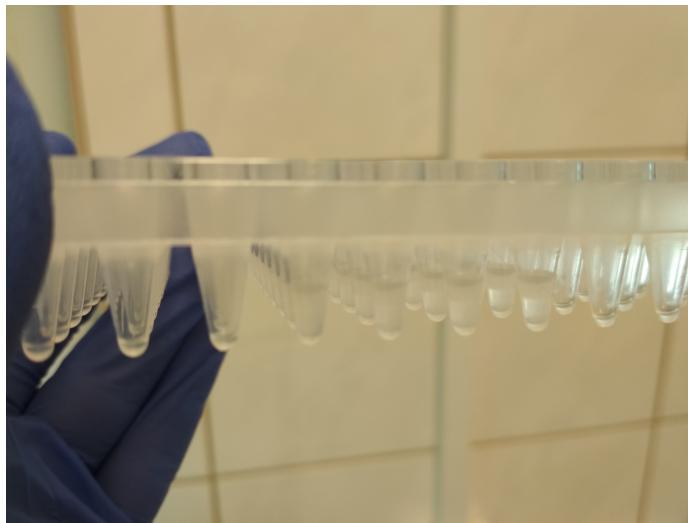


plate after droplets generation - two phases visible in each well with sample

- 9 Take the plate with droplets out of the machine and seal it with pierceable aluminium foil (170°C, 3s).

 PCR Plate Heat Seal foil piercable **Bio-rad Laboratories Catalog #1814040**

 00:00:03 sealing at 170°C

- 10 Put immediately the sealed plate into PCR cycler.

### Note

Droplets are unstable at this stage. Proceed to next step as fast as possible. After PCR droplets become stable and can be kept at fridge for some time (one day) before measurement.

11 Clean AutoDG machine, waste used consumables.

### PCR program

3h

- 12 1. 95°C –5'
2. x 40 {
  - a. 95°C – 30"
  - b. 52°C – 2'}
3. 4°C – 5'
4. 90°C –5'
5. 10°C –hold



Set ramp rate for each step to 2°C/sec!

Set reaction volume to 40ul.

 40 µL reaction volume

### Note

- After run is finished check if there are still two phases present
- Let the plate cool down (droplets will not be so sticky and will be more easy to analyse).

### Droplets reading

13 Put the plate into a metal holder, place them together into **QX200 reader**.



### Note

Switch on the reader 30 min before measurement.



Droplet reader with a plate after PCR already placed inside the metal holder

- 14 Set up the QuntaSoft experiment as follows:

Exp. type: Absolute quantification (ABS)  
Supermix: QX200 ddPCR EvaGreen Supermix  
Target1: Ch1 (for FAM labeled probes)

Define the position of each sample.

- 15 Check the levels of reader Oil and waste - green control (bottles are physically accessible from machines left side).

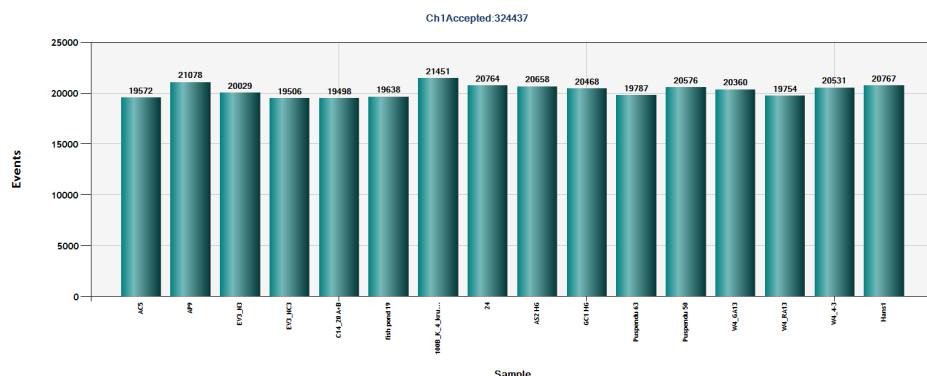
### Note

If the instrument was not in use longer than week it has to be Primed first (oil flushed).

- 16 Start measurement.

## Note

After it finishes go over the results and see how many droplets were executed for each sample. To get reliable results total droplet count should be above 12.000. We usually have got 18.000 - 20.000 droplets analyzed per sample.



an example of total droplets count (positive together with negative ones) being generated and read

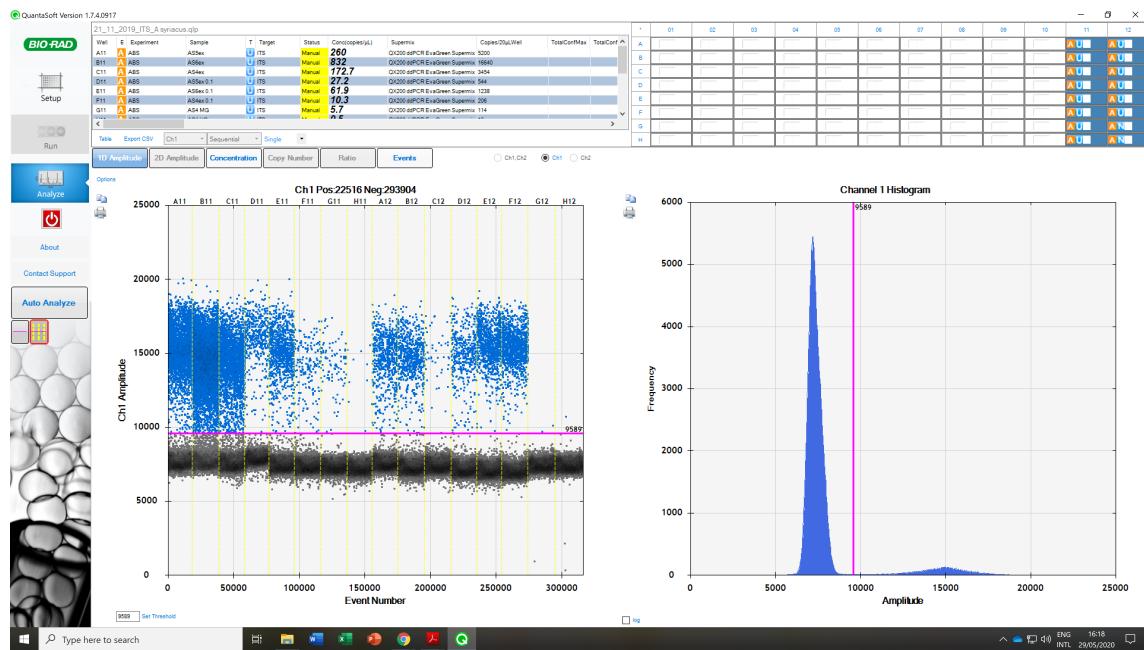
## Analysis

- 17 Set up the threshold just above negative control sample in order to distinguish positive (containing PCR products) from negative (do not contain any PCR product) droplets.



## Note

Quanta software will automatically calculate copy number of target gene for each sample by Poisson distribution algorithm. For that calculations at least some of the negative droplets are necessary. If the sample contains only positive population it can not be evaluated.



An example of ITS Fungi copy numbers data analysis. Last two samples on the left graph are negative (NTC) samples by which a threshold was set up.

- 18 Export .csv file with concentrations (copies/ul) that are posessing you the number of copies in 1ul of reaction. To obtain number of copies in 1ul of your input DNA you have to recalculate:

$$\text{no. of copies in 1ul of input DNA} = (\text{concentration value} \times 22) / \text{volume of DNA}$$