

SUPPLEMENTARY INFORMATION

Flow virometry for water-quality assessment: Protocol optimization for a model virus and automation of data analysis

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Supplementary Methods

Preparation of purified, high-titer phage stocks

Preparation of purified, high-titer phage stocks was based on Bonilla and Barr (2016)'s "Phage on tap" protocol.¹ The bacterial host was incubated overnight in ~25 mL of nutrient broth (ATCC Medium 129) at 37°C for *E. coli*, 25°C for *P. syringae*, and shaking at 80 RPM for both. A ~20-mL aliquot of the overnight culture was spiked into 250 mL of nutrient broth and incubated at the same conditions for 1 hour, after which 200 µL of phage stock (~10⁸ phage/mL) was added. The mixture was left for 5 hours at the same incubation conditions, then stored overnight at 4°C. The next day, the mixture was aliquoted into sterile 50-mL Falcon tubes. Tubes were centrifuged at 3,200 rcf for 20 minutes, after which the supernatant was removed and passed through an 0.2 µm syringe filter. For ϕ6, the supernatants were immediately combined. For T4, an additional bacterial lysis step was performed: 0.1 volume of chloroform was added to each tube, tubes were incubated for 10 minutes at room temperature, tubes were centrifuged at 3,200 rcf for 5 minutes, and the resulting supernatants were then combined. The combined supernatants were concentrated by transferring 15 mL of solution at a time to the upper reservoir of a 100 kDa Amicon® Ultra-15 Centrifugal Filter Unit and centrifuging at 3,200 rcf for 5 minutes. A wash step was performed by adding an additional 15 mL of Tris-EDTA (TE) buffer to the upper reservoir. The washed retentate was then reserved.

Phage stock quantification via plate-based culturing

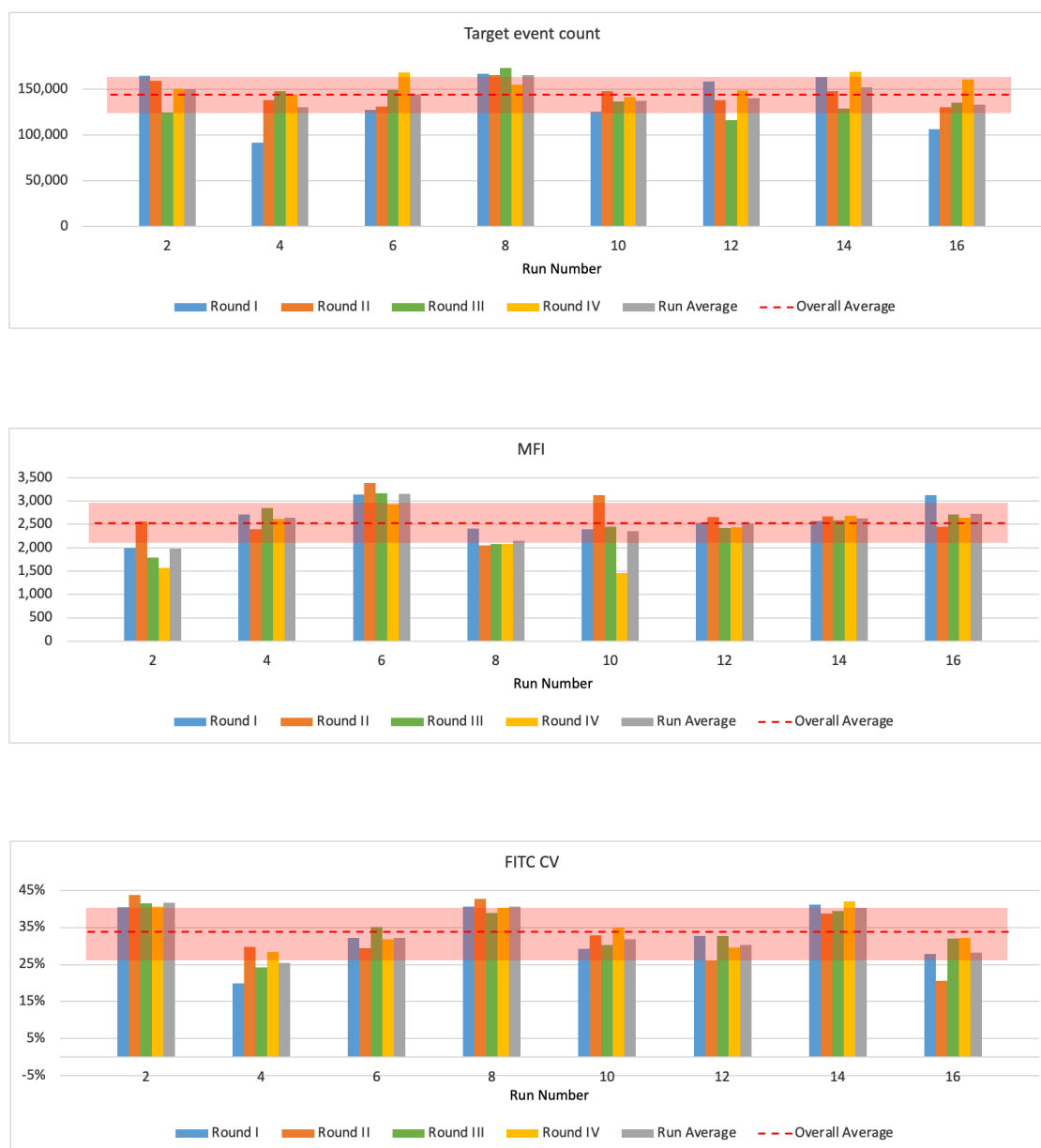
100 mm x 15 mm plates were prepared with 15 mL of nutrient agar (ATCC Medium 129) each, and glass tubes were prepared with 7 mL of soft agar "stabs"; plates and stabs were stored at 4°C. 10x dilutions of the phage stocks were prepared in TE buffer, and overnight host cultures were prepared as described above. During plating, plates were allowed to come to room temperature, and stabs were melted in a 100°C water bath for at least 2 hours. Stabs were immersed in room-temperature water until cool to touch, after which 200 µL of host and 100 µL of stock dilution were immediately added. Stabs were vortexed gently, poured onto plates, allowed to set at room temperature, and incubated at temperature overnight without shaking. Three replicates of each dilution were plated. Stock titers were determined as plaque-forming units (PFU)/mL by counting visually distinct plaques, averaging replicate results, and performing appropriate back-calculations.

Phage stock quantification via qPCR/RT-qPCR

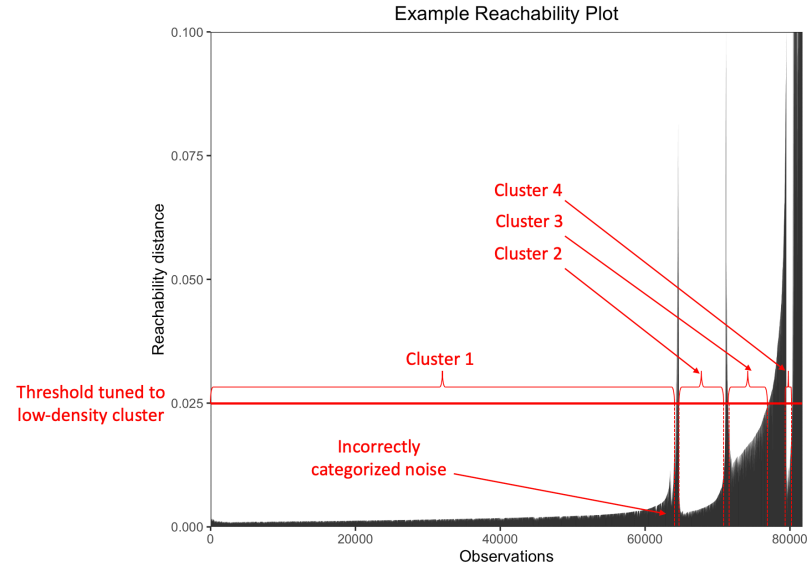
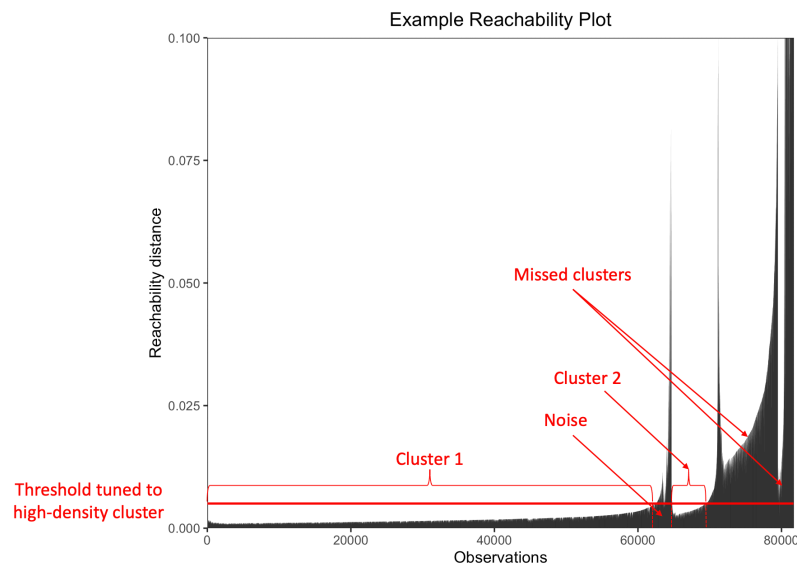
We diluted the initial stocks to an appropriate concentration in TE buffer, then extracted the diluted stock using the PureLink™ Viral RNA/DNA Mini Kit (Invitrogen™), per the manufacturer's instructions but without the use of carrier RNA. Extracts were then analyzed using qPCR for the DNA phage T4 and one-step RT-qPCR for the RNA phage ϕ6. qPCR/RT-qPCR amplifications were performed on StepOnePlus qPCR thermocyclers (Applied Biosystems). For the T4 qPCR assay, each 12-µL reaction contained the following components: 0.5 µM forward and reverse primers, 0.2 µM probe, 0.48 µL RNase-free water, 6 µL TaqMan Universal PCR Master Mix (Applied Biosystems), and 5 µL sample extract. For the ϕ6 RT-qPCR assay, each 25-µL reaction contained the following components: 1 µM forward and reverse primers, 0.15 µM probe, 0.625 µL bovine serum albumin (BSA; 25 mg/mL), 12.5 µL RNase-free water, 2.5 µL 10x Multiplex Enzyme Mix from the Path-ID™ Multiplex One-Step Kit (Applied Biosystems), 12.5 µL of 2x Multiplex RT-PCR Buffer from the Path-ID™ kit, and 5 µL sample extract. Mastermix preparation and plating were carried out in a separate location from sample loading to avoid

contamination. Each stock was assayed in triplicate, and titers were determined as gene copies (gc)/mL using six-point standard curves constructed from serially diluted plasmids. Table S8 summarizes primers, probes, and cycling conditions for the two qPCR assays performed as part of this work and Table S9 details the master standard curves used for each target.

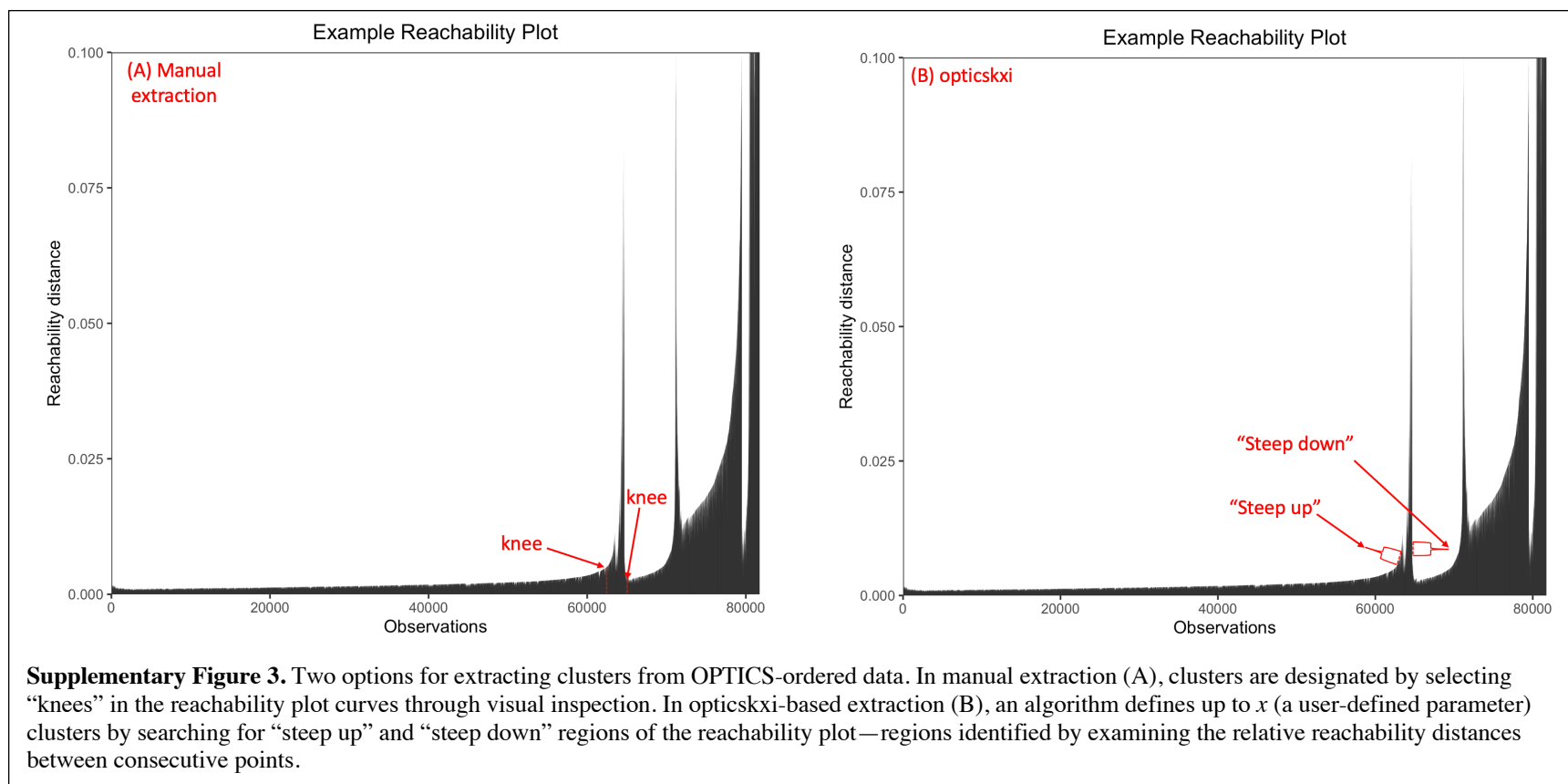
Supplementary Figures

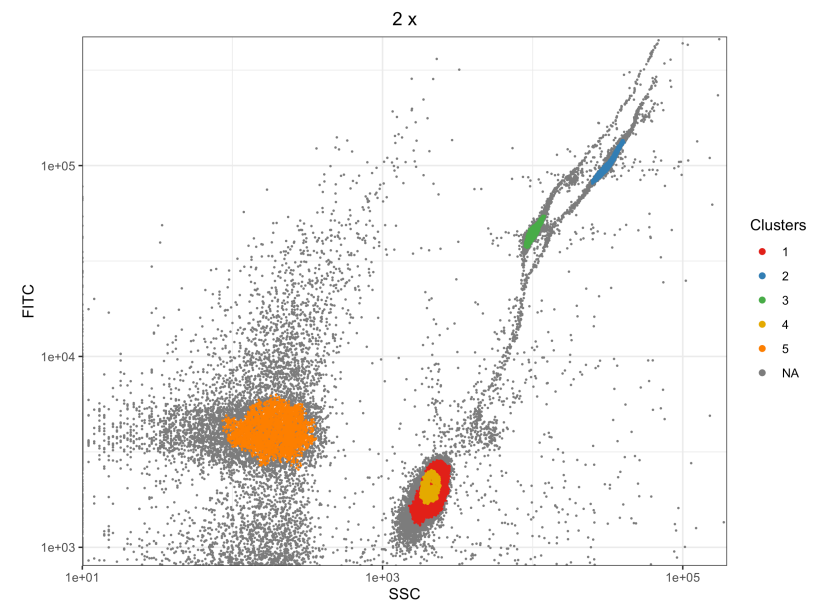
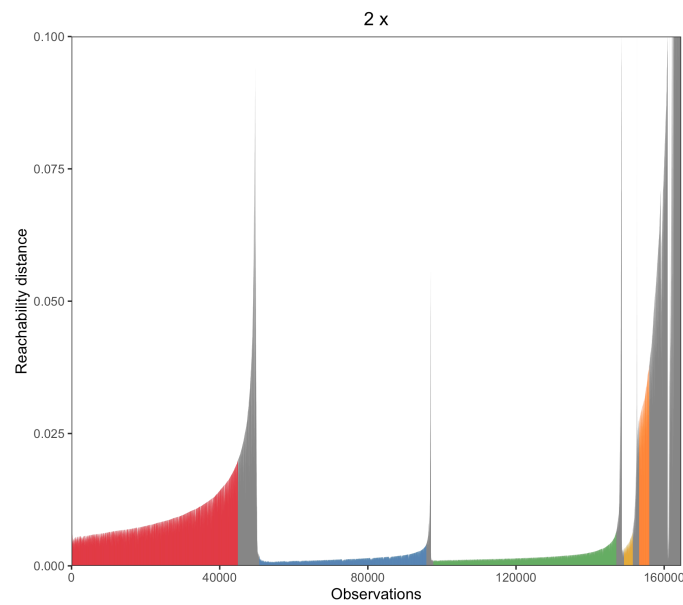
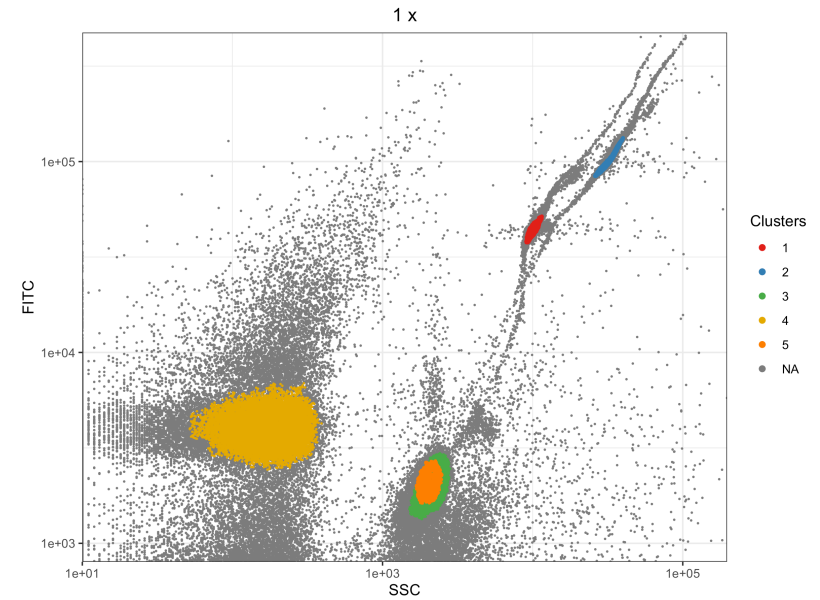
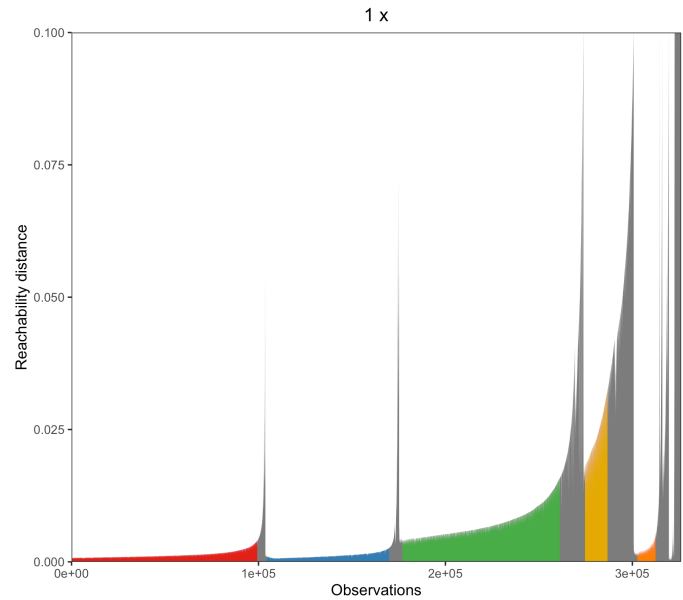


Supplementary Figure 1. Graphical comparison of optimization results for glutaraldehyde-treated runs in the T4 optimization. Shaded red region denotes one standard deviation of average.

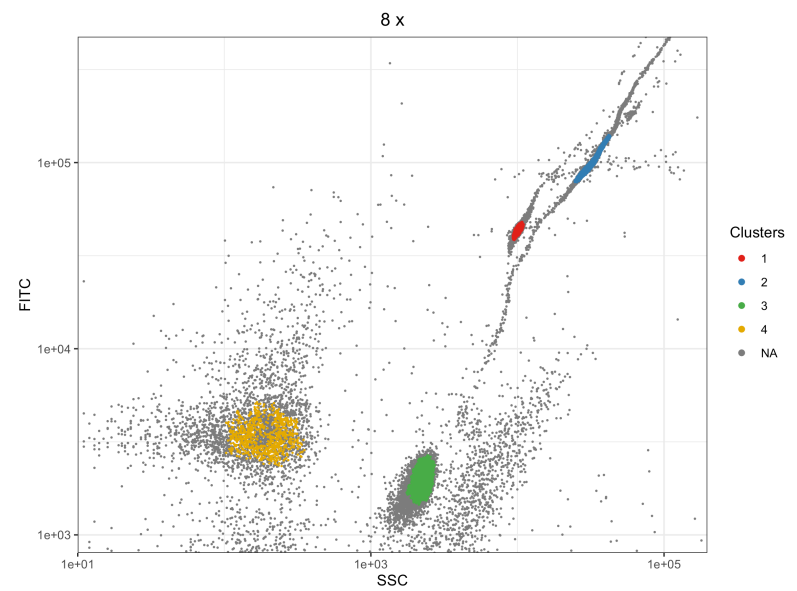
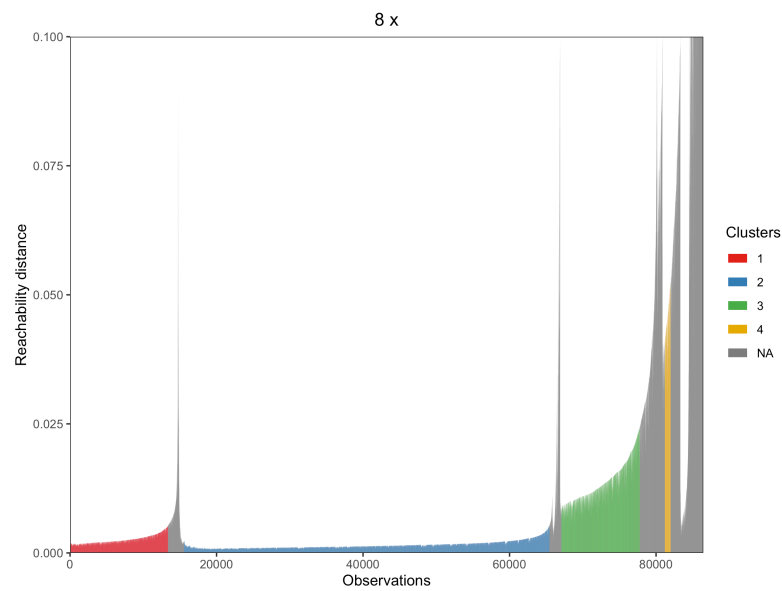
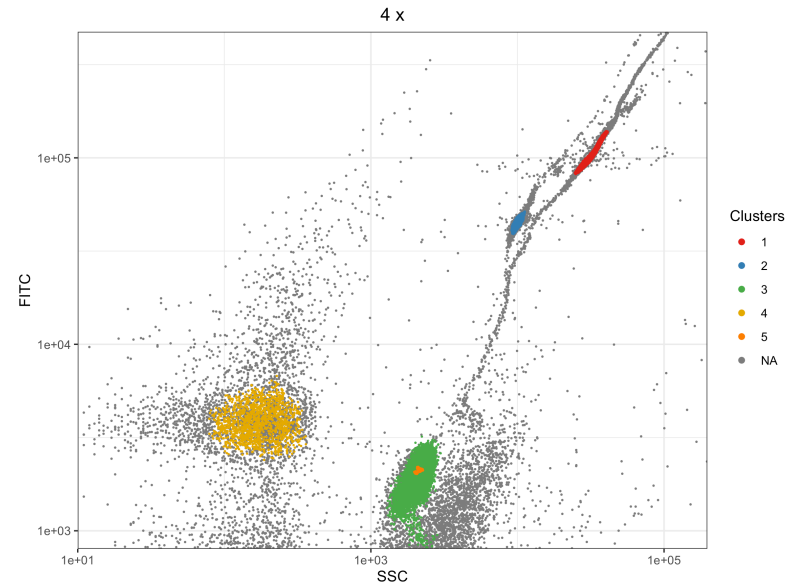
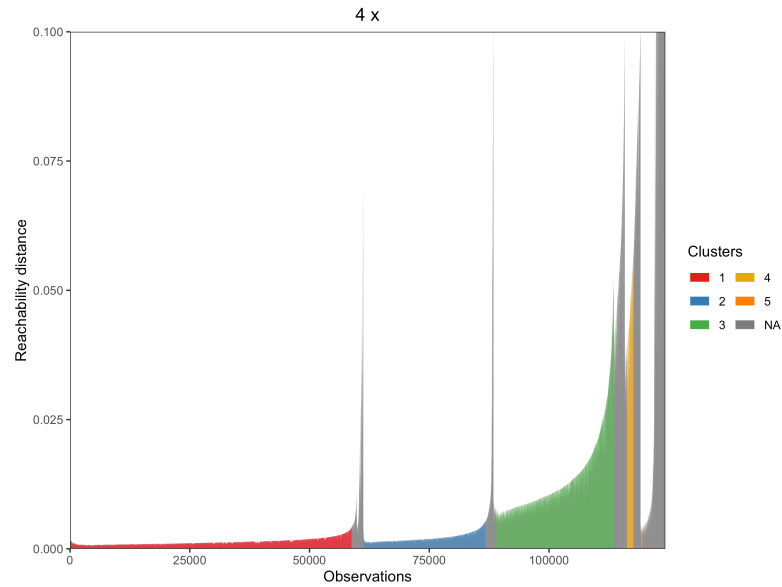


Supplementary Figure 2. Illustration of pitfalls of setting a single global threshold to extract clusters from OPTICS-ordered data. For samples containing clusters of varying densities, setting a single global threshold results in either missed clusters and/or noise points incorrectly categorized as clusters.

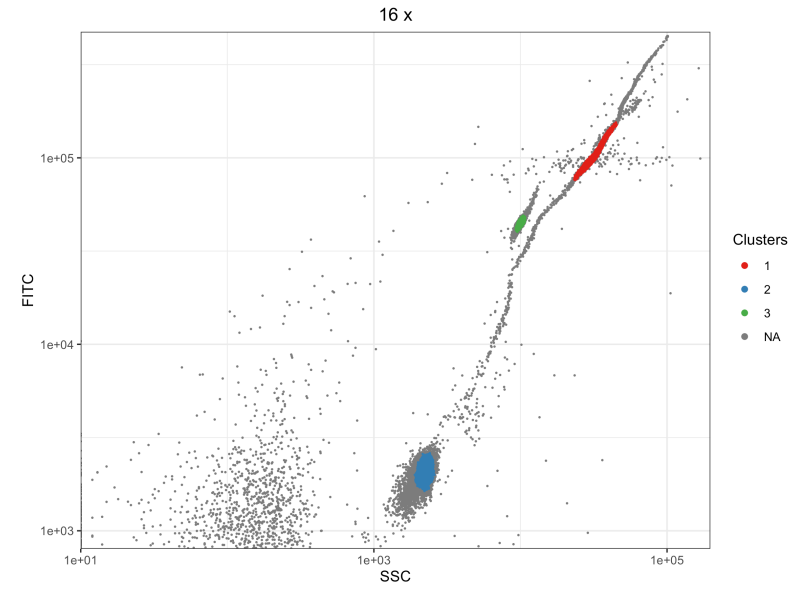
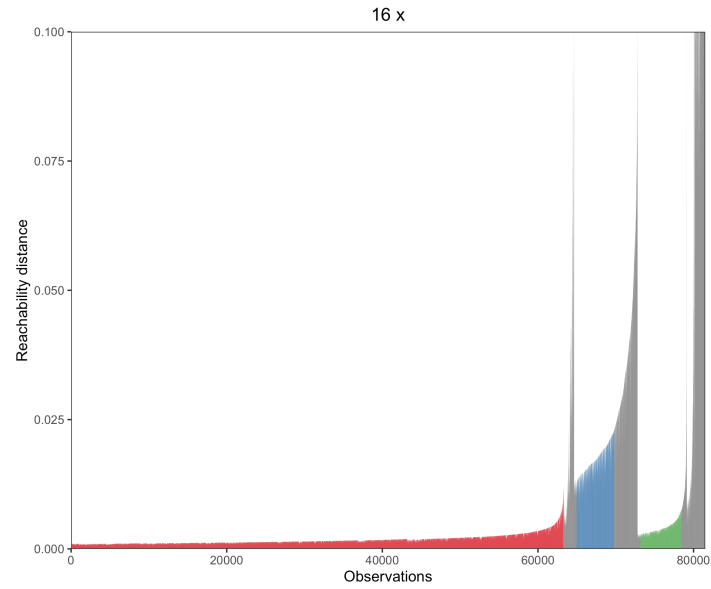




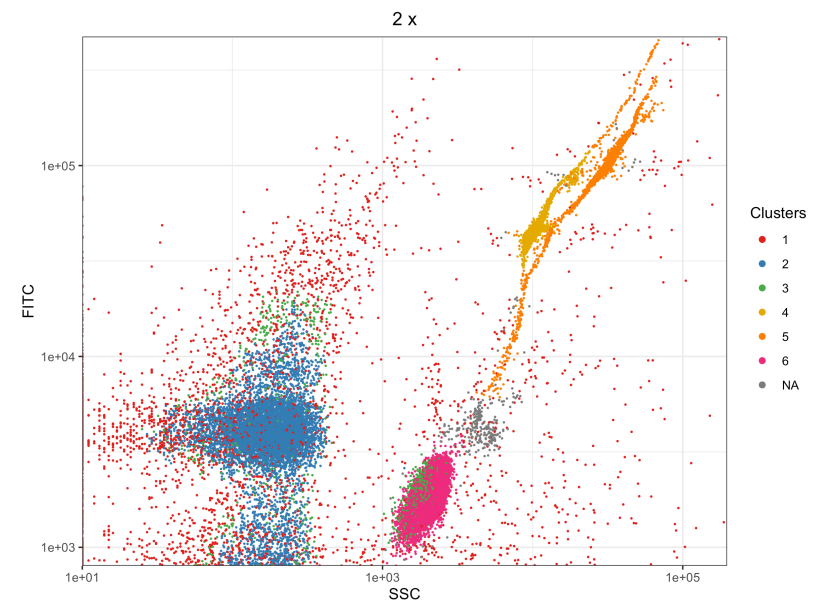
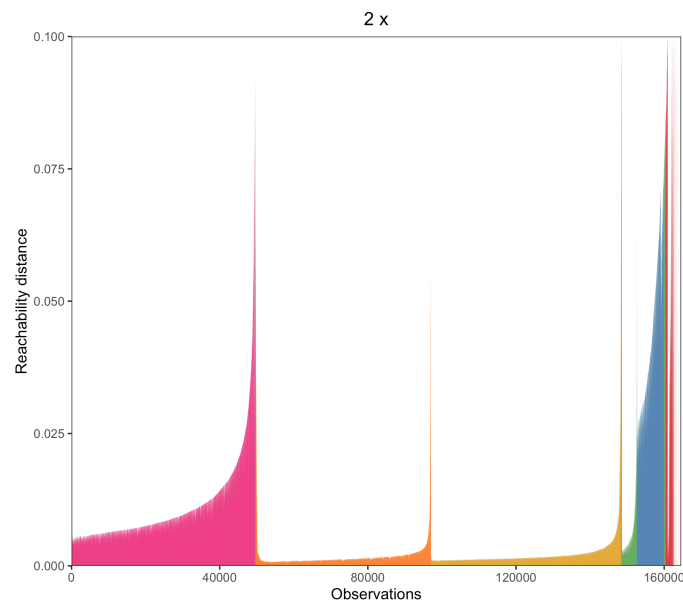
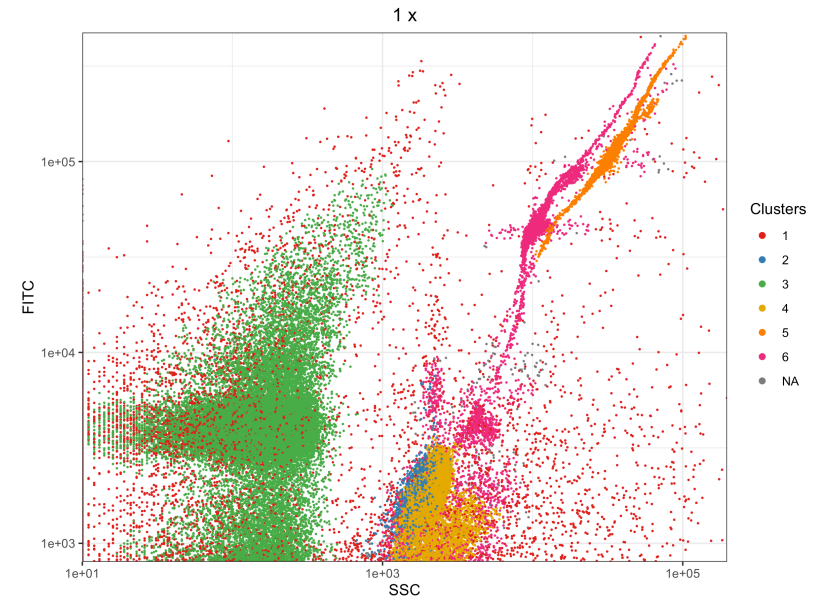
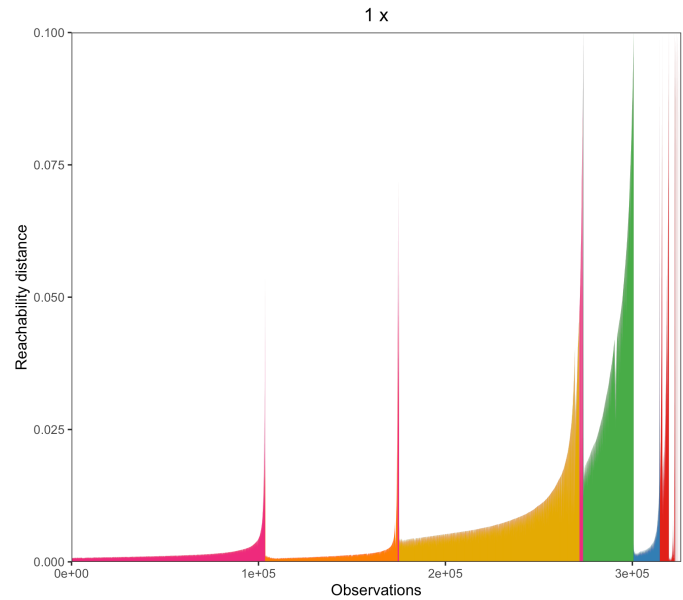
Supplementary Figure 4. Additional representative results of manually extracting clusters from OPTICS-ordered data collected from each dilution tested in mixed-target experiment (companion to Figure 4).



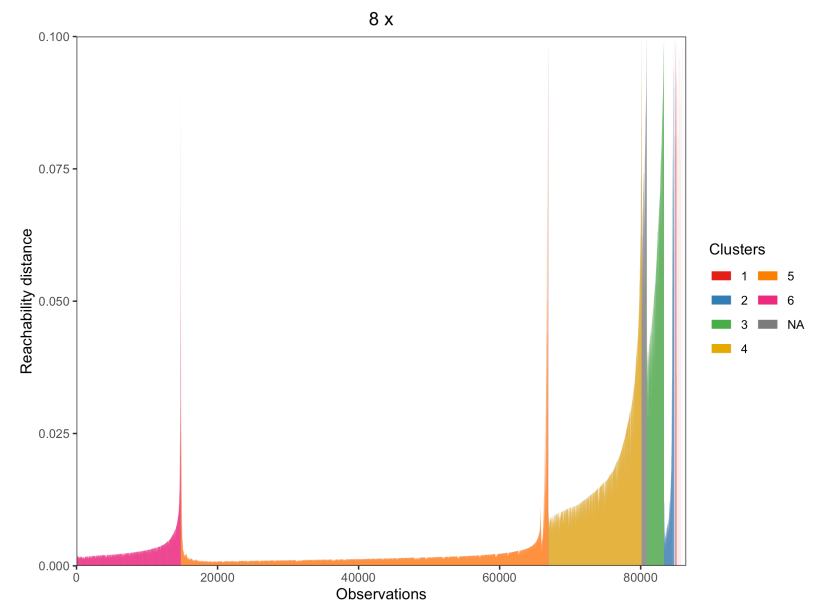
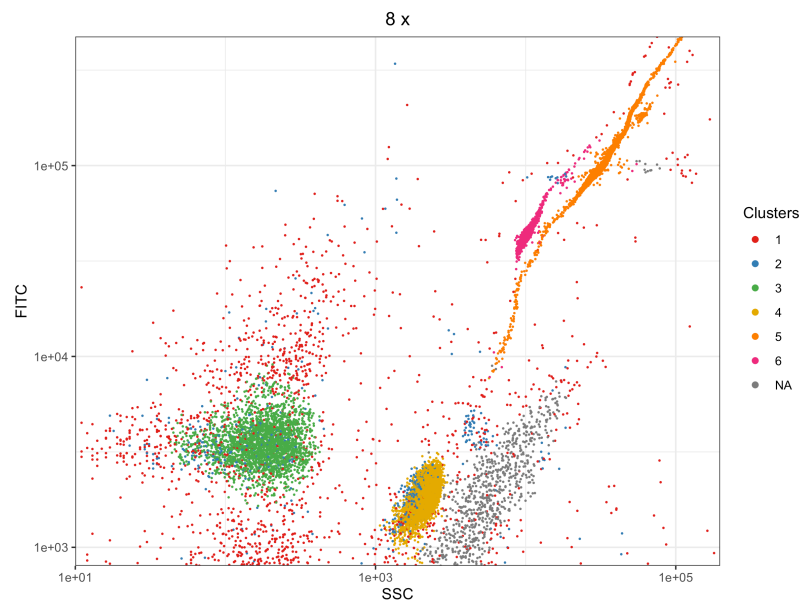
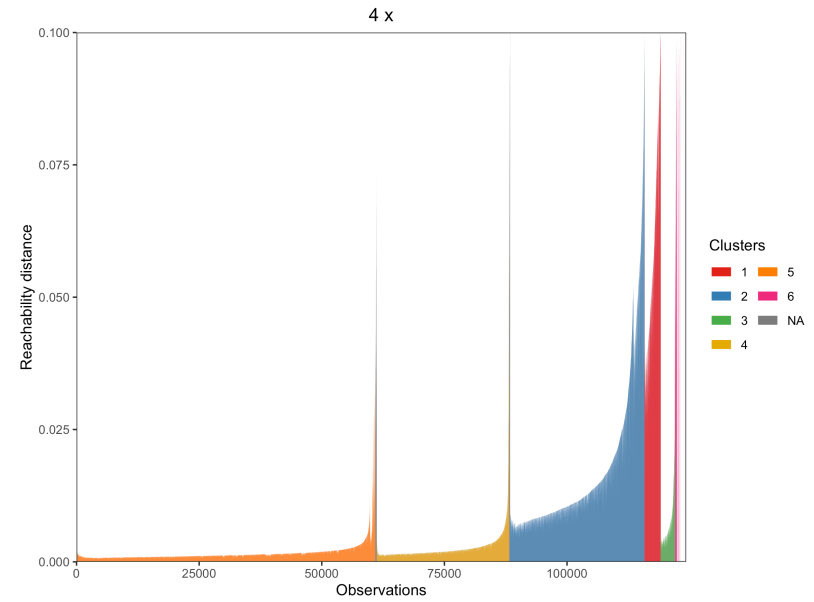
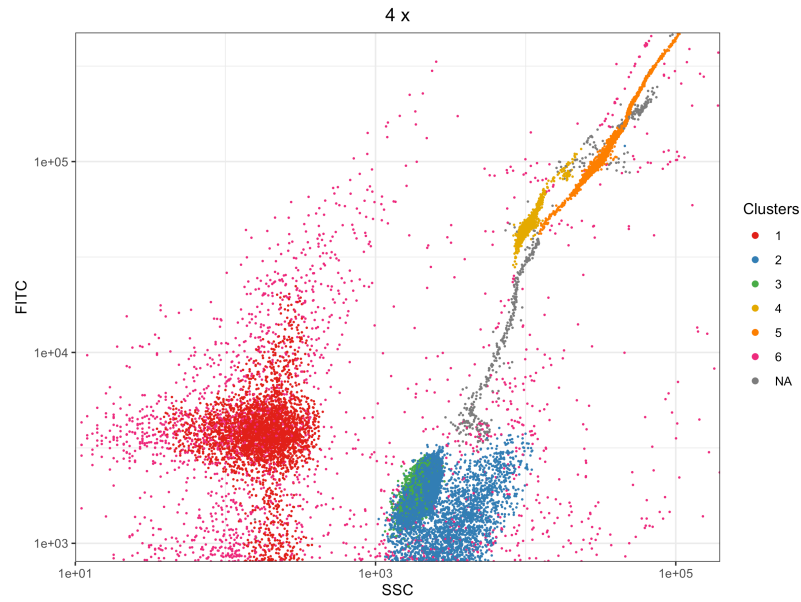
Supplementary Figure 4 (cont).



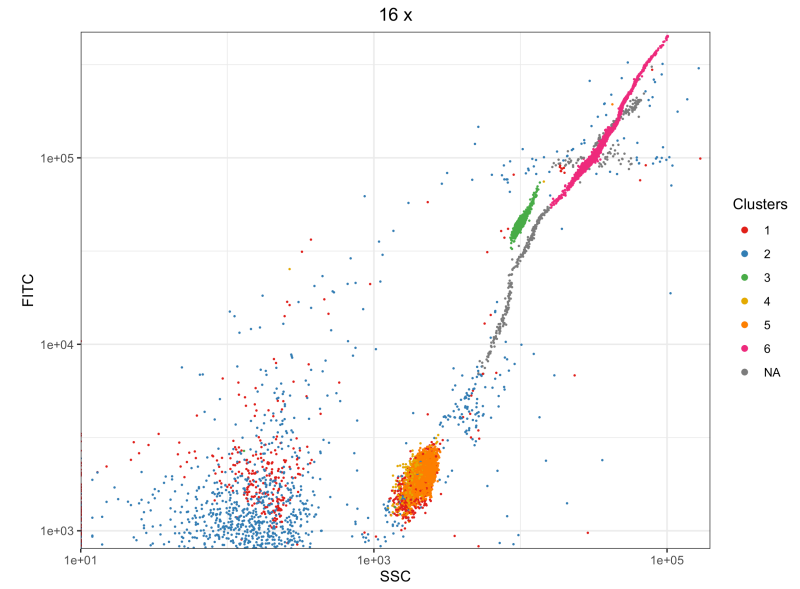
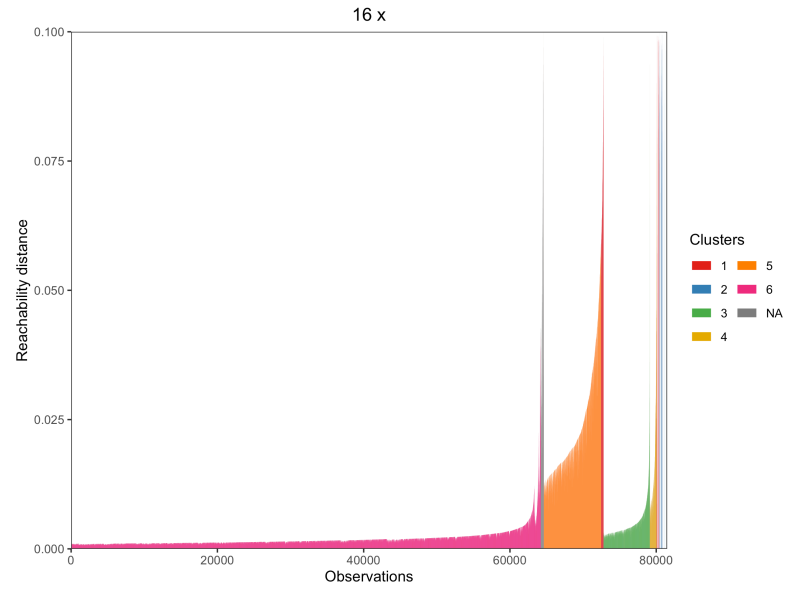
Supplementary Figure 4 (cont).



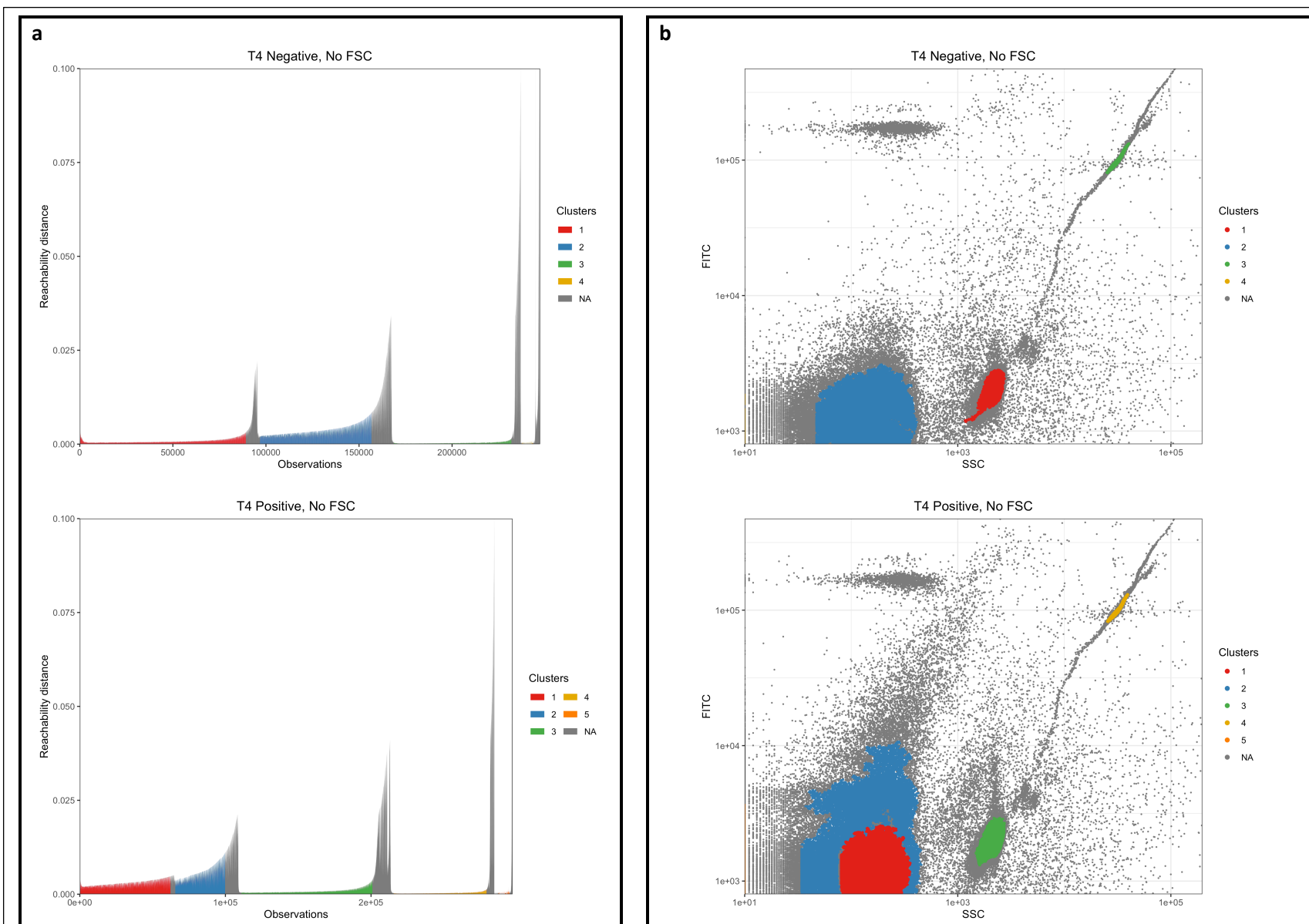
Supplementary Figure 5. Additional representative results of algorithmically extracting clusters from OPTICS-ordered data collected from each dilution tested in mixed-target experiment (companion to Figure 5).



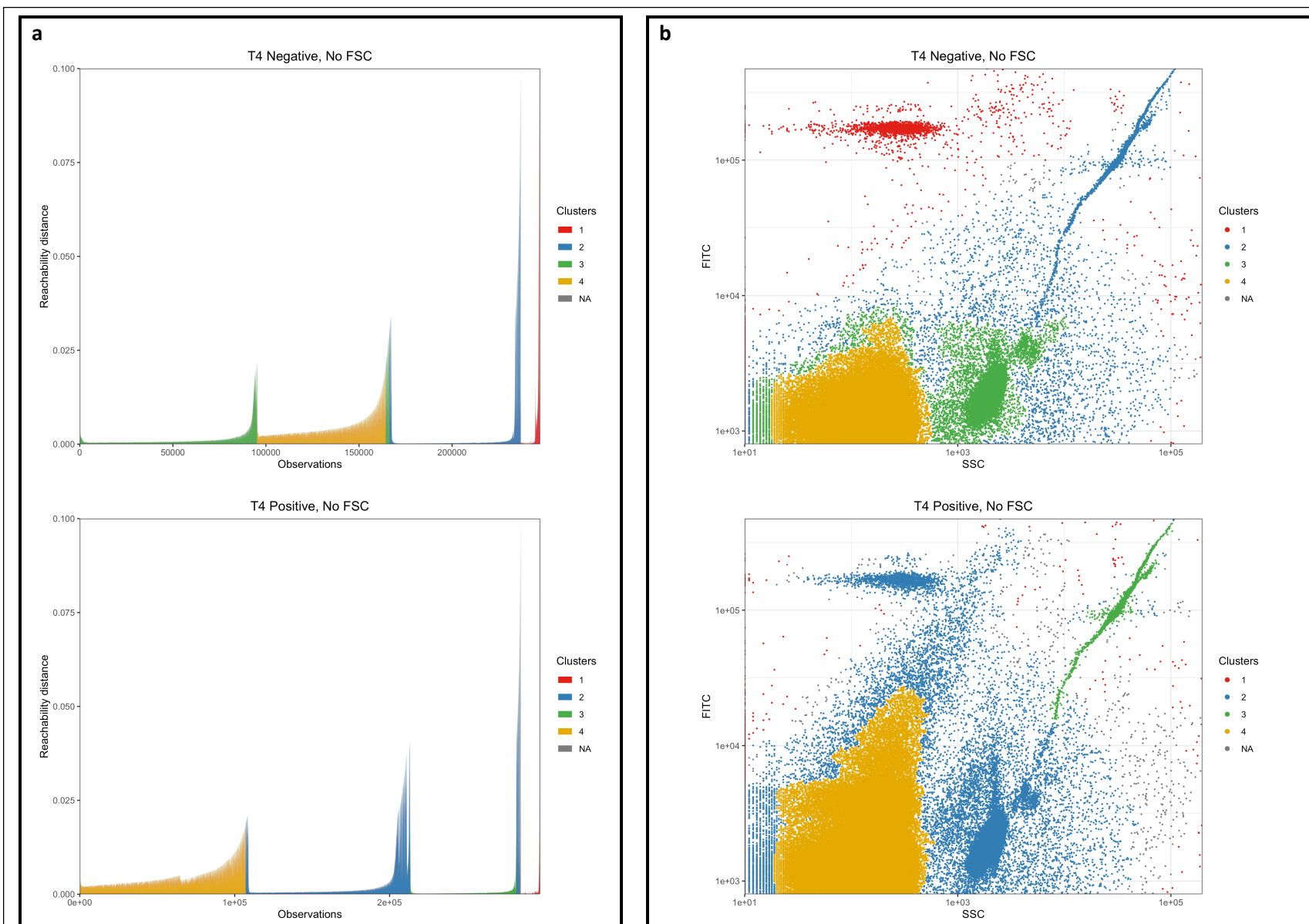
Supplementary Figure 5 (cont).



Supplementary Figure 5 (cont).



Supplementary Figure 6. Representative results of manually extracting clusters from OPTICS-ordered data collected from environmental-spike experiments, with the FSC dimension included in the OPTICS ordering. Panel a plots show the OPTICS ordering of the data from which the clusters were manually extracted. Colors highlight each manually extracted cluster. Panel b plots visualize the data as SSC vs. FITC plots, with points colored based on the cluster extraction. Cluster colors are consistent between the two plots for each of the T4-spiked and unspiked experiments, but not necessarily across experiments. Some clusters are not visually apparent on the scatterplots because they include low-SSC points collapsed onto the y-axis.



Supplementary Figure 7. Representative results of using the opticksxi algorithm to extract clusters from the same OPTICS-ordered data shown in Figure S6. Panel a plots show the OPTICS ordering of the data from which the clusters were algorithmically extracted. Colors highlight each algorithmically extracted cluster. Panel b plots visualize the data as SSC vs. FITC plots, with points colored based on the cluster extraction. Cluster colors are consistent between the two plots for each of the T4-spiked and unspiked experiments, but not necessarily across experiments. Some clusters are not visually apparent on the scatterplots because they include low-SSC points collapsed onto the y-axis.

Supplementary Tables

Supplementary Table 1. Main and two-way effects estimation from optimization experiments.

Factor	All runs			Glutaraldehyde-treated runs		
	Effect on total event count (p-value)	Effect on MFI (p-value)	Effect on FITC CV (p-value)	Effect on target event count (p-value)	Effect on MFI (p-value)	Effect on FITC CV (p-value)
Glutaraldehyde	65,402 (0.000)***	360 (0.000)***	-9.0 (0.000)***	—	—	—
Staining temperature	868 (0.696)	-47 (0.058) [†]	0.0 (0.988)	-1,860 (0.570)	-38 (0.635)	-2.7 (0.000)***
Stain type	10,330 (0.000)***	14 (0.568)	5.9 (0.000)***	4,576 (0.169)	-116 (0.156)	1.5 (0.011)*
Staining time	-3,040 (0.175)	13 (0.597)	-1.9 (0.005)**	-3,286 (0.319)	-11 (0.890)	-1.2 (0.044)*
Stain concentration	-3,052 (0.173)	-54 (0.029)*	-0.5 (0.441)	2,325 (0.478)	-64 (0.424)	1.8 (0.004)**
Diluent	1,999 (0.370)	64 (0.010)*	-1.2 (0.059)**	-7,807 (0.023)*	122 (0.135)	-4.4 (0.000)***
Glutaraldehyde/ Staining temperature	-5,290 (0.020)*	62 (0.013)*	-2.3 (0.001)***	—	—	—
Glutaraldehyde/ Stain type	-1,924 (0.388)	28 (0.258)*	-2.6 (0.000)***	—	—	—
Glutaraldehyde/ Stain time	-320 (0.885)	51 (0.039)*	0.3 (0.632)	—	—	—
Glutaraldehyde/ Stain concentration	624 (0.778)	-84 (0.001)***	0.5 (0.447)	—	—	—
Glutaraldehyde/ Diluent	-3,895 (0.084)	113 (0.000)***	-0.8 (0.205)	—	—	—
Staining temperature/ Staining time	-428 (0.847)	2 (0.938)	-0.3 (0.639)	-2,309 (0.481)	-56 (0.485)	-0.8 (0.173)
Staining temperature/ Diluent	-181 (0.935)	3 (0.886)	-0.4 (0.555)	-2,734 (0.405)	85 (0.293)	0.1 (0.888)

Significance levels: [†] = 0.05–0.1; * = 0.01–0.05; ** = 0.001–0.01; *** = 0–0.001

Supplementary Table 2. Expected event counts for targets in mixed-target and environmental-spike experiments, per effective volume (10 µL) analyzed via FCM.

Target	Approximate expected event counts						
	Mixed target					Environmental spike	
	1x	2x	4x	8x	16x	Positive	Negative
ϕ6*	100,000	50,000	25,000	12,500	6,250	—	—
T4*	20,000	10,000	5,000	2,500	2,500	20,000	—
0.2 µm beads**	20,000	10,000	5,000	2,500	1,250	20,000	20,000
0.5 µm beads**	40,000	20,000	10,000	5,000	625	—	—
0.8 µm beads**	80,000	80,000	80,000	80,000	80,000	80,000	80,000

* Based on qPCR titer; ** Based on manufacturer-provided bead concentration

Supplementary Table 3. Comparison of results from different clustering approaches applied to mixed-target FCM data.

Bucket	Clustering approach	Average event count by dilution (standard deviation)				
		1x	2x	4x	8x	16x
Viruses (T4 + $\phi 6$ + VLPs)	MG	29,209 (2,124)	9,677 (467)	4,466 (153)	3,313 (160)	952 (82)
	O:ME	12,291 (1,866)	1,973 (1,727)	591 (673)	729 (440)	0 (–)
	O:kxi	26,432 (1,738)	8,409 (516)	3,187 (344)	2,573 (237)	51 (162)
0.2 μm beads	MG	105,224 (4,191)	55,563 (2,589)	29,325 (725)	15,393 (641)	9,115 (382)
	O:ME	89,204 (5,604)	46,821 (2,777)	25,180 (2,172)	12,459 (3,525)	4,990 (502)
	O:kxi	96,874 (6,284)	50,992 (2,148)	27,235 (440)	14,108 (660)	8,005 (732)
0.5 μm beads	MG	99,845 (4,167)	52,933 (2,429)	27,925 (732)	15,225 (537)	6,363 (283)
	O:ME	94,462 (3,901)	50,640 (2,667)	26,022 (940)	13,851 (557)	4,953 (439)
	O:kxi	100,249 (4,526)	53,040 (2,467)	28,022 (652)	15,199 (542)	6,509 (702)
0.8 μm beads	MG	67,433 (2,755)	48,199 (2,190)	61,898 (1,488)	54,264 (2,141)	62,755 (2,853)
	O:ME	63,818 (2,513)	46,438 (2,128)	60,642 (1,968)	53,339 (2,195)	62,224 (2,897)
	O:kxi	79,279 (3,058)	48,993 (2,395)	63,197 (1,761)	55,281 (2,158)	63,832 (2,839)

MG = manual gating; O:ME = OPTICS: manual extraction; O:kxi = OPTICS: kxi-based extraction

Supplementary Table 4. Results from application of manual gating to environmental-spike FCM data.

Gate	Average event count by sample (<i>standard deviation</i>)	
	<i>Positive</i>	<i>Negative</i>
T4	31,203 (1,871)	9,017 (1,104)
0.2 µm beads	94,700 (3,308)	95,073 (1,925)
0.8 µm beads	66,742 (2,405)	66,732 (1,619)

Supplementary Table 5. Approximate positive phage stock titers determined by different methods.

	T4	φ6
Plate-based culturing (PFU/mL)	10 ⁷ –10 ⁸	10 ⁹ –10 ¹⁰
qPCR/RT-qPCR (gc/mL)	10 ⁸ –10 ⁹	10 ¹⁰ –10 ¹¹

Supplementary Table 6. Experiments included in fractional factorial design for staining optimization. Factor levels are detailed in Table 1.

Aliases	A	B	C	D	E	F
Factors	Glutaraldehyde	Staining temperature	Stain type	Staining time	Stain concentration	Diluent
Run number	Factor levels					
1	1	1	1	1	1	1
2	2	1	1	1	2	1
3	1	2	1	1	2	2
4	2	2	1	1	1	2
5	1	1	2	1	2	2
6	2	1	2	1	1	2
7	1	2	2	1	1	1
8	2	2	2	1	2	1
9	1	1	1	2	1	2
10	2	1	1	2	2	2
11	1	2	1	2	2	1
12	2	2	1	2	1	1
13	1	1	2	2	2	1
14	2	1	2	2	1	1
15	1	2	2	2	1	2
16	2	2	2	2	2	2

Supplementary Table 7. Confounding structures and different main and two-way effects present for the T4 optimization fractional factorial experimental design.

Estimation factor	Confounding structure	Main and two-way effects present
I_1	A + ABCE + ABCDF + DEF	Glutaraldehyde
I_2	B + ACE + CDF + ABDEF	Staining temperature
I_3	C + ABE + BDF + ACDEF	Stain type
I_4	D + ABCDE + BCF + AEF	Staining time
I_5	E + ABC + BCDEF + ADF	Stain concentration
I_6	F + ABCEF + BCD + ADE	Diluent
I_7	AB + CE + ACDF + BDEF	Glutaraldehyde/Staining temperature Stain type/Stain concentration
I_8	AC + BE + ABDF + CDEF	Glutaraldehyde/Stain type Staining temperature/Stain concentration
I_9	AD + BCDE + ABCF + EF	Glutaraldehyde/Stain time Stain concentration/Diluent
I_{10}	AE + BC + ABCDEF + DF	Glutaraldehyde/Stain concentration Staining temperature/Stain type Staining time/Diluent
I_{11}	AF + BCEF + ABCD + DE	Glutaraldehyde/Diluent Staining time/Stain concentration
I_{12}	BD + ACDE + CF + ABEF	Staining temperature/Staining time Stain type/Diluent
I_{13}	BF + ACEF + CD + ABDE	Staining temperature/Diluent Stain type/Staining time
I_{14}	ABD + CDE + ACF + BEF	N/A
I_{15}	ACD + BDE + ABF + CEF	N/A
I_{16}	I + ABCE + BCDF + ADEF	N/A

Supplementary Table 8. qPCR/RT-qPCR primers, probes, and cycling conditions used in Chapter 3.

Target	Primer/probe sequences (5'-3')		Cycling conditions	Source/Reference
T4; gp18 tail protein gene	Forward	AAGCGAAAGAAGTCGGTGAA	50°C for 2 min; 95°C for 10 min; 45 cycles of 95°C for 15s and 60°C for 1 min	Gerriets et al. (2008)
	Reverse	CGCTGTCATAGCAGCTTCAG		
	Probe	CY5-CCACGGAAATTTCTTCATCT TCCTCTGGCCGTGG-BHQ2		
φ6; P8 protein gene	Forward	TGGCGGCGGTCAAGAG	50°C for 30 min; 95°C for 10 min; 40 cycles of 95°C for 15s and 60°C for 60s	Based on Gendron et al. (2010)*
	Reverse	GGATGATTCTCCAGAAGCTGCT		
	Probe	MGB-GTCGCAGGTCTGACACT-BHQ1		

*Slight modifications made by removing several nucleotides from the primer and probe sequences and by changing the probe to MGB. These changes were made based on the advice of specialists at the UC Davis Real-time PCR Research and Diagnostics Core Facility based on needed applications for our lab.

Supplementary Table 9. Master standard curves for each target.

Target	Standard curve	R ²	Efficiency	Limit of detection (gene copies /reaction)*
T4	$y = -3.627x + 42.146$	0.99	88.67%	0.4
φ6	$y = -3.038x + 43.79$	0.99	113.39%	0.3

*Reported at a 99% confidence level.

Supplementary References

- [1] Bonilla, N. et al. Phage on tap—a quick and efficient protocol for the preparation of bacteriophage laboratory stocks. *PeerJ* **4** (2016).
- [2] Gerriets, J. E., Greiner, T. C. and Gebhart, C. L. Implementation of a T4 extraction control for molecular assays of cerebrospinal fluid and stool specimens. *J. Mol. Diagn.* **10**, 28–32 (2008).
- [3] Gendron, L., Verreault, D., Veillette, M., Moineau, S. & Duchaine, C. Evaluation of Filters for the Sampling and Quantification of RNA Phage Aerosols. *Aerosol Sci. Tech.* **44**, 893–901 (2010).

ITEM TO CHECK	IMPORTANCE	CHECKLIST
EXPERIMENTAL DESIGN		
Definition of experimental and control groups	E	Experimental groups: (i) buffer containing a mix of biological (T4 and phlo bacteriophages) and non-biological (fluorescent polystyrene beads) specimens; (ii) filtered wastewater effluent containing spiked T4 and Control groups: N/A.
Number within each group	E	964 wastewater samples were analyzed over the duration of the experimental period.
Assay carried out by core lab or investigator's lab?	D	Sample collection was performed by staff at the City of Davis and UC Davis. All sample processing and analysis was conducted in the Bichel Lab at UC Davis.
Acknowledgement of authors' contributions	D	
SAMPLE		
Description	E	Samples were 24-hour composites collected by insulated, ice-packed autosamplers deployed at the sampling zones.
Volumetric mass of sample processed	D	45 or 5 mL of wastewater, depending on the concentration method used.
Microdissection or macrodissection	E	N/A
Processing procedure	E	Samples were either processed immediately or were stored at 4°C for up to one week before processing. Prior to concentration, samples were pasteurized for 10 minutes at 60°C and then spiked with a known quantity of phlo bacteriophage. Concentration was carried out using either ultrafiltration (via 100 kDa Amicon devices) or magnetic-particle capture (via Nanotrap Magnetic Virus Particles on a Kingfisher Flex robot).
If frozen - how and how quickly?	E	Samples were not frozen.
If fixed - with what, how quickly?	E	Samples were not fixed.
Sample storage conditions and duration (especially for FFPE samples)	E	See above.
Procedure and/or instrumentation	E	Samples concentrated by ultrafiltration were extracted using either the Macherey-Nagel NucleoSpin RNA Stool kit or the Qiagen RNeasy PowerViral DNA/RNA kit. Samples concentrated using magnetic particles were concentrated using the MagMAX Microbiome Ultra Nucleic Acid Isolation Kit on a Kingfisher Flex robot.
Name of kit and details of any modifications	E	Kits listed above. The Macherey Nagel kit was used following the manufacturer's instructions for isolating total RNA with the following modifications: (1) bead beating was only carried out for 2 minutes, and (2) the DNA digestion step was omitted. The Qiagen kit was used following the manufacturer's instructions but omitting the bead beating step. Samples were eluted into 100 or 105 µL of RNase-free water.
Source of additional reagents used	D	No DNase or RNase treatment was performed.
Details of DNase or RNase treatment	E	No contamination assessment was performed.
Contamination assessment (DNA or RNA)	E	
Nucleic acid quantification	E	RNA concentrations in two ultrafiltration-processed samples with two biological replicates each (n = 4) were measured using a Nanodrop 2000 spectrophotometer (Thermo Scientific) and following the equipment's RNA Nucleic Acid quantification method. Yields and A260/A280 ratios in the 1.8-2.15 ng/µL and 1.77-2.08 ranges were obtained, respectively.
Instrument and method	E	See above.
Units (A260/A280)	D	See above.
Valid	D	See above.
RNA integrity method/instrument	E	No assessment of RNA integrity was performed.
RIN/RQ or Cq of 3 and 5' transcripts	E	N/A
Electrophoresis traces	D	
Inhibition testing (Cq dilutions, spike or other)	E	A subset of RNA extracts was used to assess template inhibition in qPCR by conducting serial dilutions. Four samples processed via particle-based capture and two processed via ultrafiltrations were tested using the PMMoV assay and undiluted, 4x, 16x and 64x, and undiluted, 3x, 9x and 27x dilutions, respectively. Two biological replicates were extracted and qPCR-assayed for both of the ultrafiltration samples. qPCR replicates and controls were run as described in the manuscript. No inhibition was observed in any of the samples tested.
Complete reaction conditions	E	Reverse transcription was performed as part of a one-step RT-qPCR process. Assay details are provided in SI Materials and Methods.
Amount of RNA and reaction volume	E	N/A
Priming oligonucleotide (if using CNP) and concentration	E	N/A
Reverse transcriptase and concentration	E	N/A
Temperature and time	E	N/A
Manufacturer of reagents and catalogue numbers	D	
Cqn with and without RT	D	
Storage conditions of cDNA	D	
If multiplex, efficiency and LOD of each assay	E	N/A
Sequence accession number	E	N1 and N2 genes from the SARS-CoV-2 genome (accession nos. MN909471; Phi accession nos. = M17461, M17462, M12921, PMMoV accession no. M01413).
Location of amplicon	D	
Amplicon length	D	N1 = 71 bp; N2 = 67 bp; phlo = 82 bp; PMMoV = 68 bp.
In silico specificity screen (BLAST, etc)	D	BLAST was used to confirm assay specificity.
Pseudogenes, retrotransposons or other homologs?	D	
Sequence alignment	D	
Secondary structure analysis of amplicon	D	
Location of each primer by exon or intron (if applicable)	E	N/A
What splice variants are targeted?	E	N/A
Primer sequences	E	See Table S2.
RT/Primer/B Identification Number	D	
Probe sequences	D	
Location and identity of any modifications	E	N/A
Manufacturer of oligonucleotides	D	
Purification method	D	
Complete reaction conditions	E	RT-qPCR amplifications were performed in 25 µL reactions on StepOnePlus qPCR thermocyclers (Applied Biosystems). Each reaction contained the following components: 0.625 µL Iovone serum albumin (BSA; 25 mg/mL), 1.875 µL primer/probe mix, 2.5 µL RNase-free water, 2.5 µL 10x Multiplex Enzyme Mix from the Path-ID Multiplex One-Step kit, 12.5 µL of 2x Multiplex RT-PCR Buffer from the Path-ID kit, and 5 µL of sample extract (substituted with 5 µL of calibration standard for positive control, and 5 µL of RNase-free water for no-template controls). Triplicate reactions were performed for each of four singleplex targets: the N1 and N2 genes of SARS-CoV-2 bacteriophage: phlo bacteriophage, and proper mild middle virus (PMMoV).
Reaction volume and amount of cDNA/DNA	E	Reaction volume = 25 µL; template = 5 µL of undiluted RNA extract.
Primer, (probe), Mg++ and dNTP concentrations	E	500 nM primers for N1 and N2; 400 nM primers for phlo; 450 nM primers for PMMoV.
Polymerase identity and concentration	E	2x Multiplex RT-PCR Buffer (ThermoFisher) containing AmpliTaq Gold ultra-pure thermostable DNA polymerase; added to achieve 12.5 µL of multiplex buffer in each 25 µL reaction.
Buffer/kit identity and manufacturer	E	See above.
Exact chemical constitution of the buffer	D	
Additives (SYBR Green I, DMSO, etc.)	E	See "Complete reaction conditions", above.
Manufacturer of plates/tubes and catalog number	E	
Complete thermocycling parameters	E	See Table S2.
Reaction setup (manual/robotic)	D	Manual.
Manufacturer of qPCR instrument	E	Applied Biosystems.
Evidence of optimization (from gradients)	D	
Specificity (gel, sequence, melt, or digest)	E	N2 sequence-verified SARS-CoV-2 (2019-nCoV) BLO plasmid controls were purchased from IDT for the N1 and N2 assays. Restriction enzyme digestion followed by gel electrophoresis was performed for the synthesized PMMoV and Phlo plasmids.
For SYBR Green I, Cq of the NTC	E	N/A
Standard curves with slope and y-intercept	E	See Table S4.
PCR efficiency calculated from slope	E	See Table S4.
Confidence interval for PCR efficiency or standard error	D	See Table S4.
σ of standard curve	E	See Table S4.
Linear dynamic range	E	1,000,000 - 2.5 gene copies/reaction (N1)
Cq variation at lower limit	E	1,000,000 - 5 gene copies/reaction (N2)
Confidence intervals throughout range	D	1,000,000 - 1,000 gene copies/reaction (PMMoV)
Evidence for limit of detection	E	5.78E+09 - 5.78E+04 gene copies/reaction (Phlo)
If multiplex, efficiency and LOD of each assay	E	0.09 (N1); 0.63 (N2); 0.06 (PMMoV); 0.10 (Phlo)
qPCR analysis program (source, version)	E	StepOne Plus v2.3
Cq method determination	E	Standard curve
Outlier identification and disposition	E	No Cq values were discarded. Outlier detection and removal later in the data-analysis pipeline is discussed in the manuscript.
Results of NTCs	E	NTC reactions were run in duplicate on each plate. Amplification of NTCs was rarely observed, and never consistently.
Justification of number and choice of reference genes	E	N/A
Description of normalization method	E	N/A
Number and concordance of biological replicates	D	One biological replicate per sample.
Number and stage (RT or qPCR) of technical replicates	E	Three technical replicates per target.
Repeatability (intra-assay variation)	E	Standard deviations of targets: 0.60 (N1); 0.82 (N2); 0.60 (phlo); 0.23 (PMMoV).
Reproducibility (inter-assay variation, %CV)	D	
Power analysis	E	
Statistical methods for result significance	E	See information on the data-analysis pipeline in the manuscript.
Software (source, version)	E	Data analysis performed using MATLAB (R2021a).
Cq or raw data submission using RAWL	D	

Table 1. MIQE checklist for authors, reviewers and editors. All essential information (E) must be submitted with the manuscript. Desirable information (D) should be submitted if available. If using primers obtained from RT/PrimerDB, information on qPCR target, oligonucleotides, protocols and validation is available from that source.

*Assessing the absence of DNA using a no RT assay is essential when first extracting RNA. Once the sample has been validated as RNA-free, inclusion of a no-RT control is desirable, but no longer essential.

**Disclosure of the probe sequence is highly desirable and strongly encouraged. However, since not all commercial pre-designed assay vendors provide this information, it cannot be an essential requirement. Use of such assays is advised against.