Electronic Supplementary Material (ESI) for Lab on a Chip.

Supporting Information

Supplemental Material S1. Custom-written algorithm for MddRPA assay

The python code used for MddRPA assay can be downloaded from Github under the following link: https://github.com/delte1821/MddRPA_analysis.py. The algorithm included following steps: 1) Detection of droplets, 2) Measurement of fluorescence intensities, 3) Plotting histogram, 4) Auto thresholding, and 5) Quantification *via* Poisson's statistics. The information of the images and calculated RNA concentrations were saved in a SQL database file.

Supplemental Material S2. Theoretical determination of the detection range of the assay

The detection range of the MddRPA assay can be calculated using the Poisson distribution, which describes the probability of observing a given number of target molecules in each partition. The formular for Poisson distribution is:

$$P(k) = \frac{\lambda^k \times e^{-\lambda}}{k!}$$

Where $P(k)$ is the probability of observing k target molecules in a partition, λ is the average number of target molecules per partition, k is the number of target molecules. The detection range is determined by the range of target concentrations for which the assay can accurately quantify the target molecules. The range is typically defined by the lower limit of detection (LoD) and the upper limit of quantification (ULOQ).

The LoD is the lowest target concentration that can be reliably detected by the assay. It is influenced by the partition volume and the number of partitions. For our MddRPA assay, the volume of each droplet is approximately 3 nL and we generate ~20,000 partitions. The LoD can be estimated using the Poisson distribution as the concentration at which 1% of partitions contain at least one target molecule:

$$P(k \geq 1) = 1 - P(k = 0) = 1 - e^{-\lambda} = 0.01$$

The ULOQ is the highest target concentration that can be accurately quantified by the assay. It is determined by the maximum number of partitions that can be reliably distinguished as positive or negative. To estimate the ULOQ, we can use the Poisson distribution to calculate the concentration at which 95% of partitions contain no more than one target molecule:

$$P(k \leq 1) = P(k = 0) + P(k = 1) = e^{-\lambda} + \lambda \times e^{-\lambda} = 0.99$$

By solving the equations above for λ, the following limits of detection can be calculated:

$$LoD = c_{low} = \frac{\lambda_{low}}{V_{droplet}} \approx \frac{0.0101\ copies}{3\ nL} = 3.36\ copies/\mu L$$

$$ULOQ = c_{high} = \frac{\lambda_{high}}{V_{droplet}} \approx \frac{0.7630\ copies}{3\ nL} = 2{,}543.33\ copies/\mu L$$

Table S1. Sequence of the forward and reverse primers as well as the probe used for MddRPA assay.

Target		Sequence 5'-3'	Amplicon size
229E	Forward primer	TGTTTGATAGTCACATTGTTTCCAAAGAGT	178 bp
	Reverse primer	TTGAATTCTAGTGCACTAGGGTTAAGAAGA	
	Probe	GGCAACACTGTGGTCTTGACTTTCAC TACT[FAM-DT]A[THF]A[BHQ1-DT] GTGACTGTGCCCAAA[C3-SPACER]	
OC43	Forward primer	TGCTGGCATTGGTTTACATTTAAAAGTTAATT	152 bp
	Reverse primer	AATCTTTTACACGCTCATAGCATTTCATCT	
	Probe	TCAGCGTGTTGATGAGAACGGTGATAAATT [TAMRA-DT]A[THF]A[BHQ-DT]TCAGTTCTTTGTTGT	
NL63	Forward primer	CGTTATTCTTTGATAGTGAGGTTAGCACTG	120 bp
	Reverse primer	TCTGCTCAATGAACTTAGGAAGGTTCTTAT	
	Probe	GTGGGTGATAATGTTCAGATTACCTATACC[CY5-DT]A[THF]A[BHQ2-DT]AATGCTTGTAGCTAA	
SARS-CoV-2 E gene	Forward primer	TGCTTTCGTGGTATTCTTGCT	45 bp
	Reverse primer	AGTACGCACACAATCGAAGC	
	Probe	[FAM] AGTTACACTAGCCATCCTTACTGC [BHQ1]	

Table S2. Comparison table of droplet digital isothermal amplification methods.

Method	Target species	Amplification method	Sensitivity (copies/μL)	Reaction time (min)	Multiplexing capability
ddLAMP [1]	DNA	LAMP	4.39	60	No
TriD-LAMP [2]	DNA	LAMP	19.8	30	Yes (2)
ddRPA [3]	DNA	RPA	10	30	No
MddRPA (Our method)	RNA	RPA	5	20	Yes (3)

References

1. S.-A. Hsieh, D. Shamsaei, D. R. Eitzmann and J. L. Anderson, *Anal. Chem.*, 2022, **94**, 11949-11956.
2. C. Wu, L. Liu, Z. Ye, J. Gong, P. Hao, J. Ping and Y. Ying, *Anal. Chim. Acta*, 2022, **1233**, 340513.
3. J. Q. Cui, F. X. Liu, H. Park, K. W. Chan, T. Leung, B. Z. Tang and S. Yao, *Biosens. Bioelectron.*, 2022, **202**, 114019.

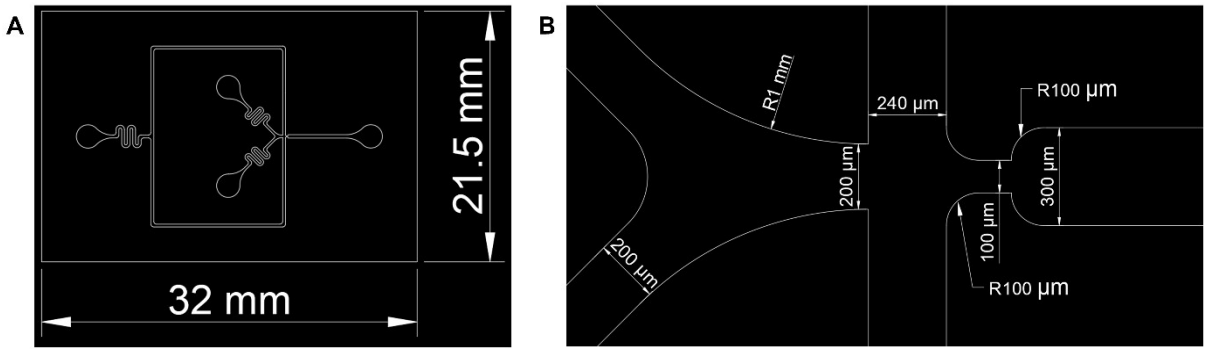

Fig. S1. Design of the droplet-based microfluidic chip. CAD drawing of the droplet-based microfluidic chip (A). CAD drawing of the cross junction for the droplet generation (B).

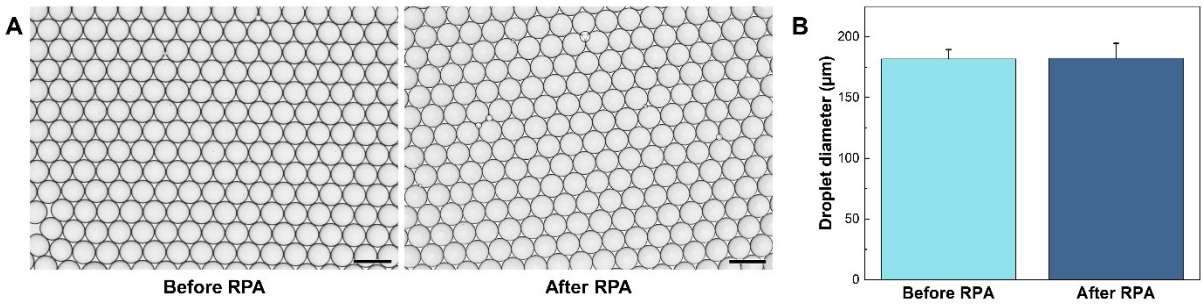

Fig. S2. Evaporation test of the ddRPA assay. Microscopy images of the droplets before RPA and after RPA (A). Graph of droplet diameter before RPA and after RPA (B). The scale bars are 300 µm.

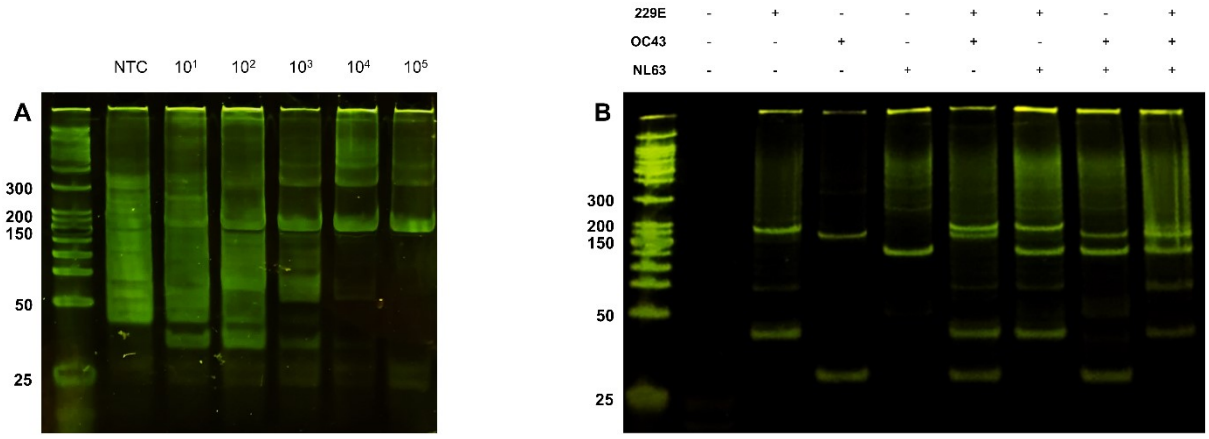

Fig. S3. Gel electrophoresis analysis of the primer set. Dynamic range test of the designed primers (A). Specificity test of the target RNAs (B).

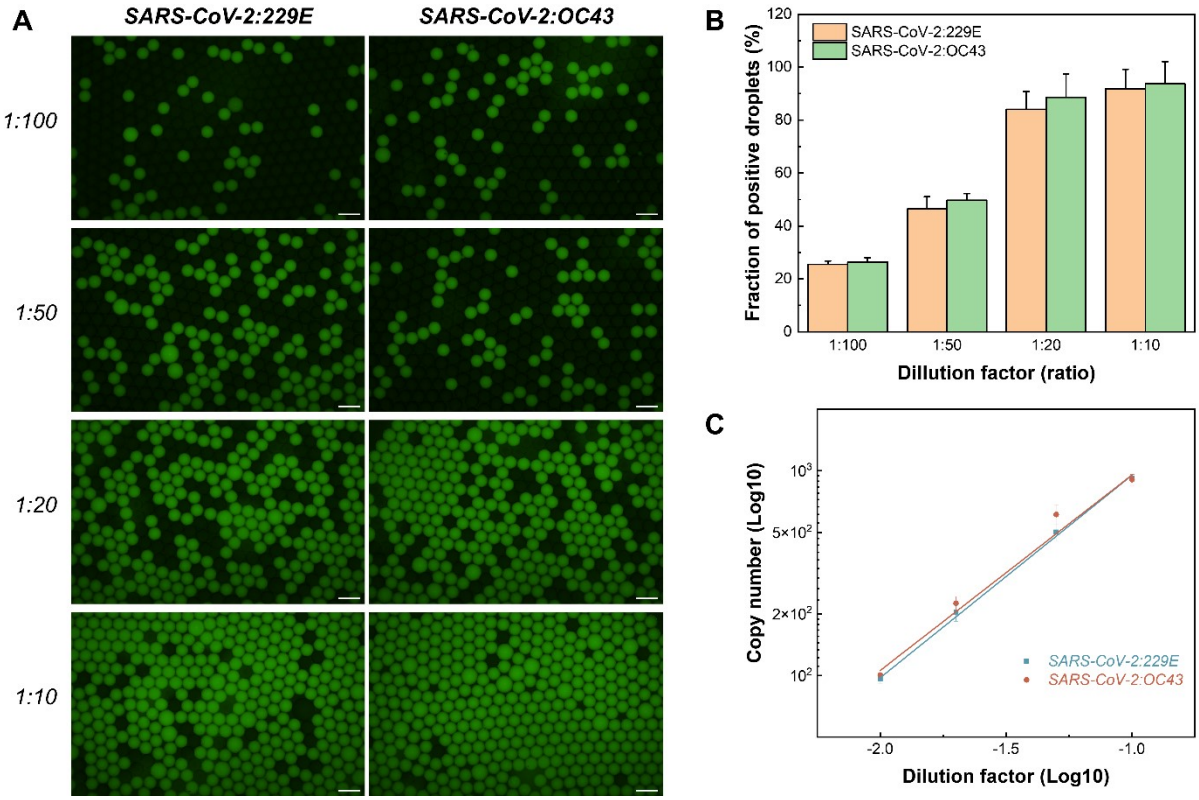

Fig. S4. Quantification analysis of SARS-CoV-2 using mock samples. Fluorescence images representing MddRPA analysis with a serial dilution of the target RNA ranging from 1:100 to 1:10 (A). The mock samples contained two types of human coronavirus (e.g., 229E, OC43). Bar graph representing the fraction of positive droplets (B). Linear regression curve showing MddRPA results using mock samples (C). The scale bars are 300 μm.