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Quantification of 16S rRNA Gene Copies Using ddPCR (probe-based assay: 338F-516P-805R)

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Protocol status: Working
We use this protocol and it's working

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Abstract

This protocol is dedicated to evaluation of 16S rRNA bacteria gene copy number using **Droplet Digital PCR technology (ddPCR)** from Bio-Rad company. This is the up-to-date modification and improvement of clasical probe based qPCR assay.

For the assay we are using universal 16S Bacteria primers and probe:

BAC338F ACT CCT ACG GGA GGC AG , target *E.coli :* 338-354, Yu et al. (2005), B&B
BAC516P* TGC CAG CAG CCG CGG TAA TA, target *E.coli :* 516-536, '
BAC805R GAC TAC CAG GGT ATC TAA TC , target *E.coli :* 785-805, '

* Probe must be dual-labelled either with 5'-6-FAM, 3'-BHQ1 or any other valid combination.

Note

What is the difference between clasical qPCR and ddPCR target gene quantification?
Among the bigest advantages of the ddPCR technique belong its high sensitivity (up to one molecule of target gene presented in input DNA) and robustness to enzyme inhibitors. Moreover, because it is an absolute quantification technique there is NO need of any internal standard for evaluation.

Guidelines

1. The crucial steps that can influence the final results a lot are **precise pipetting, mixing and dilutions**!
2. Keep in mind that ddPCR technigue 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 negatives and system will not be able to calculate copy number from that. 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 coppies of target gene. That is why we usually test several dilutions of few samples before 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
- Automated Droplet Generation oil for Probes **Bio-Rad Laboratories Catalog #**186-4110
- ddPCR™ Supermix for Probes (No dUTP) **Bio-Rad Laboratories Catalog #**1863023

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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

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

20m

2

10m

	Reagent	Final conc.	1 tube (22 µl)	plate (22 µl x 100)
	PCR H_2O		6.6	660
	ddPCR Supermix for for Probes (no dUTP)	1x	11	1100
	BAC 338F (10 µM)	0.5 µM	1	100
	BAC 805R (10 µM)	0.5 µM	1	100
	BAC 516P (10 µM)†	0.2 µM	0.4	40
	Template		2	2 × 100

† Probe must be dual-labelled either with 5'-6-FAM, 3'-BHQ1 or any other valid combination.

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 of master mix per well

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

ddPCR™ Supermix for Probes (No dUTP) **Bio-Rad Laboratories Catalog #**1863023

Note

Tip: use a mechanical or electronic dispenser (for ex. Multipette, Pipettman, or multichanel 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. 5s

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.

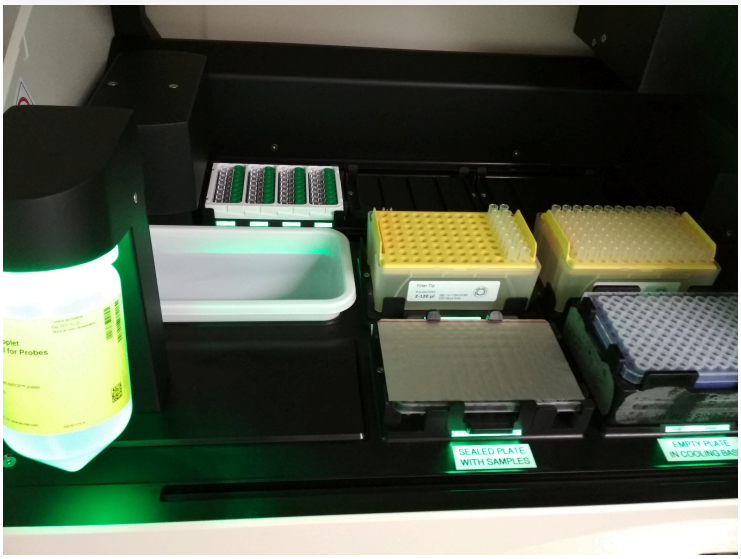
00:00:30 vortexing

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

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

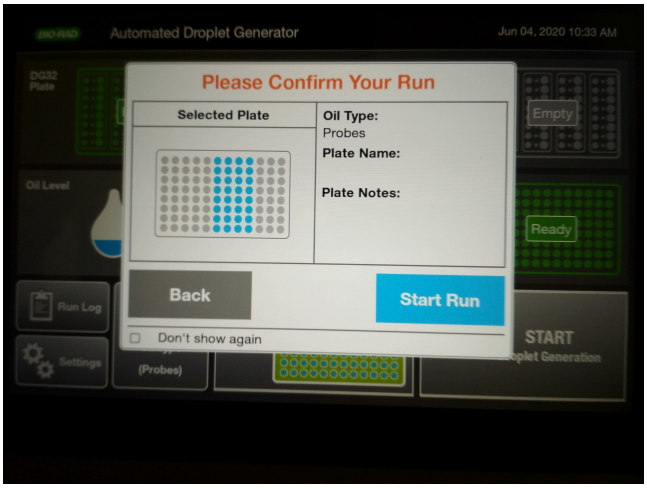


This is an example how it should look like inside the AutoDG before strating droplets generation

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

Automated Droplet Generation oil for Probes **Bio-Rad Laboratories Catalog #**186-4110

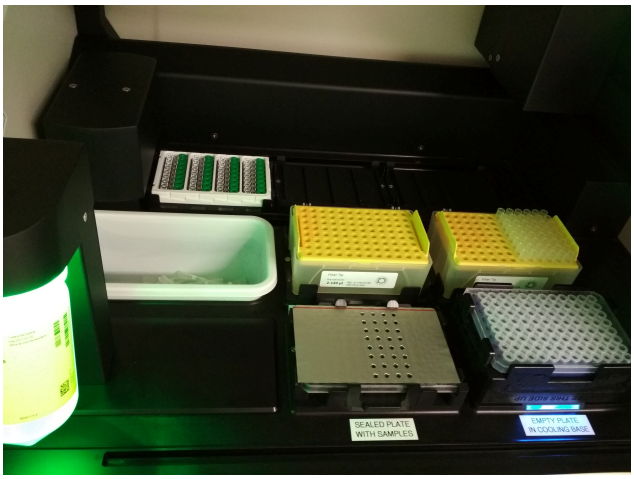
8 Choose a position of samples on touch screen and start dropplets generation. Wait after its finished.



Confirmation window will appear on AutoDG screen before procedure starts. Make sure you have chosen right position of a samples and oil type suitable for an assay.

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 dropplets and lower clear oil phase.



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

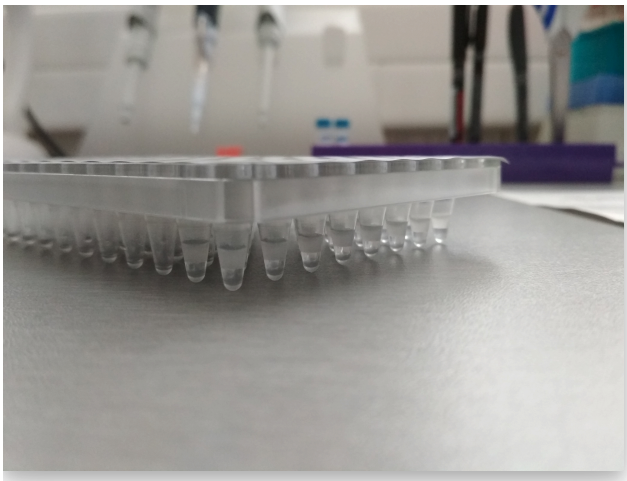


plate after droplets generation - two phases visible in each well with sample

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

12 1. 95°C –10′
2. x 40 {
   a. 95°C – 30″
   b. 60°C – 2′
   }
3. 98°C –10′
4. 10°C –hold

3h

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

30m

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: ddPCR Supermix for Probe
Target1: Ch1 (for FAM labeled probes)

Define the position of ech sample.

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

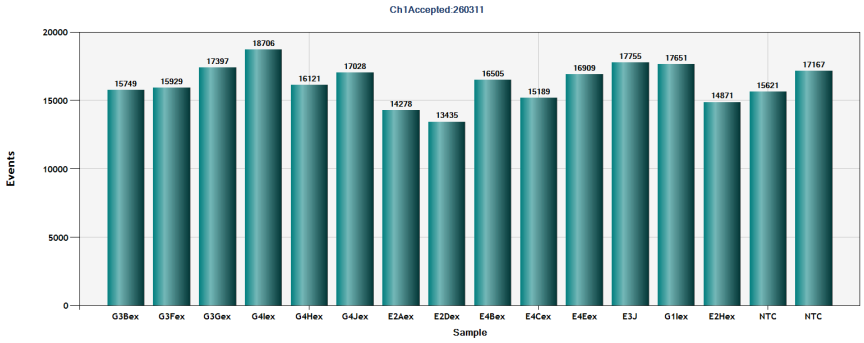
Note

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.



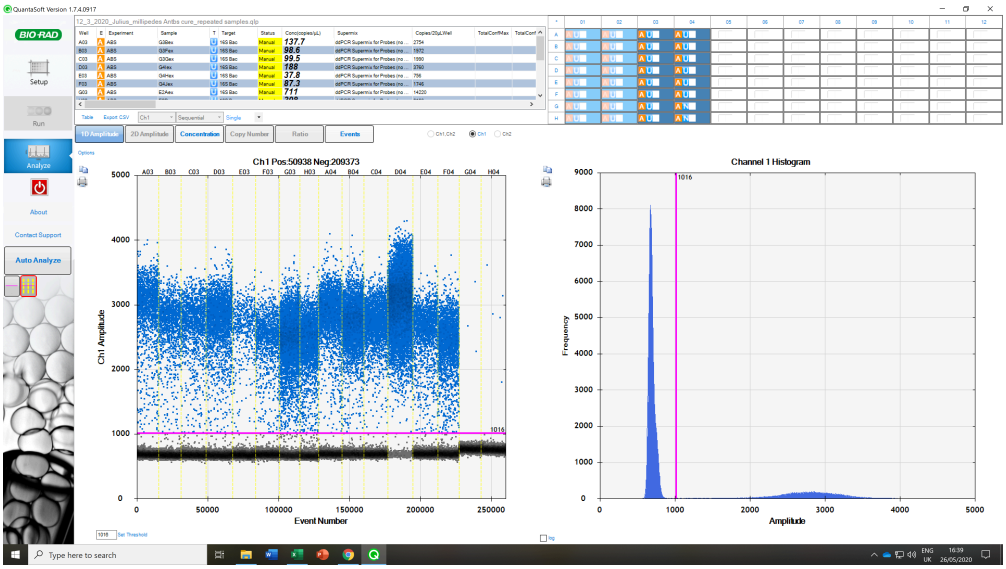
An example of total droplet counts, including positives and negatives together, for several environmental samples.

Analysis

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

Note

Quanta software will automaticaly 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 16S Bac copy numbers data analysis. Last two samples on the left graph are negative (NTC) samples by which a treshold 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:

no. of copies in 1ul of input DNA = (concentration value x 22)/ volume of DNA