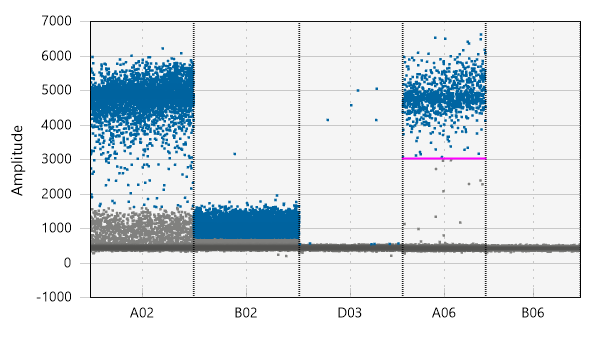
**Supplementary information for “Considerations for droplet digital PCR for environmental samples”**

**Table S1.** Web of Science (Clarivate Analytics 2018) search for environmental studies using ddPCR. For each study, the type of sample used, and the threshold determination method is reported.

|  |  |  |
| --- | --- | --- |
| **Source** | **Type of sample** | **Threshold strategy** |
| Kokkoris et al., 2021 | Fungal spores | Manual Quantasoft – Based on controls |
| Fu *et al.* 2020 | Seeds/grains | Auto Quantasoft (1.6.6) |
| Grudlewska *et al.* 2020 | Sewage/wastewater | Manual Quantasoft – 7000-8000 RFU depending on assay |
| Suttner *et al. 2020* | Watershed sediment | Auto Quantasoft (1.7.4) |
| Rosa et al., 2020 | Roots | Manual Quantasoft – Based on controls |
| Maldonado-González et al., 2020 | Soil | Auto Quantasoft - Unspecified |
| Liu et al., 2020 | Soil | Define the rain algorithm |
| Wen et al., 2020 | Soil | Define the rain algorithm |
| Martínez-Diz et al., 2020 | Soil | Auto Quantasoft - Unspecified |
| Manzari et al., 2020 | Bacteria | Manual QuantaSoft - Unspecified |
| Hansen et al., 2019 | Soil | Unspecified |
| Kokkoris et al., 2019 | Soil/plant tissue | Manual Quantasoft – Based on controls |
| Köppel *et al.* 2019 | Meat tissue | Auto Quantasoft – Unspecified |
| Cao *et al.* 2019 | Fish muscle tissue | Auto Quantasoft (1.7) |
| Shehata *et al.* 2019 | Meat | Auto Quantasoft – Unspecified |
| Cottenet *et al.* 2019 | Plant tissue | Auto Quantasoft (1.7.4) |
| Deng *et al.* 2019 | Seeds | Manual Quantasoft – Unspecified |
| Mauvisseau *et al.* 2019 | Water | Manual Quantasoft – 3500 RFU |
| Giraldo *et al.* 2019 | Plant tissue | Auto Quantasoft – Unspecified |
| Bahar *et al.* 2018 | Plant tissue | Manual QuantaSoft - High |
| Cooley *et al.* 2018 | Surface water | Manual – BioRad Rare Mutation Detection guidelines |
| Gao *et al.* 2018 | Compost | Auto QuantaSoft |
| Gobert *et al.* 2018 | Faecal | Manual QuantaSoft- Low |
| Gradoville *et al.* 2018 | Seawater | Three standard deviations above average NTC RFU |
| Grelewska-Nowotko *et al.* 2018 | Plant tissue | Manual QuantaSoft - Unspecified |
| Hamaguchi *et al.* 2018 | Coastal sediment | Auto QuantaSoft |
| Köppel *et al.* 2018 | Plant and animal tissue | Auto QuantaSoft |
| Naaum *et al.* 2018 | Meat products | Auto QuantaSoft |
| Paternò *et al.* 2018 | Wheat | Manual QuantaSoft - Unspecified |
| Paquette *et al.* 2018 | Faecal | Auto QuantaSoft |
| Steele *et al.* 2018 | Water | Manual QuantaSoft - Low |
| Selvaraj *et al.* 2018 | Plant tissue | Auto QuantaSoft |
| Wang *et al.* 2018 | Meat products | Auto QuantaSoft |
| Zhong *et al.* 2018 | Plant tissue | Auto QuantaSoft |
| Boynton *et al.* 2017 | Leaf tissue | Manual QuantaSoft - Unspecified |
| van Dorssen *et al.* 2017 | Faecal | Auto QuantaSoft |
| Hunter *et al.* 2017 | Pond water | Manual QuantaSoft - Unspecified |
| King *et al.* 2017 | Insect tissue | Manual - Low |
| Košir *et al.* 2017 | Plant tissue | Manual QuantaSoft – 1600-18500 RFU depending on assay |
| Lee *et al.* 2017 | Seawater | Auto QuantaSoft |
| Maheshwari *et al.* 2017 | Plant tissue | Auto QuantaSoft |
| McMahon *et al.* 2017 | Beef, pork, plant tissue | Auto QuantaSoft |
| Perez-Lopez *et al.* 2017 | Plant tissue | Auto QuantaSoft |
| Perry and Lee 2017 | Wheat | RainDrop Sense |
| Ren *et al.* 2017 | Meat | Auto QuantaSoft |
| Shehata *et al.* 2017 | Meat | Auto QuantaSoft |
| Singh *et al.* 2017 | River sediment | Auto QuantaSoft |
| Yang *et al.* 2017b | Wastewater | Auto QuantaSoft |
| Yang *et al.* 2017c | Faecal | Auto QuantaSoft |
| Zink *et al.* 2017 | Insect tissue | definetherain |
| Bucher and Köppel 2016 | Rice | Auto QuantaSoft |
| Cavé *et al.* 2016 | Soils and organic residues | Auto QuantaSoft |
| Cremonesi *et al.* 2016 | Cheese | Auto QuantaSoft |
| Demeke *et al.* 2016 | Plant tissue | RainDrop |
| Dobnik *et al.* 2016 | Corn | Manual - Mid |
| Gao *et al.* 2016 | Potato | Auto QuantaSoft |
| Iwobi *et al.* 2016 | Plant tissue | Auto QuantaSoft |
| Jerde *et al.* 2016 | Water | Unspecified |
| Lievens *et al.* 2016 | Plant tissue | Auto QuantaSoft |
| Palumbo *et al.* 2016a | Soils | Unspecified |
| Palumbo *et al.* 2016b | Grapes | Auto QuantaSoft |
| Porcellato *et al.* 2016 | Milk | Manual QuantaSoft - 2000-2200 RFU |
| Simmons *et al.* 2016 | Water | Auto QuantaSoft |
| Zhao *et al.* 2016 | Plant tissue | Manual - Unspecified |
| Cao *et al.* 2015 | Water and Faecal | Auto QuantaSoft |
| Doi *et al.* 2015a | Water | Manual QuantaSoft - Unspecified |
| Doi *et al.* 2015b | Water | Manual QuantaSoft – 1100 RFU |
| Te *et al.* 2015 | Water | Manual QuantaSoft - 9000 or 5400 RFU depending on assay |
| Kim *et al.* 2014 | Soil | Auto QuantaSoft |
| Nathan *et al.* 2014 | Water | Manual QuantaSoft - Unspecified |
| Rački *et al.* 201 | Plant tissue | Auto QuantaSoft or manual at 7000 RFU |
| Yang *et al.* 2014 | Faecal | Auto QuantaSoft |
| Kelley *et al.* 2013 | Nostril swab | Auto QuantaSoft |
| Morisset *et al.* 2013 | Corn | Manual QuantaSoft- Unclear |
| Roberts *et al.* 2013 | Eye swabs | Custom Perl and R scripts |



**Figure S1**. **A key to interpreting ddPCR output figures.** 1) Y axis represents the number of droplets amplified as a reflection of their fluorescence. Higher amplitude levels can be achieved with optimized cycling PCR protocol and optimized PCR reaction protocol. Higher amplitude levels result in improved cloud separation 2) X axis represents individual samples, in this example five individual samples are presented (A02, B02, D03, A06 and B06) and are named based on their location on the 96 well plate. 3) The cluster of high amplitude droplets (amplitude 4000 – 6000) is called “positive cloud” and represents positive to the target DNA droplets. 4 & 5) droplets between the “negative cloud” (see 10) and the “positive cloud” are called “rain” and may be negative or positive droplets. 6) Blue droplets are recognized as positive droplets from the automatic threshold determination system of the QuantaSoft software. While sample A02 and B02 are from a single ddPCR run, the automatic threshold treats each sample distinctly placing the “rain” as negative or “positive” respectively. 7) Low number of positive to the target DNA droplets. 8) Clean “negative cloud” 9) The pink line represents the manually set threshold. Above the line all droplets are considered as positive while bellow the line all droplets are considered as negative. 10) This sample represents a non-template control sample where there is no rain and no positive droplets. The negative cloud droplets cluster closely together creating a thick line at low amplitude. In theory, droplets that appear above this cloud in samples other that non-template controls can be potentially positive.

**Negative environmental**

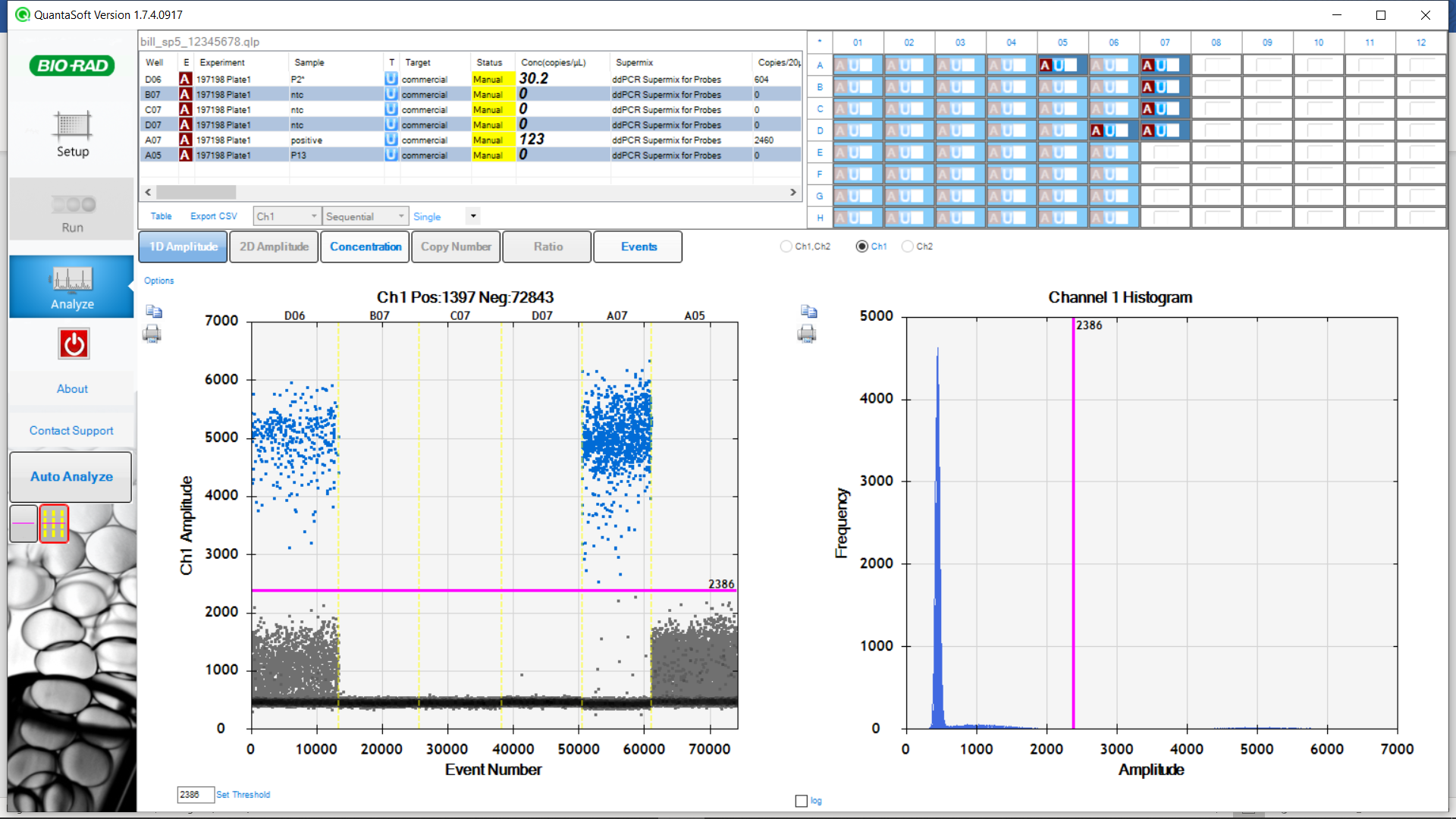
**Positive control**

**Unknown environmental**

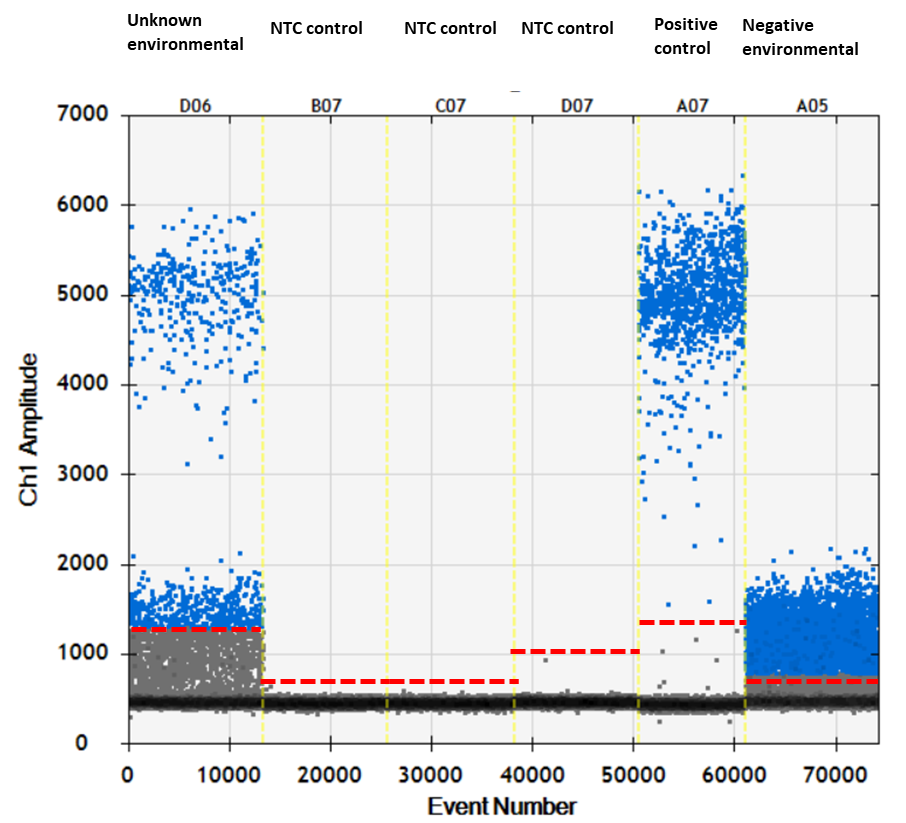
**NTC control**

**NTC control**

**NTC control**



**Figure S2. Manual threshold determination.** In order to determine the quantity of target DNA in an unknown environmental sample (well D06) we used three NTCs (wells B07, C07, D07), an organismal positive control (well A07) and a negative environmental sample (well A05). The threshold (purple line) was set manually at amplitude of 2386 to include the positive droplet cloud produced by the organismal positive control but to exclude the droplets produced by the negative environmental samples. Blue droplets represent positive droplets while grey droplets represent negative droplets. Separation of negative from positive cloud was achieved by setting the threshold manually at “2386” amplitude.



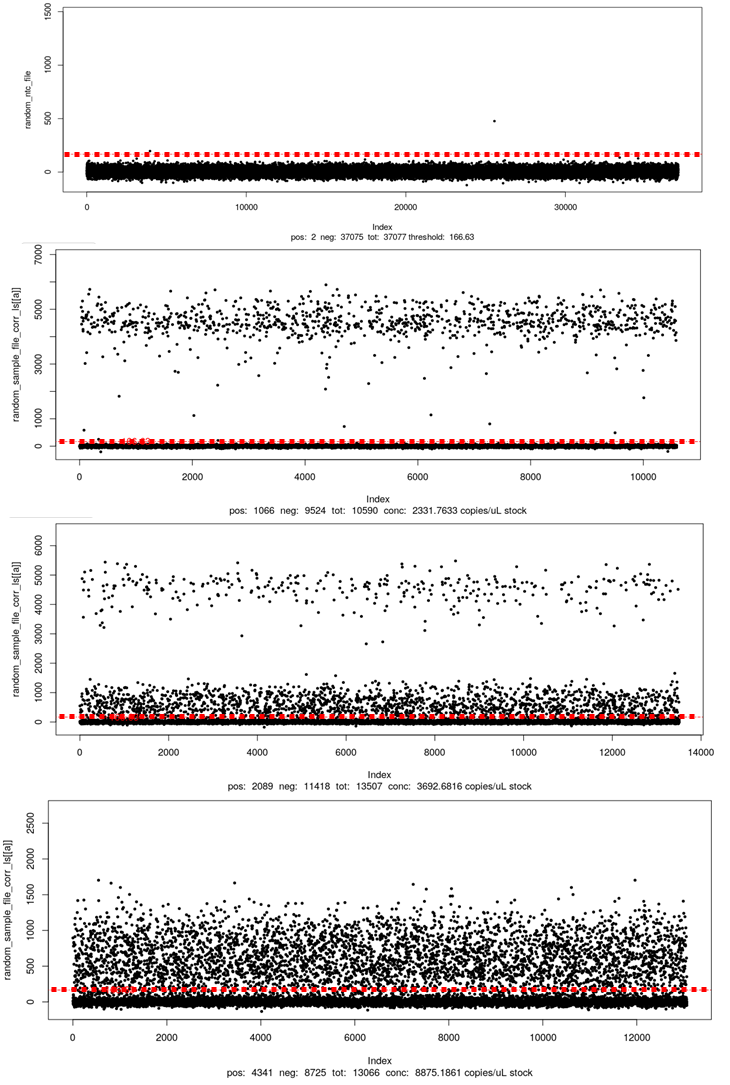
**Figure S3. Automatically determined threshold using the QuantaSoft® software.** Threshold was variable for each sample. The QuantaSoft® software algorithm failed to discriminate between true positive and negative droplets.Blue droplets represent positive droplets while grey droplets represent negative droplets.

NTC control

Positive control

Unknown environmental

Negative environmental



**Figure S4. Automatically determined threshold using the ‘ddPCRquant’ algorithm.** The ‘ddPCRquant’ algorithm merges the three NTC samples and calculates the threshold based on the dense negative droplet cloud. The threshold was set automatically at “166.63” amplitude (red line in each sample). All samples above this value were considered erroneously as positive, including the negative environmental sample.

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