Supplementary Information (SI) for Lab on a Chip.
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Supporting Information

DROP-LCMS for Wastewater Surveillance of Viral Disease

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Experimental

Wastewater sample collection and preparation

Wastewater samples used in this study were prepared and utilized as described previously.[1] Briefly, 24-hour flow-dependent composite post-grit wastewater influent samples were collected in triplicate (three parallel samples per day) from the Oakville Southwest (OSW) wastewater treatment plant (WWTP) on six distinct days and transported to the laboratory. RNA was extracted by centrifugation at 10,000 x *g* for 45 minutes and 13,000 x *g* for 1 minute at 4°C (to pellet and remove solids), followed by processing the supernatant with a RNeasy PowerMicrobiome Kit (Qiagen, Germantown, MD). A 741 bp region located in the nucleocapsid (N) gene of SARS-CoV-2 (corresponding to positions 28221 to 28961 in the viral genome) was then converted to cDNA and amplified by PCR using a pair of N200 primers (5'-TAGTCGCAACAGTTCAAGAAAT-3' and 5'-CTGGTTCAATCTGTCAAGCAG-3'). For identification by mass spectrometry, the amplified product was then converted into format appropriate for on-DMF or in-tube nested PCR by amplifying with a pair of P13 nested primers (5'-TCAGCGAAATGCA-3' and 5'-CCAAACGTAATGCG-3') to yield a 31 bp DNA product covering the characteristic P13 high-frequency mutation site that is unique to SARS-CoV-2 Omicron variant (lineage: B.1.1.529). The product sequence and mass-to-charge ratio (m/z) are shown in Table S1.

DMF device and instrument configuration and use

A Miro Canvas system was purchased from Miroculus (which was later acquired by INTEGRA Biosciences, Brannan, SF). This instrument is composed of a replaceable DMF cartridge and a base unit that is connected to the internet for remote control of the platform. The cartridge comprises a flexible bottom plate and transparent top plate coated with dielectric material and a conductive layer, respectively, and then a hydrophobic layer on each plate surface. Droplets were sandwiched between the two hydrophobic layers and driven by the electrical field generated from the electrode array on the base unit below the bottom plate. The cartridge features 19 holes designed to accept inserted pipette tips, which can be connected to integrated syringe pumps in the system via valves mounted on the lid. The lid includes manifold adapters that allow easy connection of tubes to valves. Two tubing assemblies were used (Figure S1): (i) a ‘mixing and waste assembly’, which comprises a waste collection tube connecting a pipette tip and a waste reservoir tube to a valve; and (ii) a ‘mixing assembly’, which consists of a single tube linking a valve directly to a pipette tip. The base unit includes 912 active electrodes, 10 syringe pumps, 3 thermocycler regions, and 4 magnetic areas (each with a hole on top). The volume of a “unit droplet” that covers one of the electrodes is ~7 µL. The control of each element was coded in JavaScript and executed in the online website user interface (https://app.miroculus.com/). The instrument features a webcam (Logitech C930S Pro) that is positioned above the cartridge to monitor droplets inside the cartridge. When operated with the web-based software user interface Miro-Palette, a virtual grid of electrodes is superimposed onto a live image of the cartridge to enable real-time manipulation of each electrode to control droplet position.

On-DMF PCR

The DMF PCR procedure is represented in Fig. 2, step 2 in the main text, and in Fig. S2-yellow (in more detail). PCR reaction mix was prepared in a tube by combining 100-fold diluted 741 bp amplicon template (0.5 μL), which had been generated from wastewater samples as described in the "Wastewater sample collection and preparation" section, with 22 μL UltraPure DNase/RNase-Free Distilled Water (Thermo Scientific), 25 μL Taq 2X Master Mix (New England Biolabs), and 2.5 μL P13 nested primer solution to achieve a final reaction volume of 50 μL with a primer concentration of 500 nM, followed by the addition of 75 μL protection oil (Miro-Dropgloss, Part number M-03-0001-001-01) to encapsulate the reagents and prevent evaporation. A custom five-sub-step program was developed in Miro Palette to run the PCR procedure. Briefly, (1) the reaction mix (an approximately 18-unit droplet) was loaded into the cartridge through the dispense hole in the top plate and moved across the array of electrodes to the thermocycler region. (2) The droplet was heated to 95°C and incubated for 5 minutes. (3) The droplet was driven through 30 thermal cycles, each consisting of: (i) denaturation at 95°C for 30 s, (ii) annealing at 46°C for 30 s, and (iii) extension at 68°C for 10 s. (4) The droplet was incubated at 68°C for 5 minutes. (5) The reaction was cooled to 4°C and the droplet was stored for further processing.

On-DMF DNA Purification

The DMF DNA purification procedure is represented in Fig. 2, step 3 in the main text and in Fig. S2-blue-and-green in more detail. A custom ten-sub-step protocol was developed in Miro Palette for short oligo purification using magnetic beads. Briefly, (1) the PCR product (described above) was moved to a magnetic area of the chip, underneath one of the holes on the DMF cartridge's top plate. (2) A ~26-unit droplet of bead mixture [consisting of 80 μL S3 buffer, 90 μL EM buffer, and 10 μL magnetic bead suspension (BioDynami)] was pipetted onto the sample through the hole referenced in step (1). (3) A pipette tip connected to one of the Miro Canvas's integrated syringe pumps using the mixing assembly, was inserted through the same hole, and the bead-sample mixture was mixed using the Miro Canvas's automatic mixing function for 15 cycles at a volume of 190 μL and a speed of 15 abstract units (AU). (4) After 10 minutes of incubation, the magnetic element on the Miro Canvas region was activated to pellet the magnetic beads. (5) The supernatant, along with the oil shell, was aspirated by another pipette, connected to another syringe pump using the mixing and waste assembly. (6) A ~26-unit droplet of ethanol was driven onto the beads at low velocity to minimize disturbance to the beads. Following a 1-minute incubation, the ethanol was aspirated with a speed of 15 AU using the waste pipette. (7) The beads were allowed to dry at room temperature for 3 minutes. (8) A ~7-unit droplet of water was loaded into the cartridge and then driven over the beads. (9) The Miro's mixing function was activated with a volume of 60 μL and a speed of 55 AU for 15 cycles. (10) The magnet was activated to pellet the magnetic beads, and the supernatant containing the purified DNA product was driven to an exit port, where it was collected for analysis.

In-Tube PCR and Purification

For comparison, some wastewater samples were PCR-amplified in a T100 Thermal Cycler (Bio-Rad Inc) by pipetting in tubes as described previously.[1] In most experiments, the annealing temperature was 46°C; in other experiments, this temperature was varied. Amplified samples were then purified using a manual version of the DMF magnetic bead technique described above, or by a manual solvent precipitation procedure as described previously.[1]

Optimization of DMF Temperature Control and Heating

To investigate the thermal sensitivity of the annealing step, a wastewater sample containing 44.25% wild-type P13 and 55.75% Omicron variant P13L was processed in tubes, and analyzed by PCR-HPLC-MS (as described below). Replicates were evaluated after annealing at a range of temperatures, and the ratios of Omicron/wild-type determined from the mass spectra were compared to the known ratios. Total MS signal was also recorded for each experiment. Optimal annealing temperature was determined to be 46°C.

A forward looking infrared (FLIR) camera (FLIR E8 Pro, Teledyne FLIR LLC) was used to assess and optimize temperature profiles in DMF experiments. In initial experiments, the camera was aligned over a Miro chip containing a 7-unit droplet of deionized water on a hot plate. The relationship between measured temperature (in the droplet) and programmed temperature (on the hot plate) was established and used for calibration. The camera was then mounted over the Miro Canvas. In each experiment, a 7-unit droplet of deionized water was loaded into the chip and driven to the heating element, which was programmed using the Miro Palette software. Sample FLIR data are shown in Fig. S3A, and uncalibrated droplet temperatures are shown in Fig. S3B. From this data, a calibration formula was extrapolated that allowed for robust prediction of droplet temperature in the Miro Canvas. This formula was used to precisely set the droplet temperature for all samples processed by the Miro described herein.

Design, Manufacture, and Use of DROP-LCMS Manifold

A custom manifold connecting the DMF cartridge and HPLC autosampler system was designed using AutoCAD and Fusion 360 software from two pieces – a cartridge holder (Fig. S4A) and a needle guider (Fig. S4B), with particular attention paid to ensuring compatibility with the DMF cartridge's 19 dispensing holes and the autosampler specifications. The final CAD model was exported as an STL file, which was then cross-sectioned by the Cura slicer (Ultimaker) with a triangular infill density of 20% and a resolution of 0.2 mm and printed with an Ultimaker 2 3D printer in polylactic acid (PLA) with a nozzle temperature at 220 °C. After printing, the part was polished with 5000 grit sandpaper (3M), then sterilized using ethanol and RNase AWAY (Thermo Scientific) before use (Fig. S4C-E). In experiments, after completing droplet manipulation operations, the cartridge was removed from the Miro Canvas and was assembled between the manifold's cartridge holder and needle guider, which (together) has the same size and dimensions of a 54-vial rack. The manifold bearing the cartridge was then inserted into the sample tray of a Vanquish UPLC system (Thermo Scientific) for use (Fig. S4F). The injection needle height was positioned such that it precisely reached the bottom plate of the device through the interface hole. The UPLC autosampler was then programmed to collect approximately 5 µL of the droplet at a rate of 1 µL/s.

Manifold performance was assessed by HPLC-MS in two stages. In initial tests, 5-unit droplets containing a sense (5'-ACAAACTAAATGTCTCTAAATGGACCCCAA-3') or an anti-sense oligo (5'-TTGGGGTCCATTTAGAGACATTTAGTTTGT-3') standard (purchased from Invitrogen, Thermo Fisher Scientific) were driven through the cartridge, and loaded through the manifold into the HPLC-MS where they were analyzed by HPLC-MS as described below (Fig. S4G, Table S2). In follow-up tests, manifold performance was characterized by replicate analyses of wastewater samples processed by the full DROP-LCMS procedure (Fig. S5).

HPLC-MS

Purified PCR products (prepared on-DMF or manually in tubes) were loaded into an autosampler (manually, or via the manifold), and then were separated using a Vanquish UPLC system (Thermo Scientific). The analytical column employed was an ACQUITY UPLC Oligonucleotide BEH C18 Column, with dimensions of 50 mm × 2.1 mm and a particle diameter of 1.7 µm (130Å). Mobile phase A consisted of water with 15 mM triethylamine (TEA) and 25 mM hexafluoroisopropanol (HFIP), while mobile phase B was neat methanol. The flow rate was maintained at 0.2 mL/min. The gradient profile initiated at 5% of mobile phase B for 2 minutes, followed by a linear increase to 30% B over 7 min, then a ramp to 99% B over 0.5 minutes, which was sustained at 99% B for 6 minutes. Subsequently, the gradient was reduced to 5% B within 0.5 minutes and held constant for an additional minute. Molecular identification was carried out using a Q-Exactive Orbitrap mass spectrometer (Thermo Scientific). Ionization and desolvation were facilitated by applying a spray voltage of 3.0 kV and setting the ion transfer tube temperature to 300°C. Precursor ion spectra were acquired in negative ionization mode over a mass-to-charge ratio (m/z) range of 1000 to 5000, with a resolution setting of 140,000. Representative results are shown in Fig. S6A and S7-S9. Based on the known amplified product of wild-type P13 (5'-TCAGCGAAATGCACCCCGCATTACGTTTGGa-3') and P13L (5'-TCAGCGAAATGCACTCCGCATTACGTTTGGa-3'), the mass-to-charge ratios (m/z) were determined to be 2368.90 for wild-type P13 and 2372.65 for Omicron variant P13L. The assigned proportion of Omicron variant P13L in each sample was calculated by dividing the intensity at 2372.65 by the sum of intensities at 2368.90 and 2372.65. Finally, for standards with known proportion of Omicron, error in the assignment of Omicron % was defined as the difference between the average assigned proportion and the known value.

Gel Electrophoresis

Purified, amplified samples (processed manually or by DMF), as well as a 10 bp DNA Step Ladder (Promega), were stained by the GelGreen DNA Stain (Thermo Scientific) and electrophoresed in 5% agarose gels at 90 V for 45 minutes. Gels were visualized using an E-Gel Imager (Life Technologies) equipped with an E-Gel Imager camera hood (Life Technologies) with a blue light base. Representative results are shown in Fig. S6B.

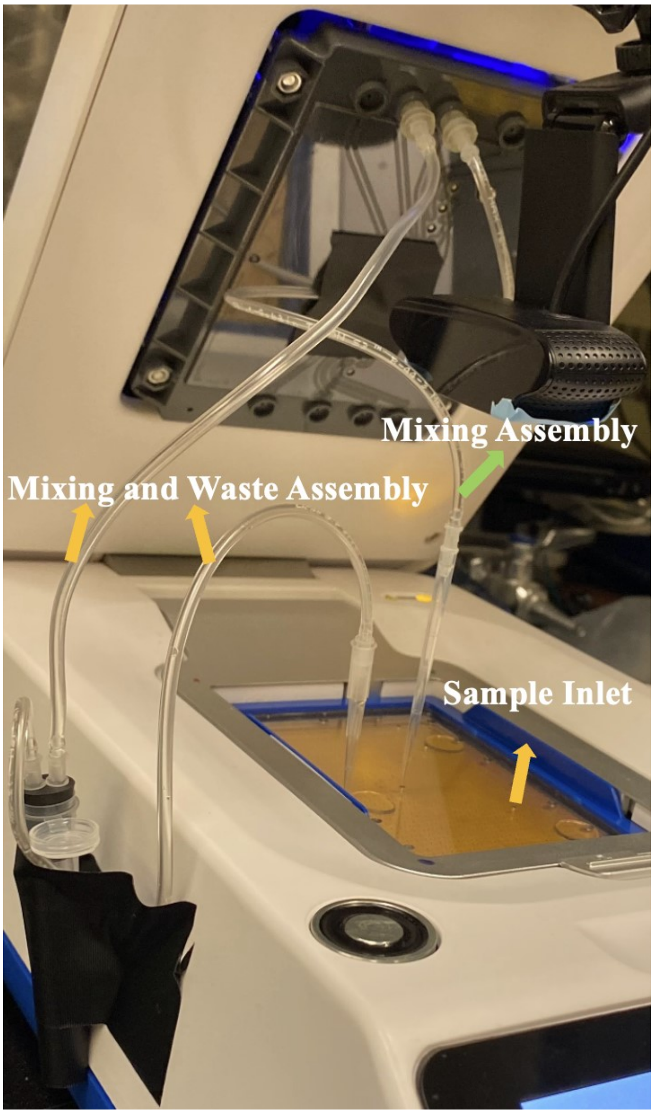

Figure S1. Picture of the Miro Canvas system interfaced with the two tubing assemblies that were used. The 'Mixing Assembly' consisted of a tube connected at one end to a manifold adapter, which allows attachment to a valve on the Miro Canvas's lid (which is connected to one of the system's integrated syringe pumps). The other end of the tube was connected to a pipette tip, which could be inserted into one of the holes on the DMF cartridge for direct droplet manipulation. The 'Mixing and Waste Assembly' comprised one tube connected to the syringe pump valve to a waste collection junction, which led to a waste reservoir, and a second tube that extended from the junction to a pipette tip for liquid handling.

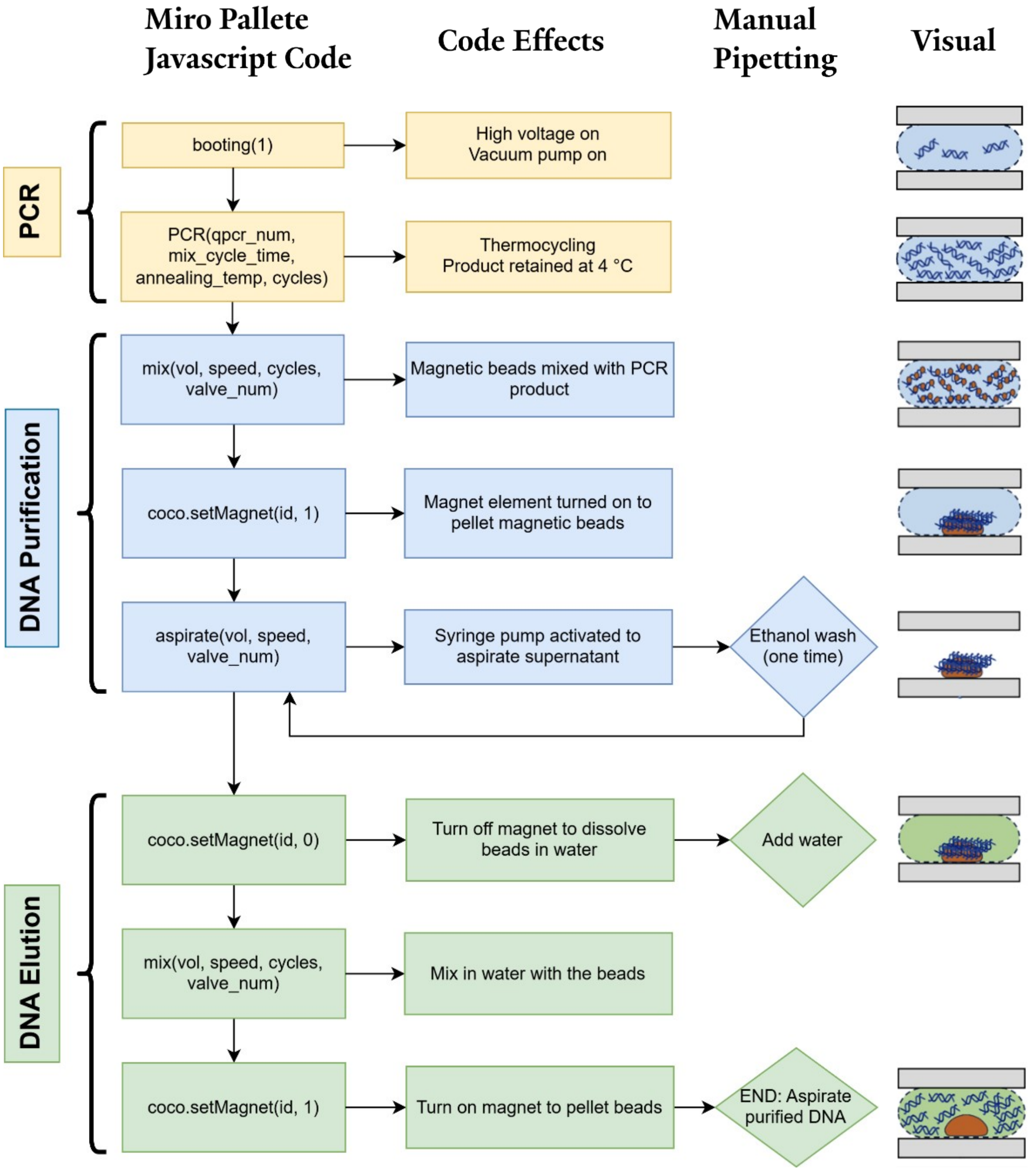

Figure S2. DMF sample processing protocol. The boxes in the first column enumerate JavaScript code snippets entered into a text terminal within the Miro-Palette UI, while the boxes in the second column illustrate the effects of each snippet of code. Diamond-shaped boxes in the third column indicate manual steps performed by the user (pipetting in/out liquids). The fourth column shows a cartoon indication of the primary process in each step.

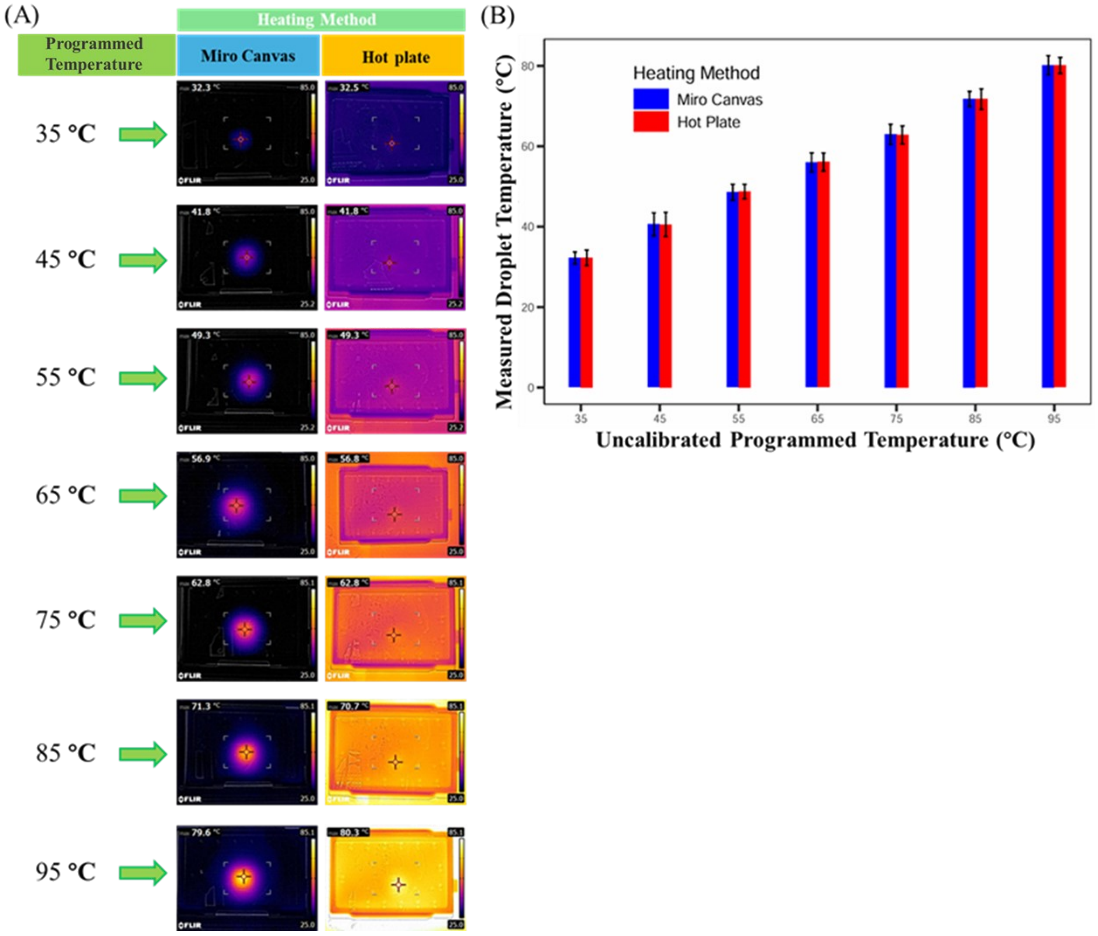

Figure S3. DMF heating element test. (A) Series of FLIR images comparing a ~7-unit water droplet on two platforms: the Miroculus Miro Canvas (left) and a hot plate (right). Each image corresponds to a matched temperature setting on both instruments, ranging from 35°C to 95°C. (B) Comparison of measured temperatures for~7-unit water droplets heated on a Miro Canvas (blue bars) and a hot plate (red bars), across set temperatures ranging from 35°C to 95°C. Error bars represent 95% confidence interval for n=3 replicates per condition. A linear regression was performed to relate the set temperature of the Miro Canvas and the FLIR-measured mean surface temperature. The resulting model, $y = 4.91 + 0.787x$, demonstrated an excellent fit with an R^2 value of 0.9999, and was used to calibrate droplet temperatures on the Miro Canvas from the FLIR-measured mean surface temperatures (as shown in Fig. 3C in the main text).

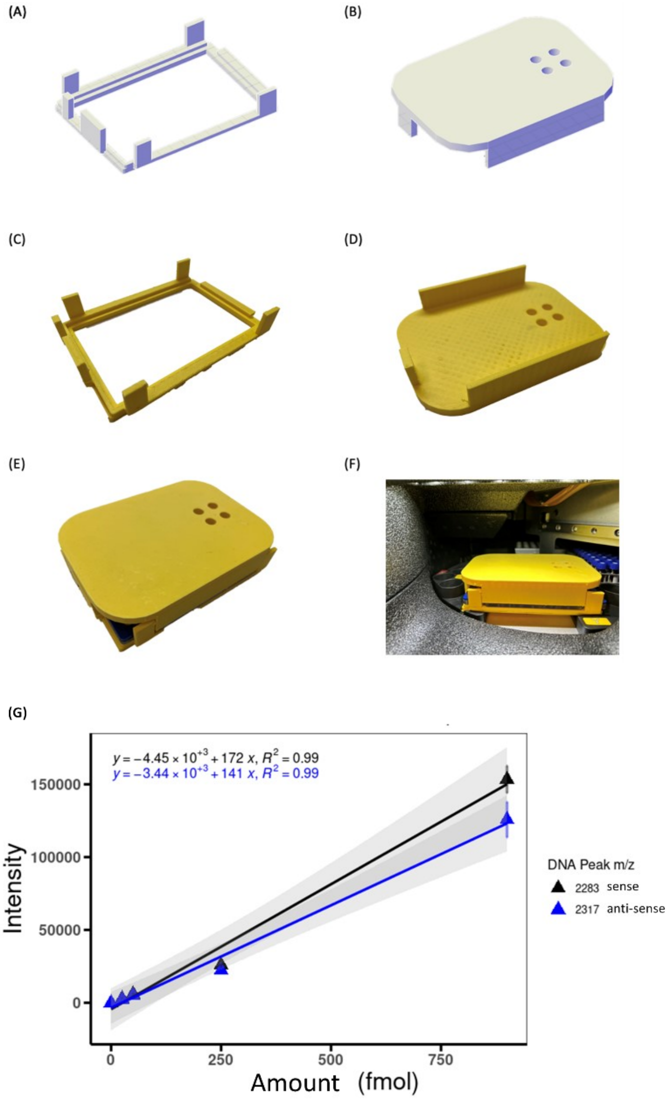

Figure S4. DROP-LCMS Manifold. CAD renderings of the (A) cartridge holder and (B) needle guider. Photographs of the (C) cartridge holder, (D) needle guider, (E) fully assembled manifold, and (F) manifold and cartridge upon insertion into the rotary sampling chamber of the Vanquish UPLC system. (G) Plot of MS intensities from droplets sampled using the DROP-LCMS manifold as a function of amount of sense (black triangles) and anti-sense (blue triangles) oligo standards. Regressions (black and blue lines) had high fidelity to the data (equations and R^2 values). Error bars represent standard deviation for n=3 replicates, and the gray shading indicates the standard error.

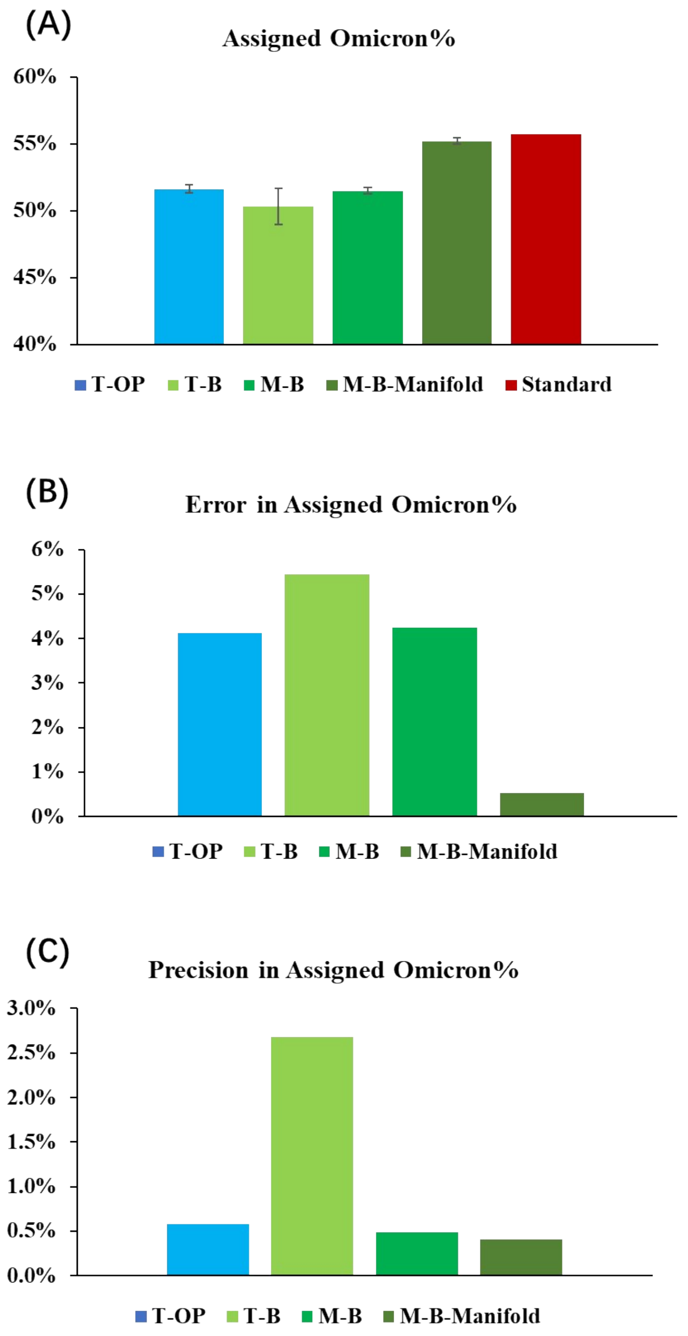

Figure S5. Sample processing accuracy and precision. (A) Plot of the average percentage of Omicron variant assigned from measurements of a wastewater standard with a known ratio (55.75%, red bar, confirmed by qPCR), treated using four different processing methods, including in-tube PCR with manual organic precipitation loaded manually into the autosampler (T-OP, turquoise bar), in-tube PCR with manual bead purification loaded manually into the autosampler (T-B, light green bar), on-DMF PCR with automated bead purification loaded manually into the autosampler (M-B, med. green bar) and on-DMF PCR with automated bead purification loaded automatically from the cartridge into the autosampler via the manifold (M-B-Manifold, dark green bar). Error bars represent ± 1 std. deviation from n=3 replicate analyses per condition. (B) Plot of the error for the data in (A). (C) Plot of the coefficients of variation for the data in (A).

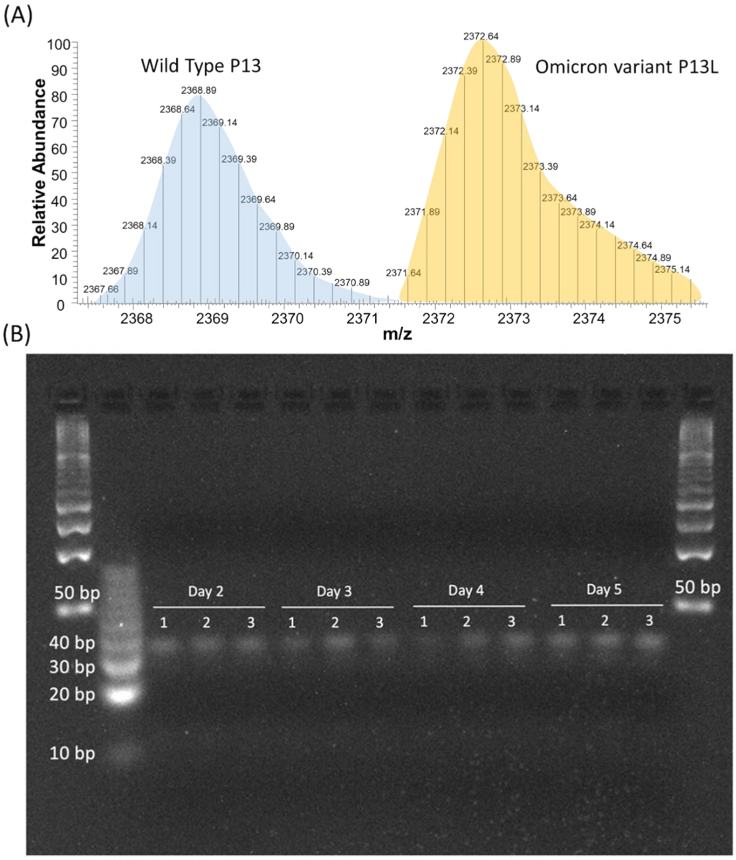

Figure S6. Verification of Omicron variant in wastewater. (A) Representative mass spectrum results used to identify the sense strand of wild-type P13 (peaks on a blue background) and Omicron variant P13L (peaks on a yellow background) in wastewater. (B) Gel electrophoresis results used to verify the chain length of the amplification product in wastewater on days 2-5.

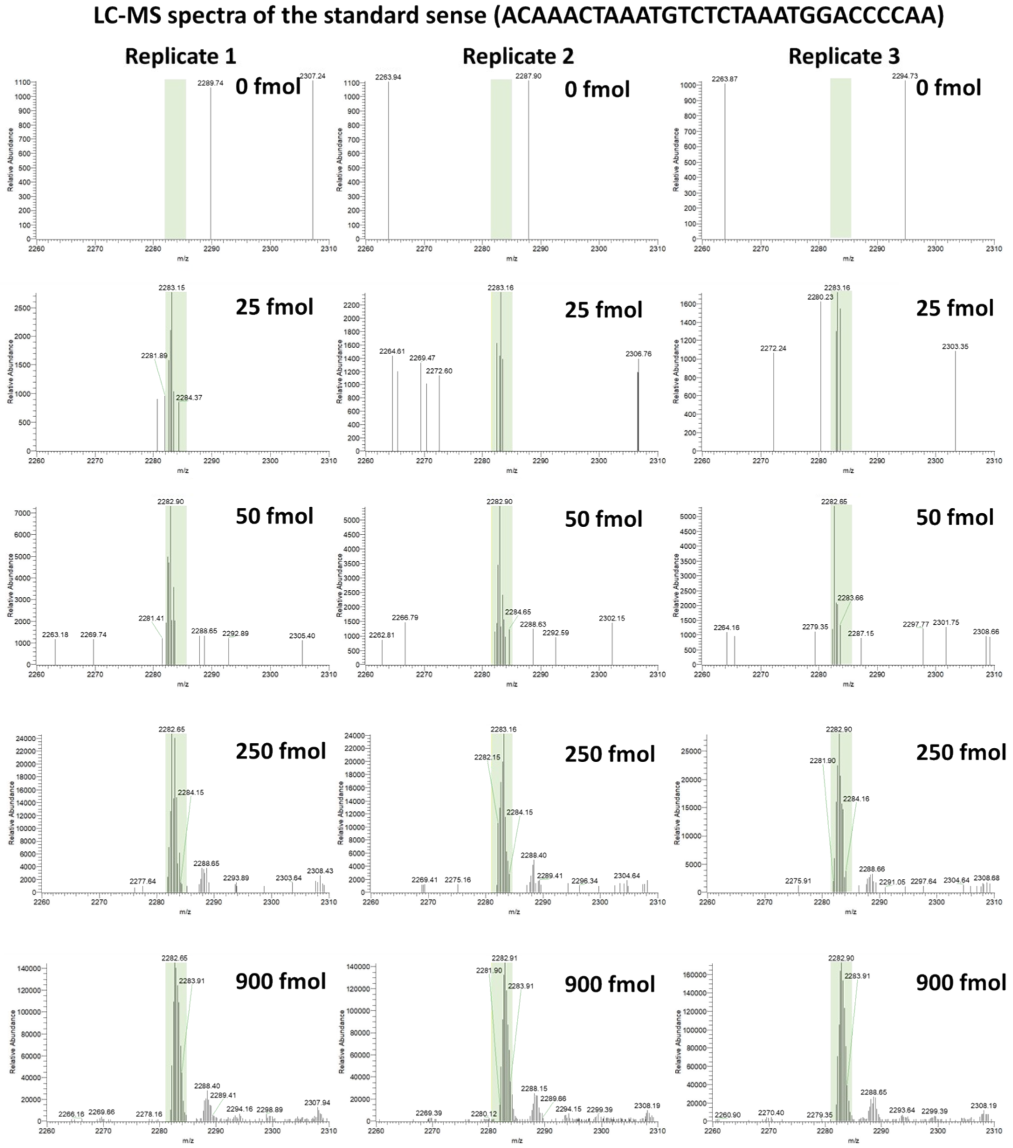

Figure S7. Representative LC-MS spectra generated from three replicates (columns 1-3) from solutions containing 0, 25, 50, 250, and 900 femtomoles (rows 1-5) of the DNA standard sense strand (ACAAACTAAATGTCTCTAAATGGACCCCAA). Green shading highlights the region of the spectrum from which intensities were extracted to generate the 'sense' data in Figure S4G.

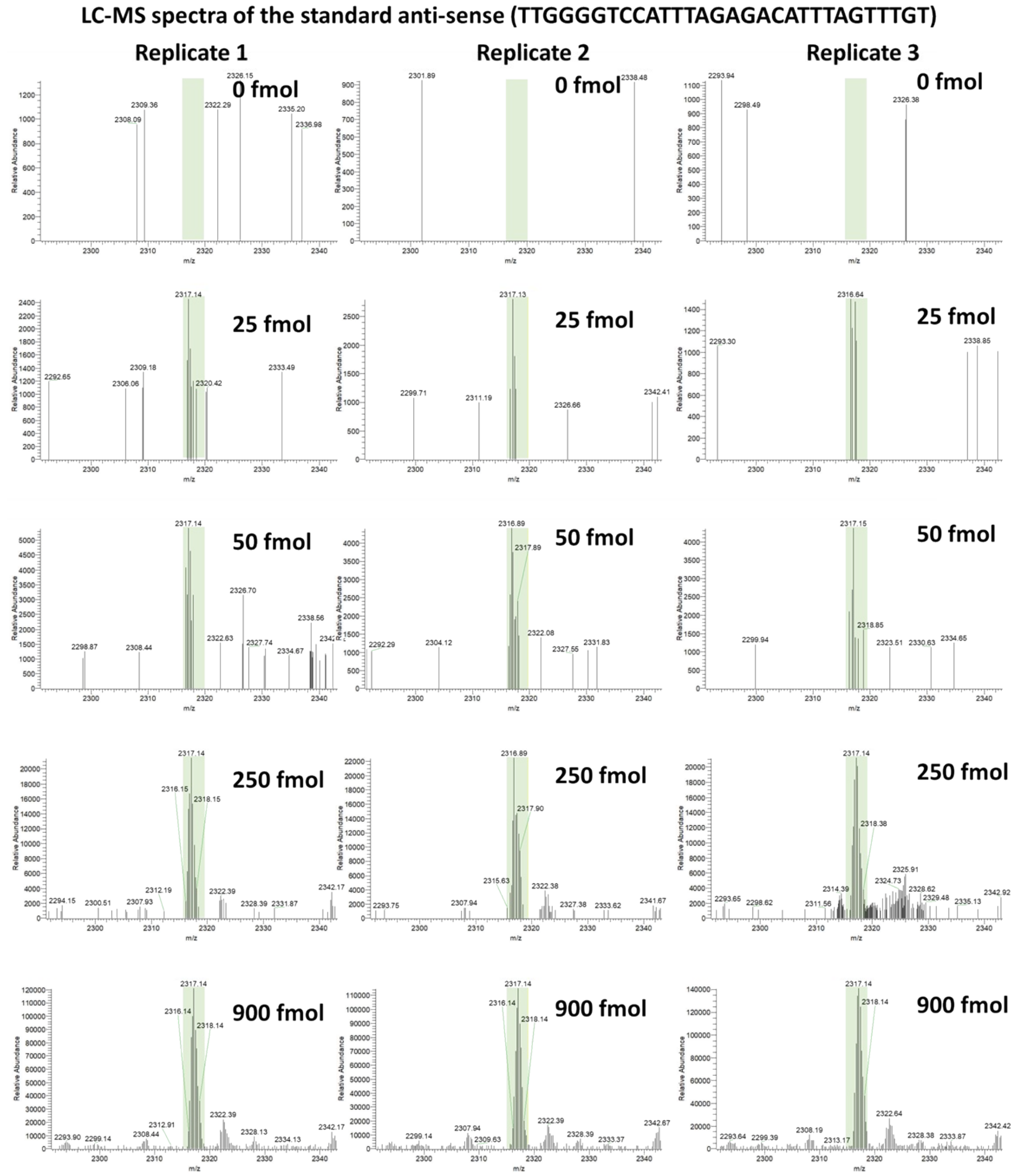

Figure S8. Representative LC-MS spectra generated from three replicates (columns 1-3) from solutions containing 0, 25, 50, 250, and 900 femtomoles (rows 1-5) of the DNA standard anti-sense strand (TTGGGGTCCATTTAGAGACATTTAGTTTGT). Green shading highlights the region of the spectrum from which intensities were extracted to generate the ‘anti-sense’ data in Figure S4G.

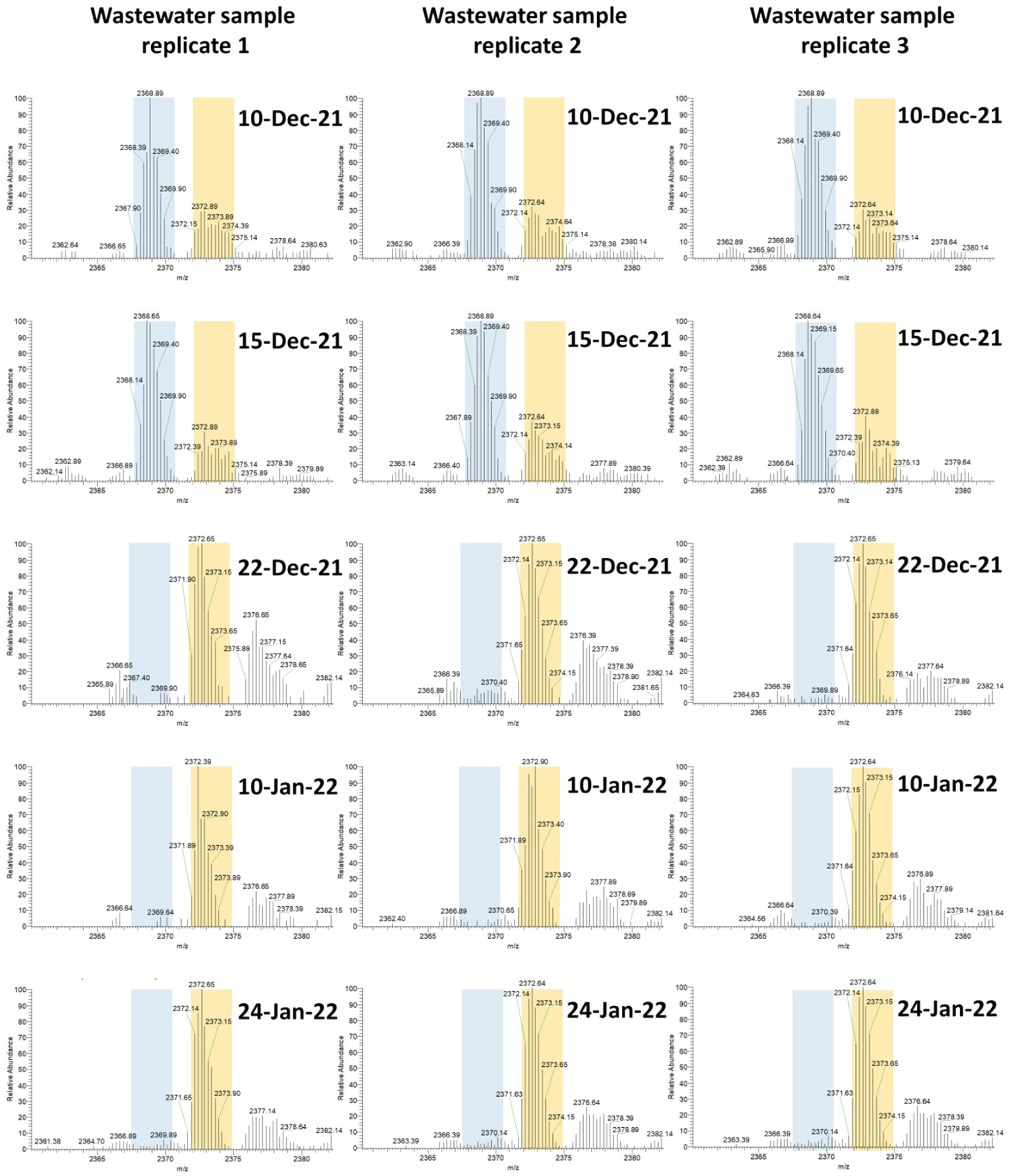

Figure S9. Representative LC-MS spectra of wastewater samples collected from three different sampling sites (columns 1-3) on Dec. 10 (2021), Dec. 15 (2021), Dec. 22 (2021), Jan. 10 (2022), and Jan. 24 (2022) (rows 1-5) analyzed using the DROP-LCMS method. Spectra regions featuring m/z corresponding to the sense strands of wild-type P13 and Omicron variant P13L are higlighted in blue and yellow shading, respectively.

Table S1. Sequence information of Omicron variant amplicons and DNA standard used in this work.

Target	**Sequence (5’→3’)**	**m/z**
Wild-type P13	TCAGCGAAATGCACCCCGCATTACGTTTGGa*	2368.90
P13L	TCAGCGAAATGCACTCCGCATTACGTTTGGa	2372.65
Standard	ACAAACTAAATGTCTCTAAATGGACCCCAA	2283.24
Standard anti-sense	TTGGGGTCCATTTAGAGACATTTAGTTTGT	2317.49

* Taq polymerase has a nontemplate-dependent terminal transferase activity that adds a single deoxyadenosine (a) to the 3′-ends of PCR products.

Table S2. Relative quantification of sense and anti-sense DNA standards using manifold-assisted HPLC-MS.

Standard Amount (fmol)	**Individual Ratio（sense amount, m/z=2283 / anti-sense amount, m/z=2317)**	**Mean Ratio**	**Accuracy (%)**	**SD**	**RSD (%)**
25	1.092 0.996 1.010	1.033	103.3	0.0518	5.01
50	1.075 0.964 1.029	1.023	102.3	0.0558	5.45
250	0.992 0.906 1.011	0.970	97.0	0.0561	5.79
900	0.993 1.085 0.949	1.009	100.9	0.0691	6.85

Supplementary References:

1. Peng, J.; Sun, J.; Yang, M. I.; Gibson, R. M.; Arts, E. J.; Olabode, A. S.; Poon, A. F. Y.; Wang, X.; Wheeler, A. R.; Edwards, E. A.; Peng, H., Early Warning Measurement of SARS-CoV-2 Variants of Concern in Wastewaters by Mass Spectrometry. *Environ Sci Technol Lett* **2022,** *9* (7), 638-644.