Version 2

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Quantification of 16S rRNA Gene Copies Using ddPCR (EvaGreen-based assay: 338F-805R) V.2

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1 Works for me dx.doi.org/10.17504/protocols.io.bm3ak8ie

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ABSTRACT

This protocol describes how to quantify 16S rRNA bacterial gene or transcript copy numbers using Droplet Digital PCR technology (ddPCR) from Bio-Rad This is an up-to-date modification of a classical bacterial enumeration qPCR-assay. This assay uses the EvaGreen™ chemistry. The primers are taken from Yu et al. (2005).

Advantages of ddPCR over qPCR

Among the biggest advantages of the ddPCR technique are its high sensitivity (down to one molecule of target gene presented in input DNA) and low sensitivity to enzymatic inhibitors. Moreover, because it is an absolute and direct quantification technique, no external standard is needed for evaluation.

Yu Y, Lee C, Kim J, Hwang S (2005). Group-specific primer and probe sets to detect methanogenic communities using quantitative real-time polymerase chain reaction.. Biotechnology and bioengineering.

ATTACHMENTS

MSDS-EvaGreen.pdf

DOI

dx.doi.org/10.17504/protocols.io.bm3ak8ie

PROTOCOL CITATION

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digital droplet PCR, 16S rRNA gene, bacteria

LICENSE

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ATTACHMENTS

MSDS-EvaGreen.pdf

GUIDELINES

1. Just like for qPCR, careful and precise pipetting, and adequate mixing and dilutions are crucial to the success of the assay.
2. Keep in mind that ddPCR works with a lower dynamic concentration range compared to qPCR does.
3. One can easily overload the reaction with too much template DNA because ddPCR requires that a certain proportion of the droplets remain empty. Ideally, a ddPCR reaction should contain between 10^1 - 10^4 copies of the target gene. If the expected copy-number range cannot be assumed in advance it is advisable to prepare several dilutions of the sample in parallel and analyse them together.
4. The ddPCR Droplet Reader processes the samples in batches of 8. Therefore, even if the total number of samples is not a multiplication of 8 all the columns in the plate must be filled with reagents.

STEPS MATERIALS

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

EQUIPMENT

NAME	CATALOG #	VENDOR
PX1 PCR Plate Sealer	1814000	Bio-rad Laboratories
Automated Droplet Generator	1864101	Bio-rad Laboratories
X200™ Droplet Reader	1864003	Bio-rad Laboratories
T100™ Thermal Cycler	1861096	Bio-rad Laboratories

SAFETY WARNINGS

See the regulations of your institute for proper handeling and disposal of DNA-intercalating dyes. The MSDS of the EvaGreen dye is enclosed this protocol.

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Advantages of ddPCR over qPCR

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Among the biggest advantages of the ddPCR technique are its high sensitivity (down to one molecule of target gene presented in input DNA) and low sensitivity to enzymatic inhibitors. Moreover, because it is an absolute and direct quantification technique, no external standard is needed for evaluation.

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BEFORE STARTING

Take all the reagents out of the freezer and allow them to reach room temperature.

Primers

1 For the assay we use the following universal 16S Bacteria primers:

Name	Direction	Sequence	Target region[1]
BAC338F	F	ACT CCT ACG GGA GGC AG	338-354
BAC805R	R	GAC TAC CAG GGT ATC TAA TC	785-805

1. Relative to *E. coli* SSU rRNA gene

PCR mixture 40m

2 20m

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

3 10m

Reagent	Final conc.	1 tube (22 µl)	plate (22 µl x 100)
PCR H_2O		8.6	860
QX200 ddPCR EvaGreen Supermix	1x	11	1100
BAC 338F (10 µM)	0.1 µM	0.2	20
BAC 805R (10 µM)	0.1 µM	0.2	20
Template		2	2 x 100

Prepare the master mix according to the number of samples (incl. at least one NTC sample) and mix for several seconds by vortexing or pipetting. Transfer mix into 96-well plate à **20 µl** .

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ddPCR 96-well plates
by Bio-rad Laboratories
Catalog #: 12001925

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

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

4 Add **2 µl DNA template** into each well. 5m

5 Seal the plate (**00:00:05** **180 °C**) with a pierceable aluminium foil. 5s

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

PX1 PCR Plate Sealer
Plate Sealer
Bio-Rad **1814000**

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PX1 Plate Sealer set-up

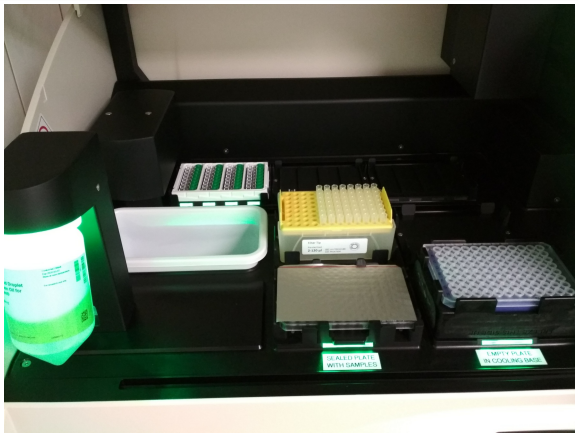
6

Let the foil cool down and mix the plate vigorously by vortexing for **00:00:30** - **00:01:00**

Droplet generation using AutoDG 1h

7 Place the cartridges, tips, sealed plate with samples and an empty 96-well plate into their appropriate positions in the QX200 AutoDG Droplet Digital PCR System (Bio-Rad). 40m

Note: Two pipette tips are needed for each sample!



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

Automated Droplet Generator
Droplet Digital PCR System
Bio-Rad 1864101

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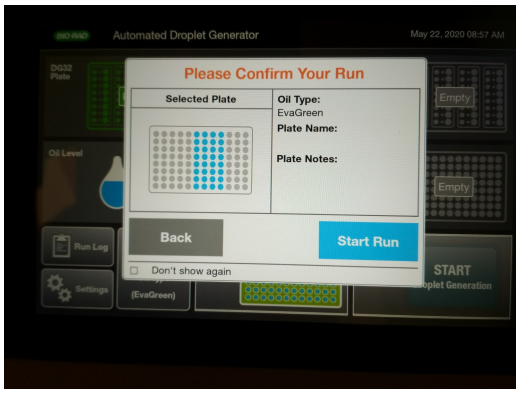
8 Make sure that the correct oil bottle—Automated Droplet Generation oil for EvaGreen—is connected to the system.

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

The droplet generation oil is prone to expire about one year after opening. Replace the oil if it has turned milky.

9 Mark the position of the samples in the plate on the touch screen and press "START" initiate the droplet generation.



A confirmation window will appear on the screen before the procedure starts. Make sure you have chosen the right positions of the samples.

After the droplet generation is finished, the system will you automatically display a message about the success or failure of the procedure. Nevertheless, even after a successful run, it is advisable to inspect the wells and ensure that two separated phases are clearly visible. Upper part with droplets and lower clear oil phase.

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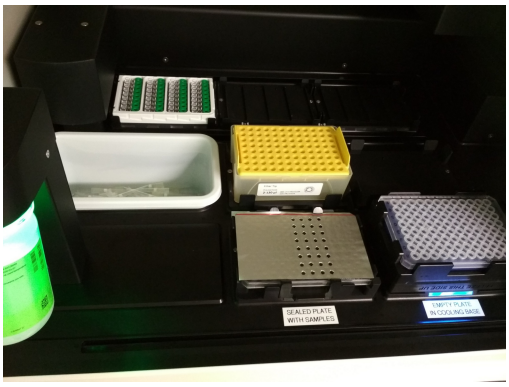
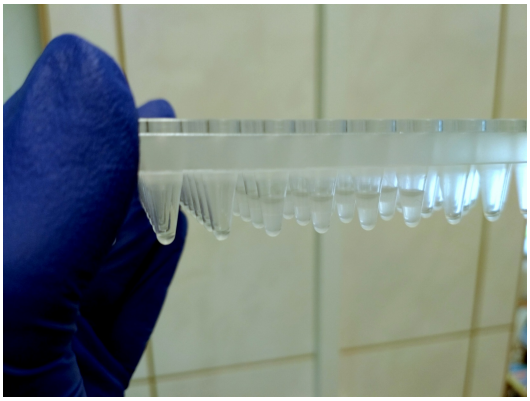


This is how it should look like inside the AutoDG after droplets generation



The 96-well plate after droplet generation: two phases are visible in each well containing a sample

10 Take the plate with droplets out of the AutoDG and seal it with pierceable aluminium foil (**170 °C** , **00:00:03**).

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

11 ⚠

Immediately place the sealed plate into PCR cycler (see below) and initiate the reaction.

The droplets are unstable at this stage. Proceed to the next step as soon as possible. Following PCR, the droplets become stable and can be kept at **4 °C** for some time (up to 24 hours) before measurement.

12 Clean the AutoDG and discard used consumables.

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PCR program

13

3h

1. **95 °C** **00:05:00**
2. x 5 {
   a. **95 °C** **00:00:30**
   b. **60 °C** **00:02:30** ' (**-1 °C** each step)
   }
3. x 35 {
   a. **95 °C** **00:00:30**
   b. **55 °C** **00:02:30**
   }
4. **4 °C** **00:05:00**
5. **90 °C** **00:05:00**
6. **10 °C** hold

Settings:

1. Set the ramp rate for each step to 2°C/sec.
2. Set the reaction volume to **40 µl** .

- After the run is finished check if there are still two phases present.
- Let the plate cool down before downstream measurement
- To maximise the droplet count, leave the plate overnight in a fridge before processing the samples in the QX200 Droplet Reader. This is because the droplets tend to stick together after the PCR step, but loose this tendency after prolonged co

T100™ Thermal Cycler
Thermal Cycler
Bio-Rad **1861096**

Droplet reading 30m

14

1h

Put the plate into a metal holder, place them together into QX200 Droplet Reader.

Switch on the reader 30 min before measurement.

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

X200™ Droplet Reader
Droplet Reader
Bio-Rad 1864003

15 **Set up the QuntaSoft experiment as follows:**

Exp. type	Absolute quantification (ABS)
Supermix	EvaGreen ddPCR Supermix
Target1	Ch1

Define the position of each sample.

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

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

17 Start the measurement.

After the count is finished go over the results and check how many droplets were counted for each sample. To get a reliable count the number of droplet should be above 12.000. On average, droplet counts range from 16.000 to 18.000.

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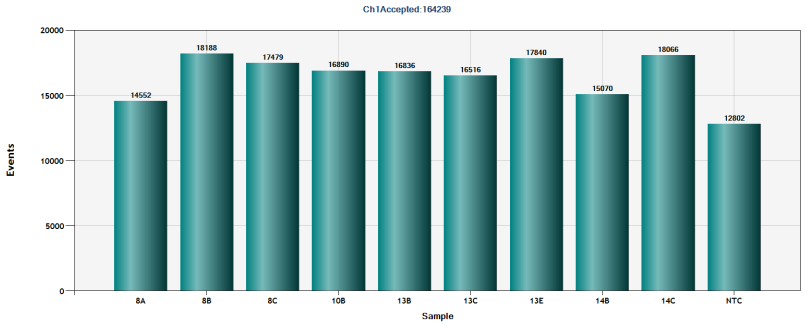


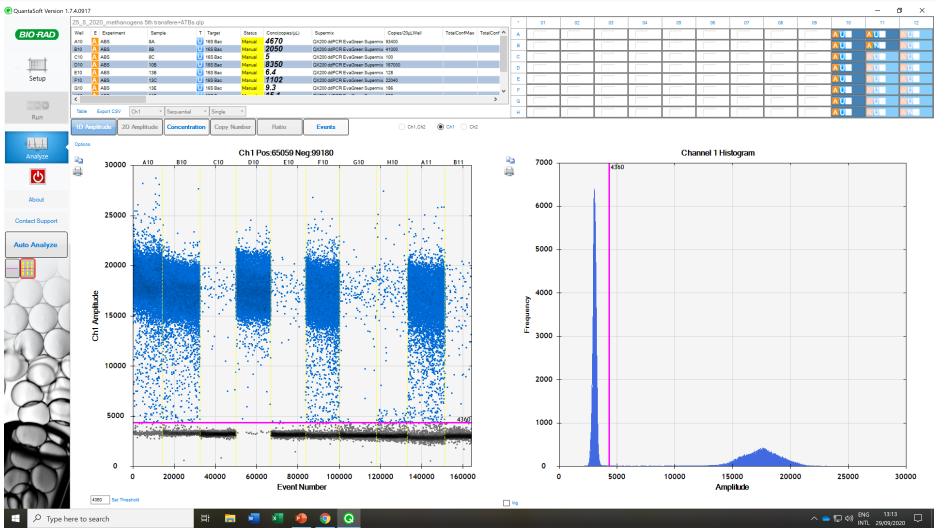
An example of total events = droplet counts (positives and negatives together) for ten samples.

Analysis

18

Set the threshold just above the negative control sample in order to distinguish positive (droplets containing PCR products) from negative (empty) droplets.

QuantaSoft will automatically calculate a copy number of the target gene for each sample using its Poisson distribution algorithm. For these calculations, a certain portion for the droplets must be negative. If the sample contains only positive droplets it cannot be evaluated properly (see an example below).



An example of 16S Bac copy numbers data analysis. The right-most sample is a negative-control sample (NTC), according to which a threshold was set up. Sample 4 (well D10) shows an overloaded sample with an insufficient number of negative (empty) droplets. The quantification for this sample is inaccurate and the sample should be repeated with a higher dilution.

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QuantaSoft Analysis Pro
Windows 7 or Windows 10, 64 bit 1.7.4
by Bio-Rad Laboratories

19 Export a CSV file with concentrations (copies μl^{-1}). To obtain the number of copies in 1 µl of template DNA use the following formula:
no. of copies in 1 µl of template DNA = ddPCR conc. x 22 / volume of template DNA

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