Supplementary Materials

S1 – Methods and data format for RT-dPCR with the naica® system

This one-step reverse-transcription PCR kit is a triplex PCR allowing amplification, detection and quantification of one sequence in the *N* gene, one sequence in the *ORF1ab* region of SARS-CoV2 and an endogenous internal control (IC) to assess the quality of the sample and extraction. These sequences are targeted by three TaqMan probes respectively labelled with a FAM, HEX and Cy®5 fluorophore.

As recommended by the kit manufacturer, the PCR mix for a single reaction contained 12.5 µL of dPCR MasterMix 1, 1 µL of dPCR Mix 2, 1 µL of COVID-19 Assay and 10.5 µL of either, P8, P16, P32, positive control, negative control or individual extract. The 25 µL of this PCR mix were loaded in the inlet ports of the Sapphire chips (Stilla Technologies, France). The chips were placed in the Geode (Stilla Technologies, France) for droplets generation, reverse transcription and PCR amplification following the kit manufacturer's instructions.

After amplification, the chips were transferred to the Prism3 (Stilla technologies, France) for fluorescence reading in the three detection channels and data were analyzed with Crystal Miner Software (Stilla Technologies, France) following the kit manufacturer's instructions.

An illustration of the resulting fluorescence dot-plots used to quantify the SARS-CoV-2 virus by RT-dPCR is shown in Supplementary Materials (Fig. S1.1 and S1.2).

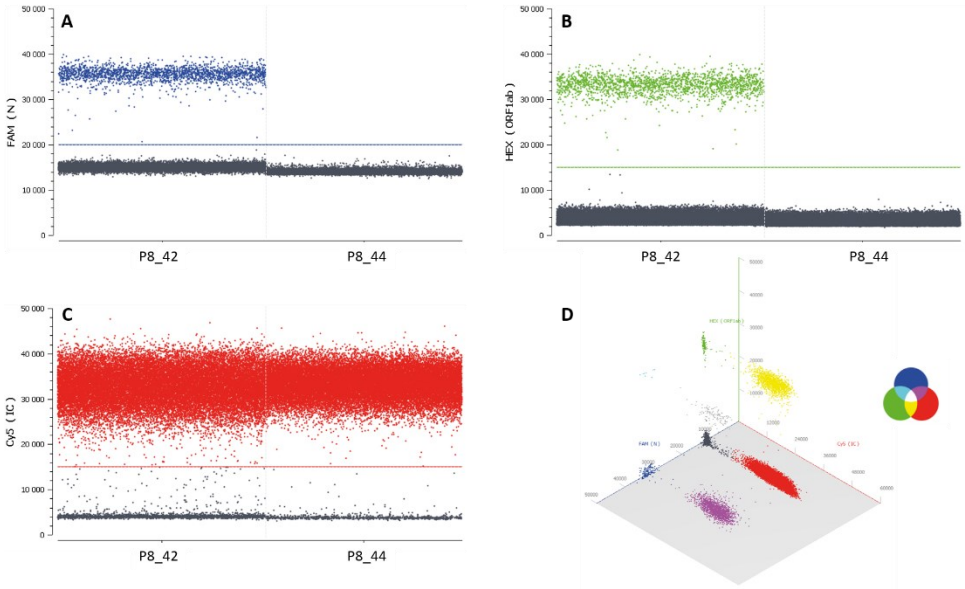

Figure S1.1: Fluorescence results as displayed in the Crystal Miner software showing 1D dot-plots of a positive (P8_42) and a negative (P8_44) pool of 8 samples in the FAM (A; blue = N gene), HEX (B; green = ORF1ab gene) and Cy5 (C; red = internal control) channels. The horizontal line marks the threshold above which droplets (represented as dots) are considered positive for the amplification of N, ORF1ab and for the endogenous internal control respectively. The thresholds are set by manufacturer at 20 000 RFUs, 15 000 RFUs and 15 000 RFUs for the FAM, HEX and Cy5 channels. (D) 3D dot-plots of P8_42: if the concentration of either target is high enough, co-encapsulation of several targets can occur in a droplet leading to the appearance of clusters for double positive droplets (cyan, yellow and purple) and triple positive droplets (light grey). Triple negative droplets, containing no target, are shown in the dark grey cluster.

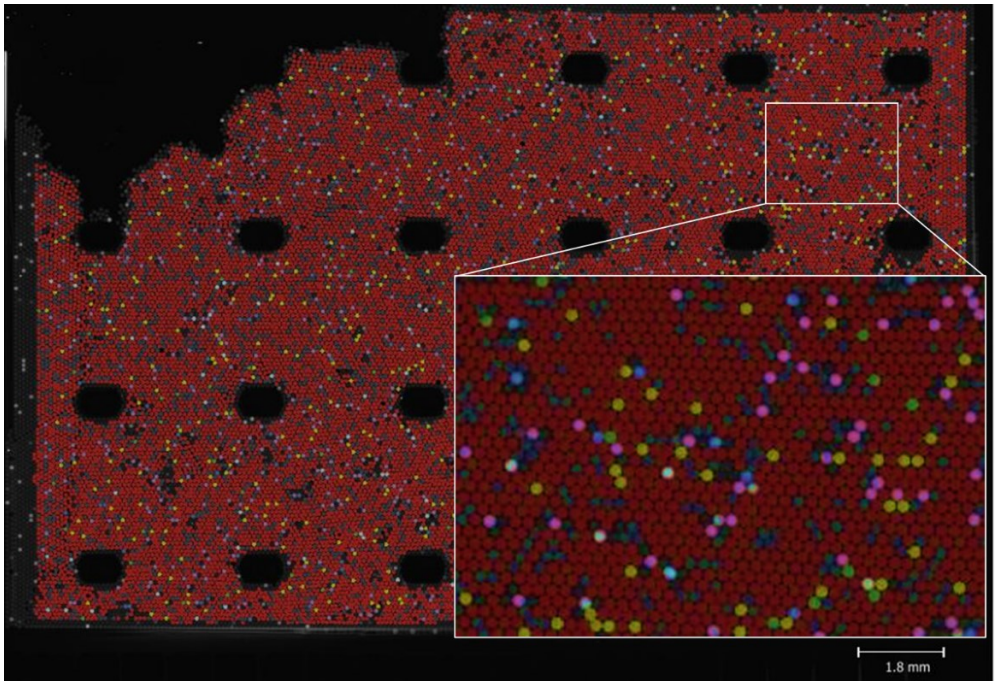

Figure S1.2: Image of the droplet crystal obtained using the naica® system on grouped extracts P8_22, including a zoom on a sub-region of the crystal. Droplet color code: dark grey: negative for all targets; blue: positive for N gene only ; green: positive for ORF1ab only; red: positive for IC (Internal Control) only; cyan : positive for N and ORF1ab genes; magenta: positive for N gene and IC; yellow: positive for ORF1ab gene and IC; white / mixed: positive for all. The droplet crystal contains 25 820 analyzable droplets, out of which 1 057, 883 and 21 121 were positive for the N, ORF1ab and IC respectively.

S2 – Limit of blank (LoB) evaluation

Method

The Limit of Blank (LoB) was evaluated for SARS-CoV-2 detection using the group testing approach used in the study on a cohort of 256 pre-epidemic nasal swab samples (negative control samples) that were collected between December 1st 2019 and January 31st 2020 and for which transport medium was stored at -20 °C within 48h after sampling.

Specimens were randomly grouped into 32 groups of 8 negative controls which were co-extracted and analyzed by RT-dPCR using the same protocol described above. The results for all 32 groups are given in Supplementary Materials. The LoB at 95% confidence level for the *N* target and the *ORF1ab* target is determined to be of 2 and 0 positive droplets respectively.

Consequently, a threshold of at least 3 positive droplets in aggregate between both the *N* target and *ORF1ab* target was used to classify a sample as positive to SARS-CoV-2 by RT-dPCR in this study.

Sample ID	**Number of droplets**	**Pos. droplets (N+ORF1ab)**	**Pos. droplets (N)**	**Pos. droplets (ORF1ab)**	**IC (cp/µL)**
Group 1	19460	0	0	0	31430
Group 2	20641	0	0	0	10985
Group 3	21449	0	0	0	4054
Group 4	20983	0	0	0	15143
Group 5	19881	1	1	0	7401
Group 6	20609	0	0	0	8783
Group 7	19044	0	0	0	6249
Group 8	20787	0	0	0	8759
Group 9	21470	0	0	0	10271
Group 10	18952	0	0	0	17578

Group 11	23424	0	0	0	8222
Group 12	22748	0	0	0	9875
Group 13	23747	0	0	0	10275
Group 14	24886	0	0	0	4266
Group 15	24079	0	0	0	10264
Group 16	24718	0	0	0	13219
Group 17	24228	0	0	0	10186
Group 18	23742	0	0	0	8431
Group 19	23880	0	0	0	7628
Group 20	23613	0	0	0	3111
Group 21	25723	0	0	0	8987
Group 22	25522	0	0	0	7411
Group 23	24781	0	0	0	6894
Group 24	7074	0	0	0	5884
Group 25	27003	0	0	0	8911
Group 26	27138	0	0	0	5552
Group 27	24425	0	0	0	7886
Group 28	27017	0	0	0	7484
Group 29	26722	0	0	0	9963
Group 30	26088	0	0	0	2492
Group 31	26747	0	0	0	5703
Group 32	26728	0	0	0	5825

Table S2: Detection results in number of positive droplets for N, ORF1ab, N+ORF1ab and IC by RT-dPCR in 32 groups of 8 pre-epidemic samples.

The pre-epidemic groups were used as negative controls for SARS-CoV-2 detection. Only one out of 32 (group 5) had one positive droplet for the *N* target out of 19881 droplets analyzed. The LOB at 95% confidence level for this RT-dPCR assay was estimated automatically by the Gene-Pi online statistical tool (https://www.gene-pi.com/statistical-tools/loblod/), considering a droplet volume of 0.548 nL as specified by the manufacturer. The

LOD at 95% confidence level is computed from the experimental LOB. The LOB for the *N* and *ORF1ab* targets are found to be of 2 and 0 droplets respectively..

Using a joint analysis, a LOB analysis can be performed for the combination of the *N* and *ORF1ab* targets. In the case, the sum of the positives for the *N* and *OFR1ab* target is used as the measure and the LOB for the sum is 2 positive droplets. Consequently, in this study, a RT-dPCR result is considered as positive when the sum of positive droplets in the *N* and *ORF1ab* targets is of 3 droplets or more (strictly greater than 2).

For details on calculation models, see:

- https://www.gene-pi.com/wp-content/uploads/2018/03/Memo_LOB_calculation_method.pdf

S3 - Results of LoD experiments

S3 - 1. Method

Evaluation of the Limit of Detection (LoD) has been performed with the commercially available inactivated virus AMPLIRUN® TOTAL SARS-CoV-2 CONTROL (SWAB) (Vircell, Spain) which has been titrated by qPCR (Altona) with a dilution range of an in-house DNA standard in a preliminary experiment. After resuspension of the vial in 500 µL nuclease-free water, the titer was evaluated at 10 copies of inactivated virus per µL of suspension.

The latter value has been used for the evaluation of the LoD which was determined by spiking 0 ("no spike"), 30, 77 and 170 copies of the inactivated virus from a fresh suspension into ten 1 mL pools of 8 randomly assembled Cobas-negative specimen. After nucleic acids extraction and elution in 50 µL, the 40 extracts were run in RT-PCR (Altona) and RT-dPCR in parallel.

S3 - 2. Results for LoD evaluation using Cobas-negative NP specimen as background sample matrix

Due to a suspected spiking error for one data point at 170 copies/mL (spiked sample negative by both RT-PCR and RT-dPCR), only 9 data points were kept in the analysis for this spiked concentration. 6 out of the 10 pools at 30 cp / mL were detected by RT-dPCR whereas only one was detected by RT-PCR. All 10 pools at 77 copies per mL were positive in RT-dPCR whereas 2 out of 10 only were detected by RT-PCR. The spikes at 170 cp / mL was detected in 9 out of 9 pools and 7 out of 9 pools by RT-dPCR and RT-PCR respectively..

the LoD for RT-dPCR was determined to be between 30 and 77 copies per mL of transport medium (6/10 pools positive at 30 copies/mL and 10/10 pools positive at 77 copies/mL, corresponding to 3 copies/reaction and 8 copies/reaction respectively) while the LoD for RT-PCR (Altona) was determined to be of more than 170 copies/mL of transport medium (7/9 pools positive at 170 copies/mL, corresponding to 34 copies/reaction). Of note, the PCR (Altona) LoD was previously evaluated at 625 copies/mL in the same laboratory and conditions (18). Full results are shown in Table S3.A.

S3 - 3. Comparison with spike-in experiments on bare transport medium as background sample matrix

Interestingly, we also report here results obtained by spiking the same amounts of inactivated virus in bare transport medium. The results are shown in Table S3.B.

While RT-dPCR results are qualitatively similar between bare transport medium and negative patient samples, RT-PCR is found to perform significantly better on bare transport medium. All spike-in samples tested positive by RT-PCR on bare transport medium including for spikes of 30 cp/mL, compared to 1 out of 10 tested positive for the same spike concentration using negative patient samples.

One explanation for the difference in performance of RT-PCR between bare transport medium and negative patient samples could be the presence of PCR inhibitors in the RT-PCR reaction, introduced when processing patient samples. Digital PCR is known to be less sensitive than RT-PCR to inhibitors (13), explaining the similar results of RT-dPCR on bare transport medium and on negative patient samples.

A

	Altona E gene	Pos. Droplets (N)	Pos. Droplets (ORF1ab)
No spike	0	0	0
	0	0	0
	0	1	0
	0	0	0
	0	0	0
	0	0	0
	0	0	0
	0	0	0
	0	1	0
	0	2	1
30 cp / mL	0	1	2
	36.3	3	0
RT-dPCR:	0	0	0
3 cp / rxn	0	3	1
	0	1	0
RT-PCR:	0	2	3
6 cp / rxn	0	0	0
	0	1	0
	0	3	3
	0	1	2
77 cp / mL	0	4	1
	35.8	4	1
RT-dPCR:	0	6	7
8 cp / rxn	0	9	8
	0	5	1
RT-PCR:	0	7	3
15 cp / rxn	36.2	6	0
	0	9	5
	0	2	1
	0	19	13
170 cp / mL	0	12	5
	34.3	25	24
RT-dPCR:	36.5	17	13
19 cp / rxn	34.2	22	17
	34.2	15	12
RT-PCR:	32.9	47	22
34 cp / rxn	34.1	8	7
	0	14	11
	33.6	36	21
	0	-1	0

Data point excluded, suspected operator error during spiking

B

	Altona E gene	Pos. Droplets (N)	Pos. Droplets (ORF1ab)
No spike	0	0	0
	0	0	0
	0	NA	NA
30 cp / mL	34.7	4	4
	33.8	1	0
	33.1	NA	NA
77 cp / mL	34.6	3	3
	33.6	6	3
	34	NA	NA
170 cp / mL	32.9	8	3
	33.2	16	6
	33.5	NA	NA

Table S3: Detection results in CT for the E gene and number of positive droplets for the N gene and ORF1ab for 0, 30, 77 and 170 copies of inactivated virus spiked either in (A) pools of 8 Cobas-negative patient's samples or (B) bare transport medium. The red color indicates a negative call (i.e. no C_T in RT-PCR or number of positive droplets for N + ORF1ab under the LoB in RT-dPCR) and the green color indicates a positive call (i.e. C_T measured in RT-PCR or number of positive droplets for N + ORF1ab above the LoB in RT-dPCR).

S4- Analysis of individual PCR and pooled dPCR discordances

PCR-/dPCR+ discordances

Three P8 groups (P8_20, P8_28 & P8_39) and one P16 group (P16_14) tested positive by RT-dPCR while containing only negative samples by individual RT-PCR testing. As P16_14 included P8_27 and P8_28, its discordance is the result of the P8_28 discordance.

To further investigate these PCR-/dPCR+ discordances, confirmatory testing RT-dPCR was performed on all individual samples from corresponding groups. For each group, one sample tested positive by individual RT-dPCR, with measured concentrations of viral RNA ranging from 128 to 2 copies per reaction for the *N* gene, and from 106 to 1 copies for the *ORF1ab* gene. The three corresponding RT-dPCR+ samples (sample IDs: 52042, 56075 and 60401) were retested on the Cobas® 6800 system and by confirmatory individual RT-qPCR using the Altona assay.

Two samples were found positive using the Altona assay with Ct values ranging between 28.4 and 33 for the *E* and *S* genes. Among them, the sample presenting the highest viral load by RT-dPCR (Sample 56075) was also found positive by the Cobas® confirmatory assay with a high Ct value of 36.7 for the *E* gene while the *ORF* gene was not detected. The remaining sample tested negative with both the confirmatory Cobas assay and the Altona assay. It had borderline levels of positive droplets in RT-dPCR (N=2; ORF1ab=1).

Based on these results and for further sensitivity discussions, samples *52042* and *56075* that tested positive by both RT-dPCR and Altona RT-PCR are considered as true positive samples. Sample 60401 is considered an RT-dPCR false positive result.

Investigation of the PCR+/dPCR- discordance

One group of 8 (P8_02) was tested negative by RT-dPCR and contained one RT-PCR+ sample (*Sample 25659*) with Ct values of 34 and 32.3 for the *E* gene and *ORF1ab,* respectively. *Sample 25659* was subsequently retested by RT-PCR on the Cobas protocol and was also re-extracted individually and retested by individual RT-dPCR. *Sample_25659* was found to be borderline negative by RT-dPCR (N=2; ORF1ab=0) but Ct values of 37.3 and 34.9 were found for *E* and *ORF* respectively in the second Cobas® assessment. An additional RT-PCR assessment with the Altona kit rendered a negative result.

Based on these results and for further sensitivity discussions, sample 25659 is considered as non-conclusive sample.

S5 – Correlation between RT-dPCR measurements and Ct values

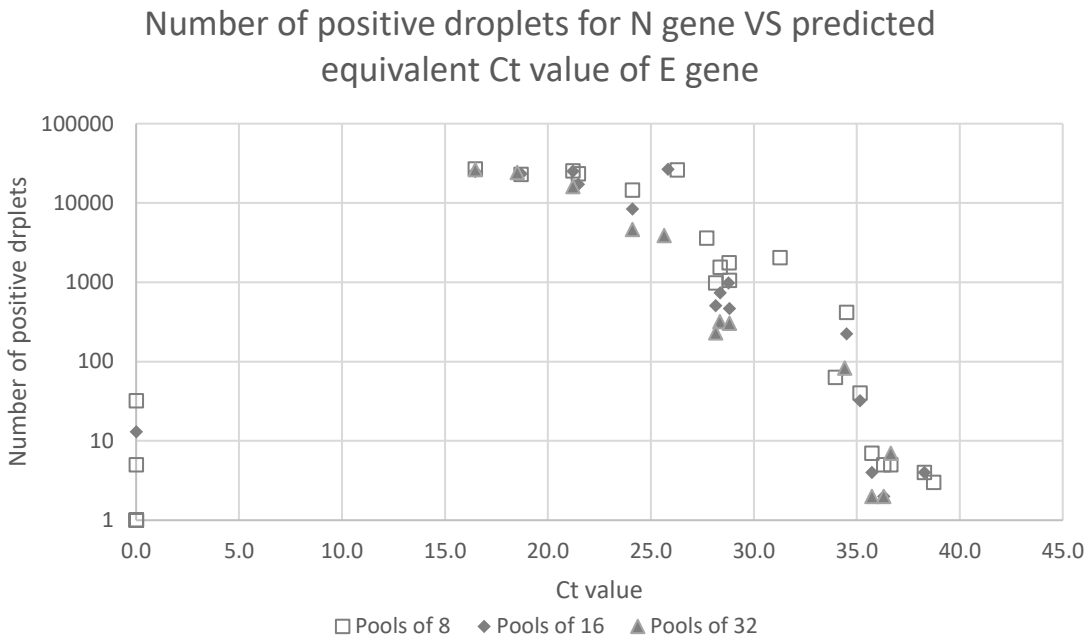

Figure S5.1: Plot of the number of positive droplets measured by RT-dPCR for the N gene target in the groups of 8, 16 and 32 versus the predicted equivalent Ct value of the E gene of an RT-PCR measurement of the group using the Cobas® SARS-CoV-2 assay. The predicted equivalent Ct value of a group is defined as an average of the Ct values of the positive samples included in the group, taking into account the logarithmic scale of the Ct value.

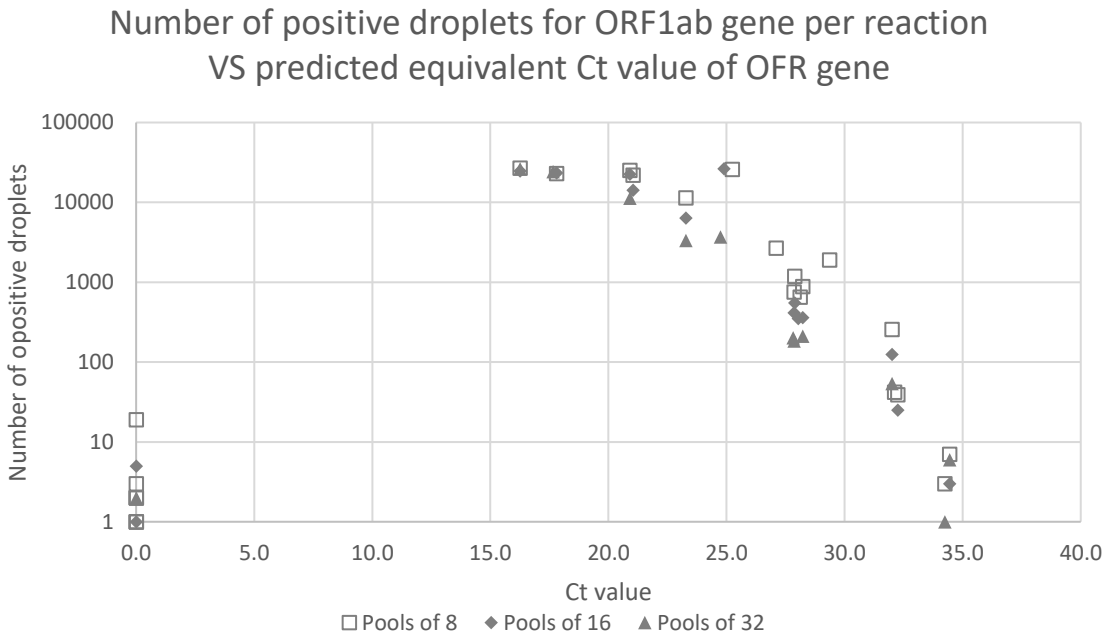

Figure S5.2: Plot of the number of positive droplets measured by RT-dPCR for the ORF1ab gene target in the groups of 8, 16 and 32 versus the predicted equivalent Ct value of the ORF gene of an RT-PCR measurement of the group using the Cobas® SARS-CoV-2 assay. The predicted equivalent Ct value of a group is defined as an average of the Ct values of the positive samples included in the group, taking into account the logarithmic scale of the Ct value.

S6 - *Suggested practical protocol for implementation of group testing by RT-dPCR*

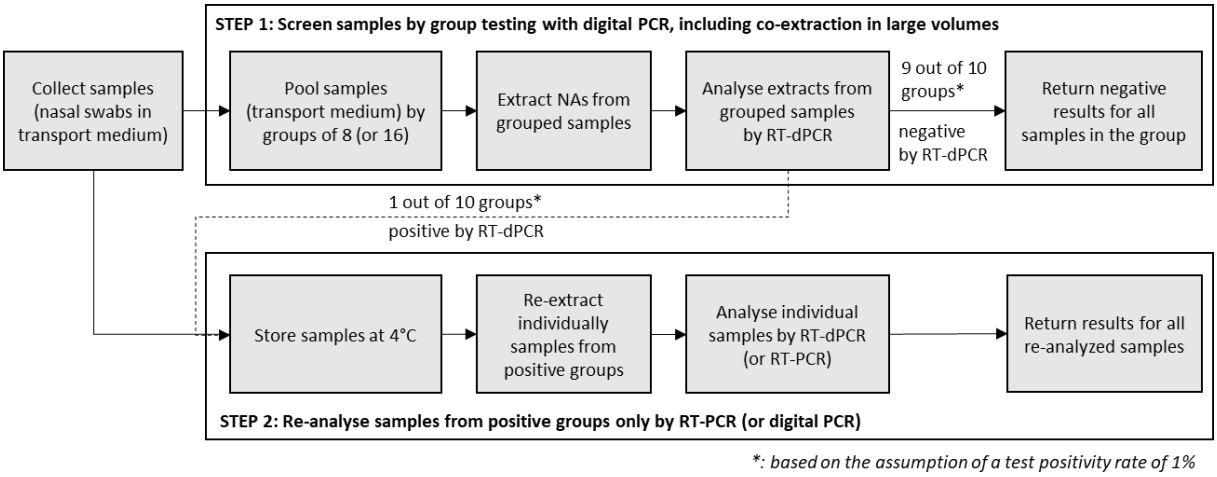

Figure S6: Graph of the suggested practical protocol for implementation of group testing by RT-dPCR

S7 - Analysis of reagent and cost savings for group testing combined with digital PCR

Generalities

q	Cost of analyzing a sample by RT-PCR (including extraction)
d	Cost of analyzing a sample pool by RT-dPCR (including extraction)
N	Number of samples to analyze
n	Number of samples per group
p	Test positivity rate
R_r	Reagent reduction factor (individual testing / group testing)
R_c	Cost reduction factor (individual testing / group testing)

Number of individual test: N

Cost of testing by individual RT-PCR: $N \times q$

Number of groups to test: $\frac{N}{n}$

Probability of group being positive: $1-(1-p)^n$

Number of tests required by group testing = Number of groups + Number of individual re-testing

$$N/n \ + N\ (1-(1-p)\text{^}n\ )$$

Cost of testing by group testing:

$$N/n \ \times p + N\ (1-(1-p)\text{^}n \ \times q$$

Analysis of reagent savings

We assume here that analyzing one individual sample by RT-PCR and analyzing one group by RT-dPCR require both the same amount of reagents, including reagents for one extraction and one PCR reaction (mastermix, primers and probes). Analysis by RT-dPCR also requires the use of a microfluidic disposable (Sapphire Chip in the case of the technology from Stilla Technology) which is not required by RT-PCR but only reagents, which have been in short supply during the pandemic, are considered here.

As a result, the group testing approach divides the volume of reagents required by a factor R_r given by

$$R_r = \frac{n}{\left(1+ n\,(1-(1-p)^n)\right)}$$

Figure S7.1 plots R_r as a function of *p* for the 3 groups sizes investigated here. For a group size of 8, reagent consumption is reduced by a factor 2 or more starting from a test positivity rate of 5% and is nearing the maximum reduction factor of 8 for test positivity of 0.1% or below.

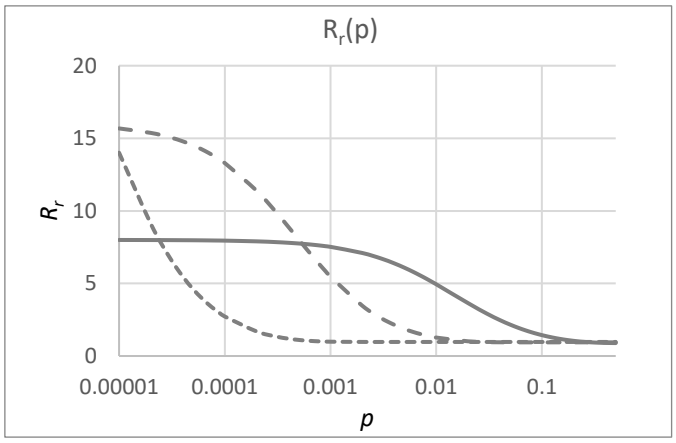

Fig. S7.1 : Plots of the reagent reduction factor R_r as a function of the test positivity rate p for 3 groups sizes: n = 8 in solid line; n = 16 in dashed line ; n = 32 in small dashed line.

Analysis of cost savings

In this analysis, we assume that analyzing one individual sample by RT-PCR as a fixed cost of q. This cost includes all costs from input of the transport medium tube in the RT-PCR analysis workflow to

result but excludes sampling costs for example. We assume that re-analyzing a sample by RT-PCR after group testing as the same cost as individual RT-PCR testing.

Similarly, we assume that analyzing one group of samples by RT-dPCR as a fixed cost of *d*. This cost includes all costs from the pooling of transport medium to the RT-dPCR result for the group and we assume that this cost is independent of the group size n.

As a result, the group testing approach divides the total cost of testing a population by a factor R_c given by

$$R_c = \frac{n}{\left(d/q + n\,(1-(1-p)^n)\right)}$$

The cost reduction factor is dependent of the group size n, the test positivity rate p and the relative cost of RT-dPCR compared to RT-PCR d/q .

For a given test prevalence ratio, R_c has a non-linear variation with the group size *n*. There is an optimal group size for which the reagent consumption and cost is minimized, as described in the literature.

However, for practical implementation, the group size will be a fixed number. For a fixed group size, R_c increases as the test positivity rate decreases, with a maximum of $\frac{n}{d/q}$ for a null positivity rate as shown on Fig. S7.1.

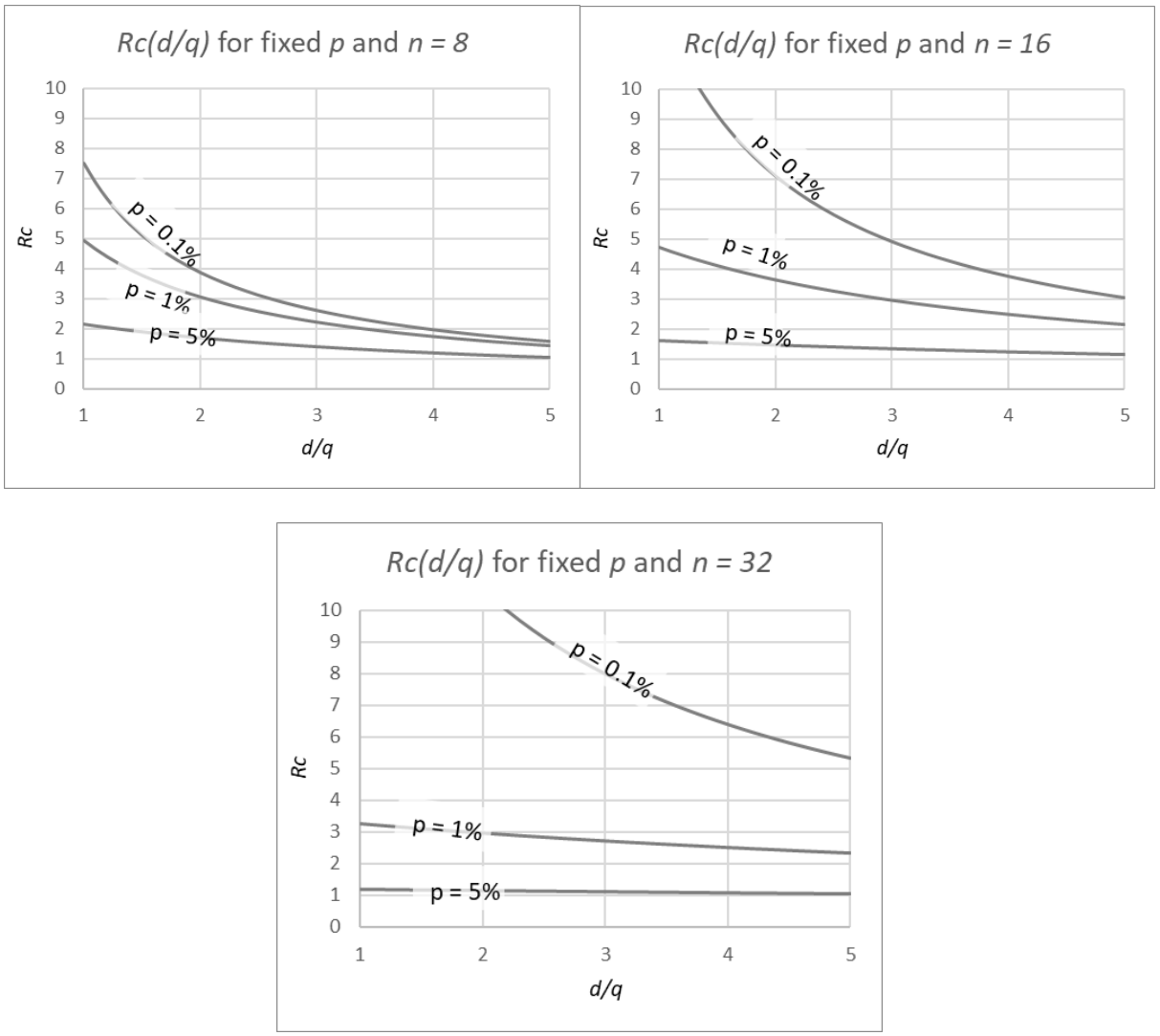

Fig. S7.2 : Plots of R_c as a function of d/q for test positivity rates of 0.1%, 1% and 5% in the case of group sizes of n = 8, 16 and 32.

Actual cost of dPCR group testing *d* is likely to vary between implementation sites with values between d/q = 2 and d/q =4. Figure S8.2 plots R_c as a function of the test positivity rate *p* for the 3 group sizes investigated.

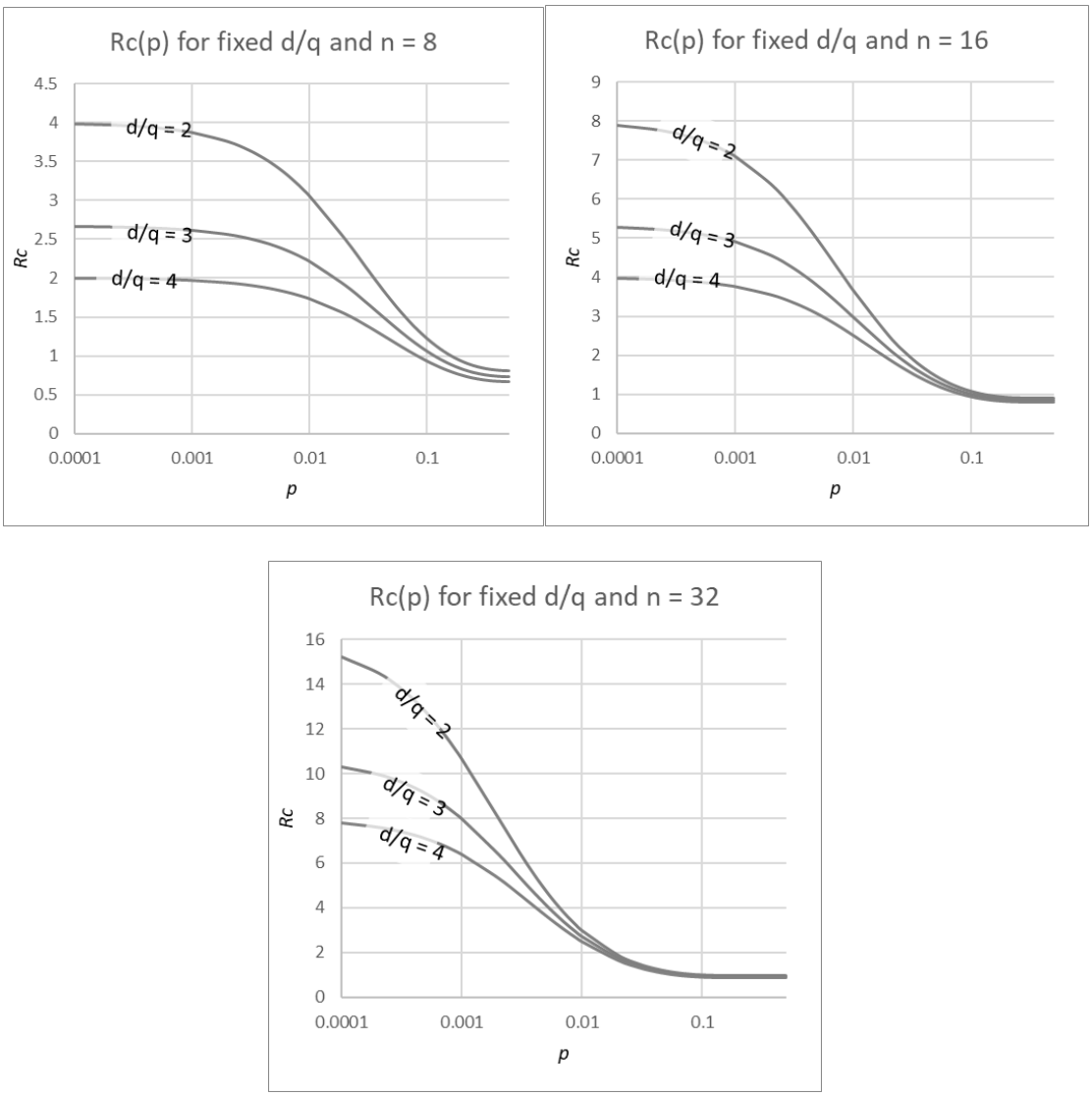

Fig. S7.3 : Plots of R_c as a function of p for dPCR costs d/q of 2, 3 and 4 in the case of group sizes of n = 8, 16 and 32.

For a group size of 8, costs are reduced ($\mathrm{R_c} > 1$) for test positivity rates of 5% or less. Cost reduction reaches a maximum value of $n\ /\ (d/q)$ as the test positivity rate near 0%, resulting to maximum reductions by a factor 2 to 4 for values of d/q between 2 and 4.